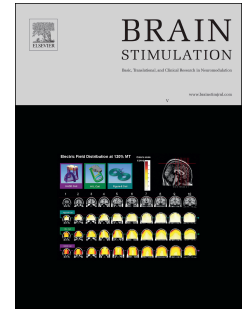


# Journal Pre-proof

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# Driving Hebbian plasticity over ventral premotor-motor projections transiently enhances motor resonance

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## Keywords

transcranial magnetic stimulation; cortico-cortical paired associative stimulation; action observation network; motor connectivity

## Highlights

- ccPAS is applied to manipulate the strength of the pathway between PMv and M1
- Enhancing PMv→M1 projections transiently increases motor resonance in M1
- This effect is indexed by an increased MEP facilitation during action observation
- No change is observed following active control and sham ccPAS protocols
- Motor resonance effects in the corticospinal system rely on the PMv→M1 pathway

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## Abstract

*Background:* Making sense of others' actions relies on the activation of an action observation network (AON), which maps visual information about observed actions onto the observer's motor system. This motor resonance process manifests in the primary motor cortex (M1) as increased corticospinal excitability finely tuned to the muscles engaged in the observed action. Motor resonance in M1 is facilitated by projections from higher-order AON regions. However, whether manipulating the strength of AON-to-M1 connectivity affects motor resonance remains unclear.

*Methods:* We used transcranial magnetic stimulation (TMS) in 48 healthy humans. Cortico-cortical paired associative stimulation (ccPAS) was administered over M1 and the ventral premotor cortex (PMv), a key AON node, to induce spike-timing-dependent plasticity (STDP) in the pathway connecting them. Single-pulse TMS assessed motor resonance during action observation.

*Results:* Before ccPAS, action observation increased corticospinal excitability in the muscles corresponding to the observed movements, reflecting motor resonance in M1. Notably, ccPAS aimed at strengthening projections from PMv to M1 (PMv→M1) induced short-term enhancement of motor resonance. The enhancement specifically occurred with the ccPAS configuration consistent with forward PMv→M1 projections and dissipated 20 minutes post-stimulation; ccPAS administered in the reverse order (M1→PMv) and sham stimulation did not affect motor resonance.

*Conclusions:* These findings provide the first evidence that inducing STDP to strengthen PMv input to M1 neurons causally enhances muscle-specific motor resonance in M1. Our study sheds light on the plastic mechanisms that shape AON functionality and demonstrates that exogenous manipulation of AON connectivity can influence basic mirror mechanisms that underlie social perception.

## Introduction

Humans are equipped with a sophisticated neural system that allows for the perception and understanding of the actions performed by other people[1–3]. This system, often referred to as the action observation network (AON), maps visual information about others' actions onto the observer's motor representations in parietal and premotor regions[4,5]. This neural mechanism, termed motor resonance, is believed to involve mirror neurons[2,6,7].

Strong evidence for motor resonance in humans comes from studies using single-pulse transcranial magnetic stimulation (TMS) over the primary motor cortex (M1)[5,8,9]. These studies have shown that action observation enhances the amplitude of TMS-induced motor-evoked potentials (MEPs), reflecting an increase in M1 corticospinal excitability specific to the muscles involved in the observed action[10–16]. Motor resonance in M1 is believed to reflect the activity of higher-order fronto-parietal nodes within the AON, exerting a modulatory effect on M1 excitability[11,17–20], mainly through the copious projections coming from the ventral premotor cortex (PMv)[21,22].

The AON is a dynamic and malleable system shaped by the coupling between perceived and executed actions[23–26]. When such coupling is experimentally altered, for example, when participants observe an action while performing another (counter-mirror training), motor resonance can reduce or reverse[27,28]. Similarly, atypical motor resonance can be induced when observing an action and stimulating the motor representation of another effector in M1[29]. According to influential accounts, this experience-dependent tuning of AON activity is the result of Hebbian associative mechanisms of spike-timing-dependent plasticity (STDP) between visual and motor representations[2,30–32]. However, despite these plastic changes are believed to occur at the level of cortico-cortical connections within the AON, direct neurophysiological demonstration that STDP can act on AON connectivity is lacking.

To address this outstanding issue, in this study, we tested whether the AON is amenable to STDP modifications in the strength of cortico-cortical connectivity with the M1. We focused on PMv – a core region of the AON – and M1, a key area where motor resonance is expressed[10–15,17–20]. Together, PMv and M1 form a thoroughly studied visuo-motor circuit, whose effective connectivity

has been extensively documented at rest, during action performance and action observation[18,33–39], suggesting that the PMv-M1 circuits could provide a valuable test-bed for investigating the plasticity of AON connections. However, none of the previous research has addressed whether strengthening PMv projections to M1 (i.e., the PMv→M1 pathway) through STDP affects motor resonance in M1.

Therefore, in this study, we took advantage of a dual-coil TMS protocol named cortico-cortical paired associative stimulation (ccPAS), originally developed by Rizzo and colleagues[40], stemming from the classical PAS protocol[41,42], to modulate offline the strength of the PMv→M1 pathway and test whether this exogenous manipulation causes a change in motor resonance.

The ccPAS is a dual-coil TMS technique modulating the synaptic efficacy of cortico-cortical connections[43–49]. The ccPAS protocol, uses two focal coils to stimulate two interconnected cortical areas and induce Hebbian STDP between them[40,50]. According to the Hebbian principle, synapses are potentiated when presynaptic neurons fire immediately before postsynaptic neurons in a coherent and repeated manner[50,51]. The ccPAS protocol mimics this pattern by repeatedly stimulating a “pre-synaptic area” immediately before the “post-synaptic area”. The inter-stimulus interval (ISI) between the two pulses is tailored to the temporal properties of the pathway connecting the two areas[41,49].

Previous studies demonstrated that the ccPAS protocol effectively induces STDP in PMv→M1 projections[52–56], affects M1 excitability[55,57–62] and motor performance relying on the PMv→M1 network[59,60,63]. Building on these findings, we hypothesize that a ccPAS protocol repeatedly activating the PMv→M1 pathway would increase the synaptic efficiency of the circuit, fostering enhanced communication within the AON during action observation, thereby causing consistent cascading effects on the expression of motor resonance in M1.

