



Cerebellar inhibition in hepatic encephalopathy

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HIGHLIGHTS

- Decreased cerebellar inhibition (CBI) suggests affection of the cerebellar efferent pathway in hepatic encephalopathy (HE).
- The extent of CBI increases with disease severity.
- The results suggest increase of the GABAergic tone in Purkinje cells with increasing severity of HE.

ABSTRACT

Objective: Previous animal work reported that hyperammonemia leads to opposing changes of GABAergic neurotransmission in terms of increase in the cerebellum and decrease in the cerebral cortex. In this study, we investigate GABAergic tone in the cerebellum in patients with hepatic encephalopathy (HE) at different stages of the disease and its relation to critical flicker frequency (CFF) and ataxia.

Methods: Cerebellar inhibition using transcranial magnetic stimulation was investigated in 15 patients with different stages of HE and 15 healthy controls. All patients were assessed using CFF and the score for assessment and rating of ataxia (SARA).

Results: Decreased cerebellar inhibition (CBI) was observed in manifest HE at interstimulus interval from 5 to 7 ms. However, the degree of CBI at 7 ms correlated significantly with disease severity measured with SARA and with CFF by trend.

Conclusion: Reduced CBI in HE patients indicates affection of the cerebellar efferent pathway. The disease severity dependent increase of CBI magnitude supports the notion of disease stage dependent increase of GABAergic neurotransmission in Purkinje cells.

Significance: The results support previous animal experiments showing increase of GABA-ergic neurotransmission in the cerebellum and decrease in the motor cortex in HE.

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1. Introduction

Hepatic Encephalopathy (HE) is defined as brain dysfunction due to acute and chronic liver disease. Accordingly, HE can be categorized into three types A, B and C. Type A occurs in patients with acute liver failure, while type B is found in patients with bypass shunts and type C, which is the most common form, develops in patients with chronic liver disease (Ferenci et al., 2002). It is well

known that HE represents a common complication in cirrhotic patients, comprising different motor and neuropsychiatric symptoms (Häussinger and Blei, 2007). The severity of HE is judged according to the clinical symptoms. *West-Haven* grading system is the most common scale used in clinical staging of HE (Cash et al., 2010). According to this scale HE severity ranged from minimal HE (mHE), which is the earliest stage of the disease and characterized by psychomotor slowing and subtle cognitive deficits without obvious clinical symptoms, to HE grade 3–4 which is the advanced form of HE and characterized by disorientation, asterixis and even coma (Bajaj et al., 2011). The incidence and prevalence of HE is difficult to determine and usually show marked geographic

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differences because of heterogeneity in the etiology of liver cirrhosis and disease severity worldwide (Moriwaki et al., 2010; Schwendimann and Minagar, 2017). HE in liver cirrhosis is considered a clinical manifestation of a low grade cerebral edema, which is developed as a consequence of hyperammonemia (Haussinger and Schliess, 2008). It has been suggested that ammonia played a significant role in the disease pathogenesis for over 50 years due to the frequent elevation of ammonia observed in patients with liver cirrhosis and its proved cellular toxicity. However, recent evidence indicates that ammonia is only one component in a multifactorial disease process (Haussinger and Schliess, 2008; Frederick, 2011; Häussinger and Sies, 2013). Cauli et al. (2009a, 2009b) reported in animal experiments that alteration of GABAergic neurotransmission caused by hyperammonemia depends on the brain region. GABAergic tone was found to be increased in the cerebellum and decreased in the cerebral cortex. This in turn contributes to various neuropsychiatric symptoms including impairment of cognitive function, alteration of consciousness, myoclonus, and ataxia (Cauli et al., 2009a, 2009b). Consistently, we recently found in a transcranial magnetic stimulation (TMS) study reduction of GABAergic neurotransmission of the motor cortex for the first time *in vivo* in patients with manifest HE (Groiss et al., 2019). TMS is a non-invasive tool for brain stimulation which has been used to study the physiology of the central nervous system. Moreover, TMS is used to identify the functional role of specific brain structures and explore the complex network dynamics and synaptic plasticity (Kobayashi and Pascual-Leone, 2003; Dayan et al., 2013). Various conditioned TMS paradigms have been reported using the paired-pulse technique. Paired-pulse stimulation is a technique to study intracortical inhibitory and facilitatory mechanisms when applied to the motor cortex. Inhibition in terms of short-interval intracortical inhibition (SICI) is supposed to be mediated by GABA-A receptors and long-interval intracortical inhibition (LICI) by GABA-B receptors. On the other hand, intracortical facilitation (ICF) and short-interval intracortical facilitation (SICF) are supposed to reflect excitatory glutamatergic circuits (Kujirai et al., 1993; Ziemann et al., 1996; Hanajima et al., 2002; Ziemann, 2003).

