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RAPID REPORT

Cerebellar modulation of human associative plasticity

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Key point

- Increases in the strength of synaptic connections in the motor cortex (long term potentiation) can be induced in humans by repetitively pairing peripheral nerve stimuli and motor cortex transcranial magnetic stimuli given 21–25 ms apart – paired associative stimulation (PAS).
- This 'associative plasticity' effect has been assumed to relate to synchronicity between sensory input and motor output, with a similar mechanism proposed to underlie effects at all inter-stimulus intervals.
- Here we show that modulation of cerebellar activity using transcranial direct current stimulation can abolish associative plasticity in the motor cortex, but only for sensory/motor stimuli paired at 25 ms, not at 21.5 ms.
- The results indicate that human associative plasticity can be affected by cerebellar activity and that at least two different mechanisms are involved in the effects previously reported in studies using PAS at different inter-stimulus intervals.

Abstract Paired associative stimulation (PAS) is a method commonly used in human studies of motor cortex synaptic plasticity. It involves repeated pairs of electrical stimuli to the median nerve and transcranial magnetic stimulation (TMS) of the motor cortex. If the interval between peripheral and TMS stimulation is around 21–25 ms, corticospinal excitability is increased for the following 30–60 min via a long term potentiation (LTP)-like effect within the primary motor cortex. Previous work has shown that PAS depends on the present and previous levels of activity in cortex, and that it can be modified by motor learning or attention. Here we show that simultaneous transcranial direct current stimulation (TDCS; 2 mA) over the cerebellum can abolish the PAS effect entirely. Surprisingly, the effect is seen when the PAS interval is 25 ms but not when it is 21.5 ms. There are two implications from this work. First, the cerebellum influences PAS effects in motor cortex; second, LTP-like effects of PAS have at least two different mechanisms. The results are relevant for interpretation of pathological changes that have been reported in response to PAS in people with movement disorders and to changes in healthy individuals following exercise or other interventions.

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Abbreviations AMT, active motor threshold; APB, abductor pollicis brevis; cDC, cerebellar transcranial direct current stimulation; HFO, high-frequency oscillation; LTP, long term potentiation; MEP, motor evoked potential; MSO, maximum stimulator output; PAS, paired associative stimulation; PC, Purkinje cell; RMT, resting motor threshold; SAI, short afferent inhibition; SEP, sensory evoked potential; SI_{1mV}, stimulus intensity required to elicit a 1 mV MEP; STDP, spike timing dependent plasticity; TDCS, transcranial direct current stimulation; TMS, transcranial magnetic stimulation; TS, test stimulus.

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Introduction

Paired associative stimulation (PAS) is commonly used to induce long term potentiation (LTP)-like synaptic plasticity in the corticospinal system (Stefan *et al.* 2000). Repeated pairing of an electrical stimulus to the median nerve with a transcranial magnetic stimulation (TMS) pulse given 21.5–25 ms later to the motor cortex leads to a long lasting increase in corticospinal excitability. Because the interval between median nerve and cortex is important, PAS effects are thought to be mediated by spike timing-dependent plasticity (STDP): if the interval is less than the latency of the N20 sensory evoked potential (SEP) then the after-effects tend to be inhibitory, whereas if it is longer they are excitatory (Stefan *et al.* 2000; Wolters *et al.* 2003). PAS is sensitive to drugs that interact with NMDA receptor activity (Stefan *et al.* 2002; Wolters *et al.* 2003); is changed by, and changes, some forms of behavioural motor learning (Ziemann *et al.* 2004; Rosenkranz *et al.* 2007); is abnormally enhanced in individuals with dystonia (Quartarone *et al.* 2003; Weise *et al.* 2006); and is depressed in people with Parkinson's disease withdrawn from medication (Morgante *et al.* 2006) and in Huntington's disease (Crupi *et al.* 2008).

Because of its timing in relation to the N20, PAS is thought to involve rapid conduction of sensory input via the dorsal column–medial leminiscal system to sensory thalamus and from there to motor cortex via either a relay in sensory cortex or perhaps direct thalamic inputs to motor cortex (Stefan *et al.* 2000; Wolters *et al.* 2003). It is usually assumed that this early arriving input is the only one that contributes to the PAS effect. If so then the only difference between PAS at 21.5 ms (PAS21.5) and 25 ms (PAS25) is the timing of subsequent TMS pulse given to motor cortex. The slight difference in timing within a few milliseconds would still mean that inputs arrived well within the LTP time window for STDP (Dan & Poo, 2006), and hence both protocols should produce similar LTP-like effects.

However, unlike animal STDP experiments in which only one pathway or one connection between pre- and postsynaptic neurons is investigated (Dan & Poo, 2006), there are several potential pathways which could convey sensory information to cortex after stimulation of median nerve. For example, PAS at short intervals (21.5 ms) could involve direct transmission of sensory information from thalamus to motor cortex (Stefan *et al.* 2000; Wolters *et al.* 2003), whereas PAS at longer intervals might also involve, for example, (1) synapses activated after post-processing of that input, or (2) later arriving inputs coming via a slower relay in sensory cortex, or even via other structures such as the cerebellum (Wiesendanger, 1973; Butler *et al.* 1992) which is known to receive strong sensory input from spinal cord and projects to cerebral cortex (Dean *et al.* 2010). In such cases, PAS elicited with short interstimulus intervals might have different properties to PAS at longer intervals.