## Materials and Methods

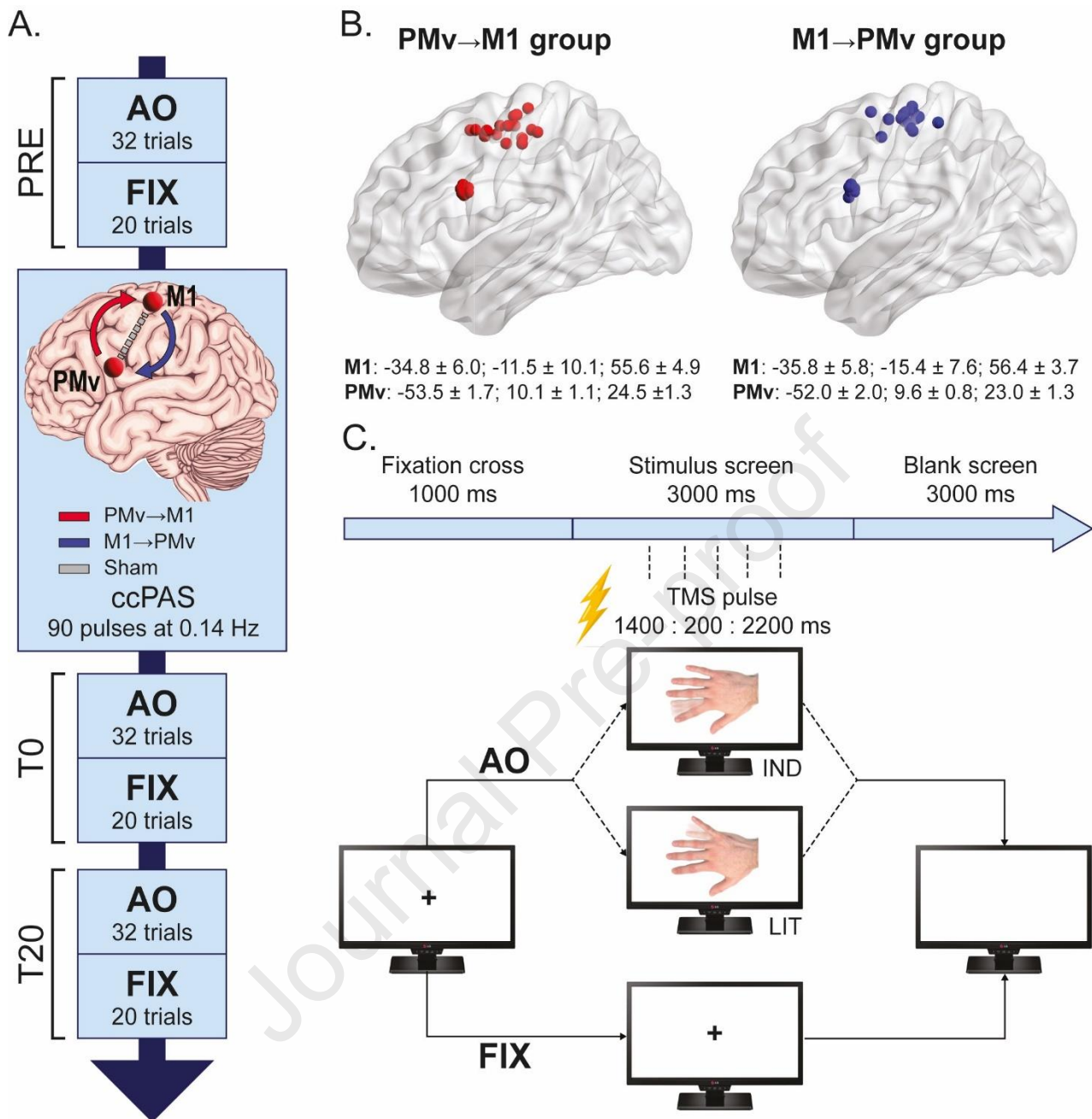
### Participants

Forty-eight right-handed healthy volunteers (26 females; mean  $24 \pm 3$  years) with normal or corrected-to-normal visual acuity and no contraindication to TMS[64] were recruited for the study (see Supplementary Materials for sample size justification). The Bioethical Committee of the University of Bologna approved the study in accordance with the ethical standards of the Declaration of Helsinki. All participants gave their written informed consent to the experiment.

### Experimental design

We adopted a mixed-design with both between- and within-subjects factors. Participants were randomly assigned to 3 groups undergoing different ccPAS protocols (Fig. 1A-B). In the experimental group (ccPAS<sub>PMv→M1</sub>), we targeted PMv and M1 to strengthening PMv→M1 projections via STDP. For the active control group (ccPAS<sub>M1→PMv</sub>), we used a stimulation of the same regions that is not expected to strengthen PMv→M1 projections, whereas, in the sham group (ccPAS<sub>Sham</sub>), no active TMS was administered.

To assess motor resonance, we recorded MEPs in 3 sessions: before (PRE), immediately (T0) and 20 minutes (T20) after the administration of a ccPAS protocol (Fig. 1A). In each session, we applied single-pulse TMS over the left M1 hand region to induce MEPs in both the right first dorsal interosseous (FDI) and abductor digiti minimi (ADM), which control abduction/adduction movements of the index (IND) and little fingers (LIT), respectively. MEPs were recorded at rest during the presentation of two action observation (AO) stimuli, i.e., abduction/adduction movements performed by a right IND or a right LIT, and a fixation cross (FIX) serving as baseline (Fig. 1C). Stimuli were adapted from[14].



