B D I	12382	WILEY	Dispatch: 8.3.16	CE: Saravanan
Journal Code	Manuscript No.		No. of pages: 11	PE: Thangaraj

Bipolar Disorders 2016

Original Article

Trait- and state-dependent cortical inhibitory deficits in bipolar disorder

Ruiz-Veguilla M, Martín-Rodríguez JF, Palomar FJ, Porcacchia P, Álvarez de Toledo P, Perona-Garcelán S, Rodríguez-Testal JF, Huertas-Fernández I, Mir P. Trait- and state-dependent cortical inhibitory deficits in bipolar disorder.
Bipolar Disord 2016: 00: 000–000. © 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.

Objectives: Euthymic patients with bipolar disorder (BD) have deficits in cortical inhibition. However, whether cortical inhibitory deficits are trait- or state-dependent impairments is not yet known and their relationship with psychiatric symptoms is not yet understood. In the present study, we examined trait- and state-dependent cortical inhibitory deficits and evaluated the potential clinical significance of these deficits.

Methods: Nineteen patients with bipolar I disorder were evaluated using the paired-pulse transcranial stimulation protocol, which assessed cortical inhibition during an acute manic episode. Cortical inhibition measures were compared with those obtained in 28 demographically matched healthy controls. A follow-up assessment was performed in 15 of these patients three months later, when there was remission from their mood and psychotic symptoms. The association between cortical inhibitory measures and severity of psychiatric symptoms was also studied.

Results: During mania, patients showed decreased short-interval intracortical and transcallosal inhibition, as well as a normal cortical silent period and long-interval cortical inhibition. These findings were the same during euthymia. Symptoms associated with motor hyperactivity were correlated negatively with the degree of cortical inhibition. These correlations were not significant when a Bonferroni correction was applied.

Conclusions: The present longitudinal study showed cortical inhibitory deficits in patients with BD, and supports the hypothesis that cortical inhibitory deficits in BD are trait dependent. Further research is necessary to confirm the clinical significance of these deficits.

Miguel Ruiz-Veguilla[a,†], Juan Francisco Martín-Rodríguez[b,†], Francisco J Palomar[b], Paolo Porcacchia[b], Paloma Álvarez de Toledo[b], Salvador Perona-Garcelán[a], Juan Francisco Rodríguez-Testal[a,c], Ismael Huertas-Fernández[b] and Pablo Mir[b,d]

[a]Grupo Neurodesarrollo y Psicosis, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio/CSIC/Universidad de Sevilla/UGC Salud Mental HVR, [b]Unidad de Trastornos del Movimiento, Servicio de Neurología y Neurofisiología Clínica, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, [c]Departamento de Personalidad, Evaluación y Tratamientos Psicológicos, Facultad de Psicología, Universidad de Sevilla, Seville, [d]entro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativ (CIBERNED), Spain

doi: 10.1111/bdi.12382

Key words: cortical inhibition – GABA receptors – longitudinal study – motor cortex – short-interval intracortical inhibition – transcallosal inhibition

Received 30 June 2015, revised and accepted for publication 8 February 2016

Corresponding author:
Pablo Mir, M.D., Ph.D.
Unidad de Trastornos del Movimiento
Servicio de Neurología y Neurofisiología Clínica
Hospital Universitario Virgen del Rocío
Avda. Manuel Siurot s/n
Sevilla 41013
Spain
Fax: +34-955923101
E-mail: pmir@us.es

[†]These authors contributed equally to this work.

Bipolar disorder (BD) is a prevalent neuropsychiatric disorder characterized by periods of elevated mood (mania or hypomania) and depression. BD is associated with significant impairment in both the physical and mental quality of life, even during euthymic periods (1). One of the main symptoms of this disorder is an impairment in the inhibition of inappropriate actions and thoughts, resulting in

overfamiliarity, impulsivity, disinhibition, and deficits in overbearing response suppression (2). These behaviors are particularly dramatic during mania. However, individuals with BD also display inhibitory deficits in depression and euthymia (3–5), suggesting that inhibitory deficits could be part of the core symptoms of BD. It has been suggested that inhibitory deficits in BD are related to impairment in cortical inhibition in which cortical γ-aminobutyric acid (GABA) inhibitory interneurons play a crucial role in inhibiting the activity of other cortical neurons (6). In this regard, experimental evidence supports an involvement of the GABA system in the pathophysiology of BD. Moreover, genetic association studies have found distinct polymorphisms of GABA receptor genes associated with BD (7, 8), and abnormalities in the expression of multiple GABA-related proteins have been reported in postmortem studies in patients with BD (9, 10).

Information about the functionality of the cortical GABAergic system can be indirectly obtained using transcranial magnetic stimulation (TMS). By using single- and paired-pulse TMS, it is possible to assess the activity of the GABAA and GABAB receptors in the motor cortex, as well as the physiological interactions between excitatory and inhibitory circuits in human subjects (11, 12). The short-interval cortical inhibition (SICI) paradigm assesses the inhibition of the motor activity induced by a conditioned magnetic stimulus that is presented shortly (2–5 msec) before another stimulus that is able to provoke a measurable motor evoked potential (MEP). This type of inhibition has been related to the activity of GABAA receptors (13). Additional measures of cortical inhibition include the cortical silent period (CSP) and long-interval intracortical inhibition (LICI), which are both measures of long-lasting intracortical inhibition and thought to be dependent on GABAB receptor activity (14, 15). Another cortical inhibitory phenomenon, transcallosal inhibition (TCI), can be assessed with TMS using either paired-pulse paradigms or via the ipsilateral silent period. Although interhemispheric corticocortical inhibitory mechanisms are not fully understood, they require the intact integrity of callosal fiber bundles, and presumably also require a preserved intracortical GABAergic inhibitory interneuron system (16, 17).

Few TMS papers have studied cortical inhibitory mechanisms in BD. Levinson et al. (18) demonstrated several cortical inhibitory deficits consisting of decreased SICI, TCI, and CSP, which suggests impaired cortical GABAergic receptor activity in patients with BD. By contrast, LICI seems to be maintained in patients with BD, as observed in a combined TMS–electroencephalography study (19). These previous studies have investigated the trait and state markers of BD by comparing cortical inhibition in euthymic patients with BD with control subjects in a cross-sectional case-control design. There is therefore the possibility of intersubject differences affecting the variability in cortical excitability, and it is difficult to investigate the underlying cortical inhibitory deficits for trait and state abnormalities and mood switching using such a design. Substance abuse is a major comorbidity in patients with BD, which may have profound clinical implications (20, 21). Substances such as cannabis, cocaine, or nicotine have been shown to alter cortical inhibition both in the non-psychiatric (22) and psychiatric population (23). Likewise, cortical excitability and inhibition is also modulated by commonly used drugs to treat BD, including mood stabilizers, benzodiazepines, and antipsychotic agents. Therefore, comorbid substance abuse or current pharmacological treatment should be taken into account when assessing cortical excitability and inhibition in BD. To address these issues, we conducted a longitudinal study with a TMS design to assess patients during the acute manic phase (BD manic) and remission from this acute phase. We also assessed the association between cortical inhibitory measures, severity of psychiatric symptoms, and lifetime and current drug use.