In the present study, we used another type of paired-pulse TMS called cerebellar inhibition (CBI). In this paradigm, a conditioning TMS pulse over the cerebellum decreases the size of motor evoked

potentials (MEPs) provoked by the test TMS pulse over the contralateral primary motor cortex at interstimulus intervals (ISIs) between 5 and 7 ms. CBI is thought to be mediated by activation of cerebellar Purkinje cells and consecutive inhibition of the dentato-thalamo-cortical pathway (Ugawa et al., 1991; Groiss and Ugawa, 2013). Previous animal experiments have reported that the terminals of the cerebellar Purkinje cells contain GABA, accordingly activation of these terminals provoke inhibitory post synaptic potentials (IPSPs) in the dentate nucleus (Sastry et al., 1997). The CBI paradigm has been used in previous studies in patients with cerebellar affection such as cerebellar cortical atrophy, spinocerebellar ataxia or cerebellar variant of multiple system atrophy, and showed reduced cerebellar inhibition due to lesion in the cerebellum or in the cerebello-thalamo-cortical pathway (Ugawa et al., 1994a, 1994b, 1994c, 1997). Furthermore, CBI decreased also if there was pure Purkinje cells dysfunction as seen in degenerative late onset ataxias such as cerebellar cortical atrophy (Ugawa et al., 1997; Grimaldi et al., 2014). Hence, the CBI TMS paradigm can be considered as valuable protocol to evaluate the integrity of the cerebello-thalamo-cortical network.

The aim of this study was to investigate alteration of GABAergic neurotransmission in the cerebellum in differently affected HE patients and to study its relation to certain clinical parameters measured by the critical flicker frequency (CFF) and ataxia score.

2. Methods

2.1. Participants

Nineteen patients with HE and fifteen healthy age-matched control participants were enrolled to the study. Four patients did not tolerate brain stem stimulation and discontinued the experiment. All participants of the study received an information sheet and written informed consent was obtained from all participants prior to the study. Patients with a recent history of alcohol abuse within 2 months, hepatocellular carcinoma, renal, or respiratory failure or neurological diseases were excluded. Patients taking psychotropic drugs within 2 months before recruitment were also excluded. Safety for TMS was assessed by a safety questionnaire (Rossi et al., 2009) and subjects with contraindications did not

Table 1
Clinical and electrophysiological characteristics of patients with hepatic encephalopathy and control participants.

Clinical features	HE Patients	Control participants	p-value
Number of participants (n)	15	15	
Age (years) ^a	64.1 ± 10.5	60.3 ± 6.2	0.23
Sex (m/f) (n)	13/2	12/3	
Etiology of liver cirrhosis (n):			
– Alcoholic	6		
– Viral hepatitis (HCV)	2		
– NASH	1		
– Cryptogenic	6		
Child- Pugh Scoring (n)			
– Class A	2		
– Class B	6		
– Class C	7		
Venous blood Ammonia in µg/dl ^a	86.36 ± 69.2		
CFF (Hz) ^a	37.4 ± 3.6	43.2 ± 2.7	< 0.001
SARA ^b	2 (0–10)		
RMT ^a	41.13 ± 11.16	40.13 ± 6.43	0.7
BS-AMT ^a	59.53 ± 10.3	49.8 ± 10.0	0.01
Test stimulus intensity (%MSO) ^a	66.33 ± 21.8	56.86 ± 13.8	0.1
Conditioning stimulus intensity (%MSO) ^a	56.46 ± 10	47.13 ± 9.6	0.01

HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; CFF, Critical flicker frequency; SARA, Score for assessment and rating of ataxia; RMT, resting motor threshold; BS-AMT, Brain stem active motor threshold.