**Figure 1.** (A) Graphical representation of experimental design showing the three ccPAS groups (ccPAS<sub>PMv→M1</sub>, ccPAS<sub>M1→PMv</sub>, ccPAS<sub>Sham</sub>) and three Sessions (PRE, T0, T20) testing MEPs during action observation stimuli (AO) and a ‘baseline’ control condition showing a fixation cross (FIX). (B) Cortical sites stimulated in the two active ccPAS groups and corresponding Talairach coordinates (mean  $\pm$  SD). For illustrative purposes, individual stimulation sites were reconstructed using BrainNet Viewer after converting Talairach coordinates into the MNI space. (C) Timeline of single-pulse TMS trials, showing an initial black fixation cross of  $2 \times 2^\circ$  of visual angle on a white background (duration: 1000 ms), followed by a stimulus screen (3000 ms) and a blank screen (3000 ms). The stimulus screen could display the same fixation cross (FIX; 20 trials), or a video-clip of a finger movement (AO; 32 trials). Each AO video-clip began with a static hand subtending a horizontal visual angle of  $13.8^\circ$  (1200 ms) and was followed by two cycles of an abduction/adduction movement (1800 ms) of the index finger (IND) or the little finger (LIT). Clips were presented in a pseudorandomized order for the factorial combination of 4 models  $\times$  2 moving fingers  $\times$  2 hand orientations  $\times$  2 repetitions. In every trial, a single TMS pulse was

delivered to M1 at five randomized intervals ranging from 2400 to 3200 ms after the beginning of the trial. This timing ensured that TMS was always administered during the observation of the finger movement in the AO condition, from 200 to 1000 ms after the movement onset. This large time window allowed us to randomize with significant variability the timing of TMS pulses, minimizing anticipation of the stimulation in the participants, while capturing motor resonance effects with muscle-specificity[9].

## Apparatus and stimuli

Visual stimuli were displayed on a 24" LED screen with a full HD resolution (1920x1080 pixels) and a refresh rate of 60 Hz, placed at 80 cm from the participant's head. MATLAB (version R2013b) and Psychophysics Toolbox controlled the presentation of the visual stimuli and triggered TMS pulses.

Each session (PRE, T0, T20) consisted of 32 AO (16 IND and 16 LIT) and 20 FIX trials, presented in two ~3-min blocks. Trials had a common structure (Fig.1C;Supplementary materials).

In the AO trials, participants were shown videos presented in a pseudorandomized order depicting two cycles of an abduction/adduction movement (IND or LIT) of two male and two female Caucasian hands. The hands were presented in palm-down position from an overhead view and rotated 90° clockwise or anti-clockwise from a first-person perspective to minimize visuo-spatial compatibility[65,66].

## TMS procedure and MEP recording

TMS was delivered through figure-of-eight iron branding coils (50-mm wing external diameter) connected to two TMS devices. To assess motor resonance, we used a Magstim Rapid<sup>2</sup> device that generates pulses with biphasic waveforms[11,16] and a Biopac MP-35 (BIOPAC Systems, Inc., CA) to record electromyographic (EMG) signal from the right FDI and ADM muscles, via Ag-AgCl electrodes with a belly-tendon montage. EMG signal was sampled at 20 kHz and band-pass filtered (30-500 Hz). In the three testing sessions (i.e. PRE, T0, T20) we applied single-pulse TMS during AO and FIX trials by placing the coil over the optimal scalp position (OSP) defined as the M1 location where TMS pulses evoked maximal MEPs in the right FDI. Stimulation intensity was set to produce MEPs of ~1 mV in the resting FDI muscle. This intensity was enough to record stable MEPs from both muscles. The coil was oriented at ~45° with respect to the midsagittal line, with the second phase of the waveform generated by the biphasic pulse inducing an optimal posterior-to-anterior

current in the brain[67,68] (Supplementary Fig.S1). The TMS inter-trial interval (ITI) was  $7000\pm 800$  ms (Fig.1C).

### Plasticity induction

During ccPAS, we administered 90 pairs of pulses at rest to the left PMv and M1 through two coils connected to two different stimulators. The ccPAS<sub>PMv→M1</sub> protocol aimed to strengthen the PMv→M1 pathway: in each pulse pair, the TMS pulse over PMv preceded the pulse over M1 by 8 ms, so that the corticocortical volley elicited by PMv stimulation reached M1 immediately before its direct stimulation, resulting in convergent activation of pre- and post-synaptic neurons, instrumental to STDP establishment [52,53,58]. This 8-ms ISI was selected according to prior PMv-M1 research that has demonstrated interactions at this timing during both resting conditions and relevant tasks, such as action preparation, execution, reprogramming, and observation[18,33–35,38,39]. Furthermore, this specific ISI had been employed to successfully modulate the PMv-M1 circuit using ccPAS[52,53,58,61,63].

To ensure that any results were not merely due to the stimulation of the two areas per se, in the ccPAS<sub>M1→PMv</sub> protocol, the order of the two pulses in each pair was reversed (i.e., M1 was stimulated 8 ms before PMv); this protocol was found to reduce the strength of PMv→M1 projections[52,58], although other studies have reported little or no effect at a functional level[60,63] or M1 corticospinal excitability[61]. The ccPAS<sub>Sham</sub> was administered with the coils tilted at 90°, resulting in an ineffective stimulation controlling for unspecific effects.

In all ccPAS protocols, the TMS pairs were delivered at a rate of ~0.14 Hz (i.e., one pair every 7 s, for a total duration of 10.5 min), well within the range used in the literature (0.1-0.2 Hz)[52,53,55,57,58], and in line with the testing phase, where pulses were delivered with an average ITI of ~7 s. M1 stimulation involved the same device (biphasic Magstim Rapid<sup>2</sup>), coil orientation and stimulation intensity (eliciting ~1 mV MEPs) utilized in the testing phase[63], see also[41,69,70]. PMv stimulation was performed with the Magstim 200, generating pulses with monophasic waveforms,

with the coil oriented to induce a current flow in the neural tissue directed toward the M1 site[54,61–63] (Fig.S1), and an intensity of 110% of the resting motor threshold (rMT)[52,53,56,58]. The rMT was determined in the initial phase of the experiment and defined as the minimal intensity that evoked at least 5 out of 10 MEPs with an amplitude  $>50 \mu\text{V}$  in the relaxed FDI[71] when targeting the OSP with the Magstim 200. A suprathreshold rather than a subthreshold PMv stimulation was preferred as the former might induce PMv-M1 connectivity changes, without affecting M1 excitability at rest (compare[52] with[61]).