Methods

Participants

A total of 19 right-handed patients with BD participated in the study (ten patients had first-episode BD; 12 males; mean age: 35.5 years; mean age at onset: 30.4 years). Patients were recruited over a two-year a period from the Inpatient Psychiatric Unit of the Hospital Universitario Virgen del Rocío Mental Health Department. Eligible patients met the diagnostic criteria for bipolar I disorder, according to DSM-IV classification standards. The participants were required to have a Young Mania Rating Scale (YMRS) (24) score ≥18. We excluded patients: (i) <18 or >65 years of age; (ii) with a history of brain trauma or neurological disease; and (iii) if they had undergone electroconvulsive therapy in the previous 12 months. All patients displayed acute mania with a YMRS >21 and none displayed major depressive symptoms. In first-episode BD, diagnosis was confirmed after one year of follow-up.

A healthy control group consisting of 28 healthy subjects was recruited by advertisement, in addition to word-of-mouth requests from staff in the research unit. Patient and control groups were matched via recruitment by age, gender, and social/occupational class. The healthy controls exhibited no past or present psychiatric or neurological disorders and had no positive family history of psychiatric disorders. Other exclusion criteria for this group were lifetime substance dependence, substance abuse during the previous month, cannabis abuse during the previous month, intellectual disability, dementia, and neurological illnesses.

Sixteen patients agreed to participate in the follow-up assessment and three withdrew during the course of the study (two of them moved to other cities after discharge and one no longer wished to participate further in the study). All patients were euthymic at the follow-up assessment, as defined by a YMRS score <7 and a Hamilton Depression Rating Scale (HDRS) (25) score <10. During the one-year follow-up, one patient displayed symptoms consistent with schizoaffective disorder, so was excluded from the analysis.

Study design

This was a longitudinal and naturalistic study that consisted of comprehensive psychopathological and TMS assessments at two points: baseline and at three-month follow-up. Baseline assessments were completed in the second week of hospital admission. Three months after hospital discharge, patients were invited to participate in a second assessment. Healthy subjects underwent a unique TMS assessment. The study protocol was approved by the local ethics committee, and all procedures followed were in accordance with institutional guidelines. Written informed consent was obtained from all subjects.

Following screening for exclusion criteria, all participants underwent a detailed examination by experienced psychiatrists to assess current mood state. BD was diagnosed using the Structured Clinical Interview for DSM-IV Disorders, researcher version with psychotic screen (26). Mood was also rated using the YMRS and the HDRS. Psychotic symptoms were assessed using the Positive and Negative Symptoms Scale (PANSS) (27). Trained interviewers administered the PANSS as part of a structured clinical interview and scored items on a scale from 1 (asymptomatic) to 7 (extremely symptomatic). Items of the PANSS were grouped using Wallwork et al.'s five-factor (positive, negative, disorganized/concrete, excited, and depressed) model (28). Higher scores in these factors imply a higher severity of psychotic symptoms.

TMS assessments were performed between 3:00 p.m. and 5:00 p.m. Doses of the atypical antipsychotic agents were transformed to chlorpromazine equivalents (29). During both assessments, benzodiazepines were withdrawn at least 15 hours prior to the TMS study. Blood samples were drawn for determination of serum lithium and valproic acid levels during clinical examinations. Effective serum lithium levels were defined as concentrations between 0.6 mEq/L and 1.0 mEq/L, while effective valproic acid levels were defined as between 50 mg/L and 100 mg/L. Patterns of cannabis and cocaine abuse were assessed using the L-section of the Composite International Diagnostic Interview (CIDI) (30). We interviewed subjects on lifetime drug use history and patterns of drug use during the previous 12 months. Urine toxicology screening (TOX/See; Bio-Rad, USA) was performed in all patients to confirm or rule out current exposure to harmful drugs. Nicotine dependence was assessed in both groups using the Fagerström test for nicotine dependence (31). A score ≥4 by combining items 1 and 4 of this test was used to identify subjects with high nicotine dependence (32).

Electromyography (EMG) recordings

Subjects were seated in a comfortable chair and surface EMG recordings were taken at the first dorsal interosseous (FDI) muscles on the side contralateral to the stimulated cortex with Ag–AgCl surface electrodes using a belly–tendon montage. EMG signals were amplified (1,000×) and band-pass filtered (bandwidth 20 Hz to 2 kHz) using a Digitimer D360 amplifier (Digitimer, UK), acquired at a sampling rate of 5 kHz through a CED 1401 laboratory interface (Cambridge Electronic Design, Cambridge, UK) and stored on a computer. The EMG traces were analyzed using customized SIGNAL software version 4.

Stimulation protocols

Single- and paired-pulse TMS of the primary motor cortex were applied using Magstim 200 magnetic stimulators (Magstim Company Limited, Whitland, UK). The stimulators were triggered through the SIGNAL software and CED 1401 board. Patients and healthy subjects were tested on the left hemisphere in a TMS session that included SICI, intracortical facilitation (ICF), LICI, and CSP. For these studies, the magnetic stimulators were connected to a standard figure-of-eight coil with an external diameter of 70 mm (peak mag-