The present experiments were designed to investigate this possibility by using transcranial direct current stimulation (TDCS) over the posterior scalp in order to interfere with cerebellar influences on sensory processing. It has been shown to have direct effects on cerebellar function (Galea *et al.* 2011; Jayaram *et al.* 2011) and may even affect cerebellar relay of sensory input to cortex. For example, Galea *et al.* (2011) found that a form of behavioural learning (visuomotor rotation during arm reaching) that is known to depend on synaptic plasticity in motor cortex can be speeded by this cerebellar TDCS (cDC), an effect attributed to modification of sensory error signals transmitted via cerebellum.

We hypothesized that cDC might be able to influence processing of sensory signals within the PAS25 time window in two ways. First, it might be able to modulate the size of any late arriving input transmitted via an indirect (transcerebellar) route (Wiesendanger, 1973; Butler *et al.* 1992). If so then we would expect it to change the PAS25 effect without influencing PAS21.5. Second, previous work has shown that unilateral cerebellar lesions reduce the amplitude of the P24 component of the somatosensory evoked potential (SEP) without changing earlier responses (Restuccia *et al.* 2001). The P24 (sometimes labelled P25) is thought to represent cortical processing of input arriving during the N20, via rapidly conducting leminiscal inputs and therefore a smaller P24 suggests that the lesion removed some tonic excitatory influence on sensory processing. It is possible therefore that cDC could change the level of this cerebellar input and indirectly interact with PAS25, but not PAS21.5, which relies on earlier inputs that are not affected by cerebellum. To probe this possibility further we tested whether cDC has any effect on leminiscal sensory input to cortex by measuring SEPs before and after cDC.

Methods

Subjects

Eighteen healthy human volunteers (six females; age, 31.8 ± 7.4 years (mean $\pm$ SD), 21–52 years) participated in the study. None of the subjects had contraindications to TMS (Rossi *et al.* 2009). All participants signed an informed consent form before participating in the experiment. The experiment conforms to the guidelines stated in the *Declaration of Helsinki* and was approved by the local Ethics Committee.

TMS and EMG recordings

TMS was delivered from a Magstim 200^2 stimulator (Magstim) every 4.5–5.5 s. A figure-of-eight coil (outer

winding diameter 70 mm) was held tangentially on the scalp at an angle of 45 deg to the midsagittal plane with the handle pointing laterally and posteriorly. Motor cortex excitability was measured as the peak-to-peak amplitude of the motor-evoked potential (MEP) generated by single pulse TMS. TMS was applied to the motor cortex representation of the right abductor pollicis brevis (APB) muscle. The motor hot spot was defined as the point where a magnetic stimulus of constant, slightly suprathreshold intensity consistently elicited an MEP of the highest amplitude. Subjects sat comfortably in a chair with both arms resting on a pillow placed on their lap. Surface electromyography (EMG) electrodes (Ag–AgCl) were placed over the right APB in a belly–tendon montage for recording the MEPs. The signals from the EMG electrodes were amplified (gain, 1000), bandpass filtered (20 Hz–3 kHz), digitized at a frequency of 5 kHz, and stored in a laboratory computer for later offline analysis by Signal software and CED 1401 hardware (Cambridge Electronic Design, Cambridge, UK).

Paired associative stimulation (PAS)

PAS consisted of 180 electrical stimuli of the right median nerve at the wrist paired with a single TMS over the hotspot of right APB muscle at a rate of 0.2 Hz. Electrical stimulation (square wave pulse; stimulus duration, 0.2 ms) was applied at an intensity of three times the perceptual threshold using a constant current generator (Digitimer, Welwyn Garden City, UK). TMS was applied at an intensity required to elicit a 1 mV MEP (SI_{1mV}). The effects of PAS given with an interstimulus interval of 25 ms (PAS25) and of 21.5 ms (PAS21.5) between peripheral and TMS stimuli were tested (see below). Both protocols have been shown previously to induce a long lasting increase in MEP amplitude (Stefan *et al.* 2000; Weise *et al.* 2006). Subjects were instructed to look at their stimulated hand and count the peripheral electrical stimuli they perceived. The MEPs evoked in the APB were displayed online during the intervention to control for the correct coil position and stored for off-line analysis.

Cerebellar transcranial direct current stimulation (cDC)

cDC was applied to the cerebellum as described previously (Galea *et al.* 2009) simultaneously with PAS. In brief, cDC was delivered with an intensity of 2 mA using a commercially available DC stimulator (Eldith-Electro-Diagnostic & Therapeutic Systems GmbH, Germany, distributed by Magstim Co., Whitland, Dyfed, UK) through saline-soaked surface sponge electrodes (25 cm^2). One electrode was centred on the right cerebellar cortex, 3 cm lateral to the inion. The other electrode was positioned on the right buccinator muscle. Anodal or cathodal cDC was delivered over the cerebellum for 15 min. It has been shown that anodal DC increases and cathodal DC decreases the excitability of primary motor cortex and cerebellum (Nitsche & Paulus, 2000; Galea *et al.* 2009). At the onset of all interventions (anodal, cathodal, and sham), current was increased in a ramp-like manner. In the sham session, anodal cDC was applied for 30 s. At the offset of TDCS, the current was decreased in a ramp-like manner.