### Neuronavigation

To target the left PMv we used a SoftTactic Navigator system (EMS s.r.l., Italy), automatically estimating Talairach coordinates from an MRI-constructed stereotaxic template based on the digitized scalp of each participant acquired using a Polaris Vicra digitizer (Northern Digital Inc., Canada). The PMv site was identified as the scalp position overlying a rostro-ventral portion of the precentral gyrus at the border with the posterior inferior frontal gyrus, at Talairach coordinates  $x=-52$ ;  $y=10$ ;  $z=24$  which was targeted in prior studies on motor resonance and action perception[17,72] as well as prior ccPAS studies[59–63]. Individual Talairach coordinates corresponding to the projection of M1-OSP and PMv on the brain surface were calculated by the SoftTactic Navigator (Fig.1B).

### Data analysis

EMG signal analysis was conducted using custom Matlab scripts. The mean MEP amplitude for each condition was computed as the peak-to-peak amplitude following removal of motor artifacts (7% of the total, see Supplementary Methods). Data from one participant was corrupted due to a technical failure, resulting in the following group sizes:  $\text{ccPAS}_{\text{PMv} \rightarrow \text{M1}}$ ,  $N=16$ ;  $\text{ccPAS}_{\text{M1} \rightarrow \text{PMv}}$ ,  $N=16$ ;  $\text{ccPAS}_{\text{Sham}}$ ,  $N=15$ .

To assess the occurrence of motor resonance before any ccPAS intervention, a preliminary mixed-factors ANOVA with the within-subjects factors Muscle (FDI, ADM) and Movement (IND, LIT) and the between-subjects factor ccPAS ( $\text{ccPAS}_{\text{PMv} \rightarrow \text{M1}}$ ,  $\text{ccPAS}_{\text{M1} \rightarrow \text{PMv}}$ ,  $\text{ccPAS}_{\text{Sham}}$ ) was performed on

MEPs in the PRE session. For this analysis, MEPs recorded in the AO condition were normalized as a percentage of the average MEP of the FIX condition (% of FIX).

To check whether ccPAS affected corticospinal excitability, MEP data acquired during the FIX condition were submitted to a mixed-factor ANOVA with the within-subjects factors Muscle (FDI, ADM) and Session (PRE, T0, T20) and the between-subjects factor ccPAS (ccPAS<sub>PMV→M1</sub>, ccPAS<sub>M1→PMV</sub>, ccPAS<sub>Sham</sub>).

In the main analysis, to test the effect of ccPAS, we computed a motor resonance index (MR index) for each muscle by subtracting the average MEP recorded in AO trials during the incongruent movement from that of the congruent movement (i.e., FDI<sub>IND</sub>-FDI<sub>LIT</sub>; ADM<sub>LIT</sub>-ADM<sub>IND</sub>) and dividing this difference to the square root of the mean of the variance of these two conditions[73,74], as follows:

$$MR\ index = \frac{\text{Mean}(\text{MEP}_{\text{congruent}}) - \text{Mean}(\text{MEP}_{\text{incongruent}})}{\sqrt{\frac{\sigma^2(\text{MEP}_{\text{congruent}}) + \sigma^2(\text{MEP}_{\text{incongruent}})}{2}}}$$

An MR index > 0 reflects muscle's higher sensitivity for the congruent observed movement, indicating motor resonance. MR index data were analyzed through a mixed-factors ANOVA with within-subjects factors Muscle (FDI, ADM) and Session (PRE, T0, T20) and between-subjects factor ccPAS (ccPAS<sub>PMV→M1</sub>, ccPAS<sub>M1→PMV</sub>, ccPAS<sub>Sham</sub>).

Lastly, the change in MR was individually computed by subtracting MR index values of PRE from those of the T0 session and averaging the resulting index across muscles, as follows:

$$\text{Mean}(\text{MR index}_{T0}) - \text{Mean}(\text{MR index}_{PRE})$$

A one-way ANOVA was used on this index to compare the effect of ccPAS protocols between groups directly.

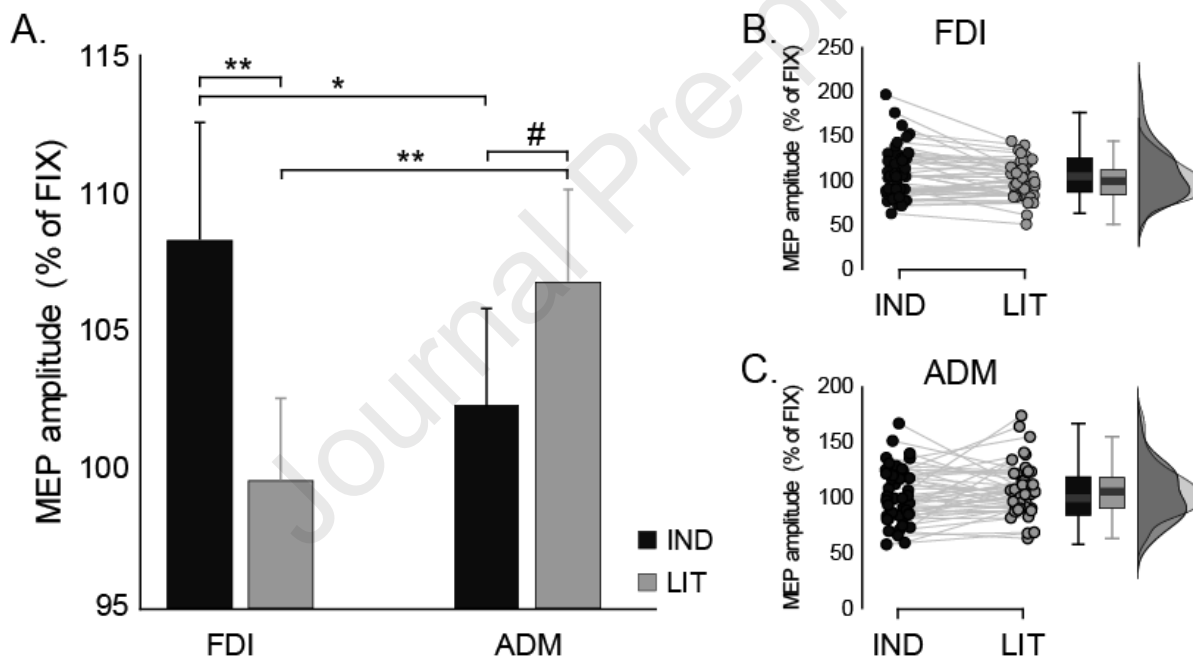
Statistical analyses were performed using the STATISTICA software (v.12; StatSoft Inc.). Post-hoc analyses were performed with the Duncan's test. ANOVA effect sizes were reported as partial eta-squared values ( $\eta_p^2$ ). *Cohen's d<sub>s</sub>* and *Cohen's d<sub>rm</sub>* indices were computed between and within post-

hoc comparisons, respectively[75]. Unless otherwise stated, values reported in the text are expressed as mean±S.D.