^a Values are shown as mean ± SD.

^b Values are shown as median (range).

Table 2

Clinical and electrophysiological characteristics of patients with hepatic encephalopathy subdivided into severity subtypes.

Clinical features	mHE	HE1	HE2
Number of patients (n)	5	6	4
Age (years) ^a	54.4 ± 9.2	73.0 ± 6.5	62.5 ± 6.5
Sex M: F (n)	4 m	5 m:1f	3 m:1f
CFF (Hz) ^a	42.9 ± 2.9	37.0 ± 0.9	34.8 ± 2.3
RMT ^a	41.6 ± 13.2	36.1 ± 8.2	48 ± 11.1
BS-AMT ^a	56.6 ± 4.7	59.1 ± 11.7	63.7 ± 14.2
Test stimulus intensity (%MSO) ^a	62.2 ± 24.9	62.5 ± 22.2	77.2 ± 18.7
Conditioning stimulus intensity (%MSO) ^a	53.6 ± 4.7	56.1 ± 11.3	60.5 ± 13.9

HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; CFF, Critical flicker frequency; SARA, Score for assessment and rating of ataxia; RMT, resting motor threshold; BS-AMT, Brain stem active motor threshold.

^a Values are shown as mean ± SD.

further participate in the study. The study was performed in accordance with the declaration of Helsinki and was approved by the local ethics committee of the Heinrich-Heine University, Düsseldorf (5179R).

The etiology of HE in all patients was liver cirrhosis, the diagnosis of liver cirrhosis was based on clinical examination, laboratory tests (blood ammonia level and liver function tests) and abdominal ultrasonography. Fibroscan (Transient elastography) and Acoustic Radiation Force Impulse (ARFI) were done in a subgroup of patients to confirm the diagnosis of liver cirrhosis. According to clinical examination, CFF and *West-Haven-Criteria*, the remaining fifteen HE patients were divided into subgroups (5 patients with minimal HE, 6 with HE 1, and 4 with HE grade 2) (Cash et al., 2010). Clinical and electrophysiological characteristic of the patients and control participants are depicted in Tables 1 and 2.

2.2. Transcranial magnetic stimulation

2.2.1. Electromyographic recordings

Subjects were seated in a comfortable chair. Pairs of Ag–AgCl surface cup electrodes (9-mm diameter) were used to record the surface electromyography (EMG) signals from the right first dorsal interosseous muscle. The active and reference electrodes were placed in a belly-tendon montage. Responses were amplified (Digitimer D360, UK), filtered from 100 to 5000 Hz, digitized with 5 kHz sampling rate and stored on a computer for offline analysis.

2.2.2. Cerebellar inhibition (CBI) paradigm

Magnetic stimulation was performed with the Magstim Bistim² (Magstim Co, Whitland, UK). The conditioning stimulus (CS) was applied over the midpoint between the mastoid process and theinion ipsilateral to the recording side with a double-cone coil with mean diameter of 110 mm. Upward current was induced in the cerebellum. Test stimulus (TS) was applied on the contralateral M1 with a round coil with mean diameter of 90 mm placed over the vertex with anteroposterior current direction (Ugawa et al., 1995; Groiss and Ugawa, 2013). In healthy individuals, the amplitude of the motor evoked potential (MEP) of TS is reduced when the interstimulus interval (ISI) between CS and TS is set to 5–7 ms. This MEP inhibition is called cerebellar inhibition (CBI) and is attributed to inhibition of the disynaptic dentato-thalamo-cortical pathway (Ugawa et al., 1995; Groiss and Ugawa, 2013).

