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Cross-modal plasticity in sensory-motor cortices and non-invasive brain stimulation techniques: new ways to explore and modulate brain plasticity

doctoral thesis by:

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Introduction

In the present doctoral thesis, I will explore whether Hebbian learning may rule the functioning of cross-modal and sensory-motor networks of the human brain. To this aim, during my doctorate, I have developed and tested two novel Paired Associative Stimulation (PAS) protocols, a class of non-invasive brain stimulation techniques in which a peripheral, sensory, stimulation is repeatedly paired with a Transcranial Magnetic Stimulation (TMS) pulse to induce Hebbian associative plasticity. The two PAS protocols presented in my thesis target sensory-motor networks with mirror functioning, exploiting a visuo-tactile (cross-modal PAS), and a visuo-motor pathway (mirror PAS), respectively.

In the first chapter of the present work, after a brief introduction to the concept of Hebbian associative plasticity, I will provide an exhaustive review of PAS protocols targeting sensory-motor systems, proposing a classification in three macro-categories: *within-system*, *cross-systems,* and *cortico-cortical* protocols, according to the characteristics of the paired stimulations.

In the second chapter, I will describe the principal properties of the Mirror Neuron System (MNS) also considering its cross-modal (i.e., visuo-tactile) characteristics and the plastic mechanisms that are been hypothesize at the ground of the development of mirror neurons' matching properties.

In the third chapter, I will introduce the cross-modal PAS (cm-PAS), a novel *cross-systems* PAS developed to exploit the visuo-tactile mirroring properties of the primary somatosensory cortex (S1) to induce Hebbian associative plasticity in such primary sensory

region. In a series of three experiments, timing dependency (*Experiment 1*), cortical (*Experiment 2*), and visual specificity (*Experiment 3*) of the protocol have been tested, by measuring changes in participants' tactile acuity. In *Experiment 3*, also possible neurophysiological changes within S1 has been assessed, recording somatosensory-evoked potentials (SEP). Then, in a fourth experiment, cm-PAS timing dependency has been further investigated, testing the hypothesis that anticipatory, predictive-like, mechanisms within S1 may play a central role in the effectiveness of the protocol.

In the fourth chapter, a second *cross-systems* PAS will be introduced: the mirror PAS (m-PAS) which exploits visuo-motor mirroring properties of the human brain. Differently from the cm-PAS, this second protocol targets visuo-motor integration within the MNS and aims at induce a novel, atypical, motor resonance phenomena (assessed recording motor-evoked potentials – MEPs – induced by TMS over the primary motor cortex – M1) following Hebbian learning. In three experiments, timing dependency (*Experiment 1*), visual (*Experiment 2*), and cortical specificity (*Experiment 3*) of the protocol have been tested. Furthermore, in the third experiment, the behavioral effects of the m-PAS are explored, using an imitative compatibility task exploiting *automatic imitation* phenomenon.

Finally, in the conclusive chapter, I will discuss theoretical, methodological, and clinical outcomes and future perspectives that arise from these two protocols and the related results.

Chapter I

Paired Associative Stimulation: advanced tool to explore sensory and motor brain plasticity

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1.1. The origins of PAS

1.1.1. A brief introduction to Hebbian associative plasticity

Since the first theorization in '*The organization of behavior*' (1949), Hebbian associative plasticity – also known as 'Hebbian learning' – has proved to be a fundamental form of plasticity in the nervous system of living beings. As Hebb himself stated: "*the general idea is an old one: that any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated' so that activity in one facilitates activity in the other*" (Hebb, 1949). Indeed, Hebbian associative plasticity claims that (*a*) temporal and (*b*) causal contingency between the response of two neurons (or two neural systems) leads, over time, to long-term potentiation (LTP) and/or long-term depression (LTD) of their synaptic efficacy (Buonomano & Merzenich, 1998; Caporale & Dan, 2008; Song, Miller, & Abbott, 2000). In the second half of the twentieth century, animal-based studies and computational models confirmed Hebb's rule, and therefore, the properties of synaptic plasticity (e.g., Bienenstock, Cooper, & Munro, 1982; Bliss & Lomo, 1973; Levy & Steward, 1979, 1983; Malinow, 1991; McNaughton, Douglas, & Goddard, 1978). However, it would be only in the 90s, thanks to the introduction of *in vitro* paired patch-clamp recordings from

monosynaptic connections between pyramidal neurons in the neocortex (Sakmann & Stuart, 1994), that the importance of temporal contingency between neurons firing could be finally demonstrated as a key factor for the successful induction of Hebbian associative plasticity. It was found that, in a homosynaptic circuit, LTP can be induced when the pre-synaptic neuron is repeatedly activated *before* the post-synaptic one, while LTD can be induced when the order of the events is reversed (i.e., the pre-synaptic neuron is repeatedly activated *after* the post-synaptic one) and the temporal window between the two stimulations has to be in the order of few milliseconds to successfully induce plastic modifications (Magee & Johnston, 1997; Markram, Lübke, Frotscher, & Sakmann, 1997). The timing dependency of neurons firing, responsible for the induction and the direction of plasticity, was translated in the concept of *spike-timing-dependent plasticity* (STDP) which encloses Hebb's classic theorization on synaptic learning and it is nowadays considered one of the main form of plasticity acting in mammalians' central nervous system (Caporale & Dan, 2008; Feldman, 2012; Markram, Gerstner, & Sjostrom, 2011).