netic field 2.2T). The coil was held tangentially to the skull with the handle pointing backwards and laterally at an angle of ~45 degrees to the sagittal plane in order to generate a posterior–anterior current in the brain. A BiStim module (Magstim Company Limited) was used to interconnect stimulators when paired-pulse TMS protocols were performed. SICI and ICF were assessed over the contralateral FDI muscle in a similar way to a previously described paired-pulse paradigm (33), whereby a subthreshold stimulus is used to condition the motor output of a suprathreshold stimulus, depending on the time interval between them. The intensity of the conditioning stimulus (CS) was 80% of the active motor threshold (AMT), defined as the minimum intensity that elicited a reproducible MEP of at least 200 μV in the tonically contracting FDI muscle in at least five out of ten consecutive trials, while subjects were contracting at approximately 20% of their maximum voluntary contraction. A constant level of muscle contraction was achieved by the use of visual feedback. The intensity of the test stimulus (TEST) was adjusted to elicit an MEP of approximately 1 mV. SICI and ICF were assessed in the same experimental block. SICI was assessed at rest at interstimulus intervals (ISIs) between the CS and a TEST of 2 msec and 3 msec, and ICF at ISIs of 10 msec and 12 msec. The presentation of TEST and CS were randomly intermingled within the SICI–ICF experimental block. For assessment of LICI, the intensity of the CS was 120% of the resting motor threshold (RMT) of the contralateral FDI muscle. RMT was defined as the minimum intensity that evoked a peak-to-peak MEP of 50 μV in at least five out of ten consecutive trials in the relaxed recorded muscle (34). LICI was assessed at rest at ISIs of 100 msec, 150 msec, and 250 msec. Ten MEPs were collected for each ISI and for the TEST. The presentation of TEST and CS stimuli were randomly intermingled within the LICI experimental blocks. For assessment of CSP, 15 single TMS pulses were applied at an intensity of 120% RMT, while patients provided a constant contraction of the FDI muscle at 20% of their maximum voluntary contraction, assisted by visual feedback. TCI was demonstrated using the dual-pulse paradigm described by Ferbert et al. (17). Briefly, two figure-of-eight coils (with an outer diameter of each half-wing of 40 mm) were used with a TEST applied to the left motor cortex and a CS applied to the right motor cortex. With these coils, RMT was again obtained at both hemispheres. The TEST stimulus was set at an intensity that, when given alone, would evoke an EMG response of 1 mV peak-to-peak amplitude. The CS was applied at 120% of the RMT. TCI was tested at four conditioning test intervals (7, 10, 45, and 75 msec), given in a random order, with a total of ten sweeps for each condition.

Statistical analysis

Student's *t*-tests for independent measures and Fisher's exact test were used to investigate differences between patients and healthy controls on demographic variables (i.e., age, gender, smoking variables, and drug abuse history variables). Fisher's exact test and paired-sample *t*-tests were used to assess changes in clinical variables at the follow-up assessment. The independent and repeated-measures *t*-tests were used to determine differences in motor thresholds and CSP. Two separate two-way fixed-effects analyses of variance (ANOVAs) were conducted for the evaluation of paired-pulse TMS protocols. To detect cortical inhibitory deficits during mania, TMS data collected from patients in this episode were compared with those obtained from healthy subjects. This analysis was carried out with *ISI* as within-subject factor and *Group* (healthy versus manic groups) as between-subject factor. Potential confounders (use of cannabis, cocaine, tobacco, and benzodiazepines) were included in a secondary model analysis if a significant group difference was seen in the primary analysis. To assess the longitudinal effects of mood episodes on cortical inhibition, a repeated-measure ANOVA with two within-subject factors, *episode* (manic and euthymia) and *ISI*, was used. Mauchly's test assessed sphericity and the Greenhouse–Geisser correction was used for non-spherical data. Significant main effects and interactions in ANOVA were followed by post-hoc independent or paired *t*-tests with Bonferroni correction. The relationship between clinical symptom severity and TMS measures in the BD group was examined using the Pearson product-moment correlation coefficient. A Bonferroni correction was applied to the correlation analysis, setting the significance cut-off at $p < 0.001$. A power analysis using the Gpower computer program (35) indicated that, given our sample size, we would expect to detect moderate effect sizes ($f = 0.25$), powered, 86%, at a significant level of $p < 0.05$.

Results

Demographic data and a history of substance use both in patients with BD and healthy subjects are displayed in Table 1. Most patients with BD were current cigarette smokers, although nicotine depen-

Table 1. Participant demographics and substance use data

	Bipolar disorder (n = 19)	Healthy controls (n = 28)	p-value
Gender, male/female	12/7	18/10	0.937
Age, years, mean ± SD	35.5 ± 11.4	33.1 ± 7.0	0.434
Current cigarette smoker, n	13	5	<0.001
FTND score, mean ± SD	3.19 ± 0.9	2.92 ± 0.4	0.168
High nicotine dependence, n	6	2	0.065
Cannabis use history, n	10	0	<0.001
Positive urine test, n	9	0	N/A
Daily smoker, n	6	–	N/A
Weekly smoker, n	4	–	
Time since last joint, days, mean ± SD	17.3 ± 27.6	–	N/A
Age at onset, years, mean ± SD	19.4 ± 4.1	–	N/A
Duration of use, years, mean ± SD	9.5 ± 5.1	–	N/A
Cocaine use history, n	4	0	0.022
Positive urine test, n	1	0	N/A
Daily cocaine user, n	2	–	N/A
3–4 days a week, n	1	–	N/A
1–2 days a week, n	1	–	N/A
Age at onset, years, mean ± SD	24.2 ± 6.7	–	N/A
Duration of use, years, mean ± SD	7.4 ± 5.5	–	N/A
Other drugs, n	0	0	N/A

FTND = Fagerström test for nicotine dependence; N/A = not applicable; SD = standard deviation.

dence measures did not differ statistically between patients and controls. Ten patients reported a history of cannabis use, while daily cannabis use was ascertained in six of them. A positive urine test for cannabis was found in nine of them. In all of these cases, time since the last cannabis exposure exceeded one week. Four patients reported a history of cocaine use. All of them, except one, reported no cocaine use in the previous year. A positive urine test for cocaine was present in one patient, who reported their last cocaine consumption to have taken place in the previous month.

Table 2 shows changes in clinical and pharmacological variables in patients during mania and euthymia. Overall, a significant improvement in manic, positive, and general psychotic symptoms was observed (all $p < 0.001$) in the follow-up assessment. Ten out of the 15 patients scored zero in the YMRS. We also detected a significant increase in the severity of depressive symptoms in the follow-up assessment. Furthermore, a significant decrease in cannabis consumption (i.e., tests positive for cannabis) was observed during follow-up.

Table 2. Clinical characteristics of patients who completed the follow-up study

	Manic episode (n = 15)	Euthymic episode (n = 15)	p-value[a]
Mood stabilizer, n			
Lithium	7	3	0.428
Sodium valproate	8	8	
Serum lithium levels, mmol/L, mean (SD)	0.61 (0.08)	0.72 (0.21)	0.336
Serum valproate levels, mg/L, mean (SD)	59.6 (23.4)	49.2 (11.2)	0.346
Antipsychotic agents, n	15	13	0.483
Antipsychotic agent doses, chlorpromazine equivalents, mean (SD)	649.8 (339.3)	595.8 (380.1)	0.392
Benzodiazepines, n	6	3	0.427
Current cigarette smoker, n	9	9	1.000
Cannabis use, n	8	2	0.020
YMRS score, mean (SD)	35.1 (7.1)	1.5 (2.8)	<0.001
HDRS score, mean (SD)	1.2 (1.6)	2.9 (2.5)	0.039
PANSS score, mean (SD)			
Positive factor	3 (1.1)	1.1 (0.3)	<0.001
Negative factor	1.3 (0.9)	1.4 (0.7)	0.309
Disorganized/ Concrete factor	3.4 (0.8)	1.3 (0.5)	<0.001
Excited factor	4.1 (0.9)	1.2 (0.3)	<0.001
Depressed factor	1.6 (0.1)	1.6 (0.7)	0.770

HDRS = Hamilton Depression Rating Scale; PANSS = Positive and Negative Syndrome Scale; SD = standard deviation; YMRS = Young Mania Rating Scale.
[a]Fisher's exact test or paired-samples *t*-tests.