2.2.3. Experimental design

First step in the experiment, we used the round coil to determine the resting motor threshold (RMT) of the FDI, which was defined as the lowest stimulator output intensity which could

evoke MEPs of 50 μ V peak-to-peak amplitude in the relaxed FDI muscle in more than 5 of 10 consecutive trials (Rossini et al., 2015). Second step, the double-cone coil was positioned centrally over theinion and the active motor threshold for pyramidal tract activation at the brain stem level (BS-AMT) was determined (Ugawa et al., 1994a, 1994b, 1994c). The threshold of the pyramidal tract activation was determined as the lowest intensity which evoked a small response in the averaged electromyographic recordings during a background tonic contraction of the right FDI (about 10 % of maximum contraction) (Ugawa et al., 1997). CS intensity was adjusted to 95% AMT. TS intensity was adjusted to evoke MEPs of 1 mV when given alone. The ISI between CS and TS were set to 4, 5, 6, 7, and 8 ms. Additionally, single TS and single CS were applied as control condition and for size ratio calculation. The experiment was performed with relaxed FDI muscle which was monitored by an oscilloscope online (Shirota et al., 2010). Signal software version 6.02 (Cambridge Electronic Design, Cambridge, UK) was used to measure the peak-to-peak MEP amplitude for each trial. Ten trials were averaged for each condition, resulting in 70 trials in total. Each condition was applied in a shuffled order, meaning the conditioned trials at different ISI and the control trials were randomly intermixed. The intertrial interval was set to 10 s ± 0.5 s.

2.3. Score for assessment and rating of ataxia (SARA)

Score for assessment and rating of ataxia (SARA) consist of eight parameters with total scores ranging from 0 to 40, which means from no ataxia to most severe ataxia. Scores for the eight parameters range as follows: parameter 1 is the assessment of gait (0–8 points), parameter 2 is assessment of stance (0–6 points), parameter 3 is assessment of sitting (0–4 points), parameter 4 is assessment of speech disturbance (0–6 points), parameter 5 is assessment of finger chase (0–4 points), parameter 6 is assessment of nose-finger test (0–4 points), parameter 7 is assessment of fast alternating hand movement (0–4 points) and finally parameter 8 is assessment of heel-shin slide (0–4 points). The motor activities of the four limbs (parameters 5–8) are bilaterally assessed and the mean values are calculated to get the total score (Schmitz-Hubsch et al., 2006). HE patients underwent this clinical scoring to search for symptoms of cerebellar dysfunction and to be correlated with the degree of cerebellar inhibition.

2.4. Measurement of critical flicker frequency threshold (CFF)

The CFF is a simple, sensitive, and valuable test for quantification of low-grade HE in patients with liver cirrhosis and useful for the detection and monitoring of HE (Kircheis et al., 2002; Sharma et al., 2007). CFF was measured using a portable, battery-powered analyzer (Hepatonorm Analyzer; nevoLAB GmbH, Maierhöfen, Germany). The measurement should be performed in a quiet, semi-darkened room without any disturbing noises.

Simply, the analyzer provoked an intrafoveal light stimulus with defined pulses of light. At the start, the analyzer was adjusted to generate a red light with a high frequency pulse (60 Hz) which gave the participant a visual perception of a continuous light. Then, the frequency was decreased gradually until the participant had the impression that the continuous light had shifted into a flickering light. The participant should be instructed to press a switch held in his hand, once he noticed the shift from continuous to flickering light. First we should be ensured that the participant well understand the test, by repeating the process for at least 5 times. Then, the procedure was repeated 10 times. The mean and the standard deviation values for each participant were calculated from the collected data (Romero-Gomez et al., 2007).

2.5. Statistical analysis

Statistical analysis was done using GraphPad Prism (GraphPad Software, CA, USA). Shapiro Wilk test was used to test for normality of the data. Student *t*-tests were used to compare RMT and BS-AMT between groups. CBI time courses for the CS-TS size ratios at each ISI were calculated for each group (Shirotta et al., 2010). The average size ratio (ASR) between ISIs of 5–7 ms were compared with Student's *t*-test to compare CBI between HE and healthy controls. One-way ANOVA with Dunnett's post hoc analysis was done in each group to compare the degree of CBI at each ISI with the baseline. The relation between CBI and severity of the disease was investigated by correlation analysis between ASR and both CFF and SARA was done using linear regression analysis. Based on the results of correlation analysis, HE subgroup analysis CBI was performed at 7 ms and compared between mHE, HE grade 1, HE grade 2, and healthy controls using one-way ANOVA with Bonferroni's post hoc test. In all analyses, a *p* value < 0.05 was considered as significant.