## Results

### Evidence of motor resonance before ccPAS

The ANOVA conducted on MEPs in the PRE session showed a significant Muscle x Movement interaction ( $F_{1,44}=13.5, p<0.001; \eta_p^2=0.23$ ; Fig.2), indexing motor resonance.



**Figure 2** – Evidence of motor resonance before the ccPAS intervention (time PRE) as shown by mean MEP amplitudes (% FIX) during the AO clips. **(A)** Muscle x Movement interaction indicating a muscle-specific motor resonance response, so that the FDI muscle was activated by the observation of index finger movements (IND) and the ADM muscle by the observation of little finger movements (LIT). Error bars denote standard error of the mean. Hashmarks and asterisks denote significance of post-hoc comparisons: # $p\leq 0.08$ ; \* $p\leq 0.05$ ; \*\* $p\leq 0.01$ . Individual MEPs, box plots, and density plots in the IND and LIT conditions are shown for the **(B)** FDI and **(C)** ADM muscles.

Post-hoc analysis revealed that MEPs were higher in the muscle congruent with the observed movement ( $FDI_{IND}=108\pm 29\%$ ;  $ADM_{LIT}=107\pm 23\%$ ) compared to the incongruent movement ( $FDI_{LIT}=100\pm 20\%$ ;  $ADM_{IND}=102\pm 24\%$ ) for FDI ( $p=0.002$ ;  $d_{rm}=0.32$ ) and marginally for ADM muscle ( $p=0.08$ ;  $d_{rm}=0.19$ ). Furthermore,  $FDI_{IND}$  response was higher than that of  $ADM_{IND}$  ( $p=0.028$ ;  $d_{rm}=0.22$ ), and  $ADM_{LIT}$  response was higher than that of  $FDI_{LIT}$  ( $p=0.009$ ;  $d_{rm}=0.33$ ).

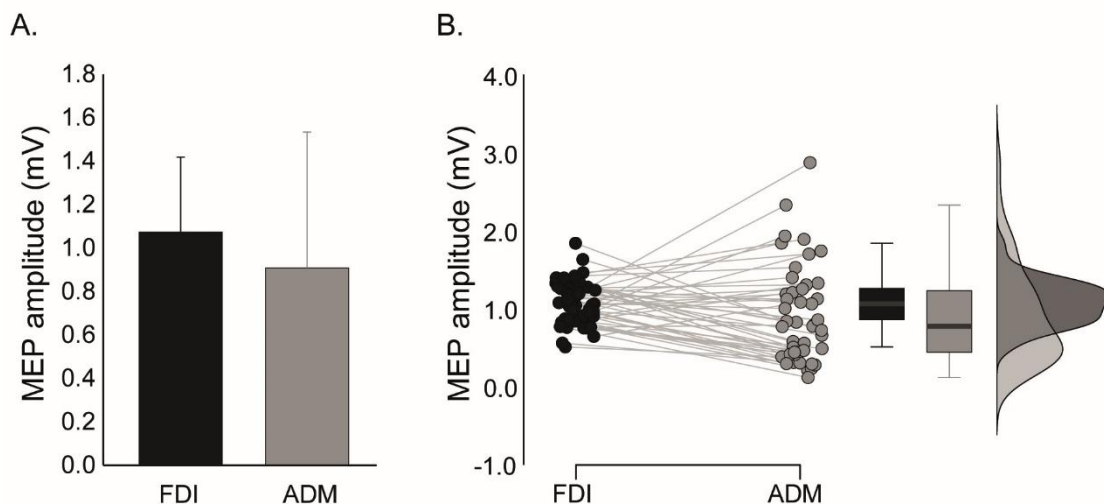
No other main effects or interactions resulted significant (all  $p\geq 0.34$ ). The null effects suggest comparable motor resonance before the ccPAS intervention (Table 1).

	PMv→M1		M1→PMv		Sham	
	IND	LIT	IND	LIT	IND	LIT
<b>FDI</b>	112 ±31% (1.08 ± 0.32 mV)	99 ±17% (0.97 ± 0.25 mV)	114 ±32% (1.16 ± 0.38 mV)	102 ±23% (1.04 ± 0.29 mV)	98 ±22% (1.09 ± 0.24 mV)	97 ±21% (1.07 ± 0.21 mV)
<b>ADM</b>	108 ±29% (0.82 ± 0.55 mV)	111 ±32% (0.81 ± 0.51 mV)	104 ±22% (0.83 ± 0.59 mV)	106 ±16% (0.84 ± 0.58 mV)	95 ±21% (1.10 ± 0.80 mV)	104 ±18% (1.15 ± 0.78 mV)

**Table1.** Motor resonance across the three groups before ccPAS administration. Means ±S.D. of normalized MEP amplitudes (% of FIX) and correspondent raw amplitudes (in mV) reported in brackets during the session PRE.

