Cerebellar-Motor Cortex Connectivity: One or Two Different Networks?

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Abstract (250 word limit)

Anterior-posterior (AP) and posterior-anterior (PA) pulses of transcranial magnetic stimulation over primary motor cortex (M1) appear to activate distinct interneurone networks that contribute differently to two varieties of physiological plasticity and motor behaviours (Hamada et al., 2014). The AP network is thought to be more sensitive to online manipulation of cerebellar (CB) activity using transcranial direct current stimulation. Here we probed CB-M1 interactions using cerebellar-brain inhibition (CBI). Transcranial magnetic stimulation (TMS) over the cerebellum produced maximal CBI of PA-evoked EMG responses at an interstimulus interval of 5ms (**PA-CBI**), whereas the maximum effect on AP responses was at 7ms (AP-CBI), suggesting that CB-M1 pathways with different conduction times interact with AP and PA networks. In addition, paired associative stimulation using **ulnar** nerve stimulation and PA TMS pulses over M1, a protocol used in human studies to induce cortical plasticity, reduced **PA-CBI** but not AP-CBI, indicating that cortical networks process cerebellar inputs in distinct ways. Finally, **PA-CBI** and AP-CBI were differentially modulated after performing two different types of motor learning tasks that are known to process cerebellar input in different ways. The data presented here are compatible with the idea that applying different TMS currents to the cerebral cortex may reveal cerebellar inputs to both the premotor cortex and M1. Overall, these results suggest there are two independent CB-M1 networks that contribute uniquely to different motor behaviours.

Significance Statement (120 word limit)

Connections between the cerebellum and primary motor cortex (M1) are essential for performing daily life activities, as damage to these pathways can result in faulty movements. Thus, developing and understanding novel approaches to probe this pathway following movement is critical to

advancing our understanding of the pathophysiology of diseases involving the cerebellum. Here, we show evidence for two distinct cerebellar-cerebral interactions using cerebellar stimulation in combination with directional transcranial magnetic stimulation (TMS) over M1. These distinct cerebellar-cerebral interactions respond differently to physiological plasticity and diverse motor learning tasks and furthermore, may represent separate cerebellar inputs to premotor cortex and M1. Overall, we show that directional TMS can probe two distinct cerebellar-cerebral pathways that likely contribute to independent processes of learning.

Introduction

Converging evidence from non-invasive brain stimulation (NIBS) and neuroimaging studies in healthy individuals and in patients with neurological damage has shown that the cerebellum plays a critical role in optimizing motor control and motor learning by refining motor inhibition (Diedrichsen *et al.*, 2005; Wolpert *et al.*, 2011). The integrity of these actions are reliant, in-part, on the connections between cerebellum and cerebral areas via cerebellar-thalamic-cortical connections, as damage of this pathway may result in ataxia or dysmetria of movements (Koziol *et al.*, 2014). These connections have also been implicated in the pathophysiology of dystonia (Argyelan *et al.*, 2009) and other conditions. Thus, gaining further insights into cerebello-cortical connectivity is important if we are to understand the mechanisms that play a role in the pathophysiology of these complex disorders, as targeting the cerebellum with NIBS may be capable of reducing their symptoms (Koch *et al.*, 2014; Porcacchia *et al.*, 2019).

In humans, it is possible to investigate cerebello-cortical interactions using the cerebellar-brain inhibition (CBI) method. If a conditioning **transcranial magnetic stimulation (TMS)** pulse is given to the lateral cerebellum, it suppresses corticospinal EMG responses evoked by TMS over contralateral **primary motor cortex (M1)** some 5 - 7 ms later (Ugawa *et al.*, 1995; Pinto & Chen, 2001). The cerebellar TMS is postulated to activate Purkinje cells which in turn inhibit neurons **the deep cerebellar nuclei**, thus reducing any ongoing activity in the di-synaptic excitatory pathway to motor cortex (Galea *et al.*, 2009b). **CBI is sensitive to behaviors where error-based learning mechanisms are required to learn new sensorimotor relationships (Schlerf *et al.*, 2015; Spampinato & Celnik, 2017; 2018). Specifically, a release of CBI follows learning, which has been suggested to reflect long-term depression of Purkinje cells (Jayaram *et al.*, 2011; Spampinato *et al.*, 2017) as described in models of motor learning (Ito, 2012). Therefore, studying these cerebellar-M1 connections can importantly identify physiological contributions arising from the cerebellum that is associated with from error-driven learning.**

TMS can also be used to provide insights about distinct subsets of interneurons within M1. Indeed, changing the direction of the current in M1 can activate independent sets of synaptic inputs to corticospinal neurons termed I-waves, **that reflect indirect depolarization of axons projecting monosynaptically (early I-waves) and polysynaptically (late I-waves) to output neurons (Ni *et al.*, 2011; Delvendahl *et al.*, 2014; Di Lazzaro & Rothwell, 2014). Posterior-to-anterior (PA)-induced currents to the central sulcus, consistently recruits early I-waves, whereas anterior-to-posterior (AP)-currents tend to solely activate late I-waves that result in delayed corticospinal activity** (Di Lazzaro & Ziemann, 2013). Recent work has shown that excitability changes produced by neurons **recruited by AP current directions** are more dependent on cerebellar activity than those produced with **a PA current direction** (Hamada *et al.*, 2014). However, these results rely on transcranial direct current stimulation (TDCS) over the cerebellum, an interventional approach that has had mixed results in eliciting changes in cerebellar-dependent learning tasks (Jalali R et al. 2017) and in triggering consistent changes in cerebellar excitability (Add Galea 2009 and Doeltgen 2015).

