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Paired Associative Stimulation drives the emergence of motor resonance

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ABSTRACT

Background: Associative plasticity, the neurophysiological bases of Hebbian learning, has been implied in the formation of the association between sensory and motor representations of actions in the Mirror Neuron System; however, such inductor role still needs empirical support.
Objective/hypothesis: We have assessed whether Paired Associative Stimulation (PAS), known to activate Hebbian associative plasticity, can induce the formation of atypical (absent in normal conditions), visuo-motor associations, reshaping motor resonance.
Methods: Healthy participants underwent a novel PAS protocol (mirror-PAS, m-PAS), during which they were exposed to repeated pairings of transcranial magnetic stimulation (TMS) applied over the right primary motor cortex (M1), time-locked with the view of index-finger movements of the right (ipsilateral) hand. In a first experiment, the inter-stimulus interval (ISI) between visual-action stimuli and TMS pulses was varied. Before and after each m-PAS session, motor resonance was assessed by recording Motor Evoked Potentials induced by single-pulse TMS applied to the right M1, during the observation of both contralateral (left) and ipsilateral (right) index-finger movements. In the second experiment, the specificity of the m-PAS was assessed by presenting a visual stimulus depicting a non-biological movement.
Results: Before m-PAS, the facilitation of corticospinal excitability occurred only during the view of contralateral (with respect to the TMS side) index-finger movements. The m-PAS induced new ipsilateral motor resonance responses, indexed by atypical facilitation of corticospinal excitability by the view of ipsilateral hand movements. This effect occurred only if the associative stimulation followed the chronometry of motor control (ISI of 25 ms) and if the visual stimulus of the m-PAS depicts a biological movement (human hand action).
Conclusions: The present findings provide the first empirical evidence that Hebbian learning induced by a PAS protocol shapes the visual-motor matching properties of the human Mirror Neuron System.

Introduction

Understanding and predicting other people's actions are crucial for optimal cognitive and social functioning, being even implied in neurodevelopmental and psychiatric disorders [1–4]. These abilities seem to be underpinned by vicarious activations: the human brain is endowed with an action-observation network, which implements a 'mirror' mechanism matching perceived actions onto one's own motor representations [5]. Driven by the discovery of mirror neurons, influential theories have tried to explain how such perceptual-motor transformation mechanism emerges: the 'adaptation', phylogenetic, account posits that mirror mechanisms are the product of genetic evolution as adaptation for action understanding [6,7]; the 'associative', ontogenetic, perspective proposes that mirror neurons develop their characteristics as a result of experience [8–10], and in particular of Hebbian learning [11,12]. So far, some empirical support linking Hebbian learning and Mirror Neuron System (MNS) functioning comes from behavioral [13,14] and computational [15,16] studies; however, a more direct causal

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evidence is still lacking, especially with respect to the possibility that the induction of Hebbian associative plasticity, which has been linked to spike-timing-dependent plasticity (STDP) observed in animals, may actually induce the emergence, or at least a shaping, of motor resonance in humans.

We addressed this issue in healthy adults by using Paired Associative Stimulation (PAS) [17], a method for the non-invasive induction of Hebbian associative (STDP-like) plasticity in sensory-motor cortices by means of the repeated pairing of peripheral (in standard protocols [18,19]: median nerve stimulation) and cortical (transcranial magnetic stimulation - TMS) stimulations. In recent years, PAS protocols are proven to be effective in inducing associative plasticity, not only within primary systems such as the motor [for a review [20]], the somatosensory [e.g. Refs. [21,22]], the auditory [e.g. Refs. [23,24]] or the visual ones [25] but also between brain regions, hence targeting cortical connectivity by pairing TMS pulses over different cortical areas (i.e., cortico-cortical PAS) [e.g. Refs. [26–32]] or by enhancing the functioning of a target area through the stimulation of a cross-modal network (i.e., cross-modal PAS) [33,69].

In the present study, we have modified the standard PAS protocol targeting the primary motor cortex (M1) [19], creating a mirror version (m-PAS): the peripheral afference is replaced by a visual stimulus showing an index-finger movement, which is repeatedly paired with a TMS pulse over M1. The effect of the m-PAS was assessed on a reliable neurophysiological index of motor resonance: the facilitation of corticospinal excitability (i.e., Motor Evoked Potentials – MEPs – by single-pulse TMS over M1) by action observation.

Under normal condition, action observation induces an increase of MEP amplitude that is specific for the muscle involved in the actual execution of the observed action (the so-called motor resonance effect) [34–36]. The effect is hemispheric-specific: the observation of unilateral hand movements recruits the contralateral motor system [37], as in the case of their execution. In a Hebbian learning account, such specificity would result from experience-based associations between the perception of the own hand action and its corresponding motor programs in the contralateral M1 [38].

So, in the first experiment of the study (*Experiment 1*), we used the m-PAS to create a new association between the visual representation of unilateral hand movements (i.e. abduction of the index-finger seen from an egocentric perspective) and the activation of the ipsilateral M1, which would be reflected by an (atypical) ipsilateral corticospinal recruitment by action observation. To assess the efficacy of the m-PAS, before and after its administration, MEPs induced by TMS over the right M1 were recorded from two muscles of the contralateral (left) hand (i.e., the *first dorsal interosseus* – FDI, used as target muscle and the *abductor digiti minimi* – ADM, used as control muscle) during the observation of right or left hands, which could be static or performing the same index-finger movement shown during the m-PAS (i.e., action-observation task). Moreover, since Hebbian associative plasticity induction, which relies on Long-Term Potentiation (LTP) [19,39], depends on the *inter-stimulus intervals* (ISI) between the paired stimulations of the PAS [17], *in Experiment 1* the temporal relationship between the observed action and the TMS pulse was varied: in one session, the ISI was of 25 ms (m-PAS_{25ms}), hence reproducing the conduction time of the corticospinal tract [40]; while in the second session, it was of 250 ms (m-PAS_{250ms}), in line with the chronometry of MNS activation [41,42].

