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Neurophysiological Signatures of Motor Impairment in Patients with Rett Syndrome

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Objective: Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder due to pathogenic mutations in the *MECP2* gene. Motor impairment constitutes the core diagnostic feature of RTT. Preclinical studies have consistently demonstrated alteration of excitation/inhibition (E/I) balance and aberrant synaptic plasticity at the cortical level. We aimed to understand neurobiological mechanisms underlying motor deficit by assessing in vivo synaptic plasticity and E/I balance in the primary motor cortex (M1).

Methods: In 14 patients with typical RTT, 9 epilepsy control patients, and 11 healthy controls, we applied paired-pulse transcranial magnetic stimulation (TMS) protocols to evaluate the excitation index, a biomarker reflecting the contribution of inhibitory and facilitatory circuits in M1. Intermittent TMS-theta burst stimulation was used to probe long-term potentiation (LTP)-like plasticity in M1. Motor impairment, assessed by ad hoc clinical scales, was correlated with neurophysiological metrics.

Results: RTT patients displayed a significant increase of the excitation index ($p = 0.003$), as demonstrated by the reduction of short-interval intracortical inhibition and increase of intracortical facilitation, suggesting a shift toward cortical excitation likely due to GABAergic dysfunction. Impairment of inhibitory circuits was also confirmed by the reduction of long-interval intracortical inhibition ($p = 0.002$). LTP-like plasticity in M1 was abolished ($p = 0.008$) and scaled with motor disability (all $p = 0.003$).

Interpretation: TMS is a method that can be used to assess cortical motor function in RTT patients. Our findings support the introduction of TMS measures in clinical and research settings to monitor the progression of motor deficit and response to treatment.

ANN NEUROL 2020;87:763–773

Rett syndrome (RTT) is the second most common cause of severe intellectual disability in females and is usually the result of dominantly acting mutations in the X-linked gene *MECP2*, which encodes methyl-CpG-binding protein 2 (MeCP2).[1] MeCP2 is expressed quite widely throughout the body, with notably high expression in postnatal neurons.[2,3] The currently used diagnostic criteria for RTT include an early neurologic regression occurring after an initially normal development that severely affects motor, cognitive, and communication skills.[4] Motor impairment constitutes the core diagnostic features of RTT, such as partial or complete loss of acquired purposeful hand skills and spoken language, the development of gait abnormalities, stereotypic hand movements, and the

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25712

Received Nov 17, 2019, and in revised form Mar 2, 2020. Accepted for publication Mar 2, 2020.

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progressive deterioration of motor abilities.[4] Although motor deficits are considered among the most debilitating symptoms of RTT individuals, little is known about the underlying pathophysiological mechanisms.

So far, the ability to model some aspects of the disease in the mouse, for instance by using knockout heterozygous female mice (ie, $Mecp2^{+/-}$), provided the most significant clues for understanding the disease. At the cellular level, electrophysiological studies by means of whole-cell patch clamp recordings showed alterations of synaptic excitability; the lack of MeCP2 induced a shift of the homeostatic balance between excitation and inhibition (E/I). Importantly, the direction of change of E/I in favor of excitation or inhibition depends on the specific brain circuit, even if recent evidence suggests that inhibition is reduced to a greater extent compared to excitation, thus enhancing the E/I ratio.[5] Interestingly, alterations in E/I balance have been shown to have consequences for cortical plasticity in neural circuits, and in this context, RTT has become one of the best disease models of abnormal synaptic plasticity.[6] For instance, deficits of long-term potentiation (LTP) synaptic plasticity were observed at layer II/III synapses of motor and sensory cortex[7]; more recently, it has been shown that motor learning–dependent changes of parvalbumin expression and structural plasticity in the primary motor cortex (M1) were impaired in symptomatic $Mecp2^{+/-}$ female mice, and such defective cortical activity correlated with the severity of motor behavioral impairments.[8] The impairment of synaptic excitability and plasticity in M1 is particularly interesting given the anatomical evidence of a selective reduction of dendritic arborizations in pyramidal neurons of layers III and V of the frontal and motor cortices in human brain autopsies with RTT.[9,10]

Although the mechanisms underlying E/I balance and cortical plasticity have been well studied in the mouse model of RTT, whether similar functional changes are present in humans with RTT is still unknown. To elucidate the physiological mechanisms associated with motor impairment in humans with RTT, we tested the function of excitatory and inhibitory circuits and the level of LTP-like activity in M1 using noninvasive brain stimulation techniques. Transcranial magnetic stimulation (TMS) was used to probe cortical excitability and plasticity in people with RTT. TMS activates human motor cortex transcranially; specifically, according to the microcircuit model,[11,12] TMS induces strong depolarization of layer II/III pyramidal and inhibitory cells that in turns leads to highly synchronized recruitment of clusters of excitatory neurons, including pyramidal neurons of layer V, that represent the major output of M1.[11,12] Protocols of paired pulse TMS may provide insights into the function of cortical inhibitory and excitatory interneurons depending on the interval between the conditioning and test stimuli,[13,14] and repetitive TMS (rTMS) evaluates LTP-like activity of central motor circuits and thus can reveal abnormalities in brain plasticity.[14,15] Herein, we used patterned rTMS, namely intermittent theta burst stimulation (iTBS), to investigate LTP within M1 (Table).[16]

We hypothesized that RTT patients would exhibit a lack of cortical plasticity together with a shift toward excitation of the E/I balance, likely due to a reduction of inhibitory mechanisms, and these alterations would scale with motor deficit. As an ancillary investigation, we assessed the serum level of insulinlike growth factor 1 (IGF-1), which is demonstrated to be reduced in a mice model of RTT.[17] In mice, the administration of IGF-1 partly reversed clinical phenotype,[17,18] restoring cortical plasticity[17] and normalizing the E/I balance.[5] In humans, the first clinical studies on the therapeutic use of IGF-1 reported promising effects[19–21]; however, recent placebo-controlled trials provided conflicting results.[22,23]

Patients and Methods

Patients and Clinical Evaluation

The study complied with the Helsinki declaration on human experimentation and was approved by the Ethical Committee of the University of Naples Federico II (n. 100/17). Parents or legal guardians of the participants gave informed consent. Participants were seen at the Child Neuropsychiatric Department or Epilepsy Center of the University of Naples Federico II between 2017 and 2018.