In the present study, we have therefore used CBI to probe directly the interaction of cerebellum with AP- and PA-networks in the cerebral cortex. Previous studies have only investigated CBI using PA stimulation over M1. Since responses to PA and AP stimulation of M1 may recruit distinct sub-populations of corticospinal neurons (Witham *et al.*, 2016) and behave differently when probed with **intra-cortical inhibition and sensory afferent inhibition (Hanajima *et al.*, 1998; Hannah and Rothwell, 2017)**, we first compared the effect of CBI on the response to PA and AP pulses. In a follow-up experiment, **we utilized a plasticity to target PA-inputs rather than AP-inputs (i.e. repetitive stimulation of PA currents)** and found that it had a differential effect on AP-CBI and PA-CBI. The results of both sets of experiments suggested that cerebellar inputs have highly specific connections with different interneuronal pathways in M1. Finally, we show how cerebellar interactions with PA- and AP- inputs are modulated by two distinct types of motor learning tasks that process input from the cerebellum in different ways.

Methods

Participants

A total of thirty-eight right-handed healthy young volunteers (26 females; mean age ± SD, 25.3 ± 3.7 years, range 18–35 years) participated in this study. None of the subjects had neurological disorders or contraindications to TMS, as well as no history migraines, psychiatric disease, metallic implants, drug or alcohol abuse (Rossi et al., 2009). Each experiment was conducted in accordance with the *Declaration of Helsinki* on the use of human subjects. **Experiment 1 (n=12)** was approved by the Johns Hopkins University School of Medicine Institutional (JHMI) Review Board and conducted at JHMI. **Experiments 2 (n= 14) and Experiment 3 (n=12)** was conducted at University College London (UCL) and approved by the ethics committee UCL. All participants at each institution were provided written informed consent before participating in the study. **The duration of each testing session lasted between 60 to 90 minutes.**

Neurophysiological Assessments

Electromyography (EMG) Recordings

Surface electrodes (Ag–AgCl) were placed over first dorsal interosseous (FDI) muscle for recording motor evoked potentials (MEPs) caused by magnetic stimulation. Subjects were instructed to maintain the hand in a relaxed position throughout the **entire** experiment while EMG activity was monitored. The signal was amplified (gain, 1000), band-pass filtered (20 Hz-3 kHz), digitized at a frequency of 2 kHz, and stored for offline analysis by CED 1401 hardware (Cambridge Electronic Design). Data was stored on another computer to complete off-line analysis using a variety of custom Matlab scripts (MathWorks, MA, USA).

Transcranial Magnetic Stimulation (TMS) over M1

We stimulated the left M1 using a 70-mm-diameter figure-of-eight coil TMS coil (Magstim 200^2) in order to elicit an MEP of the first dorsal interosseous (FDI) muscle of the right hand. We used a

neuronavigation system (BrainSight; Rogue Research) to ensure consistency of stimulation over the "hot spot" of the FDI muscle for throughout the entire. Previous works have demonstrated that changing the TMS current flow across hand area of the motor cortex elicits different descending volleys. For instance, applying posterior–anterior directed current (PA current) preferentially recruits early I-waves, whereas AP current tends to elicit late I-waves (Day *et al.*, 1989; Di Lazzaro *et al.*, 1998; Di Lazzaro *et al.*, 2001). In this study, we placed the TMS coil tangentially to the scalp to induce either (1) PA-directed currents, with the handle pointed either backward at a 45° angle to the midline or (2) AP-directed currents, by placing the coil handle 180° to the PA currents. For each coil orientation, we also determined the resting motor threshold (rMT), defined as the minimum intensity needed to evoke MEPs of ≥50 μV in 5 out of 10 trials (Rossi *et al.*, 2009). After finding rMT intensity, we determined the stimulator output intensity needed to evoke MEPs of about 1mV in peak-to-peak amplitude. For all TMS measures

Cerebellar-M1 Connectivity (CBI)

To assess changes in the connectivity between the cerebellum and M1, we used a well-established paired-pulse technique that consists of delivering a TMS conditioned stimulus (CS) over the right cerebellar hemisphere 5ms prior to administering a test stimulus (TS) over the left M1 (Ugawa *et al.*, 1995; Pinto & Chen, 2001; Daskalakis *et al.*, 2004). We stimulated the cerebellum using a 110mm double-cone coil (110mm mean diameter, Magsitm). To avoid stimulation of the pyramidal tract with the cerebellar TMS, the intensity for cerebellar stimulation was set at 5% below the brainstem active motor threshold (BaMT) as described in previous studies (Werhahn *et al.*, 1996; Galea *et al.*, 2009a). Specifically, BaMT was tested with a 110-mm-diameter double-cone coil centred over the inion with the stimulator current directed downward and was defined as the nearest 5% stimulator output that elicited a MEP of 50 μV in a slightly contracted FDI muscle. If the BaMT was not observed at 75% of the maximum stimulator output (MSO) MSO, then 70% MSO was used to avoid participant discomfort (Schlerf *et al.*, 2015). **Across all studies, we found nine individuals that displayed a BaMT response**

under 75% MSO (mean cerebellar conditioning intensity = 68.57 ± 0.75). To assess CBI, the double-cone coil was then placed over the right cerebellar cortex 3cm lateral to the inion, with the stimulator current directed downward (Hardwick *et al.*, 2014; Ginatempo *et al.*, 2019; Spampinato *et al.*, 2019). Moreover, the TS over left M1 was delivered using a 70-mm-diameter figure-of-eight coil and the intensity was set to a stimulator output that elicited ~1 mV MEP response. **This required the presence of two experimenters: one to hold the figure of eight coil over M1 guided by neuronavigation and another individual to hold the double-cone coil over the marked cerebellar target (the location was marked on the participant's head).**

To measure CBI, the averaged MEP amplitudes of 15 single-pulse TMS responses over M1 were compared to the 15 paired-test plus conditioned responses. This was done for each CBI assessment across all experiments. When changes in M1 excitability occurred either via a plasticity protocol (experiment 2) or motor learning task (experiment 3), the intensity of stimulation for the TS were adjusted to elicit similar MEP amplitudes. Trials in which any preceding background muscle activity were excluded. Moreover, any MEPs below the 50 uV threshold were excluded from the analysis.