### Effect of ccPAS on FIX trials

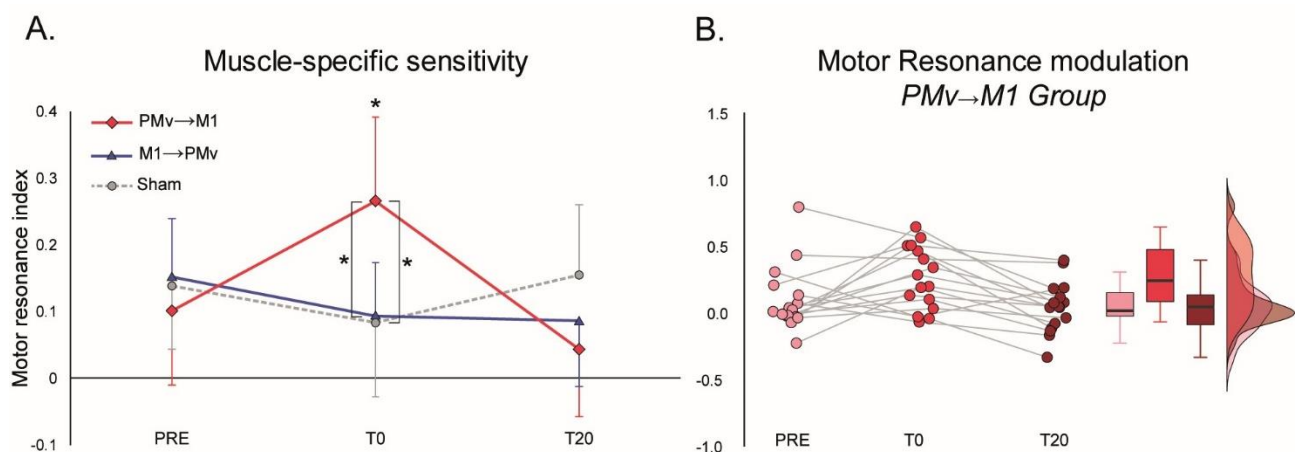
We tested whether ccPAS could induce global changes in M1 excitability over time. A mixed-factor ANOVA performed on MEP amplitudes showed a non-significant effect of the factor Muscle ( $F_{1,44}=3.27$ ,  $p=0.077$ ;  $\eta_p^2=0.07$ ; Fig.3), indicating slightly larger MEPs for the FDI ( $1.07\pm 0.32$ ) compared to the ADM ( $0.91\pm 0.63$ ). This trend is not surprising considering that the OSP was set according to the FDI representation. No other significant effects or interactions were detected ( $ps>0.15$ ; Table S3).



**Figure 3.** (A) Main effect of muscle on MEP recorded during FIX trials, indicating a trend for higher MEP amplitudes for FDI compared to ADM ( $p=0.08$ ). (B) Individual MEPs obtained from the FDI and ADM muscles of single participants with relative box plots and density plots.

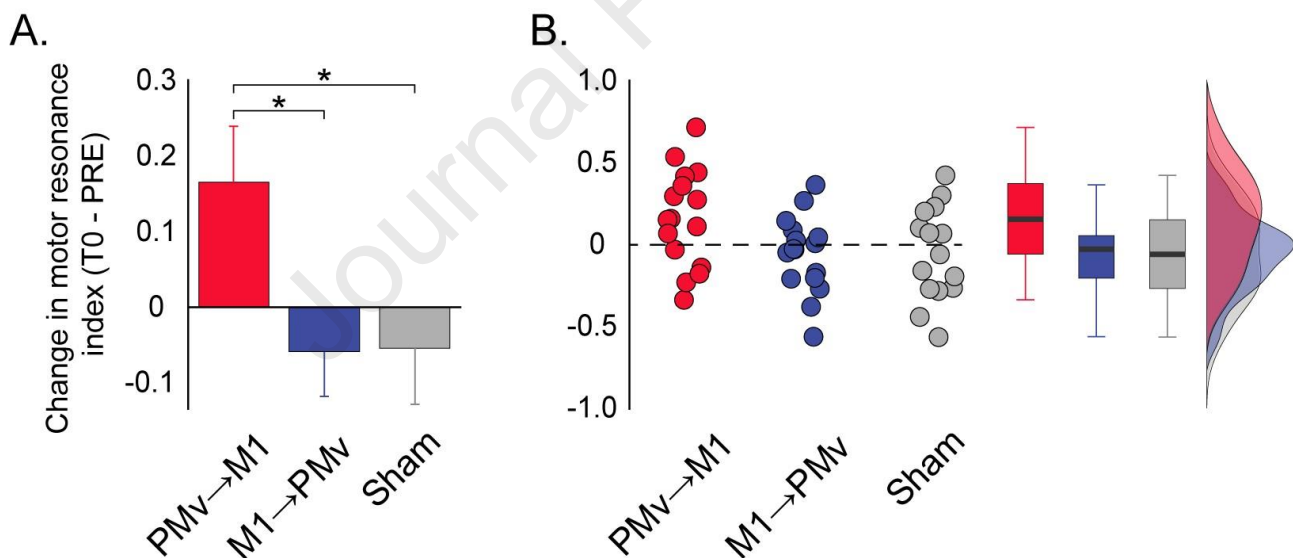
### ccPAS<sub>PMV→M1</sub> enhances motor resonance

The ANOVA performed on the MR index revealed a main effect of Muscle ( $F_{1,44}=5.61, p=0.022; \eta_p^2=0.11$ ), indicating larger sensitivity to observed actions in the FDI ( $0.19\pm 0.4$ ) than in the ADM ( $0.06\pm 0.41$ ) and, critically, a Session x ccPAS interaction ( $F_{4,88}=2.83, p=0.029; \eta_p^2=0.11$ ; Fig.4A), reflecting a change in muscle-specific sensitivity over time depending on the ccPAS protocol. Post-hoc analyses revealed that ccPAS<sub>PMV→M1</sub> led to enhanced motor resonance at T0 compared to PRE ( $p=0.037; d_{rm}=0.69$ ); the effect was no longer present at T20 ( $p=0.47; d_{rm}=0.26$ , Fig.2B). No modulation of motor resonance was detected following ccPAS<sub>M1→PMV</sub> (all  $p\geq 0.41$ ) or ccPAS<sub>Sham</sub> (all  $p\geq 0.49$ ). Moreover, while the sensitivity of the three ccPAS groups was comparable in the PRE session (all  $p\geq 0.54$ ), at T0, sensitivity was greater following ccPAS<sub>PMV→M1</sub> compared to both the ccPAS<sub>M1→PMV</sub> ( $p=0.048; d_s=0.86$ ) and the ccPAS<sub>Sham</sub> ( $p=0.042; d_s=0.88$ ). No other main effects or interactions reached significance in the ANOVA (all  $F\leq 0.89$ , all  $p\geq 0.41$ ).