Experimental parameters

The resting and active motor thresholds (RMT and AMT), MEPs, recruitment curves and short afferent inhibition (SAI) were measured. These parameters were assessed before (baseline) and for up to 30 min (T0 and T30) after PAS with cDC.

RMT was defined as the lowest intensity that evoked a response of about 50 μV in the relaxed APB in at least 5 of 10 trials (Rossi *et al.* 2009) and AMT was defined as the lowest intensity that evoked a small response (>100 μV) in more than 5 of 10 consecutive trials when subjects maintained a slight contraction of the right APB ($\sim$10% of the maximum voluntary contraction). The stimulus intensity was changed in steps of 1% of the maximum stimulator output (MSO).

Thirty MEPs were recorded with a stimulus intensity of SI_{1mV} at baseline. SI_{1mV} was kept constant throughout the experiment. The mean amplitude was calculated for the data obtained before and after PAS with cDC in each single subject.

For the recruitment curves, the intensities of the single TMS stimuli were individually expressed relative to RMT at baseline. Ten MEPs each were recorded at 100, 120 and 140% RMT. For each subject, the peak-to-peak amplitudes were measured on each single trial to calculate the mean amplitude at each stimulus intensity.

SAI was examined at ISIs of 15 ms, 20 ms and 25 ms (Tokimura *et al.* 2000). The median nerve was stimulated at wrist through bipolar surface electrodes (cathode proximal, rectangular pulse of 0.2 ms duration). Stimulus intensity was adjusted to produce a slight thumb twitch. The intensity of the test stimulus (TS) was set at SI_{1mV}. Twelve trials were recorded for each condition and randomly intermixed with 24 trials of TS alone. Stimuli were given every 4.5–5.5 s. TS intensity was adjusted after intervention, if required, in order that the MEP had the same size as at baseline. The ratio of the mean amplitude of the conditioned response to that of the TS response was calculated for each condition in each subject. These individual mean ratios were then averaged to give a grand mean ratio.

Experiment 1: modulation of PAS25 during cDC

Twelve subjects participated in a crossover study, which consisted of three randomized ordered sessions, each separated by at least 1 week (anodal-PAS25, cathodal-PAS25 and sham-PAS25). The order of physiological assessments, as described above, before and after intervention remained consistent across sessions.

Experiment 2: timing specificity of PAS modulation by cDC

Eight subjects who were also enrolled in experiment 1 participated in a crossover study, which consisted of two randomized ordered sessions, separated by at least 1 week (anodal-PAS21.5 and sham-PAS21.5). We did not evaluate the effects of cathodal cDC on PAS21.5 because the results of experiment 1 showed that anodal cDC had more consistent effects on PAS25 compared with cathodal cDC (see results). We measured RMT, AMT and MEPs before and after intervention.

Experiment 3: effects of cDC on somatosensory evoked potentials (SEPs)

To test the effect of cDC on cortical processing of sensory input, SEPs were recorded before and after anodal or sham cDC in a crossover design. Eight subjects of whom six were not enrolled in experiments 1 and 2 participated in the study. Anodal or sham cDC was performed as described above. The details for SEP recordings are described elsewhere (Hamada *et al.* 2007). In brief, before and after cDC, SEPs were elicited by electrical stimulation (square wave pulse; stimulus duration, 0.2 ms) of the right median nerve at the wrist (cathode proximal) at an intensity of 1.2 times motor threshold and at a frequency of 3 Hz using a constant current generator (Digitimer). Three recording electrodes were placed at the C3′ (2 cm posterior to C3 of International 10–20 system), the spinous process of C6 (CV6), and Erb's point with Fz reference. The impedance between the electrodes was kept below 5 kΩ. SEPs were recorded in epochs from −10 to 100 ms triggered by the electrical stimuli. The sampling rate was set at 8 kHz, and the potentials were amplified and filtered between 10 and 3000 Hz. We collected and averaged 1000 responses in each trial, and two trials were examined in each session to ascertain the reproducibility. SEPs were recorded in two sessions (before and after cDC or sham).

Amplitudes of N9 (N9 onset to N9 peak), N20 (N20 onset to N20 peak), and P25 (N20 peak to P25 peak) were measured in each trial. We also measured high frequency oscillations (HFOs) from C3′-Fz montage obtained by digitally filtering raw SEPs from 500 to 1000 Hz (Butterworth type, 12 dB/octave); two parts of the HFOs were defined as described previously (Hamada *et al.* 2007), the early HFOs (HFOs from the onset to peak of N20) and the late HFOs (HFOs later than the N20 peak). Average amplitudes of both HFOs were measured. The early subcomponent of HFOs is thought to be generated by activity of thalamus and thalamo-cortical fibres, whereas the late subcomponent is thought to be related to inhibitory interneuronal activity of sensory cortex (see review by Ozaki & Hashimoto, 2011).

Data analysis and statistics

The baseline physiological parameters are given in Table 1. The comparability of these stimulus parameters between each experimental session were tested by Student's paired *t* test (two-tailed).