Motor thresholds

The data on motor thresholds are shown in Table 3. No significant differences could be demonstrated in RMT in patients with BD ($p = 0.75$ for patients in the manic phase and $p = 0.12$ for euthymic patients). There were no statistically significant differences between healthy controls and patients with BD in the other motor thresholds obtained for right FDI muscle (all $p > 0.37$). Likewise, no significant differences in the motor thresholds were found between patients during the manic episode and during euthymia (all $p > 0.86$).

Cortical inhibition during the manic episode

SICI and ICF. A significant main effect of ISI ($F_{3,132} = 49.6$, $p < 0.001$) and ISI × group interaction ($F_{3,44} = 6.6$, $p < 0.001$) were obtained.

Table 3. TMS intensity (% of maximum stimulator output) used in the TMS assessments of patients with bipolar disorder and healthy subjects

	Healthy controls	Manic episode	Euthymic episode
Active motor threshold (right FDI muscle)	31.8 ± 1.3	32.1 ± 1.7	32.2 ± 1.8
Resting motor threshold (right FDI muscle)	40.8 ± 1.3	42.5 ± 2.1	41.2 ± 2.5
Intensity MEP 1 mV (right FDI muscle)	49.4 ± 1.9	51.4 ± 2.5	51.2 ± 2.6
Resting motor threshold (right FDI muscle)[a]	43.0 ± 1.8	42.7 ± 2.2	44.9 ± 2.3
Resting motor threshold (left FDI muscle)[a]	41.7 ± 1.4	43.9 ± 3.2	43.3 ± 2.6

Values reported as mean ± standard deviation. FDI = first dorsal interosseous; MEP = motor evoked potential; TMS = transcranial magnetic stimulation.
[a]Thresholds obtained using 40-mm coils directly connected to the stimulators.

Post-hoc analysis revealed that patients with BD during mania showed less SICI at ISIs 2 msec ($p < 0.001$) and 3 msec ($p < 0.05$), while no differences were found in ICF ($p > 0.2$) (Fig. 1A). Averaged across both inhibitory ISIs (2 msec and 3 msec), patients during mania demonstrated 33.2% less inhibition compared with healthy subjects ($t_{44} = -3.3$, $p = 0.002$). There were no differences between groups when ICF measures were pooled ($p = 0.203$). Differences in SICI measures remained statistically significant after controlling for current tobacco smoking (estimated marginal means of SICI in the BD group = 0.78, healthy group = 0.36, $p = 0.01$). We also assessed the effect of current or past cannabis use on SICI measures in patients with BD. In the multivariate model, there was a significant effect of ISI ($p < 0.001$), while neither the ISI × *current cannabis use* ($p = 0.571$) nor *current cannabis use* main factor ($p = 0.947$) reached significant levels. Similar results were obtained after including the variables *frequency of current cannabis smoking* (interaction with ISI, $p = 0.287$; main effect, $p = 0.655$), *time since last joint* ($p = 0.166$), *age at cannabis use onset* ($p = 0.639$), or *duration of cannabis use* ($p = 0.734$) on SICI. History of cocaine use did not show a significant main effect ($p = 0.482$) or interaction with ISI ($p = 0.117$). Patients on benzodiazepine treatment showed enhanced SICI at both 2 msec and 3 msec (both $p < 0.05$, as compared with patients without benzodiazepines).

Long-interval cortical inhibition. A main effect of ISI ($F_{3,135} = 17.28$, $p < 0.001$) was found, indicating a significant MEP reduction when a conditioned stimulus was applied both to controls and patients ($p < 0.001$). However, no significant main effect of group ($F_{1,47} = 0.022$, $p = 0.883$) or the interaction ISI × group ($F_{3,141} = 0.449$, $p = 0.718$) was found for this protocol.

CSP. The duration of CSP did not differ between patients with mania and healthy controls (mean CSP duration manic patients = 110.3 msec; controls = 116.09 msec; $t_{45} = 0.708$, $p = 0.483$).

TCI. One patient could not complete this protocol. Significant main effects of ISI ($F_{3,129} = 22.1$, $p < 0.001$) and group ($F_{1,43} = 7.8$, $p = 0.008$) were obtained, while the group × ISI interaction was not significant ($F_{4,43} = 1.05$, $p = 0.359$) (Fig. 1B). Planned *t*-tests showed decreased TCI in manic patients at ISIs 7 msec ($p = 0.021$) and 10 msec ($p = 0.018$). After averaging across all short-interval interhemispheric inhibitory ISIs (i.e., 7 msec and 10 msec), patients with BD displayed less inhibition than healthy controls (17.5%, $p = 0.033$). An interaction between *current tobacco smoking* and group was also detected ($F_{1,44} = 4.33$, $p = 0.044$). Post-hoc analysis revealed that smoker

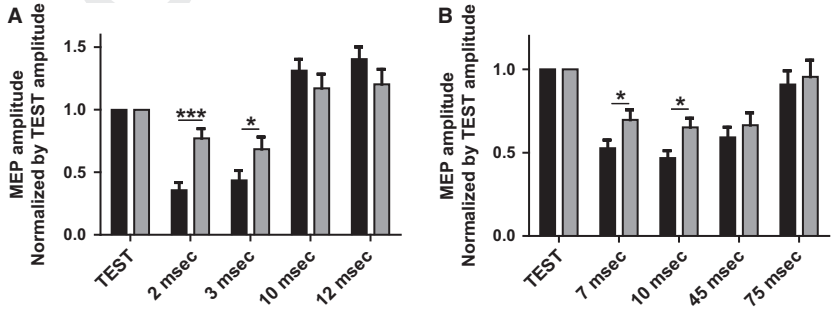

Fig. 1. (A) Short-interval intracortical inhibition and intracortical facilitation in patients with bipolar disorder and healthy subjects. (B) Transcallosal inhibition in patients with bipolar disorder and healthy subjects as a function of the interstimulus interval between the conditioning and the test stimuli. Mean ± standard error from patients during the manic episode (black bars, n = 19) and healthy controls (gray bars, n = 28). Values <1 indicate inhibition. *$p < 0.05$, ***$p < 0.001$: independent *t*-tests with Bonferroni correction for multiple comparisons. MEP = motor evoked potential.

patients with BD displayed TCI levels that were comparable with those obtained in the healthy group (p = 0.441), while TCI levels in non-smoker patients with BD were significantly reduced (p = 0.018, as compared with healthy subjects). Current tobacco smoking did not affect TCI levels in healthy subjects (p = 0.690). A current or past history of cannabis use did not show the main effect (p = 0.221) or interaction with ISI (p = 0.368). Likewise, *frequency of current cannabis smoking* did not show an effect on TCI results (interaction with ISI, p = 0.637; main effect, p = 0.257). Other variables that did not influence TCI levels in patients with BD included *time since last joint* (p = 0.993), *age at cannabis use onset* (p = 0.999), and *duration of cannabis use* (p = 0.837). A history of cocaine use did not show a significant main effect (p = 0.419) or interaction with ISI (p = 0.611). Neither the main effect of benzodiazepine (p = 0.287) nor interaction of benzodiazepine with TCI ISIs were significant (p = 0.596).