1.1.2. The 'classical' PAS targeting M1

The first *in vivo* demonstration that a form of associative, timing-dependent, plasticity also applies to human cortical systems was achieved at the very beginning of the twenty-first century thanks to the introduction of the PAS protocol (Stefan, Kunesch, Cohen, Benecke, & Classen, 2000). In their pioneering work, Stefan and co-workers found that the repeated, time-locked, pairing of electric stimulations of the right median nerve (MN) with TMS pulses over left M1 (i.e., 90 paired stimuli at a frequency of 0.05 Hz for a total duration of 30 min) led to an increase of motor-evoked potentials (MEPs) amplitude after the end of the stimulation protocol, an evidence of LTP-like plasticity induction in M1. The two paired stimulations converge and interact in the motor system: the MN stimulation indirectly

through afferent somatosensory projections to M1 and the TMS directly through the exogenous activation of M1 neurons. Crucially, associative plasticity could be induced only when the inter-stimulus interval (ISI) between the two paired stimulations matched the average conduction time of the cortico-spinal tract (i.e. about 25 ms). Besides temporal contingency, Stefan and colleagues identified three other properties considered as key markers of Hebbian associative plasticity: (*a*) topographic specificity (no excitability changes in muscles not innervated by MN), (*b*) persistency (cortico-spinal facilitation lasting 30-60 min after the end of PAS) and (*c*) reversibility (return to baseline after 60 min).

Three years later, Wolters and co-workers (2003) found that when the ISI tested in PAS was reduced (10 ms), so that the TMS activation of M1 preceded the activation of the same area by MN stimulation, PAS after-effects on MEP amplitude were reversed, leading to a decrease of cortico-spinal excitability. Thus, not only timing-dependent LTP but also timing-dependent LTD can be induced by M1-PAS, evidencing that these protocols can be successfully used to study *in vivo* STDP in the human brain (Wolters et al., 2003).

The evidence that the M1-PAS can be a very useful tool to investigate plastic properties of the human motor system had led to a spread of such protocol in the field of neuroscience and, at date, over 120 studies using such protocol have been published. Importantly, pharmacological studies showed that PAS-induced plasticity shares important features with LTP/LTD cellular models, such as the mediation of *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent Ca^{2+} channels (Malenka & Bear, 2004). Indeed, the use of drugs that are antagonists of NMDA receptors blocks the induction of LTP/LTD plasticity (Stefan, Kunesch, Benecke, Cohen, & Classen, 2002; Wolters et al., 2003), while the use of voltage-gated Ca^{2+} channels antagonists, not only blocks the induction of plasticity, but reverse its direction (Weise, Mann, Rumpf, Hallermann, & Classen, 2017). In later years also a ‘spinal’ version of the M1-PAS was introduced, in which the ISIs exploited between

the electric nerve stimulation and M1-TMS do not match the interaction time between the two stimulations at a cortical level (i.e., 25 ms for LTP and 10 ms for LTD) rather the interaction at the spinal cord level between orthodromic volleys induced by the cortical stimulation and antidromic ones induced by the peripheral electric stimulus, hence inducing associative plasticity in the corticomotoneuronal synapses of the corticospinal tract rather than in M1 (e.g., Cortes, Thickbroom, Valls-Sole, Pascual-Leone, & Edwards, 2011; Shulga et al., 2015; Taylor & Martin, 2009).

However, recently, different studies highlighted that the main drawback of the M1-PAS is the high inter- and intra-individual variability of its effects. It seems that, on average, about the 30% of the tested participants is 'PAS non-responder' and, within the same participant, PAS outcomes are not always stable (e.g., Campana, Papazova, Pross, Hasan, & Strube, 2019; López-Alonso, Cheeran, Río-Rodríguez, & Fernández-Del-Olmo, 2014; Minkova et al., 2019). Among the factors that seem to affect the emergence of PAS effects there is attention, age and gender of participants (e.g., Müller-Dahlhaus, Orekhov, Liu, & Ziemann, 2008; Pellicciari, Miniussi, Rossini, & De Gennaro, 2009; Stefan, Wycislo, & Classen, 2004), even if further research is needed to better understand the exact contribution of such variables in the effectiveness of this non-invasive brain stimulation protocol. However, it has to be noted that the variability of PAS effects is in line with one of other non-invasive brain stimulation techniques, such as transcranial direct current stimulation (tDCS) (Strube, Bunse, Malchow, & Hasan, 2015).

1.1.3. Clinical applications of the M1-PAS

Despite the issue of variability, the growing evidence of the efficacy of M1-PAS on healthy individuals (for a review, see: Carson & Kennedy, 2013; Wischnewski & Schutter, 2016) led to the application of such protocol in different clinical populations.

For instance, M1-PAS was applied to study corticospinal excitability in the lesioned hemisphere of stroke survivors (e.g., Carson & Rankin, 2018; Castel-Lacanal, Gerdelat-Mas, Marque, Loubinoux, & Simonetta-Moreau, 2007; Castel-Lacanal, Marque, & Tardy, 2009; Jayaram & Stinear, 2008, 2009; Palmer, Wolf, & Borich, 2018; Rogers, Brown, & Stinear, 2011; Silverstein et al., 2019). Overall, M1-PAS seems to be effective in enhancing corticospinal excitability of the hemiparetic upper limb when the excitatory protocol (ISI of 25 ms) is applied in chronic stroke patients (Carson & Rankin, 2018; Castel-Lacanal et al., 2007, 2009). Interestingly, Palmer and co-workers (2018) found a correlation between PAS-induced corticospinal excitability enhancement and enhanced motor performance following PAS, speculating the potentiality of the protocol to impact on motor performance of post-stroke patients, paving the way to possible rehabilitation therapies which might implement such protocol (Palmer et al., 2018). Although these promising results, other studies on stroke patients that applied the same protocol, or the inhibitory version (ISI of 10 ms), failed in replicate its efficacy (Ferris, Neva, Francisco, & Boyd, 2018; Jayaram & Stinear, 2008, 2009; Rogers et al., 2011; Tarri et al., 2018).