For RTT patients, a history and structured examination was performed for each girl by experienced examiners (P.B, C.B.) to confirm the diagnosis using consensus criteria.[24] Individuals were included if they met the consensus criteria for typical RTT,[24] carried *MECP2* mutations, and had a complete clinical assessment by means of dedicated clinical scales: the clinical severity score (CSS)[25] and the Rett Syndrome Gross Motor Scale (RSGMS).[26]

Because the main aim of the study was to evaluate the impact of motor disability on neurophysiological measures in M1, we decided to use only the motor-skill categories of the CSS, namely the hand use, motor/independent sitting, and ambulation items. Each item score ranges from 0 to 4 or 0 to 5, with 0 representing the less severe and 4 or 5 representing the most severe finding.[25] For example, in the ambulation category, a score of 2 or less indicates the ability to walk alone, whereas a score of 3 or higher indicates that the individual cannot walk unaided or is completely unable to walk. Similar divisions can be made for the hand use and motor/independent sitting category.

In addition, to further evaluate motor skills, we applied the RSGMS that measures gross motor abilities by considering 15 gross motor skills scored on a 0 to 3 scale, ranging from maximal assistance/unable (score = 0) to no assistance

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TABLE. Transcranial Magnetic Stimulation–Electromyography Measures

TMS-EMG Measures	Protocol	Putative Mechanisms	Effects of CNS Active Drugs on TMS–EMG Measures
Motor thresholds: RMT, AMT	Single pulse: the minimum TMS intensity that is necessary to elicit a liminal MEP in the target muscle, either at rest (RMT) or during slight voluntary contraction (AMT)	Cortical motor neuron voltage-gated sodium channel–mediated membrane excitability	Increased by voltage-gated sodium channel blockers (eg, carbamazepine, lamotrigine, phenytoin); decreased by NMDA-type and AMPA-type glutamate receptor antagonists (eg, ketamine)
Short-interval intracortical inhibition	Paired pulse: subthreshold conditioning stimulus and suprathreshold test stimulus applied at short interstimulus intervals of 1–5 milliseconds	$GABA_A$-mediated cortical inhibition	Increased by $GABA_A$-positive allosteric modulators (eg, lorazepam)
Intracortical facilitation	Paired pulse: subthreshold conditioning stimulus and suprathreshold test stimulus applied at interstimulus intervals of 7–20 milliseconds	$GABA_A$-mediated cortical inhibition; glutamate mediated cortical excitation	Decreased by $GABA_A$-positive allosteric modulators (eg, diazepam, lorazepam) and NMDA-type and AMPA-type glutamate receptor antagonists (eg, memantine)
Long-interval intracortical inhibition	Paired pulse: 2 suprathreshold stimuli applied at long interstimulus intervals of 50–300 milliseconds	$GABA_B$-mediated cortical inhibition	Increased by $GABA_B$ agonists (eg, baclofen, tiagabine, vigabatrin)
Intermittent theta burst stimulation	Patterned repetitive stimulation: 600 subthreshold pulses (10 bursts of triplets at 50Hz, in short trains of 2 seconds, with an 8-second pause between consecutive trains)	Glutamate-mediated LTP-like plasticity	Decreased by NMDA-type glutamate receptor antagonists (eg, memantine), L-type voltage-gated ion channel blockers (eg, nimodipine), and type 2 dopamine receptor antagonist (eg, sulpiride)

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMT = active motor threshold; CNS = central nervous system; EMG = electromyography; GABA = γ-aminobutyric acid; LTP = long-term potentiation; MEP = motor evoked potential; NMDA = N-methyl-D-aspartate; RMT = resting motor threshold; TMS = transcranial magnetic stimulation.

(score = 3).[26] By using these scores, we asked whether the magnitude of alteration in plasticity and E/I balance in M1 would be correlated with motor performance. Although CSS is considered less sensitive and reliable than RSGMS in evaluating longitudinal gross motor function, we adopted both scales to have a cross-validation of our data, providing a conceptual within-study replication that would strengthen the reliability of our results. Lastly, control data were gathered from 9 subjects with non-RTT epilepsy taking antiepileptic drugs (AED) and 11 healthy participants.

Electrophysiology

Electromyographic Recording and Focal TMS. Participants were seated comfortably in a chair reposing both hands suitably on a cushion or their lap to ensure complete relaxation. Motor evoked potentials (MEPs) were recorded by electromyography (EMG) from the right first dorsal interosseous (FDI) muscle using Ag–AgCl surface electrodes (Ambu, Ballerup, Denmark) mounted using the belly-tendon technique. The signals from the EMG electrodes were amplified, bandpass filtered (20Hz–3kHz), digitized at a frequency of 5kHz, and stored in a laboratory computer for later offline analysis by Signal software and CED 1401 hardware (Cambridge Electronic Design, Cambridge, United Kingdom). The level of baseline EMG activity was controlled by visual feedback through an oscilloscope screen and by auditory feedback through a loudspeaker. We rejected trials with involuntary EMG

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activity from FDI muscle greater than 50μV in a time window of 500 milliseconds preceding MEPs.

Focal TMS was performed using a figure-of-8–shaped magnetic coil (outer diameter of each wing 70mm) that was held tangentially to the skull with the handle pointing backward and laterally at an angle of 45° to the sagittal plane (direction of current induced in the brain: posterior to anterior). Experiments were performed by connecting the coil to a high-power magnetic stimulator with a biphasic current waveform (MagPro X100; Medtronic, Skovlunde, Denmark). The "hot spot" was defined as the optimal scalp position for eliciting MEPs of maximal amplitude in the contralateral FDI. To ensure stability of the stimulation position over the course of the experiment, the hotspot was marked directly on the scalp with a soft-tip pen.