MEP amplitudes at each time point were averaged, normalized to baseline and entered into a two-way repeated measures analyses of variance (rmANOVA) with factors 'cDC' (anodal-PAS25, cathodal-PAS25 and sham-PAS25 for experiment 1 and anodal-PAS21.5 and sham-PAS21.5 for experiment 2) and 'TIME' (T0 and T30). In order to evaluate LTP-like plasticity following PAS with cDC, one-way ANOVA was employed with a main factor of 'TIME' (baseline, T0 and T30) using absolute MEP values in each experimental session. For experiment 2, normalized MEP amplitudes were entered into three-way rmANOVA with factors 'cDC' (anodal and sham), 'PAS' (PAS25 and PAS21.5) and 'TIME' (T0 and T30). In this case, the MEP values from eight subjects in experiment 1 were used for direct comparison between PAS25 and PAS21.5. The slopes of the recruitment curve were quantified by a linear regression analysis for all data points between 100 and 140% RMT as described by others (Cirillo *et al.* 2009). RMT, AMT, the slopes of the recruitment curve, and SAI at each ISI were entered into one-way ANOVA with a main factor of 'TIME' (baseline, T0 and T30) to evaluate time course of these values. For experiment 3, amplitudes of N9 (N9 onset to N9 peak), N20 (N20 onset to N20 peak) and P25 (N20 peak to P25 peak) were measured in each trial and averaged. They were entered into three-way rmANOVA with factors 'Component' (Comp) (N9, N20, and P25), 'cDC' (anodal and sham cDC) and 'TIME' (before and after cDC). Early and late HFOs were also measured in each trial, averaged, and entered into three-way rmANOVA (within subjects factors, 'Comp' (early and late HFOs), 'cDC', and 'TIME'). The Greenhouse–Geisser correction was used if necessary to correct for non-sphericity; P values <0.05 were considered significant. Bonferroni's *post hoc* test or paired *t* tests (two-tailed) was used for further analyses. Data were analysed using software (SPSS v. 19.0 for Windows; SPSS Inc.). All data are given as means ± standard error of the mean (SEM).

Table 1. Physiological data (means ± SEM)

	RMT (%)	AMT (%)	MEP size (mV)	Test MEP (mV) for SAI		
	Baseline			Baseline	T0	T30
Experiment 1 ($n = 12$)						
Sham-PAS25	40.7 ± 2.7	32.2 ± 1.7	0.94 ± 0.09	1.05 ± 0.20	0.98 ± 0.14	1.06 ± 0.21
Anodal-PAS25	40.2 ± 2.8	31.1 ± 1.6	1.02 ± 0.07	0.85 ± 0.10	1.13 ± 0.12	1.18 ± 0.20
Cathodal-PAS25	40.9 ± 2.5	32.3 ± 1.6	1.05 ± 0.09	0.85 ± 0.10	1.04 ± 0.14	0.94 ± 0.11
Experiment 2 ($n = 8$)						
Sham-PAS21.5	40.0 ± 2.6	33.0 ± 1.3	0.92 ± 0.06	—	—	—
Anodal-PAS21.5	39.9 ± 2.5	33.5 ± 2.0	0.90 ± 0.07	—	—	—
Sham-PAS25	39.9 ± 3.0	32.9 ± 2.3	0.85 ± 0.09	—	—	—
Anodal-PAS25	40.1 ± 3.0	32.1 ± 2.2	1.02 ± 0.08	—	—	—

Experiment 3 ($n = 8$)

	N9 (μV)		N20 (μV)		P25 (μV)	
	Before	After	Before	After	Before	After
Sham	6.5 ± 1.3	7.9 ± 1.3	1.3 ± 0.3	1.5 ± 0.3	3.4 ± 0.6	3.4 ± 0.6
Anodal	8.0 ± 1.6	9.0 ± 1.8	1.3 ± 0.4	1.4 ± 0.4	3.5 ± 0.6	3.4 ± 0.6

	Early HFO (μV)		Late HFO (μV)	
	Before	After	Before	After
Sham	0.10 ± 0.01	0.12 ± 0.02	0.10 ± 0.02	0.11 ± 0.02
Anodal	0.12 ± 0.02	0.13 ± 0.02	0.14 ± 0.03	0.15 ± 0.03

RMT, resting motor threshold; AMT, active motor threshold; MEP, motor evoked potential; SAI, short afferent inhibition; PAS, paired associative stimulation; HFO, high frequency oscillation.

Results

Baseline physiological data are shown in Table 1. No differences were found between each experimental session. All subjects completed the three (experiment 1) and two (experiment 2 and 3) sessions without complications.

Experiment 1

Consistent with previous reports, sham-PAS25 induced a lasting increase in MEP size. However, anodal-PAS25 and cathodal-PAS25 did not induce any consistent changes in excitability (Fig. 1*A*). Two-way rmANOVA revealed significant effects of cDC (F (2, 22) = 11.538, $P = 0.0004$), but no significant effects of TIME (F (1, 11) = 0.118, $P = 0.737$) nor a cDC × TIME interaction (F (2, 22) = 2.249, $P = 0.129$). *Post hoc* analysis with Bonferroni's correction revealed a significant difference between sham-PAS25 and anodal-PAS25 ($P = 0.0003$) and sham-PAS25 and cathodal-PAS25 ($P = 0.021$) (Fig. 1*B*). There was no difference between anodal-PAS25 and cathodal-PAS25 ($P = 0.267$).