Debated or controversial results were also found when M1-PAS efficacy was assessed in disorders of the motor system like Parkinson's disease (e.g., Bagnato, Agostino, Modugno, Quartarone, & Berardelli, 2006; Kishore et al., 2014; Kojovic et al., 2012; Latorre, Rocchi, Berardelli, Bhatia, & Rothwell, 2019; Morgante, Espay, Gunraj, Lang, & Chen, 2006; Ueki et al., 2006), focal hand dystonia (e.g., Belvisi et al., 2013; Kang, Terranova, Hilker, Quartarone, & Ziemann, 2011; Latorre et al., 2019; Meunier, Russmann, Shamim, Lamy, & Hallett, 2012; Quartarone et al., 2003; Sadnicka, Hamada, Bhatia, Rothwell, & Edwards, 2014; Weise et al., 2017), spinal cord injury (Ling, Alam, & Zheng, 2019), Huntington's disease (Crupi et al., 2008) or Giles de la Tourette syndrome (e.g., Brandt et al., 2014; Martín-Rodríguez et al., 2015).

Finally, M1-PAS has also been applied in different neuropsychiatric conditions like schizophrenia, major depressive disorder or Alzheimer's disease, highlighting abnormalities in cortical plasticity that may be connected to the typical neurological impairments of these pathologies (i.e., learning and memory deficits) (e.g., Frantseva et al., 2008; Kuhn et al., 2016; Lahr, Paßmann, List, Vach, & Flöel, 2016; Mehta, Thanki, Padmanabhan, Pascual-Leone, & Keshavan, 2019; Player et al., 2013; Strube et al., 2016).

Overall, further research is needed to better understand the clinical potential of the M1-PAS and the possible usefulness of such protocol in motor rehabilitation. Indeed, also in the clinical population, the main weakness of this non-invasive brain stimulation protocol seems to be the high inter-participants variability of induced neurophysiological responses (e.g., Campana et al., 2019; López-Alonso et al., 2014; Minkova et al., 2019; Strube et al., 2016).

1.2. *Within-system* PAS

In the following paragraphs, I will provide a state-of-the-art on the recently developed PAS protocols targeting sensory and motor systems, to give a deepen theoretical framework in which include the two PAS protocols developed during my doctorate and described in the experimental chapters **3** and **4**. I will distinguish PAS protocols in different categories (**Figure 1.1**), namely *within-system* PAS (described in the present paragraph), *cross-systems* PAS (see **1.3**), and *cortico-cortical* PAS (see **1.4**), according to the nature of the paired

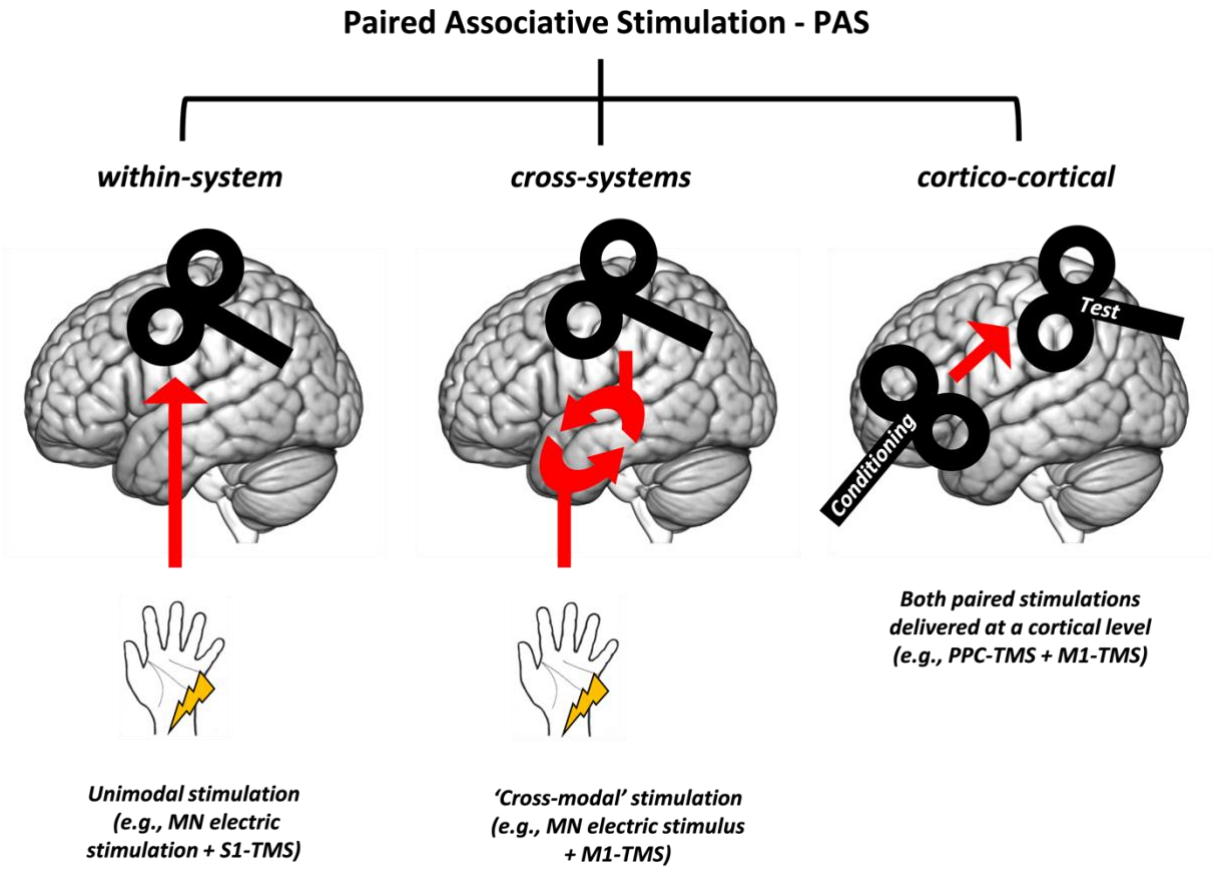

Figure 1.1. PAS protocols targeting sensory and motor areas can be divided in *within-system*, *cross-systems* or *cortico-cortical*, according to the characteristics of the paired stimulations and the cortical areas/systems activated. With this classification the standard M1-PAS should be considered a *cross-systems PAS*.