In a second experiment (*Experiment 2*), we investigated the visual specificity of the m-PAS. If the m-PAS relies on the recruitment of the MNS, the pairing of the TMS pulse with a visual stimulus showing a non-biological movement, hence not processed by the human MNS [1,43,44], should not be able to affect motor resonance neither for human actions nor for the non-biological movements. To verified this hyphothesis, in *Experiment 2*, we introduced a modified version of the m-PAS by presenting, paired with the TMS pulse, a visual stimulus showing a pair of scissors making an opening/closing movement (i.e., a non-biological movement that should not recruit the MNS; scissors-PAS_{25ms}). The effects of this protocol on motor resonance were compared to those of the m-$PAS_{25ms,}$ by using the same action-observation task depicting left hands of *Experiment 1*, as well as a scissors version of it. Now, only the ISI of 25 ms was used, based on findings from the first experiment.

Materials and Methods

Experiment 1: the m-PAS and its temporal dependency

Participants

Twenty healthy volunteers took part in the main experiment; two of them were excluded due to electromyography (EMG) artifacts leaving the final analyzed sample to 18 participants (6 males, mean age ± standard deviation – SD = 22.8 ± 1.8 years; mean education = 14.6 ± 1.7 years). They were all right-handed according to the Edinburgh Handedness Inventory [45]; none of them had contraindications to TMS [46]. The sample size was determined by means of an a-priori within-subjects repeated-measures Analysis of Variance (rmANOVA; effect size $F = .4$; Alpha Error Level: $p = .05$; Statistical Power = .95, Actual Power = .95), using the software G*Power 3.1 [47].

The study was approved by the Ethical Committee of the University of Milano-Bicocca and it was in accordance with the ethical standards of the Declaration of Helsinki. All participants gave their written informed consent to the experiment.

m-PAS

The m-PAS protocol was a modified version of the classic PAS protocol targeting the motor system [18,19] in which we substituted the electric stimulation of the median nerve with a videoclip depicting a hand movement. Each trial of the m-PAS began with the presentation of a frame depicting the dorsal static view of a right hand ("static frame", duration = 4250 ms). Immediately after its end, a second frame appeared, showing the abduction movement of the index finger of the same right hand ("action frame", duration = 750 ms). At the onset of the "action frame", a TMS pulse was delivered over the right M1 (hemisphere ipsilateral to the viewed right hand), at 120% of the participant's resting Motor Threshold (rMT; see par. 2.1.4). Real timing of the frames was checked by using a photodiode. A total of 180 trials were presented at a frequency of 0.2 Hz for a duration of 15 min [48,49].

In two different sessions, counter-balanced among participants, different ISIs were used between the index-finger movement (i.e. onset of the "action frame") and the TMS pulse: in one session, the ISI was of 25 ms (m-PAS_{25ms}), in the other session, it was of 250 ms (m-PAS_{250ms}) (Fig. 1a). During the m-PAS, the participant's hands were positioned out of view.

To ensure that participants were looking with attention to the visual stimuli, a fundamental condition for the success of a PAS protocol [50], in 15 trials out of 180, a red circle appeared on the fingernail of the moving index finger. Participants were instructed to press as faster and accurately as possible, with their right hand, the left key of the PC-mouse as soon as the circle appeared. On average, participants' accuracy at this task was of 96.8% (SD = ± 2.33%).

Trials randomization and timing of the stimuli were presented under computer control (E-Prime 2.0, Psychology Software Tool, Inc.).

Mapping motor resonance by measuring corticospinal excitability

Before and after the m-PAS, corticospinal excitability was measured by recording MEPs induced by the stimulation of the right M1, from the FDI and the ADM muscles of the left hand. MEPs were collected while participants observed videoclips showing static or moving hand stimuli (i.e., action-observation task) [[35,37], for a similar procedure see [41,49]]. Participants seated in a chair in front of a PC-screen distant approximately 57 cm from their face. Every trial began with a fixation point (a red asterisk) presented on the black background of the screen. After 5 s, the fixation disappeared and a static hand was presented for a variable duration from 1 to 3 s; then a single-frame videoclip was presented (duration = 2 s). In "movement trials", the videoclip showed the abduction movement of the index finger (the same index finger movement shown during the m-PAS), while in "static hand trials", the hand remained static. In both kinds of trials, 250 ms after the onset of the videoclip, a TMS pulse was delivered over the right M1, with an intensity of 120% of the participant's rMT [35,41,42]. The inter-trial interval was jittered between 8 and 10 s [46,52].

Two separate blocks of trials, one showing a left hand and the other one showing a right hand, were presented: in each block (each lasting 6 min), a total of 40 trials were presented in a randomized order: half (20) of the trials showed the static hand and the other half (20) the moving index finger (Fig. 1b).

To ensure that participants kept attention to the visual stimuli, in each block of the action-observation task, 8 out of 40 trials present a small (diameter: 15 pixel) colored circle that appeared on the fingernail of the index finger or of the middle finger (in a randomized order) during the third frame of the trial. Participants had to verbally report the color of the circle (which could be blue, for static hand trials, or red, for movement trials). On average, participants' accuracy at this task was of 98.5% (SD = ± 1.45%).

Trials randomization and timing of the stimuli were presented under computer control (E-Prime 2.0, Psychology Software Tool, Inc.).

TMS and EMG recording

TMS pulses were delivered during the experiment by using a figure-of-eight coil (70 mm) connected to a biphasic Magstim Super Rapid^2 stimulator (Magstim, Whitland, UK). At the beginning of each m-PAS session, the motor hotspot of left hand FDI muscle was found by moving the coil in 0.5 cm steps around the presumed motor hand area by using a slightly supra-threshold stimulus. The individual rMT was then defined as the minimum TMS intensity (expressed as the percentage of maximum stimulator output) able to elicit a MEP of at least 50 μV in the left hand's FDI 5 times out of 10 during the stimulation of right M1 [46]. On average, during the m-PAS$_{25ms}$ session, participants presented a rMT of 60.1 (SD = ± 9.7%); while during the m-PAS$_{250ms}$ session the rMT was of 59.8 (± 9.5%, vs. the TMS intensity of m-PAS$_{25ms}$, $p = .99$). TMS intensity during the experimental tasks was set at 120% of the individual rMT which induced, on average, MEPs' peak-to-peak amplitude of ≈1.8mV in the contralateral FDI muscle. For the stimulation of the right M1, the coil was always placed tangentially to the scalp with the handle hold backward and laterally at a 45° angle to the sagittal plane, thus to induce a posterior to anterior current flow [34,35,53]. The stable TMS coil placement and position during the experimental sessions were constantly monitored with a neuronavigation system (SofTaxic 2.0, E.M.S., Bologna, Italy, www.softaxic.com).