Following sham-PAS25, MEP sizes were significantly increased at T0 and T30 compared to baseline MEP values (one-way ANOVA, F (2, 22) = 8.351, $P = 0.002$)

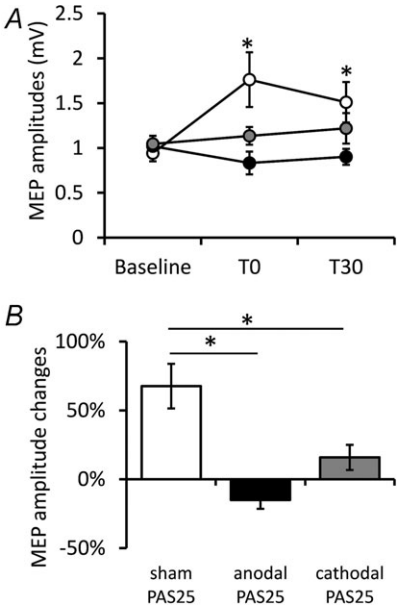

Figure 1. Modulation of PAS25 by cerebellar DC
A, mean (±SEM) amplitudes of MEPs before (baseline), immediately after (T0), and after 30 min (T30) of PAS25 with cerebellar DC (sham-PAS25, white circles; anodal-PAS25, black circles; cathodal-PAS25, grey circles). Asterisks indicate significant difference from baseline MEP sizes ($P < 0.05$ with Bonferroni's multiple correction). *B*, grand average of normalized MEPs at T0 and T30 to baseline in each session. Asterisks indicate significant difference from sham-PAS25 ($P < 0.05$ with Bonferroni's multiple correction).

(Fig. 1*A*). *Post hoc* analysis with Bonferroni's correction revealed significant increases at T0 ($P = 0.002$) and T30 ($P = 0.034$). In contrast, anodal cDC and cathodal cDC abolished LTP-like plasticity after PAS25 (anodal-PAS25, one-way ANOVA, effects of TIME, $F\ (2,\ 22) = 2.161$, $P = 0.139$; cathodal-PAS25, $F\ (2, 22) = 1.320$, $P = 0.281$) (Figs. 1*A* and *B*).

RMT and AMT did not change after any intervention (Fig. 2*A*) (one-way ANOVA, $P > 0.2$ for all conditions). The slope of recruitment curve appeared to be steeper after sham-PAS25, but not after any other intervention (Fig. 2*B*).This was consistent with a trend towards an effect of TIME ($F\ (2,\ 22) = 2.923$, $P = 0.075$) in a one-way ANOVA suggesting that the slope of the recruitment curve increased after sham-PAS25. *Post hoc* analysis with Bonferroni's correction showed a trend for an increase in slope compared with baseline at T30 ($P = 0.077$). SAI at 20 and 25 ms did not change after any intervention (Figs. 2*C* and *2D*) (one-way ANOVA, $P > 0.3$ for all conditions). The test MEP sizes before and after intervention did not differ between each time point (Table 1).

Experiment 2

To investigate whether the cDC modulation of PAS was timing specific, we also explored anodal cDC effects on PAS21.5 (Fig. 3).

As expected, sham-PAS21.5 induced a lasting increase in MEP sizes. In contrast to PAS25 with anodal cDC, however, MEP sizes also were larger after anodal-PAS21.5. Three-way rmANOVA revealed significant effects of cDC ($F\ (1,\ 7) = 11.192$, $P = 0.012$), PAS ($F\ (1,\ 7) = 12.918$, $P = 0.009$), and PAS × cDC interaction ($F\ (1, 7) = 5.839$, $P = 0.046$). There was no effect of TIME ($P = 0.639$) nor any other significant interactions ($P > 0.1$). *Post hoc* paired *t* tests revealed significant differences between sham-PAS25 and anodal-PAS25 ($t = 3.091$, $P = 0.018$) and anodal-PAS25 and anodal-PAS21.5 ($t = -3.973$, $P = 0.005$), but not for any other combination ($P > 0.4$).