stimuli of the protocol. In details, in my dissertation, I name *within-system* PAS those protocols pairing the cortical (TMS pulse) stimulus with a peripheral one of the same sensory modality (e.g., MN electric stimulation *and* S1-TMS); *cross-systems* PAS uses a sort of 'cross-modal' motor stimulation which consists in pairing a sensory stimulus with motor cortex stimulation (e.g., visual stimulus *and* M1-TMS); finally, *cortico-cortical* PAS pairs two cortical stimulations over different cerebral areas (e.g., posterior parietal cortex-TMS *and* M1-TMS). In the final section of the chapter (**1.5**), for the sake of completeness, I will also describe very recent modified PAS targeting frontal networks (and so protocols targeting associative plasticity outside sensory-motor systems). This latter class of PAS is indeed a good example of the potential of this non-invasive brain stimulation paradigm.

Considering w*ithin-system* PAS, as state above, they combine peripheral stimulations and TMS pulses targeting the same cortical system. The PAS protocol outside the motor system was first applied to the primary somatosensory cortex (S1-PAS), although, more recently, *within-system* PAS have also been used to study the plastic properties of the auditory cortex and the primary visual cortex (V1). It has to be noted that, within the proposed classification, the classical M1-PAS described in the previous paragraph is a *cross-systems* protocol since the paired, peripheral, electric nerve stimulation activates the somatosensory afferent pathway, firstly activating the somatosensory system and, subsequently, the motor one. However, two modified versions of the PAS can be considered 'entirely' motor and, thus, they will be described in this paragraph (**Figure 1.2; Table 1.1**).

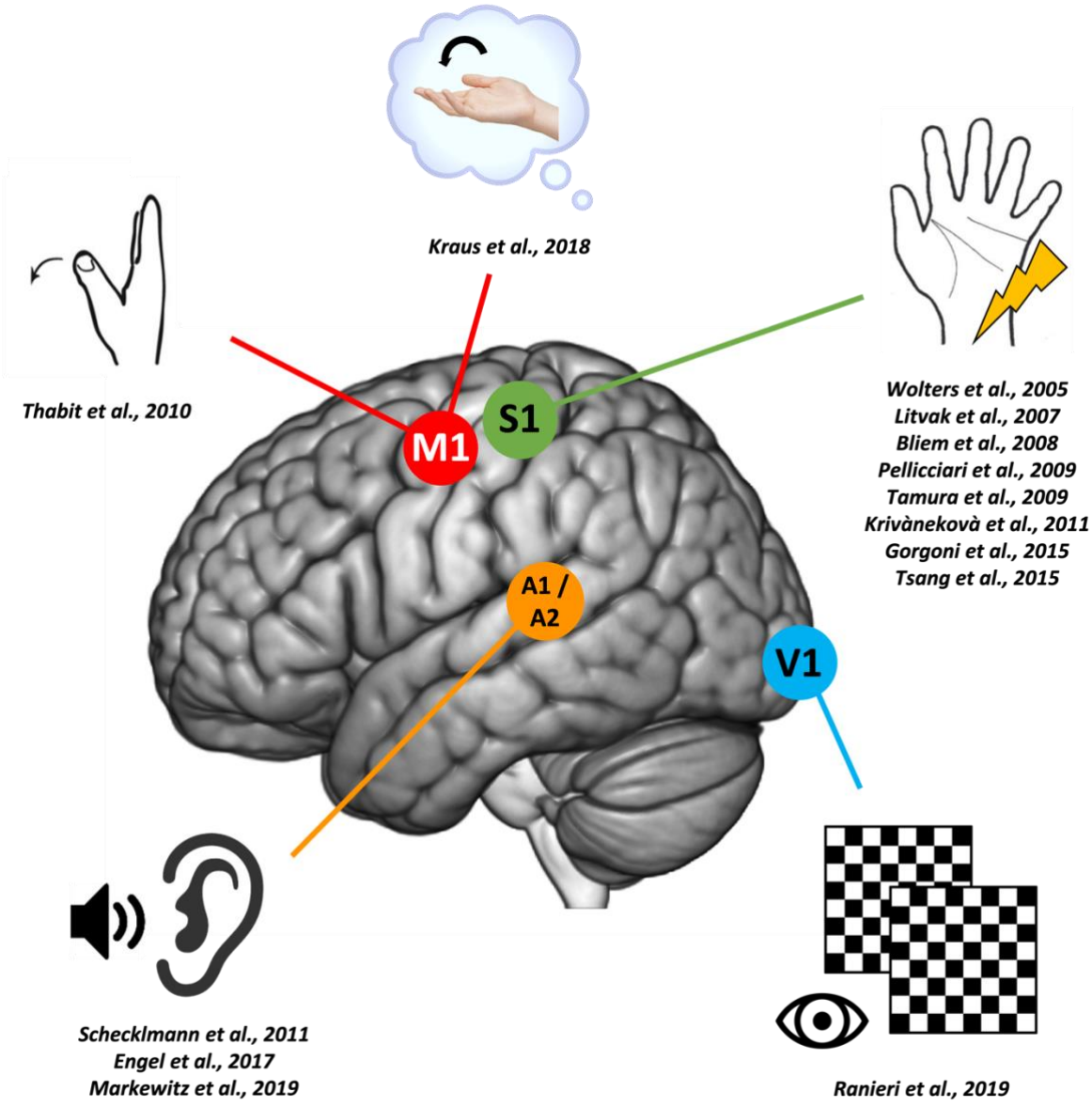

Figure 1.2. ***within-system*** **PAS**. Colored circles indicate site of the cortical stimulation (i.e., TMS) associated to the sensory, peripheral, one. Left hemisphere is depicted only for visualization purpose and does not reflect the correct hemisphere stimulated in the single study or by the single protocol. *Legend*: M1 = primary motor cortex; S1 = primary somatosensory cortex; A1 = primary auditory cortex; A2 = secondary auditory cortex; V1 = primary visual cortex.