Corticospinal excitability was measured by delivering single-pulse TMS over the right M1 while recording MEPs from the FDI and the ADM muscles of the left hand. Active electrodes (9 mm

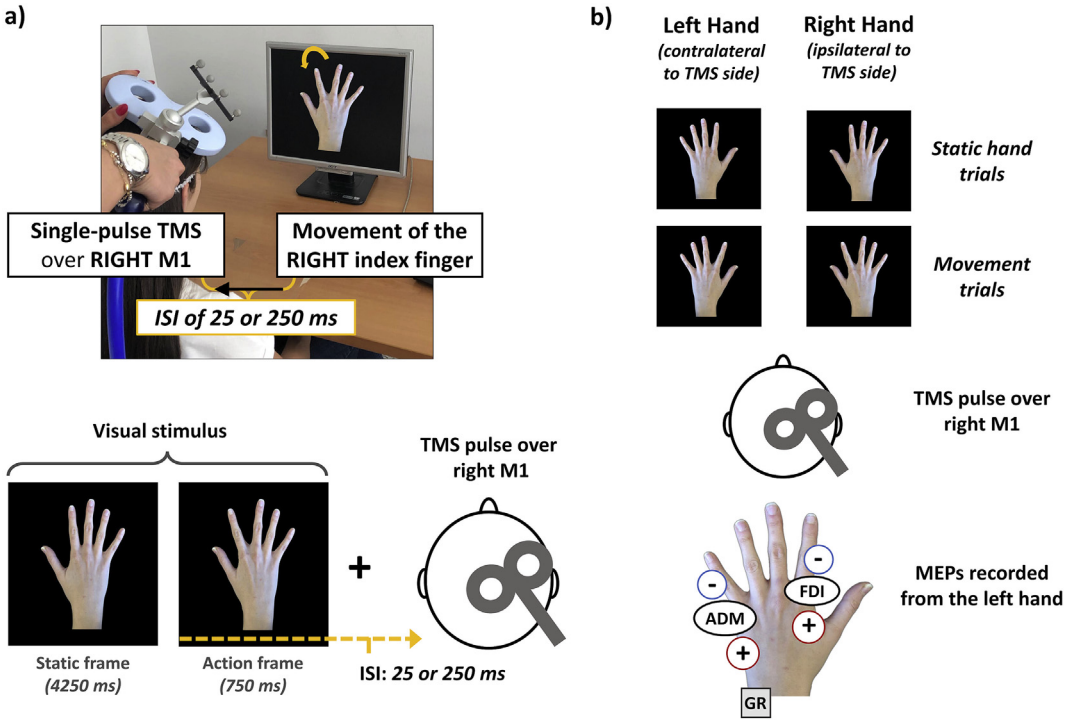

Fig. 1. *Experiment 1.* m-PAS (**a**) pairs the view of abduction movements of the right index-finger with TMS pulses over the right M1 (ipsilateral with respect to the viewed hand) applied with 25 or 250ms of delay. To assess motor resonance by action observation (**b**), MEPs, induced by single-pulse TMS over the right M1, were recorded from the FDI and ADM muscles of the left hand (contralateral to TMS hemisphere), while participants viewed a right or a left hand which was static or with an index finger performing an abduction movement (action-observation task). Red circle indicates the position of active electrodes, the blue ones the reference electrodes; the grey square represents the ground. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Ag–AgCl surface cup electrodes) were placed over the muscle bellies and reference electrodes over the metacarpophalangeal joint of the index finger, for FDI, and of the little finger, for ADM [35]. The ground electrode was placed over the left wrist. Prior to data acquisition, a visual inspection was made to guarantee that background noise from both FDI and ADM channels was smaller than 50 μV.

For MEP analysis, the signal was sampled (5000 Hz), amplified, band-pass filtered (10–1000 Hz) with a 50-Hz notch filter and stored for off-line analysis. Data were collected from 100 ms before to 200 ms after the TMS pulse (time window: 300 ms). MEPs were recorded using Signal software (version 3.13) connected to a Digitmer D360 amplifier and a CED micro1401 A/D converter (Cambridge Electronic Devices, Cambridge, UK, www.ced.co.uk).

Experimental procedure

The design of the experiment was within-participants and the experimental procedure was the same in both sessions (m-PAS_{25ms}, m-PAS_{250ms}) of the experiment. The order of the two sessions was counter-balanced among participants. Each session started with the determination of the individual rMT and the left hand's FDI hotspot. Then, motor resonance by action observation was assessed recording participant's MEPs in the two blocks (one depicting left-hands, one depicting right-hands) of the action-observation task. The order of the blocks was kept fixed within the same participant but counter-balanced among the participants. After the task, the m-PAS was administered. Immediately after its end, motor resonance was re-assessed using the same action-observation task as before. On average, a session lasted 1 h and 30 min. Both sessions were held at the same moment of the day (in the morning or in the afternoon) and at least 48 h passed between them, thus to prevent an overlapping of stimulation effects [54].

Data analysis

MEPs were analyzed off-line using the Signal software (version 3.13, Cambridge Electronic Design, Cambridge, UK). Preliminary, trials with artifacts (muscular or background noise) deviating from 200 μV in the 100 ms before TMS pulse were automatically excluded from analysis. MEPs peak-to-peak amplitude was calculated for each muscle and in each trial in the time window between 5 ms and 80 ms from the TMS pulse. In each block, trials where MEPs amplitude were ±2 SD from the mean of each condition (i.e., static hand trials, movement trials) were considered outliers and thus, excluded from analysis. On average, the 4.76% (SD = ± 1.73%) of MEPs recorded were discarded (mean number of discarded trials = 15 ± 5.5, out of 320 trials).