Cortical inhibition during euthymia

SICI and ICF. A significant main effect of ISI ($F_{3,36}$ = 10.06, p < 0.001), but not episode ($F_{1,12}$ = 0.007, p = 0.935) or interaction episode × ISI ($F_{3,36}$ = 0.056, p = 0.784), was found in the follow-up study, indicating no differences between patients during manic and euthymic phases at any evaluated condition (Fig. 2A). Current tobacco smoking in the model did not statistically alter these results (p = 0.575). Cannabis, cocaine, and benzodiazepine effects were not analyzed at the follow-up assessment owing to the small number of patients under these conditions.

Long-interval cortical inhibition. No significant main effect of episode ($F_{1,13}$ = 0.196, p = 0.665) or interaction episode × ISI ($F_{2,26}$ = 1.764, p = 0.191) was found in the follow-up study.

~~*Silent period.*~~ No significant differences were found between patients in manic and euthymic phases (t_{14} = 1.009, p = 0.331).

TCI. Analysis of differences between the manic episode and the follow-up assessment revealed a significant main factor ISI ($F_{3,39}$ = 7.94, p < 0.001), whereas neither main factor episode ($F_{1,13}$ = 1.132, p = 0.307) nor the interaction between episode and ISI ($F_{3,36}$ = 0.623, p = 0.604) were significant (Fig. 2B). A significant main effect of *tobacco* was also found in the multivariate analysis ($F_{1,13}$ = 4.86, p = 0.046). Current tobacco smokers showed an increased mean TCI compared with non-smokers.

Relationship between severity of psychiatric symptoms and measures of cortical inhibition

SICI and TCI levels (averaged across all inhibitory ISIs) correlated positively both in manic episode and euthymia. Patients with a high score in the PANSS excited factor during the manic episode displayed less SICI and TCI during both the manic episode and euthymia. PANSS disorganized factor correlated positively with the positive and negative factors of the same scale. Moreover, the PANSS depressed factor showed a negative correlation with the HDS during mania. These correlations did not reach significance after the Bonferroni correction. The full correlation matrix is presented in *Supplementary Table 1*.

We also conducted a correlation analysis with YMRS item 2, which assesses increased motor activity and energy. The score in this item correlated negatively with the degree of cortical inhibition in both manic and euthymic episodes but

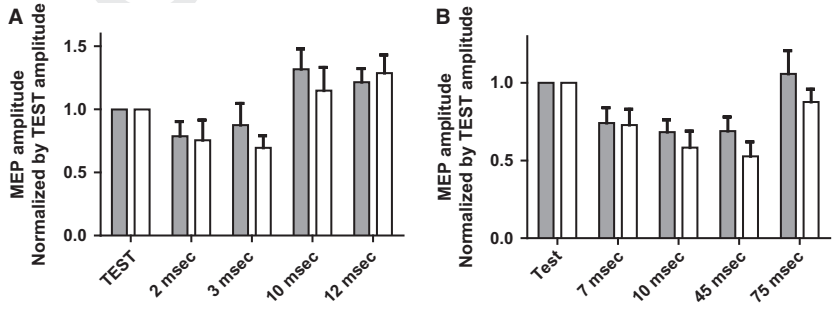

Fig. 2. (A) Short-interval intracortical inhibition and intracortical facilitation in patients with bipolar disorder during the manic episode (gray bars) and euthymia (white bars). (B) Transcallosal inhibition in patients during the manic episode and euthymia. Fifteen patients were included in this follow-up study. Values <1 indicate inhibition. No significant difference in cortical inhibition between manic episode and euthymia was found. Paired sample *t*-tests with Bonferroni correction for multiple comparison. MEP = motor evoked potential.

these did not reach significance after the Bonferroni correction (SICI mania: rho = −0.632, p = 0.006; SICI euthymia: rho = −0.562, p = 0.046; TCI mania: rho = −0.526, p = 0.065; TCI euthymia: rho = −0.409, p = 0.103).

Discussion

We found that patients with BD during mania display specific cortical inhibitory deficits – namely, altered SICI and TCI. The degree of cortical inhibition was similar during mania and euthymia, suggesting that cortical inhibitory deficits constitute a trait marker of BD. In an exploratory correlation analysis, several associations between psychopathological symptom severity and cortical inhibitory measures were detected. It should be highlighted that patients who scored high on items assessing motor hyperactivity and goal-directed behavior (i.e., PANSS excited factor items, YMRS item 2) during mania displayed higher cortical SICI and TCI deficits both in manic episodes and euthymia.

Several efforts have been made to distinguish which cognitive, emotional, and motivational alterations of BD are mood-state dependent or trait markers by examining patients across various mood states, including euthymia (36, 37). However, trait- and state-dependent cortical inhibitory deficits have not been extensively studied, despite the fact that these deficits have been linked to impulsiveness, a core symptom of BD (21, 38, 39). Indeed, clinical scales to assess BD usually include motor activity items. Thus, it may seem reasonable to evaluate trait- and state-dependent cortical excitability/inhibition as an indicator of the neuropathophysiology underlying motor deficits in mood disorders. Our results, which confirm and further extend previous observations in euthymic patients (18, 19), include several new observations on mania that provide a more complete understanding of motor inhibition deficits in BD. In particular, the longitudinal design used in our study empowered the generalizability of our data to the complete course of BD, not limited to a specific type of episode.