within-system PAS	PAS parameters	Peripheral stimulation	Cortical stimulation	ISI	Effects
somatosensory system					
S1-PAS	180 stimuli @ 0.1 Hz (30 min)	MN-electric stimulation	S1	SEP N20 +0 / -2.5 ms	↑
				SEP N20 -20 ms	↓
				SEP N20 -40 / -30 / -10 / -5 / +5 / +10 / +20 / +100 ms	∅
	600 stimuli @ 5 Hz (2 min)	MN-electric stimulation	S1	SEP N20 -2.5 ms	↑
				SEP N20 +0 / +2.5 ms	∅
auditory system					
auditory PAS	200 stimuli @ 0.1 Hz (33 min)	4 Hz tone	Auditory cortex	10 / 45 ms	↓
visual system					
visual PAS	90 stimuli @ 0.2 Hz (7 min)	visual pattern-reversal stimulus	V1	VEP P1 +25 ms	↑
				VEP P1 -25 ms	↓
				VEP P1 -50 / +50 ms	∅
motor system					
active movement PAS	240 stimuli @ 0.2 Hz (20 min)	voluntary abduction movement	M1	mean RT (in a thumb abduction task) - 50 ms	↑
				mean RT + 100 ms	↓
				mean RT -100 / +50 / +150 ms	∅
motor imagery PAS	165 stimuli @ ≈0.1 Hz (40 min)	passive opening of the hand (driven by motor imagery)	M1	0 ms	↑
				80 ms	∅

Table 1.1. *within-system* PAS. Principal *within-system* PAS targeting motor and sensory systems. Effective protocols or replicated parameters are reported. For the ISIs, I reported all the tested ones with such protocol. ↑ = excitatory effects, ↓ = inhibitory effects, ∅ = ineffective ISI.

1.2.1. Somatosensory PAS

The primary somatosensory cortex (S1) was the target of the first attempts to adapt the PAS protocol outside the motor system. The S1-PAS consists in the repeated pairing of MN stimulation and TMS over the contralateral S1, the last delivered at the latency of the first cortical MN somatosensory-evoked potentials (SEPs) component, the N20, occurring about 20 ms after the MN stimulation onset (Macerollo, Brown, Kilner, & Chen, 2018). In a standard, excitatory, protocol, the stimulation lasts 30 min, comprising 180 paired stimuli delivered at 0.1 Hz. In the pioneering study of Wolters and colleagues (2005), S1-PAS successfully enhanced the P25 component (i.e., the second cortical component of SEPs) for at least 30 min, suggesting the induction of LTP. Conversely, when the two paired stimulations were delivered synchronously (ISI of 0 ms) P25 decreased, proving evidence of LTD induction (Wolters et al., 2005). The after-effects of S1-PAS were further investigated by Litvak and colleagues two years later (Litvak et al., 2007), who showed that S1-PAS with an ISI of about 20 ms (N20 latency - 2.5 ms) was effective in decreasing tactile acuity in the contralateral index finger while increasing the little finger's one. Again, S1-PAS with 0 ms of ISI, although being able to increase tactile acuity in the index finger, did not induce any enhancement of electrophysiological components. Moreover, the authors showed a change of SEPs topographical maps, specifically at the level of a tangential source located in Broadman area 3b, suggesting plastic modification in the upper layer of S1(Litvak et al., 2007). Further investigations revealed that the neurophysiological effects of the excitatory S1-PAS (ISI of 20 ms) are modulated by age and gender, being larger in elderly (individuals aged > 60 years) and in females (Pellicciari et al., 2009).

S1-PAS was also used to study homeostatic metaplasticity within this cortex: by administrating a peripheral high-frequency stimulation (pHFS) before the PAS protocol, it was found that S1 activation state might influence the effects of PAS, although some

confirmatory studies are necessary to better understand its efficacy (Bliem, Müller-Dahlhaus, Dinse, & Ziemann, 2008). This latter evidence might explain why failures in inducing relevant modification with S1-PAS were also on record: for instance, different studies found effects only at an individual level but not at a group level (Gorgoni et al., 2015; Kriváneková, Lu, Bliem, & Ziemann, 2011).

Tsang and co-workers developed a rapid-rate PAS (Quartarone et al., 2006) targeting S1 (Tsang, Bailey, & Nelson, 2015). The high frequency (5 Hz) of administration allows to administer hundreds of paired stimuli in a very short time (e.g., 600 stimuli in 2 min), overcoming possible confounding variables for the success of the protocol such as lack of participants' attention or arousal due to the length of the standard PAS (i.e., 30 min). Tsang and colleagues aimed to investigate induced plasticity by this rapid-rate S1-PAS both on S1 and M1, thus to assess possible effects of the protocol also in nearby cerebral areas and compare them with the ones of the same rapid-rate protocol targeting M1. Rapid-rate S1-PAS led to increased S1 excitability with a trend for an enhancement of N20 and P25 components when the ISI exploited was around 20 ms (N20 latency - 2.5 ms), similar to the standard S1-PAS. Furthermore, an increase in MEP amplitude and a decrease of short-latency afferent inhibition was found, reflecting the spreading of the induced plasticity also in M1. Interestingly, these effects on motor excitability were bigger than the similar ones induced by the rapid-rate M1-PAS with an ISI of 25 ms (N20 latency + 5 ms), suggesting that rapid-rate S1-PAS might induce, overall, better plastic effects within the sensorimotor system. However, no direct comparison of rapid-rate S1-PAS effects was made with those obtained with classic, excitatory, S1-PAS, and, consequently, it's not possible to assess whether this faster version is better than the standard one (Tsang et al., 2015).