Mirror motor facilitation was computed as the difference in MEP amplitude between movement and static conditions (ΔMEPs) [e.g. Refs. [55,56]]: for both the left and the right hand trials, and for both muscles, the mean MEP amplitude in static hand trials was subtracted from MEP amplitude in movement trials. According to this index, positive values indicated motor facilitation by action observation. All subsequent analyses were conducted using such index.

Data analyses were performed with a series of within-subjects rmANOVA. A preliminary Muscle (FDI, ADM) X Session (m-PAS_{25ms}, m-PAS_{250ms}) X viewed Hand [left (contralateral to M1-TMS) hand, right (ipsilateral) hand] rmANOVA was performed in order to verify the presence of motor resonance effects in the two baseline sessions (i.e., before each m-PAS). Then, m-PAS effects were assessed through a Condition (Baseline, after m-PAS_{25ms}, after m-PAS_{250ms}) X viewed Hand [left (contralateral) hand, right (ipsilateral) hand] X Muscle (FDI, ADM) rmANOVA. Statistical significance was set at $p < .05$.

The Lilliefors corrected Kolmogorov-Smirnov test confirmed the normality of the distributions and data sphericity was confirmed by Mauchly's test in every dataset. Partial eta-squared (ηp^2) was also calculated in every rmANOVA and reported as an effect size value. Significant main effects were further explored with multiple post-hoc comparisons by applying the Bonferroni correction. If not otherwise specified, for each variable, mean ± standard error (S.E.) is reported. Statistical analyses were performed using the software Jamovi (version 1.0.8).

Experiment 2: visual specificity of the m-PAS

Participants

Twenty-two healthy volunteers took part in *Experiment 2*; two of them were excluded due to EMG artifacts, leaving the final analyzed sample to 20 participants (5 males, mean age ± SD = 22.4 ± 3.5 years; mean education ± SD = 14.6 ± 1.7 years). They were all right-handed according to the Edinburgh Handedness Inventory [45]; none of them had contraindications to TMS [46].

Experimental procedure

Materials, methods, TMS paradigms and MEPs recording procedures of *Experiment 2* were the same as *Experiment 1*. This second experiment comprised two within-subjects experimental sessions: in one session, participants underwent the same m-PAS protocol of *Experiment 1*, with visual hand actions (.e., the right hand performing an abduction movement of the index finger) paired with the TMS pulse (i.e. m-PAS_{25ms}); in another session, the visual stimulus showed a pair of scissors making an opening/closing movement (scissors-PAS_{25ms}, Fig. 2a). In both PAS protocols, the ISI between the visual stimulus and the TMS pulse was of 25 ms, given the results of *Experiment 1*. As in *Experiment 1*, before and after the m-PAS_{25ms}, the hand action-observation tasks were administered, which showed the right or the left hand (see above, and Fig. 1b). Instead, before and after the scissors-PAS_{25ms}, only the action-observation task showing the left hand was presented, along with a new scissors version of the same task. In this last task, trials could depict static or moving scissors (same kind of trials of the action-observation task; see *Experiment 1* for details, and Fig. 2b).

The order of the two sessions (m-PAS_{25ms}, scissors-PAS_{25ms}) was counter-balanced among participants and they were held at the same moment of the day (in the morning or in the afternoon). At least 48 h passed between them [54]. On average, during the m-PAS_{25ms} session, TMS was delivered with a mean intensity of 47.1% (SD = ± 7.3%) of the maximum stimulator output while, during the scissors-PAS_{25ms} session, the mean TMS intensity was of 47.4% (± 7.7%, vs. the TMS intensity of m-PAS_{25ms}, $p = .99$).

Data analysis

MEPs were analyzed off-line using the same procedure of *Experiment 1*. On average, in the action-observation tasks, outliers analysis led to discard, on average, 4.52% (SD = ± 1.49%) of MEPs recorded (mean number of discarded trials = 14 ± 4.8, out of 320). Statistical analyses were conducted with a series of rmANOVAs, following the same statistical approach of *Experiment 1*. In details, a preliminary Muscle (FDI, ADM) X viewed Stimulus (left $hand_{m\text{-}PAS}$, right hand, left $hand_{scissors\text{-}PAS}$, scissors) rmANOVA was performed in order to verify, before each PAS protocol, the presence of motor resonance selectively in the action-observation task depicting the left hand. Then, m-PAS effects were assessed through a Session (m-PAS_{25ms}, scissors-PAS_{25ms}) X viewed Stimulus (left and right hands for the m-PAS_{25ms}; left hand and scissors for the scissors-PAS_{25ms}) X Time (pre-PAS, post-PAS) X Muscle (FDI, ADM) rmANOVA. Post-hoc comparisons were corrected by applying the Bonferroni correction.

Results

Experiment 1

Motor resonance before the m-PAS

Results from the rmANOVA conducted on the baseline sessions to detect motor facilitation effects (ΔMEPs) showed a main effect of factor viewed Hand ($F_{1,17} = 11.36$, $p = .004$, $\eta p^2 = .401$) and a significant Muscle X viewed Hand interaction ($F_{1,17} = 22.15$, $p < .001$, $\eta p^2 = .566$); thus highlighting the classical, muscle-specific (FDI), motor facilitation effect at baseline induced by the observation of the index-finger movement of the left hand only (179 ± 33.5 μV; all *ps* < .004), with no difference between the two baseline sessions (m-$PAS_{25ms\_left\ hand}$ = 173.2 ± 49.2 μV *vs.* m-$PAS_{250ms\_left\ hand}$ = 184.7 ± 47.9 μV; t = −0.193, $p = .99$). No other significant main effects or interactions were found (all *Fs* < 3.3, all *ps* > .087).

Given the absence of differences between the two baseline sessions, ΔMEPs in the two baselines were averaged in the subsequent analyses.

m-PAS effects

Results from the rmANOVA showed a significant Condition X viewed Hand × Muscle interaction ($F_{2,34} = 4.31$, $p = .021$, $\eta p^2 = .202$), as well as main effects of Condition ($F_{2,34} = 6.67$, $p = .004$, $\eta p^2 = .282$) and Muscle ($F_{1,17} = 4.59$, $p = .047$, $\eta p^2 = .213$).