**Figure 4 – (A)** Motor resonance (MR) index, reflecting muscle-specific sensitivity to observed actions, displayed over time as a function of the ccPAS protocol. ccPAS<sub>PMV→M1</sub> led to enhanced motor resonance at T0 compared to PRE, while no modulation of motor resonance was detected following ccPAS<sub>M1→PMV</sub> or ccPAS<sub>Sham</sub> protocols. Error bars denote standard error of the mean. Asterisks indicate significant post-hoc comparison:  $p \leq 0.05$ . **(B)** Individual MR index, box plots, and density plots of the ccPAS<sub>PMV→M1</sub> group across the three sessions.

In line with our hypothesis, these results demonstrate that ccPAS<sub>PMV→M1</sub>, empowering PMv-M1 connectivity, enhanced motor resonance. The ANOVA conducted on the modulation index showed a main effect of ccPAS ( $F_{2,44}=3.46, p=0.04; \eta_p^2=0.14$ ; Fig.5), indicating larger modulation following ccPAS<sub>PMV→M1</sub> compared to ccPAS<sub>M1→PMV</sub> ( $p=0.035; d_s=0.83$ ) and ccPAS<sub>Sham</sub> ( $p=0.03; d_s=0.75$ ) which, in turn, were comparable ( $p=0.97$ ).



**Figure 5. (A)** Changes in the MR index at T0 (relative to Pre) as a function of the ccPAS protocol. Changes induced by the ccPAS<sub>PMV→M1</sub> protocol were greater than those induced by the ccPAS<sub>M1→PMV</sub> or ccPAS<sub>Sham</sub> protocols. Error bars denote standard error of the mean. Asterisks indicate significant post-hoc comparison:  $p \leq 0.05$ . **(B)** Individual changes in MR index of the three groups, correspondent box plots, and density plots.

## Discussion

The present study shows that ccPAS administered over PMv and M1 to strengthen directional PMv→M1 connectivity transiently enhances the effect of AO on M1 corticospinal excitability. Before ccPAS, brief clips displaying simple movements of the right index or little finger modulated left M1 corticospinal excitability tuned to the observed movements. The effect, probed by single-pulse TMS during AO, showed a high degree of muscle specificity, with FDI and ADM MEP facilitation occurring when observing index and little finger movements, respectively, thus reflecting the hallmark of motor resonance[6,8,9]. Importantly, we found that ccPAS<sub>PMv→M1</sub> enhanced motor resonance effects at T0. During ccPAS<sub>PMv→M1</sub>, PMv stimulation (first pulse) triggered a cortico-cortical volley that reached M1 neurons just before M1 stimulation (second pulse)[34,35]. This repeated paired-stimulation meeting the timing and the hierarchical organization of the PMv→M1 pathway, resulted in a consistent pre- and postsynaptic pairing, instrumental for the establishment of Hebbian STDP[50,51]. The increase in motor resonance was not observed following ccPAS<sub>M1→PMv</sub> or ccPAS<sub>Sham</sub>, suggesting it was specific to the enhancement of PMv→M1 directional connectivity[52,53,56], and not provoked by any consistent stimulation pairing the targeted areas or due to unspecific effects. The enhancement reflected a short-term expression of associative plasticity as it disappeared at T20. These findings provide unprecedented evidence that affecting PMv-M1 connectivity via exogenous manipulation of STDP transiently modulates motor resonance in humans.

The AON is a network composed of sensory and motor regions crucial for perceiving the actions of others[76–83]. It has been repeatedly shown that the stimulation of PMv and nearby inferior frontal regions affects action perception tasks[15,72,84–88], whereas M1 stimulation yielded mixed results, with some studies showing effects on AO tasks[9,89–91] and others reporting no consistent effects[77,82,92,93]. Studies on AO in monkeys have shown that both PMv and M1 contain purely motor, purely visual, as well as mirror neurons coupling observed actions with motor representations of similar actions[3,7,94,95], although they are believed to play partially distinct roles in action performance and AO alike, with PMv positioned higher than M1 in the hierarchical organization of the motor system and reflecting more abstract representations of the action's goal[3,7,96]. In turn, M1 would encode lower-level motor parameters necessary for achieving the goal and contribute to preventing unwanted reproductions of observed actions[94,97].

In line with this hierarchical organization, prior work demonstrated that activation of motor resonance mechanisms in M1 is influenced by the activity of the PMv node of the AON[11,17,18,20]. For example, in early studies, low-frequency (inhibitory) repetitive TMS over PMv was found to disrupt motor resonance in M1[11,17], while the same repetitive TMS protocol administered over M1 itself reduced M1 corticospinal excitability without impacting the magnitude of motor resonance[11]. Other studies using dual-coil TMS demonstrated that ipsilateral[18] or contralateral[19] PMv conditioning prompted or increased muscle-specific M1 corticospinal facilitation during AO. Taken together, these prior studies suggest that PMv and M1 have distinct roles in driving motor resonance over M1 corticospinal neurons, aligning with the notion of distinct neural representations in PMv and M1 mirror neurons[3,94,96,97]. Moreover, these studies support a premotor origin of M1 motor resonance[6,8,11], suggesting that visual information about observed actions is mapped onto PMv neurons, which in turn influence M1 corticospinal excitability.

Although associative plasticity is believed to forge mirror responses and shape cortico-cortical connections within the AON[2,25,29–31,98], no prior study attempted to manipulate PMv-M1 cortico-cortical connectivity via STDP to investigate the mechanism of motor resonance. Building on prior seminal TMS studies on motor resonance and leveraging the Hebbian rule, we used ccPAS to target directional PMv→M1 projections as a test-bed for studying the premotor origin of M1 motor resonance. By showing that experimental manipulation of the strength of PMv→M1 connectivity via ccPAS<sub>PMv→M1</sub> can transiently and causally enhance the effect of AO on MEP amplitudes, we demonstrate the active contribution of PMv-M1 projections in shaping motor resonance mechanism in M1. These findings provide novel mechanistic insights into the functional role of human PMv→M1 projections during AO and underscore their dynamic malleability in relation to motor resonance.