A single study using S1-PAS was conducted on a clinical population suffering from idiopathic focal hand dystonia (Tamura et al., 2009), a motor disease characterized by

uncontrolled, repetitive muscle activity, in order to deepen the involvement of S1 in this disease. By using M1-PAS, it was found that such patients suffer from abnormal associative plasticity in M1 with greater MEPs enhancement after PAS than healthy participants (e.g., Quartarone et al., 2003). Studies suggest that also S1 and sensorimotor integration processes play a key role in this disease, since highly repetitive sensory inputs following uncontrolled muscles activity may induce plasticity-based remodeling of S1 neurons receptive fields (e.g., Conte et al., 2014; Tamburin, Manganotti, Marzi, Fiaschi, & Zanette, 2002; Tinazzi, Frasson, Bertolasi, Fiaschi, & Aglioti, 1999). Results obtained by Tamura and colleagues documented electrophysiological effects after the protocol (i.e., enhancement of SEPs P25 and increase of S1 intracortical inhibition) that were greater in the patients than in the control group of healthy participants. This evidence suggests that patients with focal hand dystonia may suffer from abnormal plastic mechanisms in S1 and this may contribute to the pathophysiology of the disease (Tamura et al., 2009).

1.2.3. Auditory PAS

An auditory version of PAS was developed by Schecklmann and colleagues in 2011 to investigate plasticity mechanisms that rule the auditory system. The auditory PAS consists of the repeated pairing of an acoustic tone (duration: 400 ms, frequency: 4 kHz, intensity: 60 dB) in the right ear with a TMS pulse over the left auditory cortex. The protocol lasts about 33 min for a total of 200 stimuli, delivered at 0.1 Hz. The first study tested two different ISIs (10 ms and 45 ms), based on the assumption that the first cortical component of the long latency acoustic-evoked potentials (LLAEPs), namely P1, peaked after 50 ms from the onset of the acoustic stimulus. Thus, such ISIs should induce associative LTD because the exogenous activation of the auditory cortex by the TMS preceded the cortical activation by the acoustic stimulus. Accordingly, it was found that after both auditory PAS

the N1-P2 complex was significantly reduced, with a greater effect size after the protocol with the longer ISI. Furthermore, the reduction was present even if the acoustic stimulus had not the same frequency (i.e., 1 kHz) of the one presented during the protocol, suggesting a 'tonotopically-unspecificity' of the protocol. This unspecificity might be useful in the treatment of auditory 'phantom perception' diseases (e.g., tinnitus), in which the exact matching of a tone to the perceived auditory phenomenon is frequently difficult, compromising the effectiveness of treatments where tonotopically-specificity is required (Schecklmann et al., 2011).

In a subsequent study, the influence of tone duration was deepened (S. Engel, Markewitz, Langguth, & Schecklmann, 2017). Engel and co-workers tested two auditory PAS (ISI of 45 ms) with different lengths of the acoustic stimulus, which could be of 23 or 400 ms (the latter as in Schecklmann et al. 2011). Auditory steady-state response (aSSR), namely, a type of acoustic-evoked potentials used to assess hearing sensitivity (Korczak, Smart, Delgado, Strobel, & Bradford, 2012), was exploited as the dependent variable. Results showed that only the auditory PAS with a tone duration of 400 ms successfully reduced aSSR amplitude but only when the tone used to evoke aSSR had a frequency of 20 Hz, and not of 40 Hz. This evidence suggests that cortical plastic effects of auditory PAS may take place in the secondary auditory cortex, where the source of 20-Hz aSSR is localized, rather than the primary auditory cortex, where 40-Hz aSSRs are generated (S. Engel et al., 2017). Two years later, Markewitz and co-workers found that auditory PAS paired with the 23 ms tone had no effects on LLAEPs amplitude, probably due to the very short length of the sound, whose elaboration is implicit and prevents habituation. In fact, by using a control auditory PAS protocol pairing 400 ms tone with sham TMS, the authors found a reduction of N1-P2 complex of LLAEPs, leading to speculate an unspecific habituation effect to the acoustic stimulation (Markewitz, Engel, Langguth, & Schecklmann, 2019).

1.2.3. Visual PAS

A recent study by Ranieri and colleagues (2019) introduced a visual PAS protocol that coupled a visual pattern-reversal stimulus (a black and white checkboard) with a TMS pulse over V1 delivered at 0.2 Hz for a total of 90 trials. The timing dependency of the protocol was tested exploiting different ISIs, accordingly to the individual peak latency of the pattern-reversal visual-evoked potentials (VEPs) P1 component, which occurs, on average, after 100-120 ms from visual stimulus onset and reflects the first activation of cortical extra-striate areas (Di Russo, Martínez, Sereno, Pitzalis, & Hillyard, 2002; Jeffrey, 1971). Results showed that, in visual PAS where TMS was delivered 25 ms after individual P1 latency, VEP amplitude and VEP habituation were reduced up to ten min from the end of the protocol. Conversely, in the PAS where TMS was delivered 25 ms before individual P1 latency, VEP habituation was enhanced but no effects on VEP amplitude were recorded. Versions of visual PAS with shorter/longer ISIs (i.e., individual P1 latency + 50 ms or individual P1 latency - 50 ms) were ineffective. The effects on visual processing were not assessed (Ranieri et al., 2019).