Paired associative stimulation (PAS)

We used PAS as a protocol to induce the plastic changes in M1 (Stefan *et al.*, 2000; Wolters *et al.*, 2003). PAS consisted of 200 electrical stimuli of the right ulnar nerve paired with consecutive TMS pulses over the FDI hot spot at a rate of 0.2 hertz. Electrical stimulation was applied through a bipolar electrode using a constant current square wave pulse (duration, 1 ms) at an intensity of 3x the perceptual threshold (Digitimer, Welwyn Garden City, UK). TMS pulses were delivered with PA currents and with an intensity of 120%RMT. We opted to test PAS pairings with the electrical stimuli preceding the PA-M1 TMS by 21.5ms, as this interval has been suggested to not depend on the cerebellum (Hamada *et al.*, 2012). **Indeed, the aftereffects of PAS applied at longer intervals (i.e. 25 ms) can be blocked with cerebellar transcranial direct current stimulation, whereas the effects of**

PAS 21.5ms remained unchanged. Thus, it is speculated that the afferent pathway from the stimulated nerve to M1 traverses the cerebellum in PAS 25, whereas PAS 21.5 is thought to represent direct interactions of leminscal inputs.

Behavioural Tasks

Sequential Visuomotor Isometric Pinch-Force Task (SVIPT)

We used the Sequential Visual Isometric Pinch Task (SVIPT) to assess motor skill learning (Reis *et al.*, 2009; Marquez et al., 2013; Cantarero *et al.*, 2015; Spampinato and Celnik, 2017; 2018). Participants were seated in front of a vertical 20-inch computer screen monitor and held a force transducer between the thumb and the index finger of the right hand. **Isometrically squeezing the force transducer controlled the rightward movement of an on-screen cursor**. The objective of the task was to move the cursor between a HOME position and 5 targets (HOME-1-HOME-2-HOME-3-HOME-4-HOME-5). To move the cursor effectively, participants had to learn the logarithmic relationship between pinch force production and cursor movement. We quantified the amount of motor skill learning (SVIPT) by assessing changes in the speed-accuracy trade-off function (SAF). To do this, we used the following equation to estimate SAF throughout performance is the skill measure:

$$Skill\ Measure = \frac{1 - \text{error rate}}{\text{error rate}(\ln(\text{movement time})^{b})}$$

where error-rate was defined as the proportion of unsuccessful trials (defined as the amount of trials with at least one under- or over-shooting movements), movement time was calculated as the total trial time (time between movement onset and when the cursor reached the final target), and parameter b was fixed at 5.424, a value determined from an independent sample of subjects who performed the same task **(for more details, ref to the supplementary text of Reis *et al.*, 2009)**. Error-rate and movement time parameters were averaged for each block consisting of 30 consecutive trials.

Sequence Training

Participants performed on a 9-element sequence task requiring responses to visually cued boxes on a computer-screen (Spampinato & Celnik, 2018). They were instructed to generate responses to the sequentially ordered visual cues by pressing their index finger on a directly mapped computer key. Sequence targets were displayed using a horizontal display of three square stimuli, representing a direct left to right mapping of three neighbouring keys ('Z' leftmost, '*X*' *middle*, '*C*' rightmost). The following sequence was presented: 'CZXZCXCXZ' and amount of and order of sequence trials was identical for all subjects. A trial began with a fixation cross, which was displayed for 2 s. Participants were then instructed to respond to the cued-stimulus responded as quickly as possible. Importantly, only once the correct response was selected, the next target in the sequence became immediately highlighted. In other words, if an incorrect response was made, the sequence was paused and only resumed following the appropriate key response. After 10 consecutive trials, participants were given feedback on their performance (average movement time and error-rate) and were informed to improve their score on the subsequent trials.

Experimental Procedures

Experiment 1

We tested how applying cerebellar stimulation at varying inter-stimulus intervals (ISIs) to distinct M1 coil orientations (i.e. probing PA vs AP currents) affected CBI. Thus, we tested both AP- and PA-CBI at ISIs of 3, 5 and 7ms. Specifically, we selected these intervals as the suppressive effects measured between ISIs of 5 and 7ms have suggested to be cerebellar in nature (Werhahn *et al.*, 1996; Fisher *et al.*, 2009) (Figure 1a). Intervals above 7ms are likely to result in suppression from peripheral nerve fibres in the brachial plexus (Werhahn *et al.*, 1996; Hardwick *et al.*, 2014), whereas intervals less than 5ms do not produce strong suppressive effects likely due to amount of time needed for cerebellar stimulation to hit cortical targets. Thus, 3ms was used as a control. For each coil orientation, we recorded 15 responses for all four conditions: one corresponding to the test stimulation (TS) over M1

alone and for CBI measured the three different ISIs. The magnitude of CBI was computed as the ratio of the conditioned MEP over the unconditioned MEP.

Experiment 2

To determine if the two separate cortico-neuronal networks process cerebellar inputs in distinct ways, we administer a plasticity protocol to M1 PA-inputs. To do this, we measured AP-CBI and PA-CBI at their preferential ISI, prior to and following standard paired associative stimulation (PAS) plasticity protocol (Figure 2a). Importantly, we administered the repeated pairs of electrical stimuli to the **ulnar** nerve and PA-TMS at an interval of 21.5 (i.e. PAS at 21.5) since this technique can modulate the plasticity of PA-M1 excitability without affecting cerebellar activity (Hamada M et. al 2014). Fifteen responses were recorded for the orientation-related ISI condition (e.g. 5 ms for PA, 7 ms for AP) and fifteen responses for the TS alone.

Experiment 3

Subjects participated in a counter-balanced crossover design in which participants trained on the SVIPT in one session and on the Sequence Training in another session (Figure 3a). The order of training sessions was randomized and separated by at least 48 hours. For each motor learning task, participants completed 150 trials (5 blocks; 1 block = 30 trials) where movement times and accuracy measures were recorded. In addition to behavioural measurements, we investigated how learning two distinct motor tasks affected AP-CBI and PA-CBI at their preferential ISIs (5 and 7 ms respectively). To do this, we measured CBI (assessed with the different M1 current inputs) prior to, during and after individuals learned each task.