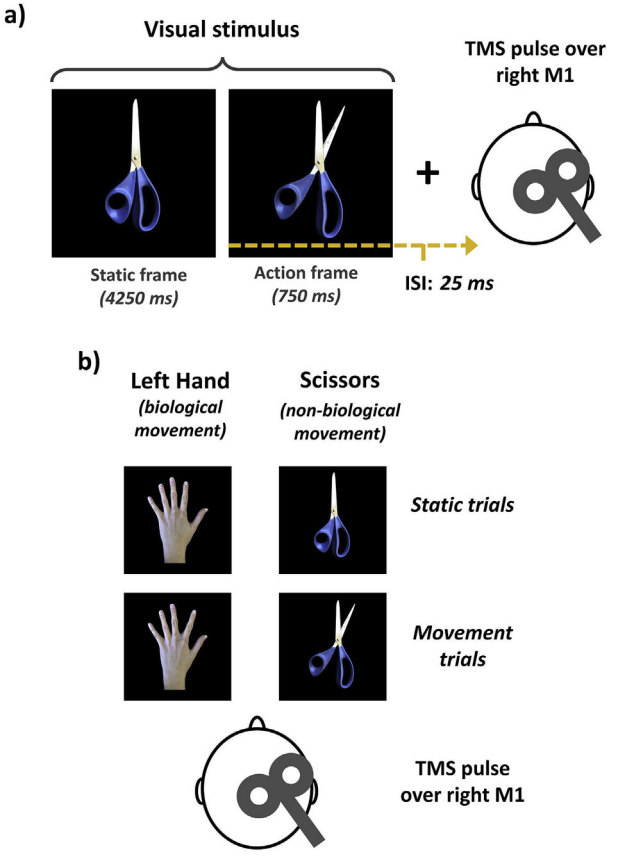

Fig. 2. *Experiment 2*. Scissors-PAS_{25ms} (**a**) pairs the view of an opening/closing movement of scissors (towards the left side, hence in the same direction of the abduction movement of the left-hand index finger of the m-PAS) with TMS pulses over the right M1 applied with 25 ms of delay. In the scissors-$PAS_{25\ ms}$, motor resonance by action observation (**b**) was assessed recording MEPs, induced by single-pulse TMS over the right M1, while participants viewed a left hand, or a pair of scissors, which could be static or could move (i.e., abduction movement of the index finger for the left hand; opening/closing movement to the left side for the scissors).

No other significant effect was found (all *Fs* < 3.09, all *ps* > .06, see Table 1).

The Muscle X Condition X viewed Hand interaction was further explored with two separate rmANOVA, one per each muscle. For the FDI muscle, this analysis showed significant main effects of the factors Condition ($F_{2,34} = 6.25$, $p = .005$, $\eta p^2 = .269$) and viewed Hand ($F_{1,17} = 6.23$, $p = .023$, $\eta p^2 = .268$), and of Condition X viewed Hand interaction ($F_{2,34} = 6.35$, $p = .005$, $\eta p^2 = .272$): ipsilateral motor facilitation by the view of right hand movements emerges after the administration of the m-PAS_{25ms} (225.2 ± 51.6 μV), as compared to baseline (−24.3 ± 37.2 μV; t = −4.415, $p < .001$) and after m-PAS_{250ms} (21.9 ± 50.1 μV; t = −3.596, $p = .009$). Importantly, the m-PAS_{25ms} effect was comparable to the typical motor resonance effects for left (contralateral to the TMS side) hand movements detected in every session ($Baseline_{left\text{-}hand}$ = 179 ± 33.5 μV, m-$PAS_{25ms\_left\text{-}hand}$ = 128.1 ± 45.8 μV; m-$PAS_{250ms\_left\text{-}hand}$ = 78.3 ± 65.0 μV; all *ps* > .064). As expected, in the baseline, motor facilitation effects were present only during the observation of left hand movements (t = −3.816, $p = .006$) (Fig. 3a and b).

For the ADM muscle, the rmANOVA showed no significant effects of factors Condition ($F_{2,34} = 2.82$, $p = .074$, $\eta p^2 = .142$), viewed Hand ($F_{1,17} < .01$, $p = .994$, $\eta p^2 < .001$) and their interaction viewed Hand X Condition ($F_{2,34} < .01$, $p = .991$, $\eta p^2 = .001$) (Fig. 3c).

Experiment 2

Motor resonance before PAS

The rmANOVA showed a significant Muscle X viewed Stimulus interaction ($F_{3,57} = 3.49$, $p = .021$, $\eta p^2 = .155$), as well as a main effect of both factors Muscle ($F_{1,19} = 5.17$, $p = .035$, $\eta p^2 = .214$) and viewed Stimulus ($F_{3,57} = 7.9$, $p < .001$, $\eta p^2 = .294$). Post-hoc analysis showed that, in both sessions, motor resonance effects were found only in the FDI muscle during the observation of left (contralateral to TMS) hand movements, while no facilitation effects were found during the observation of right (ipsilateral) hand movements and of scissors movements (m-PAS_{25ms}: left hand movements = 235.4 ± 39.2 μV, *vs.* right hand movements = −30.1 ± 54.2 μV; t = 4.369, p < .001; scissors-PAS_{25ms}: left hand movements = 234.7 ± 71.8 μV *vs.* scissors movements = 21.4 ± 49.6 μV; t = 3.509, $p = .018$).

PAS effects

Results from the rmANOVA showed a significant Session X viewed Stimulus X Time × Muscle interaction ($F_{1,19} = 5.33$, $p = .032$, $\eta p^2 = .219$; see Table 2 for all main effects and interactions). This quadruple interaction was further explored with two separate rmANOVAs, one per each muscle.

For the FDI muscle, the following effects reached the significance level: Session X viewed Stimulus X Time ($F_{1,19} = 8.25$, $p = .01$, $\eta p^2 = .303$), viewed Stimulus × Time interaction ($F_{1,19} = 20.52$, $p < .001$, $\eta p^2 = .519$) and viewed Stimulus ($F_{1,19} = 10.22$, $p = .005$, $\eta p^2 = .35$). No other statistically significant effect was found (all *Fs* < 1.76, all *ps* > .2).