Measures of Motor Thresholds and Intracortical Inhibitory/Excitatory Balance. Resting motor threshold (RMT) was determined as the minimum stimulator intensity needed to produce a response of at least 50μV in the relaxed FDI in at least 5 of 10 consecutive trials. Active motor threshold (AMT) was calculated during a mild tonic contraction (approximately 20% of maximal contraction) as the lowest intensity evoking 5 MEPs of at least 200μV in 10 consecutive trials.[27] In the case of RTT patients, muscle contraction was obtained by placing a weight in the outstretched, supinated hand, with the arm adducted at the shoulder and flexed at the elbow to about 90°; for less cooperative patients, muscle contraction was elicited using the traction reflex. In addition, to check if muscle contraction gave reliable AMT results in RTT patients, we normalized the AMT value with respect to the RMT and compared this ratio among the 3 groups.

To assess inhibitory/excitatory balance in M1, we applied 2 paired-pulse TMS protocols: short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF). SICI is supposed to be mediated by $GABA_A$-ergic intracortical circuits, and ICF is mediated by glutamatergic intracortical circuits, possibly alongside a reduction in GABAergic inhibition (see Table).[13]

SICI and ICF were determined by setting the conditioning stimulus (CS) intensity to 95% AMT and delivering the CS before the test stimulus (TS). For both paradigms, the unconditioned MEP (TS) was adjusted to evoke an MEP of ~0.5mV amplitude in the right FDI muscle. A previous study showed that SICI and ICF can be observed with TS intensity of 0.5mV.[28] SICI was recorded at interstimulus intervals (ISIs) of 2 and 3 milliseconds,[29] and intracortical ICF was determined at ISIs of 10 and 15 milliseconds; then they were expressed as the mean peak-to-peak amplitude normalized with respect to the TS.[30] Subsequently, the average of normalized SICI and ICF, over the different ISIs, was measured for each patient. To express the balance between cortical inhibitory and facilitatory interneuronal function, an excitation index was developed and expressed in the following formula:

$$\text{Excitation index} = \frac{ICF}{ICF - SICI}$$

Lastly, we applied the long-interval intracortical inhibition (LICI) that is supposed to be mediated by $GABA_B$-ergic intracortical circuits within M1 (see Table).[13,31] LICI was investigated by implementing 2 suprathreshold stimuli, with the CS adjusted at 120% of the RMT, with ISIs of 100 and 150 milliseconds (Fig 1).[32] For all paired-pulse paradigms, 15 trials were recorded for each condition and randomly intermixed with 15 trials of TS alone (0.2Hz ± 10%). Complete voluntary muscle relaxation was monitored audio visually by high-gain EMG (50μV/division). Trials contaminated with voluntary activity were discarded from the analysis.[33]

Assessment of Cortical Plasticity after iTBS. We applied iTBS using the well-known paradigm introduced by Huang et al.[16] It consisted of bursts of 3 pulses at high frequency, 50Hz, repeated at intervals of 200 milliseconds, delivered in short trains lasting 2 seconds, with an 8-second pause between consecutive trains, for a total of 600 pulses (see Fig 1). The stimulation intensity for iTBS was set at 80% AMT. To assess corticospinal excitability before iTBS, single MEPs were recorded using a stimulus intensity adjusted to produce MEP amplitude of approximately 0.5mV in the relaxed FDI muscle. For each subject, 20 MEPs were recorded, and the peak-to-peak amplitudes were measured to calculate the mean amplitude.

After the interventions, corticospinal excitability changes were monitored by collecting 12 MEP responses (0.2Hz ± 10%) every 2 minutes following the intervention for up to 30 minutes (15 blocks, starting with 2 minutes of rest, then 1 minute measurement, 1 minute rest, and so on; see Fig 1).[34,35] We decided to adopt a high temporal resolution of corticospinal excitability assessment after iTBS for a better estimation of the different patterns of motor cortex plasticity across the groups over time.[34,35] The average duration of the whole experiment in a single subject was 55 minutes: 20 minutes for the evaluation of motor thresholds and inhibitory/facilitatory circuits and 35 minutes for the assessment of motor cortex plasticity.

IGF-1 Measurement

Serum IGF-1 concentration was determined by a solid-phase, enzyme-labelled chemiluminescent immunometric assay (IMMULITE 2000; Siemens Healthcare Diagnostics,

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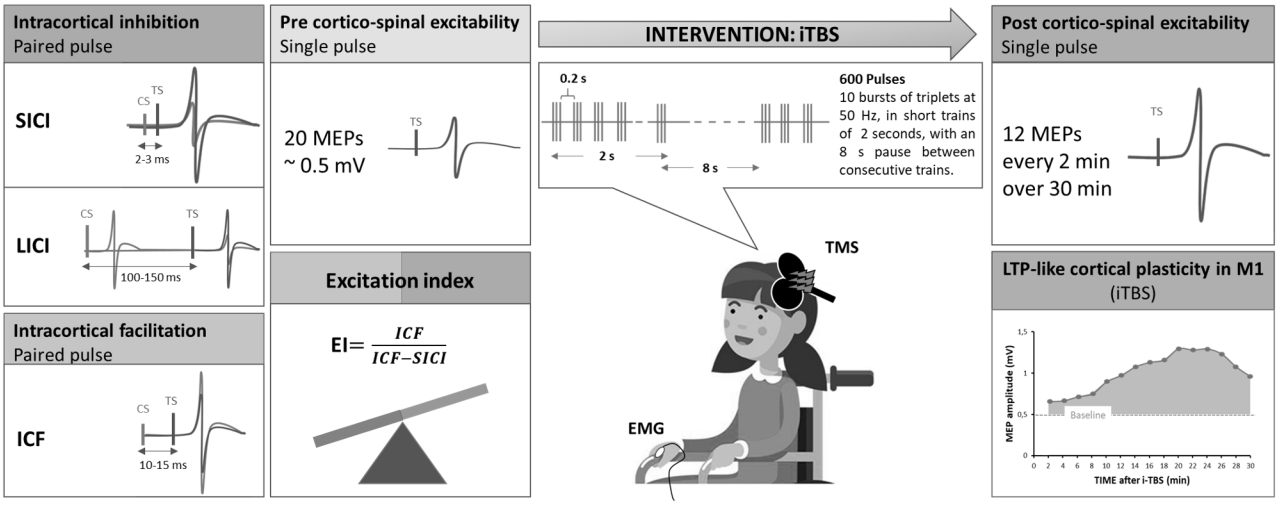