Data Analysis

For all data statistical analyses, SPSS (IBM; Version 20) was used and effects were considered significant if $p \leq 0.05$. All data are given as means ± SEM. We used separate polynomial nested repeated measures of ANOVA ($ANOVA_{RM}$) for all behavioural and physiological measures. When

significant differences were identified, we used Bonferroni-Holm corrected *post hoc* analysis to account for multiple comparisons.

Experiment 1 and 2: To determine changes in CBI in experiment 1, we used $ANOVA_{RM}$ with within-subject factors ORIENTATION (PA, AP) and ISI (3ms, 5ms, 7ms). **To determine whether PAS modulates baseline M1 excitability and CBI responses between the two coil orientations, we performed $ANOVA_{RM}$ with factors TIME (PRE, POST) and ORIENTATION (PA, AP).**

Experiment 3: To assess the performance of the SVIPT, we measured differences in the skill measure by using $ANOVA_{RM}$ with TIME (Block1, Block2…Block5) as within-factors measure. **To assess sequence learning, we compared differences in the online error-rate and movement time. We used two separate $ANOVA_{RM}$ for these measures with TIME (Block1, Block2…Block5) as the within-subject factor.** To determine changes in CBI between the two coil orientations, we used separate **$ANOVA_{RM}$ with between subjects factor GROUP (Training, Random) and within-subject factors TIME (PRE, P1, P2) and ORIENTATION (PA, AP). Here, GROUP represents whether individuals were given a learnable task (Training) compared to ones given a randomized version of the task (Random; i.e. no motor learning is expected). TIME represents distinct stimulation time points before training (PRE), after one block of training (P1) and at the end of five training blocks (P2) This was done separately for each task.**

Results

Experiment 1 Results

PA-CBI measured at 5ms ISI and AP-CBI at 7ms ISI elicits the largest CBI response

We investigated the effect of M1 coil orientation (PA and AP currents) on cerebellar-M1 connectivity responses by measuring CBI at different inter-stimulus-intervals (ISIs; Figure 1b). $ANOVA_{RM}$ revealed a significant main effect of ISI ($F_{2, 44} = 17.947$, $p = 0.001$) and ISI × ORIENTATION interaction ($F_{2, 44} = 6.556$, $p = 0.003$). Post-hoc analysis revealed CBI responses measured with PA-currents at an ISI of 5ms and 7ms were significantly different when compared to measures of CBI at an ISI of 3ms (respectively, $p = 0.001$ and $p = 0.017$). On the other hand, for AP current inputs to M1, CBI was only observed at an ISI of 7ms when compared to an ISI of 3ms ($p = 0.001$), **while no difference was found between 5ms and 3ms ($p = 0.117$). This indicates that M1 AP-currents are more responsive to cerebellar stimulation when a longer interval is given between stimulation sites.** Interestingly, post-hoc analysis also revealed differences between the coil orientations at different ISIs. Here, we found that CBI measured at 5 ms was more prominent for PA-currents ($p = 0.039$), whereas AP-currents elicited a stronger effect at 7 ms ($p = 0.025$). Importantly, we found no evidence of CBI for either M1 current direction when measured at an ISI of 3ms ($p = 0.361$). The results from experiment 1 demonstrate that assessing cerebellar-M1 connectivity at different timings between TMS pulses and with different coil orientations over M1, elicits distinct CBI responses.

Insert Fig 1 here

Experiment 2 Results

PA-PAS modulates only PA-CBI

In the next experiment, we sought to determine whether this result reflects distinct processing of cerebellar inputs within M1. To do this, we measured PA-CBI vs. AP-CBI at their preferential ISI, prior to and following standard PA-paired associative stimulation (PAS). We asked whether applying standard PAS 21.5 would result in orientation-specific changes in cerebellar-M1 connectivity (**Figure**

2). Importantly, we selected PAS 21.5 rather than PAS 25, as this method is shown to depend on **PA current directions, but not AP current directions** (Hamada

M et al. 2014), and moreover does not alter general cerebellar excitability that would likely modulate both PA- and AP-CBI.

We first assessed whether PAS 21.5 specifically modulated M1 excitability assessed with PA current directions. ANOVA$_{RM}$ revealed a significant main effect of TIME ($F_{1, 14}$ = 4.838, p = 0.047) and TIME × ORIENTATION interaction ($F_{1, 14}$ = 5.121, p = 0.041). Post-hoc analysis revealed that PAS 21.5 increased M1 excitability when measured with PA currents (p = 0.008) whereas M1 excitability measured with AP currents remained unchanged (p = 0.493). We then compared PA- and AP-CBI prior to and immediately after PAS 21.5 (Figure 2c. **ANOVA$_{RM}$** revealed a significant main effect of TIME ($F_{1, 14}$ = 4.484, p = 0.043) and TIME × ORIENTATION interaction ($F_{1, 14}$ = 6.556, p = 0.029). We found that PAS 21.5 **decreased** PA-CBI (p = 0.04), but did not modulate AP-CBI (p = 0.47), indicating that CB-M1 interactions are different for the two M1 neural networks. Importantly, this change was not due to the PAS-induced M1 excitability changes as we adjusted the stimulator intensity to evoke an MEP with a peak-to-peak amplitude of ∼1 mV (S1mV) when recoding CBI for each current direction and time-point (**see Figure 2c inset**). **ANOVA$_{RM}$ confirmed that our adjustment of the test pulse intensity was valid across CBI time points as differences in test pulse amplitudes were found for factors ORIENTATION ($F_{1, 14}$ = 6.556, p = 0.639) or TIME ($F_{1, 14}$ = 6.556, p = 0.563).**

Insert Fig 2 here

Experiment 3 Results

Participants learned the Skill and Sequence Tasks

To assess SVIPT skill learning, we quantified the skill score (see Equation 1), which incorporates the movement time and error-rate for each block of 30 trials. **ANOVA$_{RM}$** revealed a significant effect of BLOCK ($F_{4,44}$ = 7.173; $p < 0.001$). Specifically, post-hoc analysis showed individuals were better at performing the last block of training compared to the initial block ($p < 0.045$), indicating that

participants improved skill performance within the training session (Figure 3B). Additionally, to assess learning in the sequence task, we calculated the average movement time and error-rate for each training block of 30 sequence trials (Figure 3C). **$ANOVA_{RM}$** revealed that participants were able to improve their movement time ($F_{4, 44}$ = 61.859; $p < 0.001$), without compromising changes in their performance error-rate ($F_{4, 44}$ = 0.619; $p = 0.651$).