Altered cortical inhibition has been observed in severe psychotic and mood disorders, in spite of the concomitant elevated peripheral and brain GABA levels (40, 41). Therefore, a plausible hypothesis for the mechanism involved in cortical inhibitory deficits seen in BD would involve alterations in the GABA receptor system. Pharmacological studies suggest the involvement of GABAA receptors in SICI assessed in the primary motor cortex. Therefore, and in agreement with Levinson et al. (18), deficits in SICI could evidence an impairment in fast inhibitory cortical processes likely to involve GABAA receptors (42). We also found that benzodiazepines modulated the SICI level in patients with BD during mania – that is, patients taking benzodiazepines during the manic episode displayed similar SICI levels to healthy controls, while patients without this treatment displayed significantly reduced SICI levels. The effect of benzodiazepines on SICI could not be tested during euthymia as only three patients were taking benzodiazepines at follow-up. The enhancing effect of benzodiazepines on SICI is consistently reported in the pharmaco-TMS literature (43) and could underlie the findings in BD reported here. Therefore, it is possible that the SICI reduction observed in the manic phase could have been even higher than that obtained in the current study if patients had not been on benzodiazepine treatment. This possibility does not make our main findings void as manic patients still show deficits in SICI, and this does not change during euthymia.

In addition to deficits found in SICI, interhemispheric inhibition at short ISIs (7 msec and 10 msec) was also found to be abnormal both in manic and euthymic episodes. Although the exact mechanisms mediating TCI remain under investigation, there is a tentative consensus on the involvement of transcallosal glutamatergic pathways linking with pyramidal tract neurons through GABAergic interneurons (44). In humans, TCI is a robust phenomenon and occurs over a wide range of ISIs (6–50 msec). However, emerging evidence suggests that TCI elicited at short ISIs is mediated by different mechanisms than that elicited at longer intervals (45). Animal studies have suggested that the early lasting form of TCI is mediated by GABAA receptors (46). Nevertheless, data in humans are inconclusive (47, 48). Another potential factor that may underlie TCI deficits in BD regards anatomical alterations in the corpus callosum, such as the reduced callosal width documented in this population (49, 50).

Substance use was not controlled in our study. Therefore, the effect of these variables on cortical inhibitory measures was analyzed separately in multivariate analyses. Consistent with other studies, patients with BD displayed high rates of current and lifetime cannabis, cocaine, and tobacco use (20, 51). The analysis of cannabis and cocaine use showed no effect of these substances on TMS measures in BD during mania. We also observed a significant decrease in the number of patients without cannabis and cocaine use during follow-up (e.g., eight patients reported that they had stopped smoking cannabis in recent months, which was further confirmed by the urine drug test, and no

patient consumed cocaine during follow-up). The effects of these substances on TMS measures were not analyzed during euthymia owing to the small number of users. Nevertheless, the fact that cortical inhibitory deficits persist during euthymia, with a dramatic decrease in drug users, suggests that cannabis and cocaine use did not significantly affect our results.

Nicotine dependence was assessed in both groups using a validated questionnaire. According to the results of this assessment, nicotine dependence was no different between patients with BD and controls. However, daily cigarette smokers were more prevalent in the BD group than in healthy controls and, therefore, this factor could potentially have influenced our results for cortical inhibition. Interestingly, the multivariate analysis showed that tobacco smokers during the manic episode showed TCI levels comparable with those of controls, whereas non-smokers displayed abnormal levels in this variable. This enhancing effect of tobacco on TCI remains in euthymia. No effect of nicotine on SICI levels was observed. A relationship between nicotine consumption and changes in cortical excitability has previously been documented in chronic smokers without psychiatric disorders (22, 52). It has been suggested that these changes in cortical excitability could be mediated as a direct effect of nicotine on cholinergic inhibitory circuits at the cerebral cortex, which would thus regulate neocortical excitability. In our study, we did not observe this effect of nicotine on cortical excitability in healthy controls, which appeared to contradict previous studies. However, the number of smokers in our control group was small. Thus, we cannot formally rule out that the effect of nicotine that we observed in the BD group was a consequence of the higher number of smokers. However, it is important to note that non-smoking patients with BD showed remarkable cortical inhibitory deficits, indicating that nicotine dependence was not a critical factor in our results.

The severity of mood and psychotic symptoms during mania was not associated with the severity of cortical inhibitory deficits in our sample, supporting the hypothesis that cortical inhibitory deficits are not a state-dependent finding in BD (18). In the current exploratory correlation analysis, we also found an inverse relationship between the PANSS excited factor and cortical inhibition measures. Additionally, symptoms associated with motor hyperactivity were correlated negatively with the degree of cortical inhibition. In spite of the lack of statistical significance after Bonferroni correction, these results suggest a specific relationship between the clinical features of BD (hyperactivity, motor disinhibition) and cortical inhibition. Nevertheless, these preliminary data should be studied carefully and validated in a larger sample and with other measures of motor activity (e.g., actigraphy).

Study limitations

These findings should be interpreted with caution, given the limitations of the study. First, the number of subjects studied was relatively small. Our power analysis revealed that, with the available sample size, we could detect only moderate effect sizes. Hence, while our sample size was adequate to detect differences in SICI and TCI, it may not have been adequate to detect smaller but true differences that could have existed in other parameters – e.g., ICF, LICI, or CSP. In addition, no repeated measures were performed in the healthy group. We should note that the healthy control group was compared only with patients with BD during mania as, to the best of our knowledge, that comparison had not been previously reported in the literature. This comparison also served to detect which cortical inhibition variables showed deviations from normality in patients with BD that we should follow-up more carefully in the second assessment when patients are in clinical remission. Ideally, a second TMS assessment in the healthy group would have provided information about individual variability in this group during the timeline of our follow-up study. Nevertheless, several studies have consistently shown good test–retest reliability of cortical inhibition measures in healthy subjects [for example (53)]; therefore, we did not expect too much variation in our measures in our control sample.

Conclusions

Taken together, the results of the current study show specific cortical inhibitory deficits in patients with BD. Our data suggest that these deficits are trait dependent rather than associated with mood state. Our findings also suggest a relationship between specific clinical features of BD and reduced GABAergic transmission in the primary motor cortex. Further investigation will be necessary to confirm this association and to establish the prognostic value of cortical inhibitory deficits in the course of BD and response to therapy.

Acknowledgements

This study was supported by grants from the Ministerio de Economía y Competitividad de España (SAF2007-60700); the Instituto de Salud Carlos III (PI10/01674, CP08/00174, PI13/01461), the Consejería de Economía, Innovación, Ciencia y

Empleo de la Junta de Andalucía (CVI-02526, CTS-7685), the Consejería de Salud y Bienestar Social de la Junta de Andalucía (PI-0377/2007, PI-0741/2010, PI-0437-2012), the Sociedad Andaluza de Neurología, the Jacques and Gloria Gossweiler Foundation, the Fundación Alicia Koplowitz, and the Fundación Mutua Madrileña. JFM-R was supported by the 'Sara Borrell' Programme from the Instituto de Salud Carlos III; IH-F was supported by the PFIS Programme from the Instituto de Salud Carlos III; and MR-V was supported by the Grant BAE 09/90088.