1.2.4. Motor PAS

At date, also a couple of *within-system* versions of M1-PAS have been tested. One of them was developed by Thabit and co-workers (2010) and named '*movement-related cortical stimulation*'. This PAS protocol consists of the repeated coupling of a TMS pulse over left M1 and a right-hand thumb abduction movement made by the participant, for a total of 240 stimuli delivered at 0.2 Hz. Firstly, participants were trained on a visuo-motor task which required a button-press with the right thumb every time a visual cue appeared: the participants' mean reaction time at such task was used to set the critical ISI of the PAS protocol. Results showed that when the cortical pulse over M1 was delivered 50 ms before

the participants' voluntary thumb abduction movement (which timing was indexed using the participant's mean reaction time in the motor task), this 'active movement' PAS enhanced MEP amplitude; conversely, if TMS over M1 was delivered 100 ms after the voluntary movement, MEP amplitude was reduced. These STDP-like effects lasted for 20 min after the end of the protocol and they were found only in the muscle involved in the targeted movement (*abductor pollicis brevis*). Such neurophysiological effects were paralleled by behavioral changes: motor responses with the right hand at a simple reaction time task were shortened after the PAS protocol in which TMS was delivered before the voluntary movement (Thabit et al., 2010).

Another *within-system* PAS targeting M1 was developed starting from a sort of associative brain-computer interface (BCI). The first attempts to develop a BCI exploiting associative mechanisms (and, thus, Hebbian plasticity) didn't involve TMS and relied only on the association between an electric stimulation of a muscle nerve with an endogenous activation of the motor system driven by motor imagination (e.g., Mrachacz-Kersting & Aliakbaryhosseinabadi, 2018; Mrachacz-Kersting, Kristensen, Niazi, & Farina, 2012). However, a particular type of motor PAS exploiting an associative BCI was developed by Kraus and colleagues (2018). This 'motor imagery' PAS pairs a motor imagination-driven passive opening of participants' left hand, achieved using a robotic orthosis, with a TMS pulse over right M1 (Kraus et al., 2018). The protocol consists of 15 blocks of 11 paired stimulations, for a total duration of 40 min. During PAS, participants were instructed to perform a motor imagination task (i.e., opening of the hand following a visual cue) and, every time a sensorimotor event-related desynchronization in the EEG beta band was detected, i.e., a marker of motor imagination (Bauer, Fels, Vukelić, Ziemann, & Gharabaghi, 2015), a TMS pulse over right M1 was administered. Two ISIs (0 ms or 80 ms) between the two paired stimulations were tested. Results showed that when the passive hand movement

and the cortical stimulation were synchronous (ISI of 0 ms), there was a significant increase of cortico-spinal excitability and additional recruitment of corticospinal neurons (enhanced MEP amplitude and area). Conversely, the asynchronous PAS protocol (ISI of 80 ms) did not affect corticospinal excitability. Furthermore, if the same pattern of paired stimulations was delivered without the concurrent motor imagination task, no effects emerged, suggesting that this kind of PAS relies on imagery-induced M1 activation rather than the sensorimotor feedback from the passive hand movement (Kraus et al., 2018).

1.3. *Cross-systems* PAS

Cross-systems PAS refers to those PAS protocols where the two paired stimulations belong to different sensory systems (**Figure 1.3; Table 1.2**). It has to be noted that the two protocols that I have developed during my doctorate can be considered part of this class of PAS (see **Chapters 3** and **4**). Generally speaking, at date, *cross-systems* PAS are been developed to influence M1 plasticity by accessing such area with different sensory modalities (i.e., auditive, visual, and through painful stimulation).