The significant Session X viewed Stimulus × Time interaction was then split in two separate rmANOVAs, one for each PAS session. With respect to the m-PAS_{25ms} session, it showed a significant viewed Stimulus × Time interaction ($F_{1,19} = 31.32$, $p < .001$, $\eta p^2 = .622$): as in *Experiment 1*, a motor resonance effect induced by the observation of right hand movements emerged after the m-PAS_{25ms} (Pre-PAS = −30.1 ± 54.2 μV *vs.* Post-PAS = 193.8 ± 34.1; t = −3.731, $p = .004$); crucially, the magnitude of this effect was comparable to that found for the left hand (Pre-PAS = 235.4 ± 39.2 μV; Post-PAS = 88 ± 53.9 μV; all *ps* > .331). No other significant difference was found (all *ps* > .117) (Fig. 4a, left

Table 1
m-PAS effects in *Experiment 1*: results from the rmANOVA conducted on the ΔMEPs.

Factor/Interaction	F	p	ηp^2
Condition	6.67	.004	.282
viewed Hand	2.12	.164	.111
Muscle	**4.59**	**.047**	**.213**
Condition X Muscle	1.32	.279	.072
viewed Hand X Muscle	2.18	.158	.114
Condition X viewed Hand	3.09	.059	.154
Condition X viewed Hand X Muscle	**4.31**	**.021**	**.202**

panel, and the Supplemental Fig. 1 for individual data). The effect of factors viewed Stimulus ($F_{1,19} = 3.64$, $p = .072$, $\eta p^2 = .161$) and Time ($F_{1,19} = 0.59$, $p = .454$, $\eta p^2 = .03$) was not statistically significant.

The rmANOVA conducted for the scissors-PAS_{25ms} showed only a main effect of viewed Stimulus ($F_{1,19} = 7.65$, $p = .012$, $\eta p^2 = .287$), but neither of the factor Time ($F_{1,19} = .21$, $p = .656$, $\eta p^2 = .011$) nor of the viewed Stimulus × Time interaction ($F_{1,19} = 1$, $p = .33$, $\eta p^2 = .05$). Thus, the scissors-PAS_{25ms} was unable either to affect motor resonance for human actions, or to induce a facilitation effect for non-biological movements (Fig. 4a, right panel; Supplemental Fig. 1).

For the ADM muscle, the rmANOVA did not show any significant main effect or interactions (all *Fs* < 4.25, all *ps* > .053, Fig. 4b).

Discussion

The results of the present study show the efficacy of the m-PAS protocol, documenting that it is possible to promote novel visuo-motor associations in the human MNS through the induction of plastic mechanisms that rely on Hebbian associative plasticity.

Firstly, in both experiments, we find the typical, contralateral, motor facilitation effect at baseline: before m-PAS, motor resonance emerges only when viewing contralateral (left) hand movements, and it is specific for the FDI muscle [37,57], while it does not occurs when viewing ipsilateral (right) hand movements or the opening/closing movement of a pair of scissors [1].

The key finding is the emergence of motor facilitation contingent upon the observation of ipsilateral (right) hand movements selectively after the m-PAS_{25ms}, but not after the m-PAS_{250ms} (*Experiment 1*) or after the scissors-PAS_{25ms}, pairing motor cortical stimulation with the view of non-biological movements (*Experiment 2*). Additionally, no effect was induced by the PAS on MEPs recorded from ADM, confirming that motor resonance follows somatotopic rules [35,42].

In *Experiment 1*, following m-PAS_{25ms}, the observation of index-finger movements of the right hand causes motor facilitation, which was absent in the baseline, and still absent after m-PAS_{250ms}. Noteworthy, the magnitude of m-PAS_{25ms}-induced motor facilitation is comparable to the 'normal' motor facilitation for