Disclosures

The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

References

1. Gutiérrez-Rojas L, Gurpegui M, Ayuso-Mateos JL, Gutiérrez-Ariza JA, Ruiz-Veguilla M, Jurado D. Quality of life in bipolar disorder patients: a comparison with a general population sample. Bipolar Disord 2008; 10: 625–634.
2. Mehta UM, Basavaraju R, Thirthalli J. Mirror neuron activity and symptom severity in drug-naive mania – a transcranial magnetic stimulation study. Brain Stimul 2014; 7: 757–759.
3. Henry BL, Minassian A, Patt VM et al. Inhibitory deficits in euthymic bipolar disorder patients assessed in the human behavioral pattern monitor. J Affect Disord 2013; 150: 948–954.
4. Bora E, Yucel M, Pantelis C. Cognitive endophenotypes of bipolar disorder: a meta-analysis of neuropsychological deficits in euthymic patients and their first-degree relatives. J Affect Disord 2009; 113: 1–20.
5. Lombardo LE, Bearden CE, Barrett J et al. Trait impulsivity as an endophenotype for bipolar I disorder. Bipolar Disord 2012; 14: 565–570.
6. Benes FM, Berretta S. GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. Neuropsychopharmacology 2001; 25: 1–27.
7. Massat I, Souery D, Del-Favero J et al. Excess of allele1 for alpha3 subunit GABA receptor gene (GABRA3) in bipolar patients: a multicentric association study. Mol Psychiatry 2002; 7: 201–207.
8. Otani K, Ujike H, Tanaka Y et al. The GABA type A receptor alpha5 subunit gene is associated with bipolar I disorder. Neurosci Lett 2005; 381: 108–113.
9. Torrey EF, Barci BM, Webster MJ, Bartko JJ, Meador-Woodruff JH, Knable MB. Neurochemical markers for schizophrenia, bipolar disorder, and major depression in postmortem brains. Biol Psychiatry 2005; 57: 252–260.
10. Bielau H, Steiner J, Mawrin C et al. Dysregulation of GABAergic neurotransmission in mood disorders: a postmortem study. Ann N Y Acad Sci 2007; 1096: 157–169.
11. Hallett M. Transcranial magnetic stimulation: a primer. Neuron 2007; 55: 187–199.
12. Ziemann U. TMS and drugs. Clin Neurophysiol 2004; 115: 1717–1729.
13. Di Lazzaro V, Oliviero A, Saturno E et al. Effects of lorazepam on short latency afferent inhibition and short latency intracortical inhibition in humans. J Physiol 2005; 564: 661–668.
14. Werhahn KJ, Kunesch E, Noachtar S, Benecke R, Classen J. Differential effects on motorcortical inhibition induced by blockade of GABA uptake in humans. J Physiol 1999; 517(Pt 2): 591–597.
15. Sanger TD, Garg RR, Chen R. Interactions between two different inhibitory systems in the human motor cortex. J Physiol 2001; 530: 307–317.
16. Meyer BU, Roricht S, Grafin von Einsiedel H, Kruggel F, Weindl A. Inhibitory and excitatory interhemispheric transfers between motor cortical areas in normal humans and patients with abnormalities of the corpus callosum. Brain 1995; 118(Pt 2): 429–440.
17. Ferbert A, Priori A, Rothwell JC, Day BL, Colebatch JG, Marsden CD. Interhemispheric inhibition of the human motor cortex. J Physiol 1992; 453: 525–546.
18. Levinson AJ, Young LT, Fitzgerald PB, Daskalakis ZJ. Cortical inhibitory dysfunction in bipolar disorder: a study using transcranial magnetic stimulation. J Clin Psychopharmacol 2007; 27: 493–497.
19. Farzan F, Barr MS, Levinson AJ et al. Evidence for gamma inhibition deficits in the dorsolateral prefrontal cortex of patients with schizophrenia. Brain 2010; 133: 1505–1514.
20. Cassidy F, Ahearn EP, Carroll BJ. Substance abuse in bipolar disorder. Bipolar Disord 2001; 3: 181–188.
21. Swann AC, Dougherty DM, Pazzaglia PJ, Pham M, Moeller FG. Impulsivity: a link between bipolar disorder and substance abuse. Bipolar Disord 2004; 6: 204–212.
22. Lang N, Hasan A, Sueske E, Paulus W, Nitsche MA. Cortical hypoexcitability in chronic smokers? A transcranial magnetic stimulation study. Neuropsychopharmacology 2008; 33:2517–2523.
23. Wobrock T, Hasan A, Malchow B et al. Increased cortical inhibition deficits in first-episode schizophrenia with comorbid cannabis abuse. Psychopharmacology 2010; 208: 353–363.
24. Young RC, Biggs JT, Ziegler VE, Meyer DA. A rating scale for mania: reliability, validity and sensitivity. Br J Psychiatry 1978; 133: 429–435.
25. Hamilton M. A rating scale for depression. J Neurol Neurosurg Psychiatry 1960; 23: 56–62.
26. First MBS, Gibbon M, Williams JBW. Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition with Psychotic Screen. New York: Biometrics Research, New York State Psychiatric Institute, 2002.
27. Kay SR. Positive-negative symptom assessment in schizophrenia: psychometric issues and scale comparison. Psychiatr Q 1990; 61: 163–178.
28. Wallwork RS, Fortgang R, Hashimoto R, Weinberger DR, Dickinson D. Searching for a consensus five-factor model of the Positive and Negative Syndrome Scale for schizophrenia. Schizophr Res 2012; 137: 246–250.
29. Woods SW. Chlorpromazine equivalent doses for the newer atypical antipsychotics. J Clin Psychiatry 2003; 64: 663–667.
30. Robins LN, Wing J, Wittchen HU et al. The Composite International Diagnostic Interview. An epidemiologic Instrument suitable for use in conjunction with different diagnostic systems and in different cultures. Arch Gen Psychiatry 1988; 45: 1069–1077.
31. Heatherton TF, Kozlowski LT, Frecker RC, Fagerstrom KO. The Fagerstrom Test for Nicotine Dependence: a revision of the Fagerstrom Tolerance Questionnaire. Br J Addict 1991; 86: 1119–1127.
32. de Leon J, Diaz FJ, Becona E, Gurpegui M, Jurado D, Gonzalez-Pinto A. Exploring brief measures of nicotine dependence for epidemiological surveys. Addict Behav 2003; 28: 1481–1486.