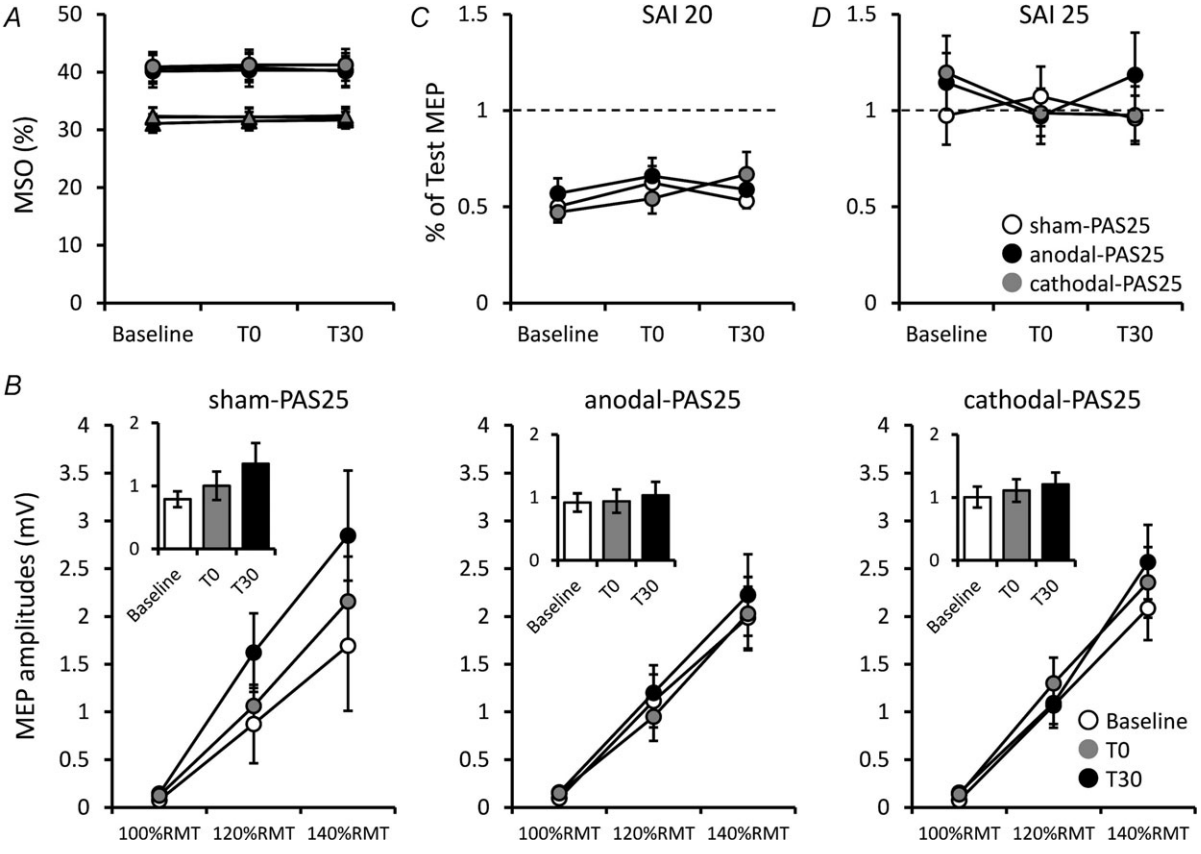

Figure 2. Motor thresholds, recruitment curves, and short afferent inhibition.
A, RMT and AMT before and after each intervention. RMT, circles; AMT, triangles. White, sham-PAS25; black, anodal-PAS25; grey, cathodal-PAS25. No significant effect of TIME in each condition was found. *B*, the recruitment curve before and after intervention (left, sham-PAS25; middle, anodal-PAS25; right, cathodal-PAS25). After sham-PAS25, there were strong trend for the effects of TIME ($F\ (2,\ 22) = 2.923$, $P = 0.075$) indicating time-dependent increase of slope of the recruitment curve. *Post hoc* analysis with Bonferroni's correction showed strong trend for the increase in the slope after T30 ($P = 0.077$). *C* and *D*, SAI changes in each condition (*C* for ISI at 20 ms; *D* for ISI at 25 ms). No significant effects of TIME in each condition were found.

Following sham-PAS21.5, MEP sizes were significantly increased at T30 compared to baseline (one-way ANOVA, F (2, 14) = 4.255, P = 0.036) (Fig. 3). *Post hoc* analysis with Bonferroni's correction revealed a significant increase at T30 (P = 0.041), but not at T0 (P = 0.178). Likewise, one-way ANOVA revealed significant effects of TIME (F (2, 14) = 4.653, P = 0.028) for anodal-PAS21.5. *Post hoc* analysis showed a significant increase of MEP at T30 (P = 0.029), but not at T0 (P = 0.195). Finally, RMT and AMT did not change after any intervention (one-way ANOVA, $P > 0.2$ for all conditions).

Experiment 3

To investigate whether cDC modulation of PAS was due to changes in excitability of sensory cortex and/or thalamus, we measured the amplitude of SEPs and HFOs before and after anodal cDC (Table 1). Three-way rmANOVA revealed significant a main effect of 'SEP Comp', but no significant effect of 'TIME', 'cDC', nor any other interactions (Table 2). We conclude that cDC had no effect on the early components of the median nerve SEP.

Table 2. ANOVA results for SEPs and HFOs (Experiment 3)

	SEP		HFO	
Factor	*F* value	*P* value	*F* value	*P* value
Comp	13.299	0.008	0.161	0.7
cDC	0.549	0.483	4.233	0.079
Time	4.388	0.074	1.897	0.211
Comp × cDC	1.051	0.341	2.566	0.153
Comp × Time	3.933	0.086	0.012	0.917
cDC × Time	0.225	0.650	0.244	0.636
Comp × cDC × Time	0.105	0.901	0.059	0.816

Discussion

We found that plasticity induced by PAS25 was blocked by concurrent anodal or cathodal TDCS over the cerebellum (cDC). In addition the effect was timing specific since plasticity induced by PAS21.5 was not blocked by anodal cDC. Below we consider the possible mechanisms of these effects and their implications for interpretation of previous studies using PAS and for future research.

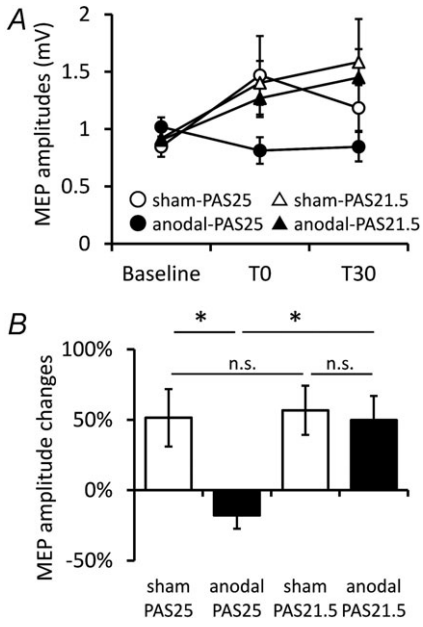

Figure 3. Modulation of PAS25 and PAS21.5 by anodal cDC
LTP-like plasticity of PAS25 was blocked, whereas that of PAS21.5 was unaltered. Asterisks, $P < 0.05$ with paired t tests.