3. Results

3.1. Baseline characteristics

Data from fifteen HE patients and fifteen control subjects were analyzed, and their baseline characteristics are shown in Table 1.

Shapiro Wilk test of normality revealed that the data were normally distributed, accordingly we used parametric test for analysis. There was no significant age difference between HE and control groups. CFF was significantly lower in patients with HE compared to the control group. Considering TMS characteristics measured in both groups, absolute CS intensity was higher in the HE group compared to the control group, which is attributed to increase in BS-AMT in HE patients. There was no significant difference in RMT and TS intensity between both groups (Table 1).

3.2. Cerebellar inhibition

The mean time course of CBI among either the total HE group or the respective HE subgroups and the control group are presented in Fig. 1A and C, respectively. Student's *t*-test revealed less CBI in HE patients compared to healthy controls ($p = 0.0001$) (Fig. 1B). Analysis of data by One-way ANOVA with post hoc Dunnett's test indicated reduction in CBI compared to baseline only in healthy controls at ISI 5, 6, 7 and 8 ms, but not in patients within the HE group (Fig. 1A). However, HE subgroup analysis revealed different degrees of CBI between groups. One-way ANOVA with post hoc Bonferroni comparisons between HE subgroups disclosed reduced CBI at 7 ms for patients with mHE compared to both HE grade 2 and healthy controls ($p = 0.01$ and $p = 0.002$, respectively). There was no difference in degree of CBI between patients with HE grade 2 and healthy controls (Fig. 1D).