33. Kujirai T, Caramia MD, Rothwell JC et al. Corticocortical inhibition in human motor cortex. J Physiol 1993; 471: 501–519.
34. Berardelli A, Rona S, Inghilleri M, Manfredi M. Cortical inhibition in Parkinson's disease. A study with paired magnetic stimulation. Brain 1996; 119(Pt 1): 71–77.
35. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007; 39: 175–191.
36. Martinez-Aran A, Vieta E, Reinares M et al. Cognitive function across manic or hypomanic, depressed, and euthymic states in bipolar disorder. Am J Psychiatry 2004; 161: 262–270.
37. Almeida JR, Versace A, Hassel S, Kupfer DJ, Phillips ML. Elevated amygdala activity to sad facial expressions: a state marker of bipolar but not unipolar depression. Biol Psychiatry 2010; 67: 414–421.
38. Campbell S, MacQueen G. An update on regional brain volume differences associated with mood disorders. Curr Opin Psychiatry 2006; 19: 25–33.
39. Swann AC, Pazzaglia P, Nicholls A, Dougherty DM, Moeller FG. Impulsivity and phase of illness in bipolar disorder. J Affect Disord 2003; 73: 105–111.
40. Ongur D, Prescot AP, McCarthy J, Cohen BM, Renshaw PF. Elevated gamma-aminobutyric acid levels in chronic schizophrenia. Biol Psychiatry 2010; 68: 667–670.
41. Brady RO Jr, McCarthy JM, Prescot AP et al. Brain gamma-aminobutyric acid (GABA) abnormalities in bipolar disorder. Bipolar Disord 2013; 15: 434–439.
42. Rabow LE, Russek SJ, Farb DH. From ion currents to genomic analysis: recent advances in GABAA receptor research. Synapse 1995; 21: 189–274.
43. Ziemann U, Reis J, Schwenkreis P et al. TMS and drugs revisited 2014. Clin Neurophysiol 2014; ???: ???–???.
44. Perez MA, Cohen LG. Interhemispheric inhibition between primary motor cortices: what have we learned? J Physiol 2009; 587: 725–726.
45. Reis J, Swayne OB, Vandermeeren Y et al. Contribution of transcranial magnetic stimulation to the understanding of cortical mechanisms involved in motor control. J Physiol 2008; 586: 325–351.
46. Kawaguchi Y. Receptor subtypes involved in callosally-induced postsynaptic potentials in rat frontal agranular cortex in vitro. Exp Brain Res 1992; 88: 33–40.
47. Irlbacher K, Brocke J, Mechow JV, Brandt SA. Effects of GABA(A) and GABA(B) agonists on interhemispheric inhibition in man. Clin Neurophysiol 2007; 118: 308–316.
48. Daskalakis ZJ, Christensen BK, Fitzgerald PB, Roshan L, Chen R. The mechanisms of interhemispheric inhibition in the human motor cortex. J Physiol 2002; 543: 317–326.
49. Lloyd AJ, Ali HE, Nesbitt D, Moore PB, Young AH, Ferrier IN. Corpus callosum changes in euthymic bipolar affective disorder. Br J Psychiatry 2014; 204: 129–136.
50. Brambilla P, Nicoletti M, Sassi RB et al. Corpus callosum signal intensity in patients with bipolar and unipolar disorder. J Neurol Neurosurg Psychiatry 2004; 75: 221–225.
51. Jackson JG, Diaz FJ, Lopez L, de Leon J. A combined analysis of worldwide studies demonstrates an association between bipolar disorder and tobacco smoking behaviors in adults. Bipolar Disord 2015; 17: 575–597.
52. Grundey J, Freznosa S, Klinker F, Lang N, Paulus W, Nitsche MA. Cortical excitability in smoking and not smoking individuals with and without nicotine. Psychopharmacology 2013; 229: 653–664.
53. Du X, Summerfelt A, Chiappelli J, Holcomb HH, Hong LE. Individualized brain inhibition and excitation profile in response to paired-pulse TMS. J Motor Behav 2014; 46: 39–48.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Correlation analysis (Pearson's correlation) between severity of psychiatric symptoms during mania and cortical inhibition measures

Author Query Form

Journal: BDI
Article: 12382

Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.
Many thanks for your assistance.

Query reference	Query	Remarks
1	AUTHOR: Please confirm that given names (red) and surnames/family names (green) have been identified correctly.	
2	AUTHOR: Please provide city name for fourth affiliation.	
3	AUTHOR: is my change here OK?	
4	AUTHOR: please also add the city and state of manufacture	
5	AUTHOR: Please provide city name for Digitimer	
6	AUTHOR: Please give manufacturer information for SIGNAL software version 4: company name, town, state (if USA), and country	
7	AUTHOR: By " The presentation of TEST and CS were randomly intermingled" do you mean "The presentations of TEST and CS were randomly intermingled" or " The presentation of TEST and CS was randomly intermingled"?	
8	AUTHOR: please see earlier query about "presentations" or "presentation"	
9	AUTHOR: should this be "for both hemispheres"?	
10	AUTHOR: is my change here OK	
11	AUTHOR: could you please supply the name of the manufacturer and the city, state (if relevant) and country of manufacture for this progra	
12	AUTHOR: should this be "in SICI"	
13	AUTHOR: is there a reason why three asterisks are being used rather than two? It looks very strange – can we change "***" to "**"	
14	AUTHOR: do you mean "LICI" , as previously defined	
15	AUTHOR: can we change this to "for any evaluated condition"	
16	AUTHOR: in the legend to Figure 2, coud you please add the fact that the values represent the mean ± standard err	
17	AUTHOR: again, do you mean "LIC	
18	AUTHOR: please define "HD	
19	AUTHOR: is this change OK?	
20	AUTHOR: is my change here OK	

21	**AUTHOR: I'm not sure I understand the second half of this sentence - do you mean "... in patients with BD, and we should follow up these patients carefully in a second assessment, when they are in clinical remission"**	
22	**AUTHOR: Please provide the volume number, page range for reference [43**	

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	··· under matter to remain	New matter followed by
Insert in text the matter indicated in the margin		or (x2)
Delete	through single character, rule or underline or through all characters to be deleted	or (x2)
Substitute character or substitute part of one or more word(s)	through letter or through characters	new character or new characters
Change to italics	under matter to be changed	
Change to capitals	under matter to be changed	
Change to small capitals	under matter to be changed	
Change to bold type	under matter to be changed	
Change to bold italic	under matter to be changed	
Change to lower case	Encircle matter to be changed	
Change italic to upright type	(As above)	
Change bold to non-bold type	(As above)	
Insert 'superior' character	through character or where required	or under character e.g. or
Insert 'inferior' character	(As above)	over character e.g.
Insert full stop	(As above)	
Insert comma	(As above)	
Insert single quotation marks	(As above)	or and/or or
Insert double quotation marks	(As above)	or and/or or
Insert hyphen	(As above)	
Start new paragraph		
No new paragraph		
Transpose		
Close up	linking characters	
Insert or substitute space between characters or words	through character or where required	
Reduce space between characters or words	between characters or words affected	