FIGURE 1: Schematic overview of experiment. Before the intervention, participants underwent motor threshold assessment, namely resting and active motor thresholds (not shown), intracortical inhibitory (short-interval intracortical inhibition [SICI], long-interval intracortical inhibition [LICI]), and facilitatory circuit (intracortical facilitation [ICF]) evaluation by means of paired-pulse transcranial magnetic stimulation (TMS) protocols. In addition, to evaluate the balance between facilitation and inhibition within the motor cortex, we computed the excitation index (EI), expressed here as the ratio between ICF and SICI. Lastly, just before the application of the intermittent theta burst (iTBS), corticospinal excitability was evaluated by recording 20 motor evoked potentials (MEPs) at around 0.5mV of amplitude. Following the intervention (horizontal *arrow*) subjects paused for 2 minutes, and post-iTBS corticospinal excitability was established by obtaining MEP responses (15 blocks consisting of 12 MEP responses each, with each followed by 1 minute of rest) up to 30 minutes after intervention. CS = conditioning stimulus; EMG = electromyography; LTP = long-term potentiation; M1 = primary motor cortex; TS = test stimulus.

Tarrytown, NY). IGF-1 concentration was expressed as standard deviation score (SDS) according to the normative data provided by the manufacturer. SDS ≤ −2.5 was considered abnormal.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics v.22.0 for Windows (IBM, Armonk, NY). Normal distribution was verified by means of Kolmogorov and Smirnov test. One-way analysis of variance (ANOVA) was applied to compare age, motor thresholds, and excitation index in the 3 groups: RTT patients, epilepsy controls, and healthy subjects. The same test was also applied to ensure that amplitude of TS for different paired-pulse paradigms (SICI–ICF and LICI) and MEP amplitudes before iTBS did not differ across groups. Then the effect of SICI–ICF and LICI (normalized values) were compared with a 2-way mixed-model ANOVA, with "ISI" as within-subjects factor and "group" as between-subjects factor. When dealing with iTBS, a 2-way mixed-model ANOVA was performed on MEP amplitude expressed as percentage of change in comparison to baseline, with "time" as within-subjects factor and "group" (RTT, epilepsy controls, and healthy controls) as the between-subjects factor. If a significant main effect was obtained, group differences were examined with post hoc tests (Bonferroni correction for multiple comparisons). The Greenhouse–Geisser method was used to correct for nonsphericity whenever necessary.

Correlation between IGF-1 (SDS), clinical scores (disease duration, CSS motor score, RSGMS), and the main neurophysiological parameters (excitation index, mean amplitude change of MEP after iTBS, and the mean inhibition at LICI) were evaluated using Pearson correlation coefficient. Alpha inflation due to multiple comparisons was controlled according to Bonferroni approach when appropriate. Effects were considered significant if $p < 0.05$. All data are presented as mean ± standard error of the mean (SEM) if not stated otherwise.

Results

Participants

Fourteen young adults with RTT were recruited (mean age = 22.64 ± 2.12 years); 11 had full TMS testing, and 3 participants were excluded because of high motor thresholds (Supplementary Tables 1 and 2). Nine people with epilepsy taking AEDs (E1–E9; mean age = 25.11 ± 2.56 years) and 11 healthy participants (mean age = 22.64 ± 1.75 years) were recruited as control groups. All healthy participants and epilepsy controls were right-handed females. Supplementary Table 1 shows demographic information, genetic diagnosis (for those with RTT), epilepsy diagnosis (for epilepsy controls), and medication at time of testing. There was no significant difference in age among the 3 groups (1-way ANOVA $F_{2, 33} = 0.386$, $p = 0.683$).

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Motor Thresholds

One-way ANOVA comparing motor thresholds in all 3 groups showed significant differences for both RMT ($F_{2,\ 30}$ = 21.734, p < 0.001) and AMT ($F_{2,\ 30}$ = 19.925, p < 0.001); post hoc analysis confirmed a significant difference only between patients (RTT and epilepsy controls) and healthy subjects (all p < 0.001), with RMT and AMT higher in patients (RTT group: RMT = 60.09 ± 3.86, AMT = 49.36 ± 3.29; epilepsy controls: RMT = 60.89 ± 1.87, AMT = 50.22 ± 1.69) with respect to healthy participants (RMT = 37 ± 2.39; AMT = 30.36 ± 2.09). Interestingly, motor thresholds of the 2 RTT patients not taking AEDs were within the normal limits (RMT = 38 and 48, upper limit <50; AMT = 33 and 38, upper limit <42; see Supplementary Table 2). Overall these results confirm the well-known effect of AEDs on increasing motor thresholds.[13,14,36] In addition, muscle contraction gave reliable AMT results in RTT patients; paired t test showed that AMT values were consistently lower than RMT in each participant (t test: p < 0.001), and AMT values normalized with respect to RMT were almost identical among the 3 groups (RTT: 0.82 ± 0.02, epilepsy controls: 0.82 ± 0.02, healthy participants: 0.83 ± 0.03), as confirmed by 1-way ANOVA ($F_{2,\ 30}$ = 0.026, p = 0.975).