PA-CBI decreases early in skill learning, whereas AP-CBI reduced only late in skill learning

We compared the amount of cerebellar-M1 connectivity changes for each orientation (PA-CBI, AP-CBI) prior to skill learning (Pre), following one-block of training (P1) and after five blocks of training (P2) (Figure 3D). $ANOVA_{RM}$ revealed a significant CBI changes for TIME ($F_{2,44} = 5.807$; $p = 0.006$) and TIME x GROUP interaction ($F_{2,44} = 7.801$; $p = 0.001$). PA-CBI showed a selective reduction following early skill learning when compared to baseline ($p = 0.006$), and in addition was significantly different compared to value of AP-CBI early-on in learning the task ($p = 0.037$). On the other hand, AP-CBI specifically reduced at the end of skill training when compared to both baseline ($p = 0.013$) and P1 ($p = 0.024$), and furthermore was found significantly different from the PA-CBI changes found at the end of training ($p = 0.047$). This result suggests that the reduction of CBI assessed for each coil-orientation is sensitive to the amount of training individuals undergo when acquiring a new skill.

PA-CBI is reduced only early during motor sequence learning

As previous investigations showed that CBI changes in more simplistic motor sequence learning mimic the changes found in more complex motor skill learning (Spampinato & Celnik, 2018), we also test whether AP-CBI and PA-CBI changed differently when learning a simple sequence. We observed a significant TIME x GROUP interaction of these factors ($F_{2, 26}$ = 3.49, $p < 0.05$). Here, we found that only PA-CBI **was significantly reduced** following early sequence training (P1) when compared to baseline responses ($p = 0.013$). This effect was also significantly different from AP-CBI changes following early

sequence learning (*p = 0.036*). These results indicate that only cerebellar-M1 connectivity measured with PA over M1 is sensitive to learning motor sequences (Figure 3D).

Insert Fig 3 here

Discussion

The present study directly assessed cerebellar-M1 connectivity using CBI. We found that output from cerebellum interacts differentially with the M1 networks activated preferentially by PA and AP current pulses. This is, **in-part**, consistent with prior investigations that used cerebellar tDCS as an indirect method to probe cerebellar-M1 interactions (Hamada *et al.*, 2014) . Specifically, we found that CBI produced maximum suppression of MEPs evoked by PA pulses at an interstimulus interval of 5ms whereas it was maximum at 7ms for AP-MEPs. Moreover, paired associative stimulation of **ulnar** nerve input with PA-MEPs suppressed CBI of PA-MEPs, but had no effect on AP-MEPs. Finally, CBI to the AP- and PA-sensitive networks was modulated in unique ways depending on the type of motor skill task individuals were required to learn. We argue below that this is consistent with the idea that different cortical motor circuits are involved in different behaviours and that they interact with input from the cerebellum in distinct ways.

CBI of AP- and PA-evoked MEPs: Two Different Pathways?

The cerebellum is a critical part of the motor network involved in learning new skills that integrate both motor and cognitive components. This notion is supported by the known mass connections the cerebellum has to both motor and non-motor regions of the cerebral cortex through cerebello-thalamo-cortical pathways (Kelly & Strick, 2003; Bostan *et al.*, 2013). Moreover, evidence from electrophysiological recordings has shown these pathways are quite complex: the axons of these pathways can terminate on both excitatory and inhibitory neurons (Na *et al.*, 1997; Daskalakis *et al.*, 2004), via di-synaptic or polysynaptic connections (Yamamoto *et al.*, 1984; Futami *et al.*, 1986; Holdefer *et al.*, 2000) and innervate across cortical layers I, III, V and VI (Ando *et al.*, 1995; Na *et al.*, 1997). Anatomically, cerebellar projections to leg and arm areas of M1 arise in different parts of the dentate nucleus, as do projections to premotor and other frontal regions (Dum & Strick, 2003).

Cerebellar TMS is unlikely to be very selective and probably engages all of these intricate pathways, perhaps via recruitment of Purkinje cells. Since these have a suppressive influence on deep cerebellar

nuclei (Celnik, 2015), cerebellar-TMS will tend to reduce any ongoing activity in these connections to M1. Our results show that the time at which this has maximum effect on M1 is different for the networks activated by PA- and AP-current pulses and is compatible with the idea that there are separate connections from cerebellum onto these networks with different conduction times. It should be noted that the latency of MEPs evoked by AP-stimulation is 2-3 ms longer than for PA-stimulation, which is similar to the difference in optimal CBI timing. However, if the time of maximum CBI had been related to MEP latency, then we would have expected the best interval for CBI of AP-MEPs would have been 2-3 ms earlier than CBI of PA-MEP rather than 2-3 ms later.