Both PAS21.5 and PAS25 are accepted techniques for induction of LTP-like changes in the motor cortex (Stefan *et al.* 2000; Weise *et al.* 2006). Although PAS25 has been more frequently used (Kujirai *et al.* 2006; Nitsche *et al.* 2007; Rosenkranz *et al.* 2007; Cirillo *et al.* 2009), in particular for studies in patients with neurological disorders such as dystonia and Parkinson's disease (Quartarone *et al.* 2003; Ueki *et al.* 2006), there has not previously been any suggestion that the techniques differ in efficacy or mechanism. In fact it has been implicitly assumed that rapidly conducted dorsal column–medial leminiscal input is responsible for the facilitation at both timings (Stefan *et al.* 2000; Wolters *et al.* 2003; Weise *et al.* 2006). However, the spread of timing intervals, between 1–2 and 5 ms after the initial N20, means that later arriving input could travel through synaptic relays quite distinct from those involved in the classic N20. Observations on short interval afferent inhibition (SAI) are consistent with the idea that sensory input can have both early and late effects on motor cortex. A single electrical pulse to the median nerve suppresses the response to a subsequent TMS pulse applied at 20 ms. However the inhibitory effects decline at longer intervals (Tokimura *et al.* 2000) and are replaced by facilitation at around 25 ms (Fischer & Orth, 2011). The neuronal mechanisms for this gradual shift from inhibition to facilitation have not been well documented but it is suggestive evidence for multiple, time-dependent effects of sensory input on motor cortex.

We have shown that the LTP-like effects of PAS can be blocked by cDC when the interval between peripheral and motor cortical stimuli is long (25 ms), but not when it is shorter (21.5 ms). This strongly suggests that there are separate mechanisms mediating the effects of PAS at these two interstimulus intervals and the PAS25 effect is dependent upon the cerebellum.

TDCS over the cerebellum (cDC) could affect the response to PAS in a number of ways. One possibility is that cDC modulates processing within the sensorimotor cortex and/or thalamus by changing tonic levels

of activity in the cerebello-thalamo-cortical pathway. To test these possibilities we measured SEPs and HFOs before and after anodal cDC. The main components of the SEP relate to cortical processing of input arriving via fast conducting leminiscal pathways. Early HFOs are thought to reflect activity in thalamus and/or thalamo-cortical fibres, whereas the late HFO subcomponent might be related to the activity of inhibitory interneurons in superficial layers of sensory cortex (Ozaki & Hashimoto, 2011). There was no evidence that either SEP or HFOs were significantly modulated by cDC compared to sham. The result strongly suggests that excitability changes within sensorimotor cortex and/or thalamus are unlikely to contribute to cerebellar modulation of associative plasticity.

Alternative possibility to explain the timing specific PAS modulation of cDC is that sensory signals to the motor cortex arriving at 25 ms but not at 21.5 ms are conveyed by a longer pathway which includes the cerebellum, and that these sensory signals are directly modulated by cDC. This view is supported by the fact that the cerebellum receives sensory information (Dean *et al.* 2010), and that patients with cerebellar degeneration have abnormal sensory motor integration (Tamburin *et al.* 2003). Also it has been suggested in animal experiments that indirect sensory pathways to motor cortex with inputs arriving later than the direct leminiscal route involve cerebellum (Wiesendanger, 1973; Butler *et al.* 1992). The pathways might contribute little to the conventional SEP, especially if the input has greater temporal dispersion than the fastest leminiscal input and hence any changes in transmission would not have been evident in our SEP data. It is possible therefore that sensory input to cortex, arriving via this transcerebellar route, contributes to PAS at 25 ms; anodal and cathodal cDC might interfere with this pathway and therefore reduce the effect of PAS25.

The suggestion that the mechanism of PAS25 effects is cerebellar dependent assumes that the effect of cDC is only active upon the cerebellum (Galea *et al.* 2009), but we acknowledge that there are other possibilities. First, it is important to consider if the effects we have observed could be due to a general change in motor cortical excitability induced by cDC. However, any general change in excitability or even the non-specific skin sensation that can occur during cDC could not account for the timing specific occlusion of PAS that we saw. In addition, Nitsche *et al.* (2007) showed that directly changing cortical excitability by giving TDCS over M1 during PAS25 leads to homeostatic effects: concurrent anodal TDCS reversed the effect of PAS25 to inhibition, whereas cathodal TDCS prolonged the facilitatory effect of PAS25. Not only do these effects differ from the present results they also cannot account for the timing specific modulation of PAS that we observed. Also, basic measures of motor cortical excitability, such as the steepness of recruitment curves, or RMT, which can influence effects of PAS, are not altered immediately after cDC (Galea *et al.* 2009). Although we cannot completely exclude the possibility that these after-effects differ from the changes that occur during TDCS, it seems unlikely given previous results. Nitsche *et al.* (2005) found that after effects of TDCS on M1 on measures of cortical excitability were similar to those seen during concurrent application of TDCS (Nitsche *et al.* 2005, 2007)