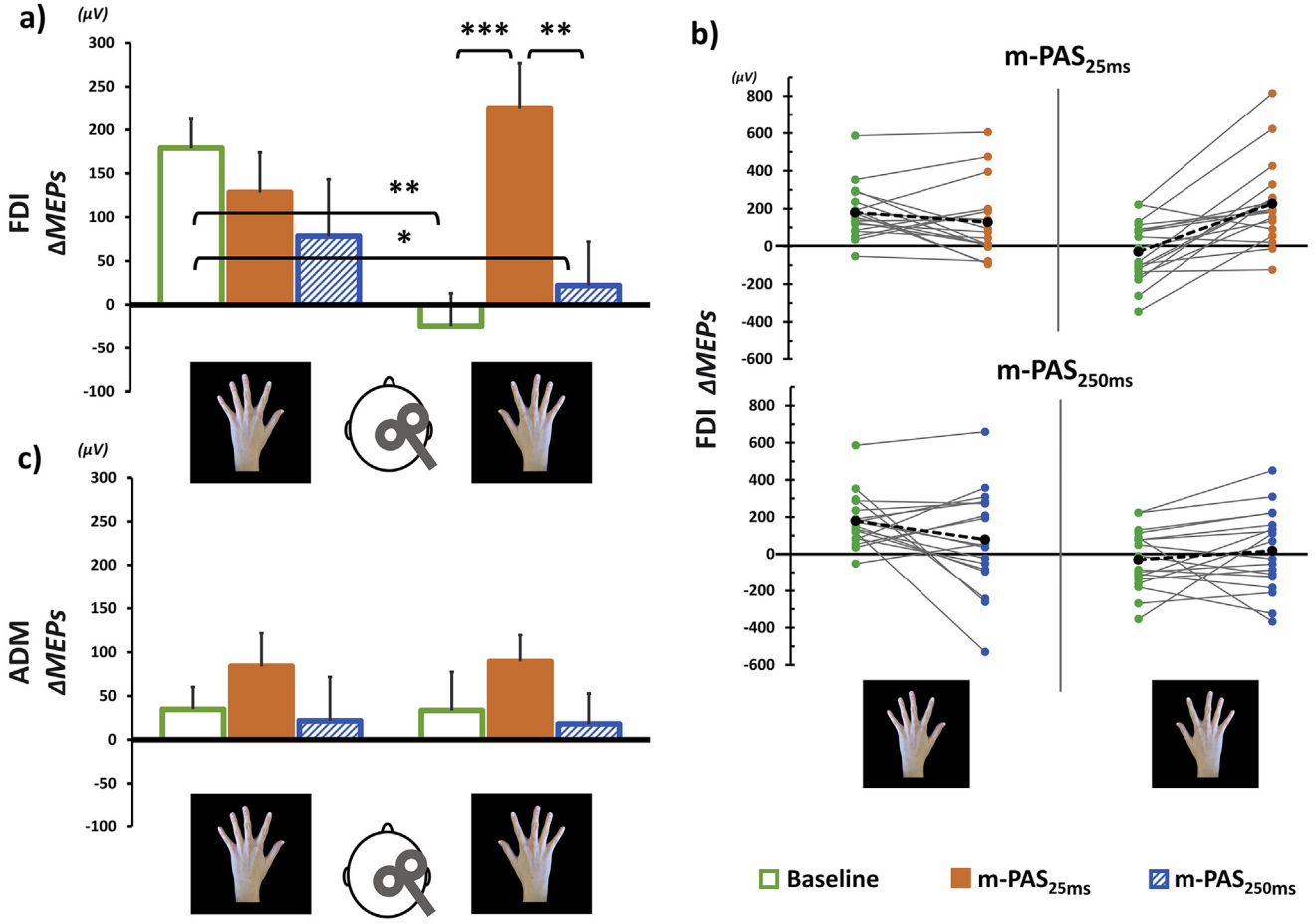

Fig. 3. Timing dependency of the m-PAS (*Experiment 1*). After m-PAS_{25ms} (orange bars and circles), observation of index-finger movements of the right hand brought about a facilitation of MEPs recorded from the left FDI, induced by TMS of the right M1 (ipsilateral with respect to the viewed hand). Mean (**a**) and individual (**b**) ΔMEPs from FDI and mean ΔMEPs from ADM (**c**) before (green blank bars and circles) and after m-PAS (blue striped bars and circles = m-PAS_{250ms}). In individual plots, the black dotted line indicates the mean. Error bars = S.E. * p < .5; ** p < .01; *** p < .001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
PAS effects in *Experiment 2*: results from the rmANOVA conducted on the ΔMEPs.

Factor/Interaction	F	p	ηp^2
Session	1.509	.234	.074
viewed Stimulus	**9.504**	**.006**	**.333**
Time	.015	.905	.001
Muscle	**9.951**	**.005**	**.344**
Session X viewed Stimulus	1.259	.276	.062
Session X Time	.383	.543	.02
viewed Stimulus X Time	**16.081**	**< .001**	**.458**
Session X Muscle	1.208	.285	.06
viewed Stimulus X Muscle	**7.368**	**.014**	**.279**
Time X Muscle	.049	.827	.003
Session X viewed Stimulus X Time	**6.245**	**.022**	**.247**
Session X viewed Stimulus X Muscle	.999	.33	.05
Session X Time X Muscle	.844	.37	.043
viewed Stimulus X Time X Muscle	**10.188**	**.005**	**.349**
Session X viewed Stimulus X Time X Muscle	**5.328**	**.032**	**.219**

contralateral hand movements detected in the baseline, which remains unaffected by m-PAS. The hemispheric-specific motor resonance likely develops from the extraction of a statistical relationship between our own actions and their sensory consequences. The m-PAS$_{25ms}$ is able to create novel links between visual and motor representations, teaching motor neurons to respond to the view of unusual (here ipsilateral) motor programs. We also highlight Hebbian learning sensitivity for veridical temporal causality: the ISI between the visual event and the motor cortical activation by TMS must follow the corticospinal chronometry (25 ms) to allow an experience of the temporal visuo-motor contingence that features motor control [40]. Conversely, if the transcranial activation of M1 follows the chronometry of MNS activation (250 ms) [41], no visuo-motor association can be created. Hebbian learning driven by the m-PAS is, therefore, a bottom-up, plastic, process that starts with the induction of associative