A recent modelling study suggests that whereas PA pulses preferentially activate the synaptic terminals of layer 5 pyramidal neurons located in the rostral lip of the central sulcus, AP pulses activate terminals in the crown of the gyrus that could originate in premotor regions (Aberra *et al.*, 2018). If so then it is possible that PA-CBI directly targets the excitability of M1 layer 5 pyramidal neurons and reduces the amplitude of PA-evoked MEPs. In contrast, AP-CBI could target excitability of neurons in premotor cortex that project to M1. Reducing the tonic level of excitation in these pathways could then reduce the excitability of the gyral M1 neurons that receive input from the synaptic terminals of premotor axons activated by AP-pulses. The additional time for cerebellar inputs to traverse the premotor cortex would mean that AP-MEPs would be maximally suppressed at longer ISIs than PA-MEPs. Retro-virus tacking studies in non-human primates have revealed cerebellar projections to prefrontal, dorsal and ventral premotor, supplementary motor and parietal areas (Kelly & Strick, 2003) and in macaques inputs have been demonstrated from Purkinje cells to area F2r, an area considered similar to dorsal premotor cortex in humans (Hashimoto *et al.*, 2010). **Moreover, projections of the cerebellar dentate to the arm areas of M1 and premotor cortex are anatomically divided as distinct output channels** (Middelton & Strick, 2000; Bostan *et al.,* 2014) **Therefore, it is conceivable that at least two distinct connections (i.e. premotor and M1) from the cerebellum can be realized by changing the current direction applied with TMS.**

Although the time for optimal CBI differed, the level of suppression of AP- and PA-MEPs was very similar. At first sight, this seems contrary to a previous result of Hamada and others who found that TDCS over cerebellum suppressed AP-MEPs more than PA-MEPs, suggesting that CBI is stronger to AP networks. However, it is difficult to compare the two results. TDCS can affect M1 excitability through the entire complex network of cerebellar inputs to M1. Its effect on MEPs is the sum of all these effects. In contrast, CBI only gives information on the most rapid connections, making it impossible to compare the two.

Evidence from paired associative stimulation (PAS)

In the second experiment, we examined the effect of PAS 21.5 on CBI. The facilitatory after-effects of PAS 21.5 are thought to depend on PA-networks rather than AP-networks (Hamada *et al.*, 2012; Hamada *et al.*, 2014). As expected, we found modulation of PA-CBI with no effect on the amount of CBI tested with AP-MEPs, confirming the selectivity of PAS 21.5 to PA-networks. However, we found that this intervention suppressed the PA-CBI effect. We can only speculate on the mechanism involved in this. It may relate to the fact that the PAS method repeatedly pairs a single volley of afferent input that is carefully timed to arrive just prior to the TMS pulse. However, other inputs, for example those from the cerebellum, occur randomly and have no specific timing relationship to TMS or afferent input. It could be that under these circumstances, the strength of these inputs is reduced by PAS with the effect that CBI is no longer effective. Whatever the mechanism, the results again suggest that there are two distinct cerebellar-cortical neuronal networks.

Evidence from Motor Learning

Previous investigations have suggested that separate interneuron circuits within M1 have distinct roles in how they contribute to learning a variety of motor behaviours (Hamada *et al.*, 2014). Specifically, cerebellar-dependent error-based forms of learning engage interneurons activated by AP M1 TMS. Here, we had individuals learn two distinct motor tasks that likely involve the cerebellum in different ways and tested if it changed the CBI connectivity to AP- vs PA- networks. One task required

individuals to learn only a sequence of movements, whereas the other task required individuals to learn how to control a new device in a novel environment (i.e. sensorimotor-map), along with performing a sequence of movements. The latter skill has been suggested to rely more heavily on a cerebellar-dependent error-based learning in order to learn the dynamics of the skill task (Diedrichsen *et al.*, 2010; Taylor & Ivry, 2014). Indeed, although learning a sequence of movements can elicit changes in cerebellar excitability, these changes were previously only found to modulate activity at the initial stage of learning (Spampinato & Celnik, 2018). The results of the present study are consistent with prior studies as **the PA-CBI effect changes early-on** for both skill tasks whereas **AP-CBI is reduced** only after individuals begin to optimize their performance of the complex skill, suggesting that the motor learning processes engaged in this task differentially recruit the pathways probed with directional TMS.

A possible explanation for the present results is that PA- and AP-inputs to cortical-spinal neurons have different roles in learning the different tasks. For instance, the early changes in PA-CBI occur during a period in which performance is initially quite poor. This could be a time during which cerebellar involvement in both tasks could reflect activity of an error correction mechanism (Shadmehr *et al.*, 2010), supporting the idea that the cerebellum updates motor commands during error-dependent learning (Herzfeld *et al.*, 2014). The later changes in AP-CBI perhaps indicate that performance is being optimized. The cerebellum has been implicated in "automatizing" behaviour (Balsters *et al.*, 2013) in tasks where extended practice lessens the overall cognitive demand to perform a task (i.e. performed entirely implicitly) (Doyon & Benali, 2005). Interestingly, recent work in rodents has revealed that firing rates within the cerebellum and premotor cortex become strongly coupled only as behavioural performance improves, although they are initially dissimilar (Wagner *et al.*, 2019). The results would therefore be consistent with our hypothesis that AP-CBI operates via cerebellar inputs to premotor cortex. As the premotor cortex has been previously found as a neural substrate flexible for recombination and efficient encoding of complex motor behaviours, it is possible that inputs to this

region from the cerebellum may help shape the overall representation of the task in a behaviour where learning a new sensorimotor mapping is required.

Implications and Limitations

These results do not come without some limitations. First, it remains unclear exactly what and where TMS stimulates. Thus, the idea that **currents applied in AP direction** over M1 may recruit pre-motor inputs remain speculative. Future studies may consider investigating the effect of applying a "virtual" lesion to pre-motor areas to see if pathways interacting with AP currents are specifically modulated. Second, we cannot disentangle whether the differences in AP- and PA-CBI found here reflect two anatomically distinct sets of cerebello-cortical fibres or if the differences found here are due to the same cerebellar input being processed differently by two distinct populations of cortical neurons. Finally, our explanation as to why these two distinct circuits behave differently when assessing interactions between PAS and motor learning is speculative and will require further study. Despite these limitations, we suggest that two distinct cerebellar-cerebral interactions can be disentangled using cerebellar stimulation in combination with directional TMS over the cerebral cortex. Our hypothesis is that these pathways can contribute to independent processes of learning, depending on the behaviour individuals are required to learn. They also present a novel approach to probing connections between the cerebellum and cerebral cortex that can be used in future patient studies to understand the pathophysiology of diseases in which the cerebellum is implicated to play a functional role.