Previous work suggests that TDCS over the rear of the scalp produces its effects by influencing the cerebellum (Galea *et al.* 2009, 2011; Jayaram *et al.* 2011), but there are a number of other neighbouring structures that could be influenced including brainstem pathways, and via their connections sensorimotor cortex and thalamus. At this stage, we have no clear evidence that cDC does not induce certain excitability changes in the brainstem. Thus, it is possible that excitability of sensory systems in the brainstem, such as medial lemniscus, spinothalamic tract, and cuneate nucleus, is altered during stimulation, leading to less effective sensory transmission to cortex. However, the brainstem MEP threshold and size, blink reflex and ipsilateral MEPs are not altered after a period of cDC (Galea *et al.* 2009). In addition, if brainstem sensory pathways were influenced by cDC, we would have expected PAS25 and PAS21.5 to be similarly modulated by cDC. The fact that cDC modulation of PAS was timing specific suggests this possibility is unlikely.

We found the response to PAS25 to be abolished by both anodal and cathodal cDC, which often are reported to have opposite effects on other parts of the cortex (Nitsche & Paulus, 2000). One explanation for the similar effects of anodal and cathodal stimulation is that any changes in the baseline excitability, either positive or negative, of Purkinje cell (PC) might significantly affect the efficiency of sensory transmission. The situation in the present experiments is not unusual since anodal and cathodal TDCS have been reported to have similar effects on adaptation motor learning (Orban de Xivry *et al.* 2011), cortical excitability changes induced by concurrent motor task (Antal *et al.* 2007), and working memory (Ferrucci *et al.* 2008).

Finally our results are consistent with the recent study showing that preconditioning by theta burst stimulation (TBS) protocol over the cerebellum could change the response to PAS25; inhibitory (i.e. continuous) TBS enhanced PAS25, whereas excitatory (i.e. intermittent) TBS suppressed the response to PAS25 (Popa *et al.* 2012). Although in the present study cathodal and anodal cDC had similar effects it is difficult to directly compare these results. Firstly, the timing for cerebellar modulation was different from ours (preconditioning *vs.* concurrent). In addition, although the interval between peripheral stimulus and TMS is identical in the two studies (25 ms), the inter-pair interval at which PAS is delivered is quite different (200 ms *vs.* 5 s). Also, TBS and TDCS could

affect the cerebellum in different ways, or at different locations. Early evidence suggests that cerebellar TBS changes motor cortical excitability (Koch *et al*. 2008, 2009), while cerebellar TDCS modulates cerebellar output without directly changing motor cortex excitability (Galea *et al*. 2009). This could be, for example, because TBS and TDCS affect different parts of the cerebellum; the part affected by TBS might have a tonic effect on motor cortex, whereas that affected by cerebellar TDCS may not have such an effect. Nevertheless, taken together these two sets of data are strong evidence that effects of PAS25 (but not PAS21.5) are cerebellar dependent.

Implications

The present findings provide important implications for research which has used PAS in the past. For example, PAS-induced plasticity using both PAS21.5 and PAS25 is enhanced in patients with organic dystonia (Quartarone *et al.* 2003; Weise *et al.* 2006). As both forms of PAS protocol have been considered to be interchangeable, these results are interpreted as a consequence of pathologically enhanced plasticity within sensorimotor cortex (Quartarone *et al.* 2003; Weise *et al.* 2006). However, cerebellar dysfunction has been shown to play a potentially important role in dystonia (Neychev *et al.* 2008; Teo *et al.* 2009; Sadnicka *et al.* 2012), and therefore it is possible that there is a dual mechanism for abnormalities in PAS response seen in dystonia, with the PAS25 effect potentially influenced by cerebellar dysfunction. Similar arguments can be put forward for abnormalities in PAS response reported in other conditions such as Huntington's disease (Crupi *et al.* 2008) and Parkinson's disease (Morgante *et al.* 2006). There are a number of reports showing that response to PAS is influenced by prior behavioural training (Ziemann *et al.* 2004; Rosenkranz *et al.* 2007). The effects are often interpreted as being due to changes in PAS-evoked plasticity in cerebral cortex. The present results show that modulation of PAS effects can occur because of changes in the cerebellum, a structure implicated in several forms of motor learning (Galea *et al.* 2011), providing an additional way in which PAS effects and motor learning might interact.

Conclusions

This study provides evidence that the cerebellum is involved in PAS-induced plasticity in a timing-specific manner. It has been generally accepted that PAS at short intervals (21.5 or N20 latency) and PAS25 share similar mechanisms in terms of induction of human associative plasticity. Instead, the present results provide evidence that PAS21.5 and PAS25 have different characteristics with important implications for research which uses PAS to investigate the pathophysiology of neurological disorders or the effects of behavioural learning.

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Author contributions

M.H. and J.C.R. conceived and designed the experiments. M.H., G.S., N.M. and A.S. collected the data. All authors participated in the analysis and interpretation of the data and in the drafting of the manuscript. All authors approved the final version of the manuscript. The authors declare no potential conflicts of interest relating to the subject of this report.

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