Intracortical Inhibitory and Facilitatory Circuits and the Excitation Index

For SICI–ICF, mixed-model ANOVA yielded a group effect ($F_{2,\ 28}$ = 4.241, p = 0.025), and post hoc comparisons showed that RTT patients exhibited an overall altered modulation for intracortical and facilitatory circuits tested by SICI–ICF with respect to the other 2 groups (p < 0.022). As expected, we also showed a main ISI effect ($F_{2.15,\ 60.27}$ = 56.657, p < 0.001; Greenhouse–Geisser correction: ε = 0.718) because MEPs were inhibited at short ISIs (ie, 2 and 3 milliseconds), whereas for longer ISI (ie, 10 and 15 milliseconds) the inhibition was replaced by facilitation (Fig 2). Instead, the interaction ISI × group did not reach any statistical significance ($F_{4.31,\ 60.27}$ = 0.903, p = 0.474; Greenhouse-Geisser correction: ε = 0.718). To determine the balance between inhibitory and facilitatory circuits in M1, an excitation index was developed. The excitation index was higher in RTT patients (2.72 ± 0.44, ANOVA: $F_{2,\ 30}$ = 9.979, p = 0.003) compared to epilepsy controls (1.69 ± 0.08, p = 0.007) and healthy participants (1.39 ± 0.10, p = 0.002; see Fig 2). Taken together, these findings suggest a dynamic shift in the balance between facilitatory and inhibitory circuits in RTT, with a preponderance to net motor cortex hyperexcitability, likely due to reduced GABAergic activity.

Impairment of GABAergic activity was also confirmed by LICI, showing a main effect of group ($F_{2,\ 28}$ = 8.265, p = 0.002). Post hoc testing revealed that only RTT group exhibited an overall reduction of the inhibition's magnitude probed by LICI (all p < 0.002; Fig 3). Mixed-model ANOVA also showed a main effect of ISI ($F_{1,\ 28}$ = 7.851, p = 0.009) providing stronger inhibition at 100 milliseconds than at 150 milliseconds for all participants (31.49 ± 7.9 vs 43.79 ± 8.87). On the

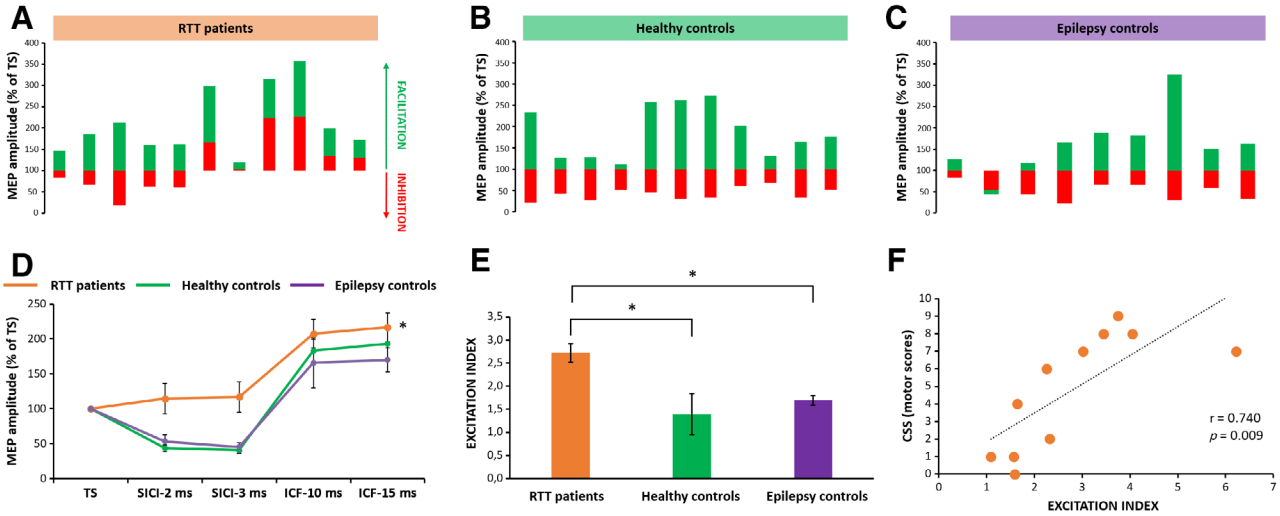

FIGURE 2: Balance between short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) circuits in patients with Rett syndrome (RTT), epilepsy, and healthy controls. Average of SICI (red bars) and ICF (green bars) expressed as a percentage of test stimulus (TS) in individual (A) RTT patients, (B) healthy controls, and (C) epilepsy controls. Group average data normalized with respect to TS for each interstimulus interval of SICI and ICF (D) showing the lack of inhibition in RTT patients (orange line). The excitation index, a biomarker reflecting the contribution of inhibitory and facilitatory circuit activity, is significantly increased in RTT patients compared to the other 2 groups, suggesting a shift toward cortical excitation (E). A nonsignificant trend was evident for the correlation between the excitation index and motor score indexed by the clinical severity score (CSS) (F). *Statistically significant. MEP = motor evoked potential; TS = test stimulus.