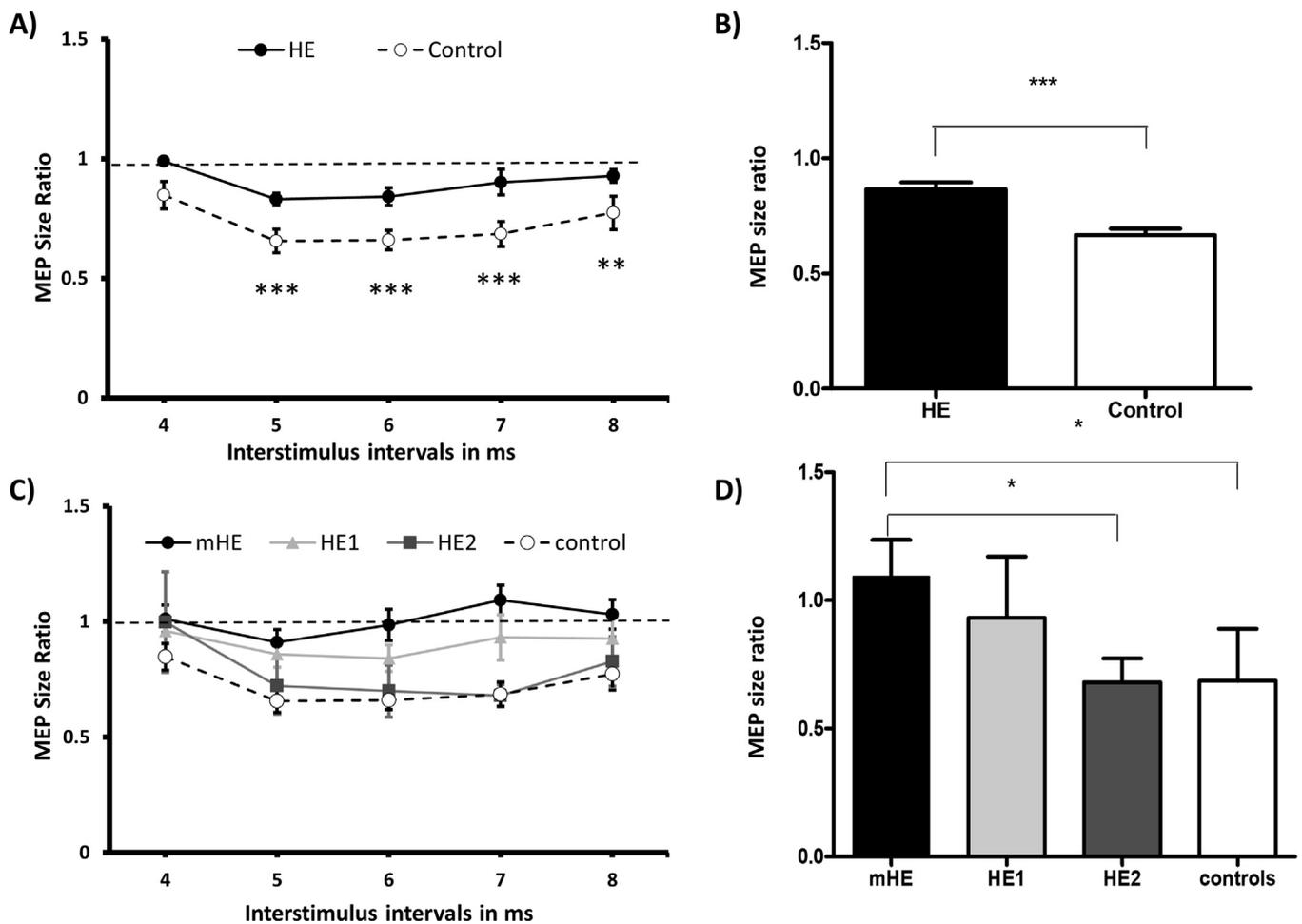


Fig. 1. Upper row shows comparison between overall HE patients and healthy controls. Lower row depicts subgroup analysis. (A) Mean time courses of CBI. Healthy controls showed significant inhibition at ISIs 5–8 ms compared to baseline while in HE no significant inhibition was found. (B) Difference of averaged (ISI 5–7 ms) inhibition between HE patients and healthy controls. (C) Mean time courses of CBI revealing disease stage dependent inhibition in HE patients. (D) Disease stage dependent inhibition in patients with HE. Differences were found between mHE patients and HE2 patients, as well as mHE patients and healthy controls. Error bars represent SEM.

Table 3

Correlation analysis between clinical parameters (CFF and SARA) and the MEP size ratio at ISIs of 5, 6 and 7 ms.

MEP size ratio at each ISI	CFF	SARA
5 ms	$p = 0.1$ $r = -0.2$	$p = 0.3$ $r = 0.6$
6 ms	$p = 0.3$ $r = 0.1$	$p = 0.1$ $r = -0.7$
7 ms	$p = 0.07$ $r = 0.4$	$p = 0.01$ $r = -0.7$

3.3. Correlation analysis

Correlation analysis was done between the clinical parameters (CFF and SARA) and the MEP size ratio at each ISI in the target duration (5, 6 and 7 ms) (Table 3). Correlation was found only at the ISI 7 ms where, we found negative correlation between MEP size ratio and SARA score ($r = -0.7$, $p = 0.01$). Moreover, MEP size ratio and CFF showed a trend for a positive correlation ($r = 0.4$, $p = 0.07$) (Fig. 2 A and B). For the other ISIs we did not find any correlation (Table 3).