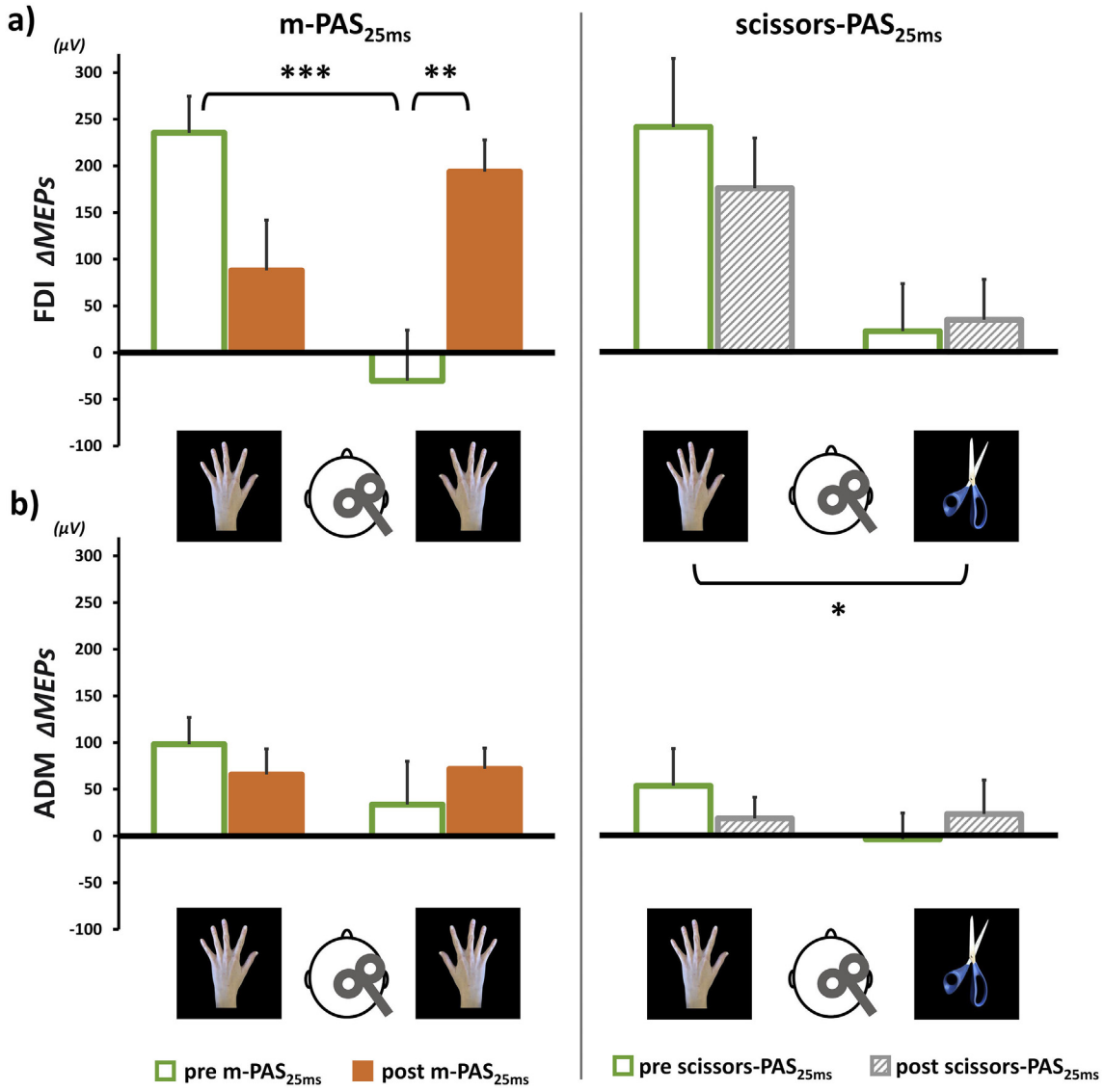

Fig. 4. Visual specificity of the m-PAS (*Experiment 2*). Before both PAS protocols (green blank bars), only the view of index-finger movements of the left hand induces a motor resonance effect on MEPs recorded from the left FDI. The m-PAS$_{25ms}$ (orange bars) induces a facilitation of MEPs recorded from the left FDI by the view of right, ipsilateral, index-finger movements. The scissors-PAS$_{25ms}$ (grey striped bars) did not affect motor resonance. Mean ΔMEPs from FDI (**a**) and ADM (**b**) before and after m-PAS$_{25ms}$ (left panel) and scissors-PAS$_{25ms}$ (right panel). Error bars = S.E. * p < .5; ** p < .01; *** p < .001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

plasticity only if we are exposed to visuo-motor association dealing with the time course of action execution, rather than that of its visual input [38]. In the superior temporal sulcus (STS), spiking of neurons representing the vision of an action occurs later than that of the premotor neurons that trigger the same action [58]; such latency reflects the likelihood of STS activation occurrence based on past sensory-motor contingencies. Probably, only long-lasting exposure to novel visuo-motor associations might drive the formation of new mirror representations in STS, along with the creation of predictive forward connections to premotor areas [38,59].

The speculation that the associative plasticity induced by the m-PAS likely occurs within the MNS is confirmed by the results of *Experiment 2*. During m-PAS, only the observation of biological movements is effective in inducing atypical motor resonance phenomena; conversely, the repeated observation of non-biological movements (scissors-PAS_{25ms}) does not promote the emergence of novel visuo-motor associations for the view of a tool (i.e., the pair of scissors). This evidence strongly supports the specific recruitment of the MNS during m-PAS, and thus our conclusion that Hebbian associative plasticity within the MNS mediates the formation of visuo-motor associations.

It has to be noticed that our results (i.e., the emergence of a novel motor resonance phenomenon) suggest the induction of Hebbian associative LTP-like plasticity within the MNS. However, to be able to hypothesize the involvement of STDP [12,15], the induction of LTD (mirrored by a loss of motor resonance) should be proved when a different timing between the two paired stimulations is exploited [39]. Hence, further studies using a wider range of ISIs between the visual stimulus and the TMS pulse of the m-PAS may be conducted to better define the neurophysiological properties of the associative plasticity induced by our novel PAS protocol.

Regardless the precise neurophysiological mechanism that led to the emergence of the new motor resonance effect, a key aspect of our protocol is that, unlike what happens in other sensorimotor trainings based on action-observation, such as the *counter-mirror* protocols [e.g. Refs. [13,51,60]], the m-PAS allows to control and investigate temporal and cortical variables which may play a fundamental role in the development and in the functioning of the MNS; variables that behavioral-only protocols cannot take into account. This is possible thanks to the use of a focal technique such as the TMS and the fact that the movement's observation is purely passive. Furthermore, the m-PAS could be a promising rehabilitation tool, for example in all the therapies based on mirror feedback or on action observation [e.g. Refs. [61–64]], due to the ease of administration, not requiring any active, voluntary movement from the patient and its relative short length (i.e., 15 min). It may also help to better understand the plasticity mechanisms responsible for the effectiveness of such therapies which are still debated and controversial [65].

In conclusion, the present study shows that Hebbian associative plasticity induced by PAS protocols can be used to shape MNS matching properties, evidencing its malleability in human adults. Certainly, further research has to be conducted to better explore the role of other cortical areas of the MNS [5,66], as well as of factors influencing the effectiveness of the visual biological movement depicted (e.g., the viewed from egocentric *vs.* allocentric perspectives; possible *vs.* impossible human body movements; human vs. robot movements) [35,67,68], but we believe that the m-PAS can be a very promising non-invasive protocol to shed light on the neurofunctional bases of the human MNS.

Declaration of interest

The authors declare no competing interests.

Data statement

Datasets, analysis, tasks and stimuli used in the experiment are publicly archived at the Open Science Framework (OSF): https://osf.io/3ujkv/

Further information will be available from corresponding authors on reasonable request.

CRediT authorship contribution statement

Giacomo Guidali: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Maíra I.S. Carneiro:** Investigation, Formal analysis, Writing - review & editing. **Nadia Bolognini:** Conceptualization, Methodology, Supervision, Project administration, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2020.01.017.

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