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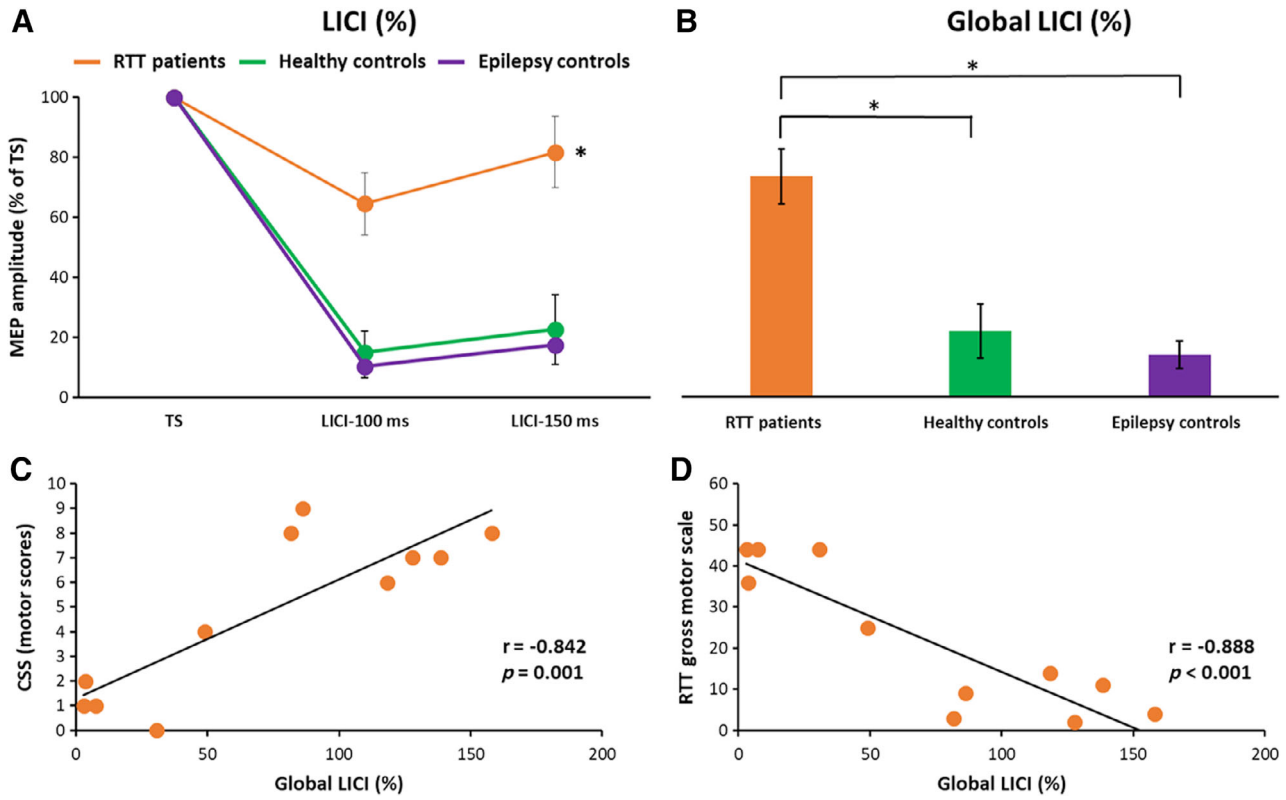

FIGURE 3: Clinical correlates of neurophysiological abnormalities in patients with Rett syndrome (RTT). Group data for long-interval intracortical inhibition (LICI) confirms the lack of inhibition in RTT patients for each interstimulus interval (ISI) (A) and for the global LICI (B), obtained by averaging ISIs at 100 and 150 milliseconds. Clinical motor scores (clinical severity score [CSS] and RTT Gross Motor Scale) correlate with the amount of inhibition in the primary motor cortex (M1), indexed by the global LICI (C, D). The higher the amount of inhibition, the better the motor phenotype was. Significant correlations are indicated by a bold continuous regression line ($p < 0.004$ after Bonferroni correction for multiple comparisons). *Statistically significant. MEP = motor evoked potential; TS = test stimulus.

contrary, the interaction ISI × group did not reach any statistical significance ($F_{2,\ 28} = 0.449$, $p = 0.643$).

Cortical Plasticity Induced by iTBS

Baseline mean MEP values (pre-iTBS) did not differ across groups (1-way ANOVA: $F_{2,\ 30} = 1.081$, $p = 0.353$). Regarding corticospinal excitability after iTBS, the mixed-model ANOVA showed a significant group effect ($F_{2,\ 28} = 5.687$, $p = 0.008$), suggesting a different modulation of excitability enhancing effect of iTBS among groups. Specifically, post hoc comparisons revealed that RTT patients did not exhibit the physiological enhancement of corticospinal excitability following iTBS (all $p < 0.014$; Fig 4 and Supplementary Table 2). ANOVA also revealed a significant effect of time ($F_{14,\ 392} = 2.765$, $p = 0.001$), indicating a different modulation of MEP amplitudes over time. Lastly, the interaction time × group did not show any statistical significance ($F_{28,\ 392} = 1.485$, $p = 0.056$).

Clinical Correlates of Neurophysiological Abnormalities in RTT

To evaluate the clinical significance of the described neurophysiological abnormalities, we performed correlation analyses between the motor scores and the main neurophysiological parameters. Significant correlations ($p < 0.004$ after Bonferroni correction for multiple comparisons) were obtained contrasting CSS motor scores with the global mean inhibition indexed by LICI ($r = 0.842$, $p = 0.001$) and with the overall gain of corticospinal excitability after iTBS ($r = -0.805$, $p = 0.003$). We observed the same significant results for the RSGMS (LICI: $r = -0.888$, $p < 0.001$; iTBS: $r = 0.800$, $p = 0.003$). On the contrary, a nonsignificant relationship was evident for the excitation index (CSS–motor: $r = 0.740$, $p = 0.009$; RSGMS: $r = -0.708$, $p = 0.015$) and for the correlation between disease duration and the mean corticospinal excitably gain after iTBS ($r = -0.677$, $p = 0.022$). The remaining correlations showed a nonsignificant trend, either contrasting neurophysiological measures with disease duration (all $p > 0.052$) or age (all $p > 0.084$). These results suggest that motor disabilities in RTT patients impact negatively on the motor cortex plasticity and the efficacy of inhibitory circuits within M1. Instead, the lack of significant results with disease duration and age might be due to the small sample size or age range.