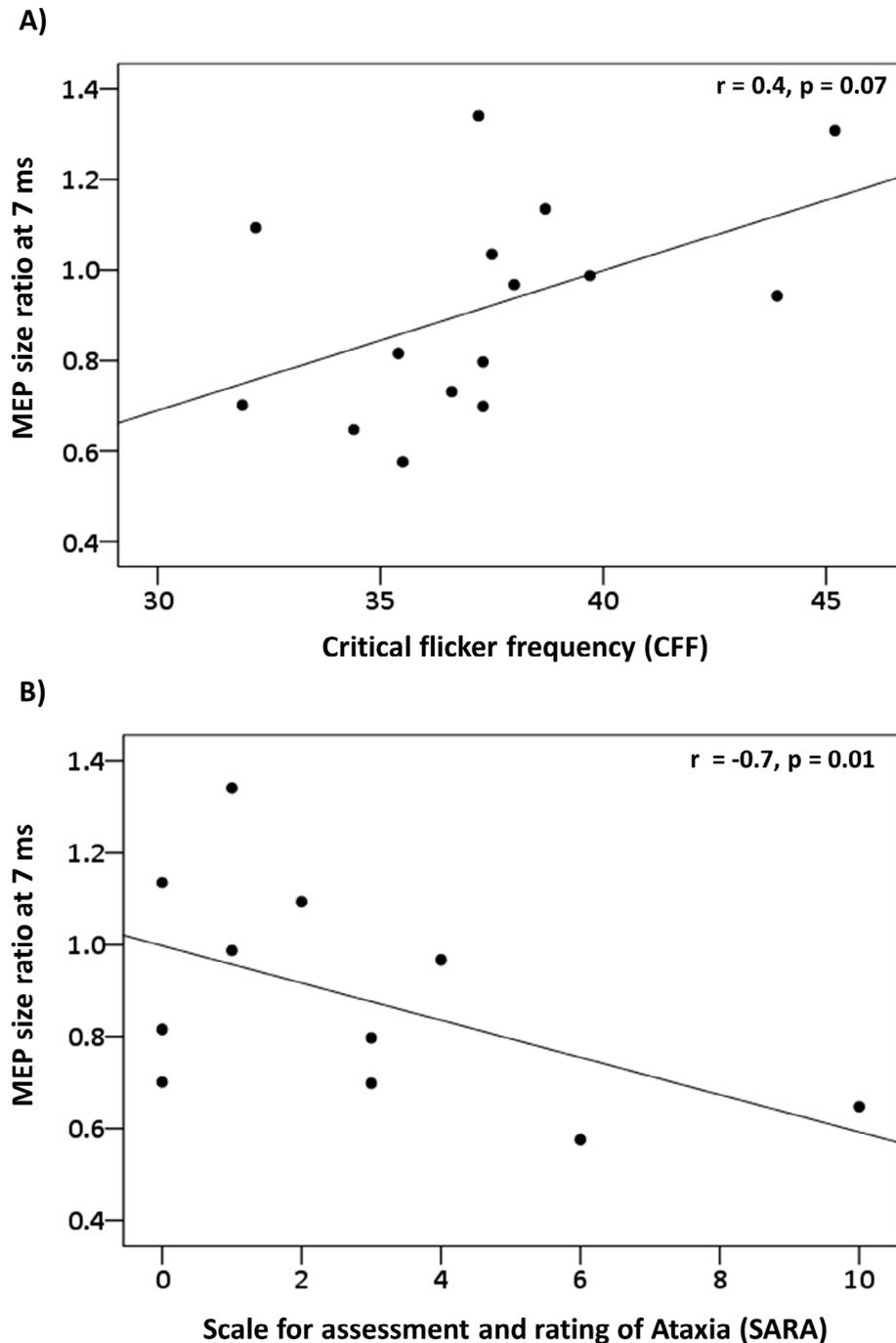


Fig. 2. Correlation analysis between (A) MEP size ratio at ISI 7 ms and CFF. (B) MEP size ratio at ISI 7 ms and clinical SARA score. Insets illustrate the results of the linear correlation analyses (black lines).

4. Discussion

Our study has two main findings. First, overall CBI was decreased in patients with HE suggesting involvement of the cerebellar efferent pathway to motor cortex in HE disease. Second, CBI in HE patients increased gradually with increase of disease severity consistent with a stage dependent increase of GABAergic neurotransmission in Purkinje cells.

Using CBI TMS protocol, the results of our study showed that CBI is decreased in HE patients. Cerebellar TMS was used for the first time in patients with HE. It is considered a sensitive tool to investigate the cerebello-cortical pathway, which turned out to be affected in HE patients. Cerebellar TMS is believed to activate Purkinje cells leading to inhibition of the dentate nucleus consecutively resulting in suppression of the contralateral motor cortex (Ugawa et al., 1991; Groiss and Ugawa, 2013). CBI TMS protocol has been proved its usefulness through many previous experiments. It was affected in lesions involving either the cerebellum or the dentato-thalamo-cortical pathway (Ugawa et al., 1997; Groiss and Ugawa, 2012). Accordingly, the present results suggest an affection somewhere within the cerebello-thalamo-cortical pathway in HE. Since CBI protocol allows only for the investigation of the complete cerebello-thalamo-cortical pathway the exact localization of the site of affection within this pathway still needs to be shown (Groiss and Ugawa, 2012). However, one area likely affected in HE may be the middle cerebellar peduncles (MCP). Consistently, earlier studies repeatedly reported on the presence of hyperintense lesions in the MCP in T2 MRI images in HE patients with ataxia (Uchino et al., 2004; Furukawa et al., 2009). Another possibility would be the affection of the dentate nucleus of the cerebellum as shown previously in a patient with chronic liver cirrhosis presented with encephalopathy, extrapyramidal symptoms and ataxia, where the MR finding showed abnormal signal foci in the dentate nucleus (Lee et al., 1998).