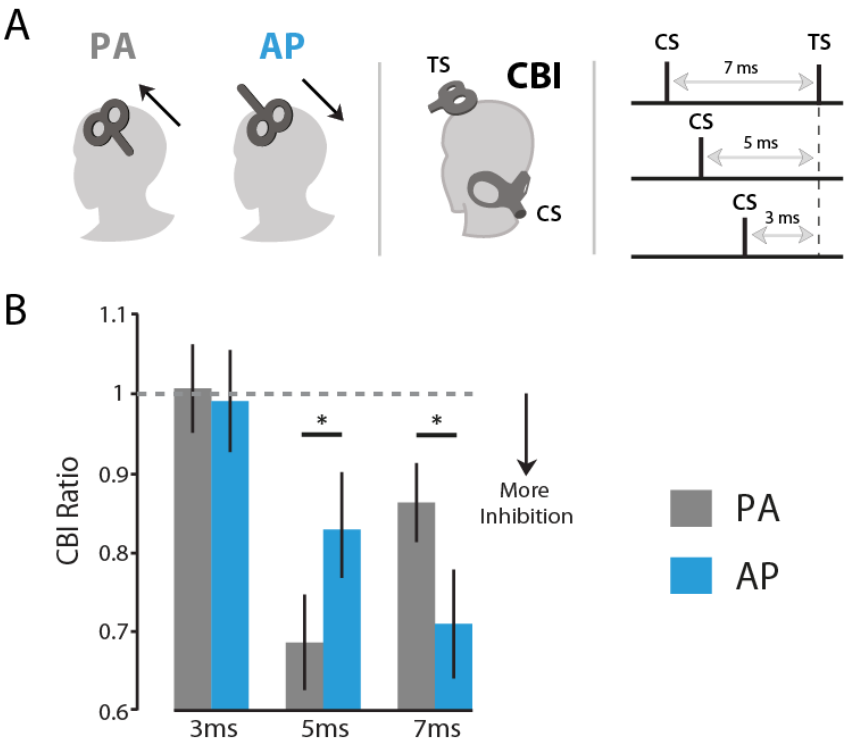

Figure 1.

Effects of different current directions applied over M1 on cerebellar-M1 connectivity (CBI)

(A) Schematic representation of experiment 1. This experiment tested how applying cerebellar stimulation (conditioning stimulus; CS) at varying inter-stimulus intervals prior to applying a test stimulus (TS) to distinct M1 coil orientations (PA vs AP currents) effected CBI. Thus, we tested both PA- and AP-CBI at ISIs of 3, 5 and 7ms. (B) Bar graphs and vertical error bars depict the mean ± SEM of the CBI ratio. X-Axis represents different inter-stimulus intervals (ISI: 3, 5 and 7ms) applied for both PA and AP-CBI. *y*-Axis shows CBI as the ratio of the conditioned vs. the unconditioned MEP Ratio values < 1 represent inhibition, whereas ratios > 1 represent facilitation. CBI measured with the different M1 currents were matched for test MEP amplitude values ~1mV. Here, we found that PA-CBI measured with an ISI of 5ms elicited stronger CBI than AP-CBI. On the other hand, when measured at 7ms, AP was produced a significantly larger effect than PA-CBI. Importantly, we did not find any evidence of CBI for either M1 current direction when measured at 3ms ISI since this interval is presumably too short to elicit any cerebellar effects to cortical regions.

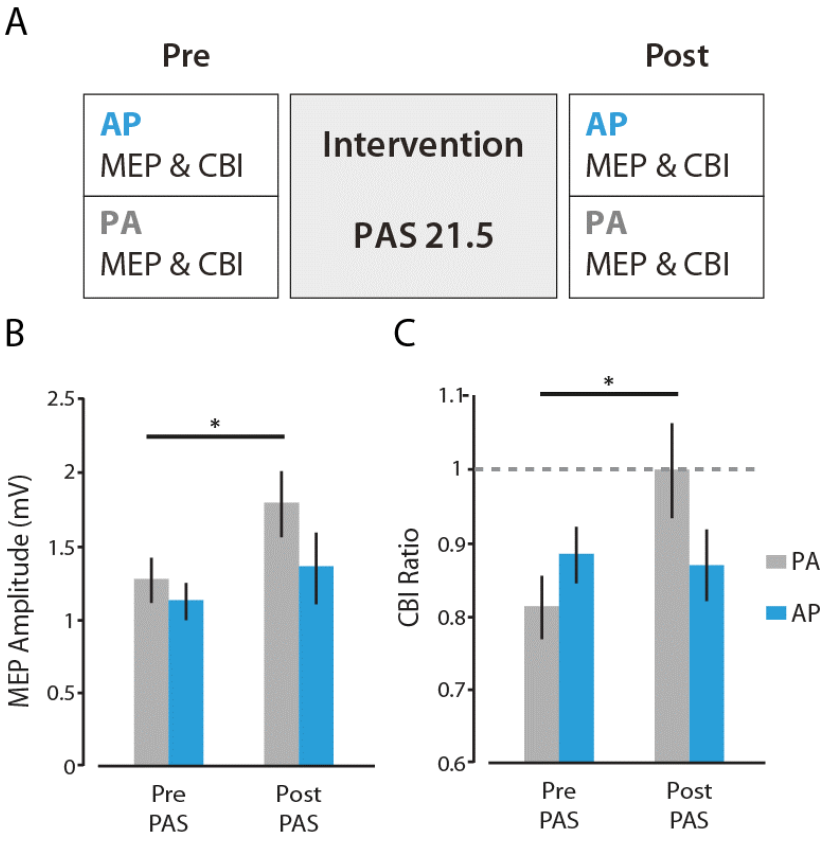

Figure 2.