IGF-1 Levels

IGF-1 levels, expressed as SDS, were within the age range for all RTT patients (range = −2.1 to 2.06). No significant

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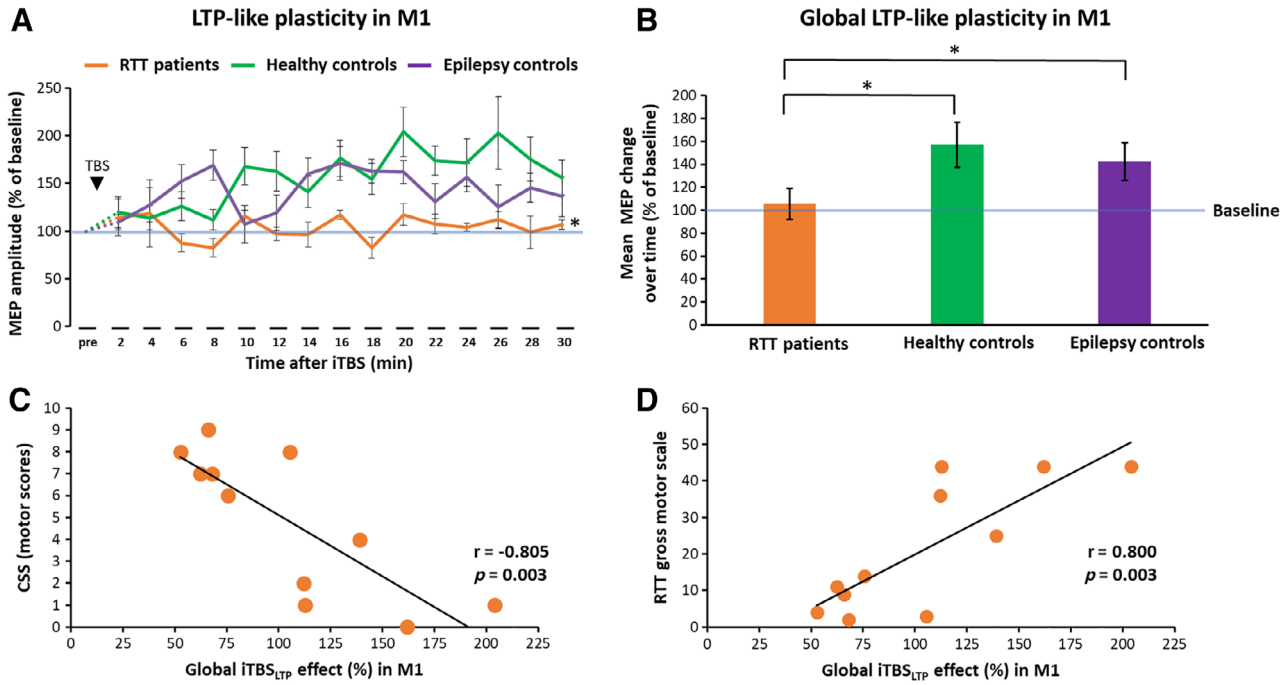

FIGURE 4: Long-term potentiation (LTP)–like plasticity in the primary motor cortex (M1) of Rett syndrome (RTT) patients. Time course of motor evoked potential (MEP) amplitude change over time. (A) Each line represents the group average in MEP responses normalized to pre–intermittent theta burst stimulation (iTBS). Note a significant loss of long-term potentiation (LTP)-like plasticity in M1 only in RTT patients. Arrowheads represent time of iTBS intervention. Small gaps in the x-axis indicate interruptions for each 1-minute break. (B) Synopsis of the overall MEP change (normalized to baseline) after iTBS in each group, confirming the lack of LTP-like plasticity in RTT group (orange bar) with respect to healthy controls (green bar) and epilepsy patients (violet bar). The gain of M1 excitability after iTBS scaled with motor performance, indexed by motor items of the clinical severity score (CSS) and the RTT gross motor scale (C, D). Significant correlations are indicated by a bold continuous regression line ($p < 0.004$ after Bonferroni correction for multiple comparisons). *Statistically significant. TS = test stimulus.

correlation was found when contrasting IGF-1 levels with neurophysiological parameters (excitation index: $r = 0.68$, $p = 0.844$; iTBS$_{LTP}$: $r = 0.77$, $p = 0.821$; mean LICI: $r = 0.085$, $p = 0.805$) and clinical scores (CSS: $r = 0.473$, $p = 0.088$; RSGMS: $r = -388$, $p = 0.170$).

Discussion

To the best of our knowledge, this is the first study showing abnormalities of the E/I balance and LTP-like plasticity in M1 of humans with RTT. These alterations were associated with a greater degree of functional motor disabilities, suggesting a pathophysiologic role of these functional changes.

E/I Balance Shifts toward Excitation in M1 of RTT

The dysfunction of excitatory and inhibitory motor circuits contributes to the development of cortical hyperexcitability in RTT. Specifically, there was a reduction of SICI along with an increase in ICF, suggesting a disinhibition of intracortical circuits in RTT group. The excitation index, which captures the balance between short-latency interneuronal inhibition and long-latency facilitation, was significantly shifted toward an excitatory drive in patients with RTT. The precise mechanisms underlying the development of hyperexcitability in M1 remain unresolved. Interestingly, although different synapses in distinct parts of the brain are differentially modulated upon loss of MECP2, recent preclinical evidence has suggested a common direction of change in E/I balance in favor of excitation.[5,6] Intracellular recordings in cortical neuron reveal that inhibition and excitation are both reduced in Mecp2 knockout mice, but inhibition is reduced to a greater degree, thus enhancing the E/I ratio.[5] In addition, in vivo functional measurements of inhibitory conductance in adult Mecp2 knockout mice, along with reduced responses of parvalbumin-expressing (PV+) interneurons, consistently revealed reduced inhibition in cortical circuits.[5] PV interneurons are powerful regulators of pyramidal neuron activity and appear to be critical regulators of the E/I balance in human neocortex.[37]

Importantly, our neurophysiological results seem to confirm that the increase of E/I ratio might be due to the reduced efficiency of inhibitory circuits within M1. ICF has been demonstrated to be decreased by GABAergic agonists that would conversely increase SICI.[13] Consequently, the decrease of SICI together with the increase in ICF could be partly consistent with the disinhibition of layer V pyramidal neurons, resulting in an enhanced corticospinal output.