Another possible explanation for this lack of CBI in HE patient is atrophy or degeneration of the Purkinje cells in this disease resulting in disinhibition of the dentato-thalamo-cortical pathway. This interpretation may be consistent with *in vitro* experiments providing evidence of cerebellar degeneration with significant loss of Purkinje cell caused by gliosis in rats with hyperammonemia. (García-Lezana et al., 2017). However, loss of Purkinje cells is expected to increase with disease progression (Balzano et al., 2018), which would in turn lead to stronger disinhibition in more severely affected patients.

However, in our study HE subgroup analysis revealed increased CBI in HE grade 2 in comparison to mHE, the degree of inhibition in HE grade 2 being comparable with healthy controls. This speaks against above interpretation of Purkinje cell loss but could rather reflect increased GABAergic tone in Purkinje cells. A possible explanation why we do not see stronger inhibition in HE2 compared to healthy controls is that it may partially be masked by the affection somewhere within the dentato-thalamo-cortical pathway, possibly in the MCP in HE. Consistent with this idea we observed a correlation between the degree of CBI and the degree of ataxia in terms of SARA score suggesting a gradual increase of inhibition with increasing disease severity. Furthermore, the correlation analysis revealed a trend for correlation between CFF and degree of CBI, also supporting the notion that increase of GABAergic tone in the Purkinje cells relates to disease severity. However, we can not fully rule out some kind of imbalance in inhibition at early stages of the disease, maybe due to some compensatory mechanisms, surprisingly normalizes at later stages of the disease.

Our results are also consistent with our previous work showing decreased GABAergic inhibition in the motor cortex in manifest HE patients (Groiss et al., 2019). Here, we investigated motor cortex

excitability of patients with HE at different disease stages using paired pulse TMS protocols and found stage dependent reduction of short-interval intracortical inhibition suggesting reduced GABAergic inhibition (Groiss et al., *in press*). It is conceivable that increased cerebellar GABA-ergic inhibition leads to disinhibition of the disynaptic cerebello-thalamocortical pathway resulting in reduced GABA-ergic inhibition of the motor cortex. This idea is also supported by earlier animal experiments, which revealed increased GABAergic neurotransmission in the cerebellum and decreased GABAergic neurotransmission in the cerebral cortex (Cauli et al., 2009a, 2009b). Cauli et al. explained the increased GABAergic tone in the cerebellum of hyperammonemic rats by the increased extracellular GABA and tetrahydrodeoxy-corticosterone, a neurosteroid that enhances GABA_A receptor activation, as well as the decreased GABAergic tone in the cerebral cortex as a result of decreased amount and sensitivity of functional GABA_A receptors (Cauli et al., 2009a, 2009b).

A limitation of our study is the small sample size for subgroup analysis. However, patient recruitment was limited due to the fact that BS-AMT was quite high in patients with HE and therefore, rather high intensities for cerebellar stimulation was necessary. Unfortunately, this led to relevant discomfort in some patients and reduced tolerability of the measurements.

In conclusion, reduction of cerebellar inhibition in HE patients suggests affection somewhere within the cerebello-thalamo-cortical pathway likely due to lesions of the middle cerebellar peduncles. However, the degree of cerebellar inhibition increased with disease severity supporting the notion that the increase of GABAergic tone in the Purkinje cells is dependent on the disease stage. The increased GABAergic tone in Purkinje cells leading to increased CBI may be balanced or masked by affection within the dentato-thalamo-cortical tract, both of which are affected in HE patients. These results are in accordance with previous data from animal studies suggesting an increase of the GABA-ergic tone at cerebellar level and a decreased GABA-ergic tone in the motor cortex.

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Conflict of interest

TJB, MB, MJ, DF, AA, DH, AS and SJG declare no conflicts of interest.

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