Paired associative stimulation (PAS) only modulates PA-CBI and not AP-CBI

(A) Schematic representation of experiment 2. For each current direction, M1 excitability and CBI was measured prior to and after participants received paired associative stimulation (PAS). PAS effects on (B) M1 excitability and (C) CBI. Bar graphs and vertical error bars depict the mean ± SEM, where data presented with grey colours represent PA-currents applied to M1 and blue colours represent AP-currents. *y*-Axis shows the (A) MEP amplitudes and (B) CBI ratio values, while the X-axis displays these values prior to (pre) and after (post) administration of PAS protocol. (A) We found that only MEP amplitudes assessed with PA currents significantly increased with PAS. (B) Similarly, we show that PA-CBI, and not AP-CBI, is decreased due to the PAS protocol. Importantly, this change was not due to the PAS-induced M1 excitability changes (i.e. results above) as we adjusted the stimulator intensity to evoke an MEP with a peak-to-peak amplitude of ~1 mV (S1mV) when recording CBI for each current direction and time-point (inset).

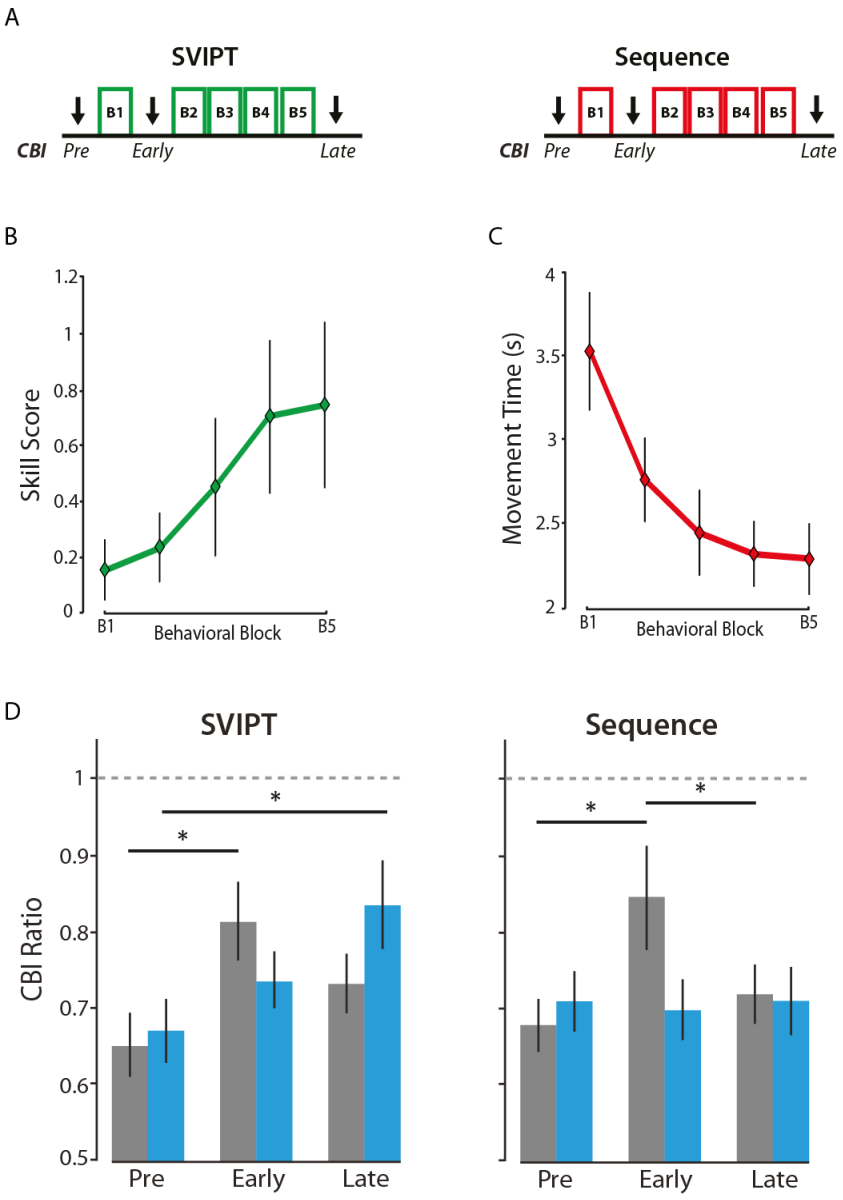

Figure 3.

PA- and AP-CBI changes with learning distinct motor tasks

(A) Schematic representation of the experiment. Participants completed five blocks consisting of 30 trials of a motor skill task (green) and of a simple sequential task (red). Cerebellar connectivity to both AP- and PA sensitive interneurons was assessed prior to training (Pre) and after the first (P1) and final training behavioural block (P2). (B-C) Behavioural Results. (B) Skill Task Results. The X-axis depicts training blocks (average of 30 trials) and the y-axis represents the average skill measure scores. Higher values depict participant's ability to improve their speed-accuracy trade-off performance of the task.

The data represent the mean ± SEM for each block. (C) Sequence Learning Results. The X-axis depicts training blocks (average of 30 trials) and the y-axis represents the average movement time to perform the entire sequence. Of note, participants were able to improve their movement time while maintaining the same level of accuracy. (D) Cerebellar-M1 connectivity changes. Bar graphs and vertical error bars depict the mean ± SEM of the CBI ratio when measured with AP-M1 (grey) and PA-M1 (blue) currents at each stimulation time-points (Pre, P1, P2). Dashed horizontal line depicts the normalized unconditioned MEP amplitude and the dashed vertical line represents the separation between different motor learning tasks. Of note, CBI for each M1 current direction was measured at their preferred ISI (i.e. 7ms for AP-CBI; 5ms for PA-CBI). We found decreases in PA-CBI that occurred early on for both the motor skill and sequence tasks, however, AP-CBI reduced only later as individuals optimized their motor skill performance.

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