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Deficits of intracortical inhibitory circuits also have been confirmed by the reduction of LICI, which, according to pharmaco-TMS studies, is supposed to be mediated by $GABA_B$ network.[13] We found that the amount of LICI scaled with clinical motor scores; namely, the worse the motor performance, indexed by the CSS motor scale and RSGMS, the lower the magnitude of inhibition. Dysregulation of PV inhibitory interneuron expression, observed in M1 of Mecp2 knockout mice, also correlated with the severity of motor behavioral impairments.[8]

Reductions of central motor conduction time and of cortical silent period assessed by TMS have been previously reported in patients with RTT and mainly explained by degeneration of inhibitory circuits.[38–40] Specifically, the authors suggested a possible "upstream" disorder, involving cortical inhibitory interneurons and consequently influencing the outflow of the pyramidal cells in M1.[38] Therefore, we reason that the shortening of the central motor conduction time could be in line with our findings of altered intracortical inhibitory circuits, as suggested by the reduced magnitude of LICI and the increased excitation index.

Lastly, we also observed higher motor thresholds in our patient groups, that is, RTT and epilepsy controls. It is well known that anticonvulsant medication might elevate motor thresholds.[13,14,36] This may account for the higher thresholds in patients relative to healthy subjects, but it seems unlikely that it explains the difference in E/I balance and motor cortex plasticity seen between the 2 similarly treated patient groups. This observation also should be considered when assessing previous results of motor threshold level in RTT patients.[38,40] Specifically, the study by Krajnc and Zidar[40] showed elevated motor thresholds even in those RTT patients not taking AEDs, whereas in our study motor thresholds were normal. Differences in the results of their study versus ours might be due to methodological dissimilarities, such as the use of a different target muscle (abductor digiti minimi vs FDI), TMS pulse waveform (monophasic vs biphasic stimulation), and RMT assessment method (100μV vs 50μV). In addition, the study of Eyre and colleagues[38] showed opposite findings—lower motor thresholds in RTT patients compared to healthy controls–suggesting a possible impairment of inhibitory controls on pyramidal neurons in M1. In this multicenter study, which includes a larger sample of RTT patients not taking AED, we could reach a definite conclusion.

Loss of Motor Cortex Plasticity Is Associated with Motor Deficits in RTT

The current study demonstrates robust evidence for deficit of LTP-like cortical plasticity in the M1 of RTT patients. An important observation is that motor cortex plasticity impairment parallels motor deficit being more seriously affected in patients with severe motor symptoms. Interestingly, our results are consistent with those of previous studies conducted in mice, where the deficit in cortical synaptic plasticity appeared with the onset of overt RTT-like symptoms. In these studies, the investigation of LTP alterations has been consistently described in the hippocampus[41–44] and less frequently in M1.[7] Synaptic plasticity deficit in the hippocampus can be observed in very mildly symptomatic male mice, and with symptom progression these subtle abnormalities in synaptic plasticity become more evident.[43] These results, together with our findings, strongly suggest that the loss of cortical plasticity is strictly associated with the progression of neurological dysfunction in humans as well. They also add new evidence supporting the idea that the deficit of MeCP2 impairs functional synaptic plasticity in the maturing nervous system and not during brain development.

Importantly, growing consensus suggests the role of inhibitory circuits in regulating human motor cortical plasticity.[45–47] Therefore, in RTT, defects in cortical inhibitory connectivity might also explain alteration in motor plasticity. Investigators recently demonstrated that altered activity and connectivity of GABAergic PV interneurons impaired structural and functional plasticity in M1.[8] Specifically, Mecp2 knockout mice displayed an atypical upregulation of PV interneurons in M1 that was associated with the severity of motor behavioral impairments.[8] In addition, consistent with a reduction in inhibition received by pyramidal neurons, monocular deprivation induced an abnormally prolonged plasticity in visual cortex of $Mecp2^{+/-}$ female mice.[17,18] Similarly, parvalbumin-specific deletion in mice led to immature adult visual cortical plasticity,[5] which was restored by enhancing inhibition via intracerebral infusion of diazepam, a $GABA_A$ receptor agonist.[48] Importantly, IGF-1, which is considered to play a role in modulating neural plasticity and cortical excitatory transmission in mice,[5] was within the normal values in RTT patients and did not correlate with our neurophysiological metrics. These findings are in line with clinical trials on the therapeutic use of IGF-1 in RTT patients, showing normal serum and cerebrospinal fluid levels of IGF-1 before treatment.[19,21]

Conclusions and Outlook

Abnormal cortical synaptic plasticity and E/I balance seem to be a prominent feature of RTT and a range of related neurodevelopmental disorders. Dysfunction of GABAergic signaling can be considered as the common thread underlying cortical abnormalities and associated symptoms.[49,50] Here we have shown the relationship between motor symptom severity and alteration of neurophysiological

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metrics of M1. This association raises the possibility of using some neurophysiological parameters as a biomarker of disease progression or to monitor the efficacy of new therapeutic interventions. For instance, LICI, which is a short paradigm (ie, around 5 minutes to accomplish), was a very sensitive metric, being highly associated with motor deficit, and was easy to perform.

In addition, because severity of symptoms, including motor dysfunction, is particularly high in late childhood and adolescence,[51] the concomitant use of drugs and non-pharmacological therapies such as noninvasive brain stimulation protocols (ie, rTMS or transcranial direct current stimulation) for overcoming decreased plasticity or altered E/I balance in M1 seems to be compelling. Important seminal work in RTT animal models showed the possibility of achieving prolonged survival and reversibility of disease phenotypes with gene reinstatement, even into adulthood. These results seemingly make RTT one of the more tractable neurodevelopmental disorders as far as potential for disease modification and improvement.[42,43,52]

Acknowledgment

We thank all study participants, their families, and the association NOI Insieme Rett. We thank V. Baiano, F. Rusciano, and A. Gallotta for technical assistance.

Author Contributions

R.D. and P.B. contributed to the conception and design of the study; R.D, C.B., F.M., and V.D.L. contributed to the acquisition and analysis of data; and R.D., P.B., A.C., L.T., and S.C. contributed to drafting the manuscript and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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