Anatomical and physiological studies on the homologue PMv area of monkeys show that this region is densely interconnected with M1 via glutamatergic cortico-cortical projections[21,22,99] through which the former exerts a powerful influence on the latter's activity[22].

Notably, these PMv projections synapse onto both inhibitory and excitatory interneurons in M1, thus providing a mechanism for modulating specific corticospinal representations. Prior studies using

dual-coil TMS have clarified that PMv→M1 projections can shift from inhibition to facilitation depending on the task at hand[34,35]. For example, during grasping preparation, muscle-specific PMv→M1 projections are facilitated depending on the type of grasp[34], and similar muscle-specific modulations have been reported during AO[18]. More broadly, PMv→M1 influences are state-dependent, however, they also depend on stimulation parameters used to probe them, such as the ISI and the intensity of the PMv conditioning[36,37]. Importantly, while prior ccPAS<sub>PMv→M1</sub> studies using subthreshold PMv conditioning have demonstrated modulations at the level of M1 corticospinal neurons (e.g.[61]), in this study, we employed suprathreshold PMv stimulation[52,53,56,58], which proved to affect PMv→M1 connectivity without modulating M1 corticospinal excitability[52]. Accordingly, we found no net effect of ccPAS during FIX trials over time (Table S1), suggesting that the documented changes in motor resonance could be attributed to STDP of PMv→M1 projections rather than local changes in M1 corticospinal excitability.

The level at which ccPAS effects occurred has implications for understanding the mechanisms of motor resonance in M1. The discovery of pyramidal mirror neurons in the monkey PMv, which directly project to the spinal cord[96,100], has raised the possibility that this class of neurons drives changes in M1 corticospinal excitability as observed in TMS-MEPs during AO. While we do not rule out the possibility that PMv could directly influence descending pathways[101], resulting in a modulation of MEP amplitude during AO, our study supports a more indirect premotor-motor pathway, highlighting the active role of PMv→M1 projections in carrying information about observed actions to M1 corticospinal neurons. Also, in light of the existence of mirror neurons in the monkey M1[94,97,102], it could be proposed that these neurons are tuned based on PMv→M1 projections.

Motor response to TMS over M1 can be affected by spatial compatibility between the position of visual cues and the observer's effectors[28]. To rule out an account of our results in terms of spatial compatibility, we rotated AO stimuli to make them orthogonal relative to the observer's hand[65,66]. Thus, we can assume that our MEP measurements truly reflected motor resonance, and ccPAS<sub>PMv→M1</sub> enhanced this mechanism instead of space-related visuo-motor associations.

Our study does not allow us to speculate on whether the modulation of motor resonance was uniquely due to improved synaptic efficacy of the PMv→M1 projections or rather the consequence of a broader modification of connectivity weights in the wider AON. Indeed, using the same ccPAS<sub>PMv→M1</sub> protocol, Johnen and colleagues[53] reported increased functional connectivity within a broader dorsolateral network for motor programming in which PMv-M1 are embedded. Additionally, studies have reported compensatory plasticity in remote network nodes following repetitive TMS, suggesting a redistribution of functional weights to offset the induced imbalance[17,103]. Therefore, one may argue that our effects could result from an altered connectivity affecting the wider AON.

In this study, we focused on a key node of the AON, specifically the PMv. Yet, it is important to clarify that the plastic effects we observed should not be considered selective for the AO domain, even though undeniably influenced processes within it. The PMv plays a significant role in several visuomotor functions, including transforming geometric properties of objects into an appropriate hand configuration for grasping and manipulation[34,35,38] and, accordingly, prior studies have shown improved grasping-related hand performance following ccPAS<sub>PMv→M1</sub> interventions[57,60,63]. Thus, while we provided causal evidence of an increase in motor resonance resulting from the manipulation of PMv→M1 projections, suggesting an enhancement of AON signals to M1, we acknowledge that the impact may extend beyond the domain of AO, and other visuomotor functions may also have been influenced by ccPAS manipulation.

In our study we did not distinguish between early and late phases of motor resonance. By examining M1 activity from 200 to 1000 ms from movement onset, we ensured to capture the feature of muscle specificity that distinguishes motor resonance from unspecific arousal responses[9]. However, prior work has also shown that while the earliest components of motor resonance are stimulus-driven, later components (>300 ms) are affected by top-down regulation, visuomotor training, and contextual information [28,66,104–106]. Consequently, future investigations employing ccPAS could shed light on whether these early and late components of motor resonance rely on distinct cortico-cortical pathways.

Finally, while we administered distinct ccPAS protocols between groups, future research may consider adopting a fully repeated-measures design to address the issue of inter-subject variability and protocol specificity. Yet, it is important to note that our randomized design does not hinder our ability to draw conclusions on the functional role of the PMv-M1 circuit in motor resonance. Indeed, our study provides the first direct evidence of short-term associative plasticity between a key node of the AON and the area directly expressing its processing, namely the PMv and M1. Our findings demonstrate that the neural pathway connecting PMv to M1, i.e., the PMv→M1 pathway, exhibits sensitivity to Hebbian STDP manipulations of cortico-cortical connectivity. This sensitivity underscores the pathway's functional malleability and its causal role in facilitating motor resonance during AO.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## List of abbreviations

ADM	Abductor Digiti Minimi
AON	Action Observation Network
ccPAS	Cortico-Cortical Paired Associative Stimulation
EMG	Electromyography
FDI	First Dorsal Interosseus
ISI	Inter-Stimulus Interval
ITI	Inter-Trial Interval
M1	Primary Motor Cortex
MEP	Motor Evoked Potential
MSO	Maximal Stimulator Output
OSP	Optimal Scalp Position
PMv	Ventral Premotor Cortex
rMT	Resting Motor Threshold
STDP	Spike-Timing-Dependent Plasticity
TMS	Transcranial Magnetic Stimulation

## References

## Highlights

- ccPAS is applied to manipulate the strength of the pathway between PMv and M1
- Enhancing PMv→M1 projections transiently increases motor resonance in M1
- This effect is indexed by an increased MEP facilitation during action observation
- No change is observed following active control and sham ccPAS protocols
- Motor resonance effects in the corticospinal system rely on the PMv→M1 pathway

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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