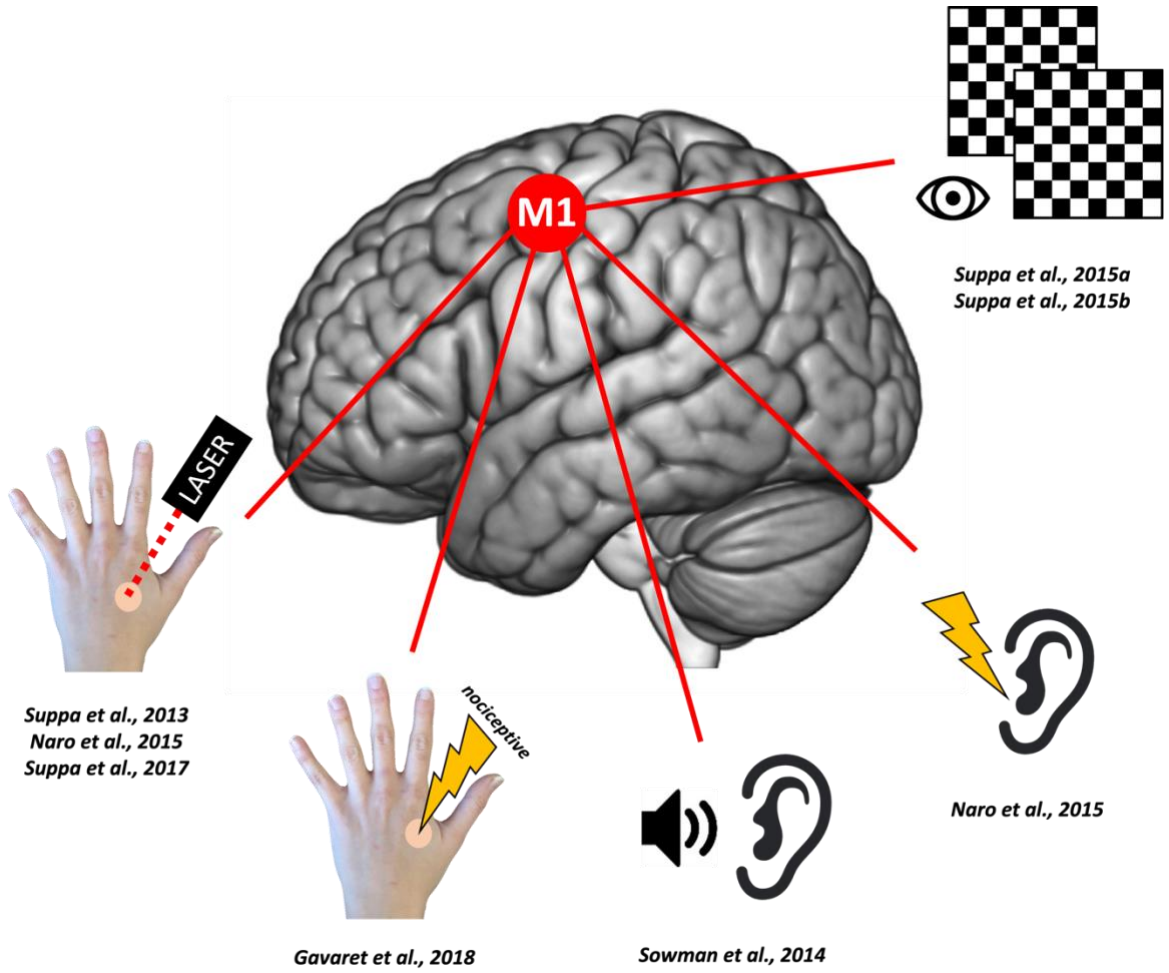

Figure 1.3. *cross-systems* PAS. Colored circles indicate site of the cortical stimulation (i.e., TMS) associated to the peripheral one. Left hemisphere is depicted only for visualization purpose. *Legend*: M1 = primary motor cortex.

cross-systems PAS	PAS parameters	Peripheral stimulation	Cortical stimulation	ISI tested	Effects
auditorymotor PAS	200 stimuli @ 0.2 Hz (17 min)	speech sound	M1	100 ms	↑
	600 stimuli @ 5 Hz (2 min)	electric stimulation of the ear nerve	M1	LLAEP N1 + 50 ms	↑
visuomotor PAS	600 stimuli @ 1 Hz (10 min)	visual pattern-reversal stimulus	M1	VEP P1 + 100 / + 120 ms	↑
				VEP P1 + 40 ms	↓
				VEP P1 +60 / +80 / +140 ms	∅
laser PAS	90 stimuli @ 0.1 Hz (15 min)	laser stimulation	M1	N1 LEP + 50 ms	↑
				N1 LEP +0 / +100 / +200 ms	∅
pain PAS	90 stimuli @ 0.1 Hz (15 min)	electric-nociceptive stimulation	M1	PREP N2 - 40 ms	↓
				PREP N2 +10 / +30 / +50 ms	∅

Table 1.2. *cross-systems* PAS. Principal *cross-systems* PAS targeting motor and sensory systems. Effective protocols or replicated parameters are reported. For the ISIs, I reported all the tested ones with such protocol. ↑ = excitatory effects, ↓ = inhibitory effects, ∅ = ineffective ISI.

1.3.1 Auditory-motor PAS

The auditorymotor PAS was developed to access the motor cortical system through audition: indeed, it is well-know that auditory inputs can influence motor activation (e.g., Fadiga, Craighero, Buccino, & Rizzolatti, 2002; Skipper, Nusbaum, & Small, 2005) as the transverse temporal gyrus, where the auditory cortex is located, and the central sulcus, where M1 is located, are widely interconnected, both functionally and anatomically (e.g., Baumann et al., 2007; Skipper & Hasson, 2017; Tourville, Reilly, & Guenther, 2008). A first version of the auditorymotor PAS was introduced by Sowman and colleagues (2014) by pairing an auditory stimulus, which consisted of the auditory presentation of the word 'Hey' (intensity: 80 dB), with a TMS pulse over right M1. A single ISI of 100 ms was tested according to a pilot study which demonstrated that the greater MEPs enhancement occurred at this time point after the onset of the auditory stimulus. A total of 200 stimuli were delivered at 0.2 Hz (total duration: 17 min). This auditorymotor PAS increased corticospinal excitability up to 15 min after the end of the protocol, suggesting the induction of associative LTP plasticity in M1 (Sowman, Dueholm, Rasmussen, & Mrachacz-Kersting, 2014).

A similar protocol was used by Naro and co-workers (2015) in patients with disorders of consciousness (DoC). According to the severity of DoC (i.e., unresponsive wakefulness syndrome *vs.* minimally consciousness state), patients may show residual preservation of auditory processing, involving higher-order associative areas (Boly et al., 2004, 2011). Hence, the authors examined whether plastic effects induced by an audiomotor PAS protocol might be different according to the severity of DoC. This alternative version of auditory-motor PAS paired transauricular repetitive electric stimulations of the right ear nerve with TMS pulses over left M1 delivered at 0.5 Hz in 3 blocks of 200 paired stimuli. The exploited ISI corresponded to the individual LLAEP N1 latency plus 50 ms. After the end of the protocol, DoC patients with minimally conscious state showed an increase of MEP

amplitude and potentiation of audiomotor integration markers (i.e., conditioned MEPs after the presentation of a sine tone burst). Conversely, more severe DoC patients, with unresponsive wakefulness syndrome, did not show any improvement or modification following PAS, probably due to a critical connectivity impairment in the audiomotor integration pathways. A group of healthy controls was also tested, showing the same pattern of results of DoC patients with minimally consciousness state, highlighting the effectiveness of the protocol also in the healthy population (Naro, Leo, Cannavò, et al., 2015).

1.3.2. Visuo-motor PAS

In humans, visual information accesses frontal areas through the superior longitudinal fasciculus, a key cortico-cortical white matter pathway that connects occipital areas with premotor ones and is involved in numerous visuomotor integration processes (e.g., Makris et al., 2005; Thiebaut de Schotten, Urbanski, Valabregue, Bayle, & Volle, 2014). Following this pathway, the visual information takes about 100-150 ms from the first elaboration in V1 to reach premotor areas and the motor system and this stream of information is fundamental for simple and complex motor behaviors guided by vision (Casali, Casarotto, Rosanova, Mariotti, & Massimini, 2010; Ledberg, Bressler, Ding, Coppola, & Nakamura, 2007). A visuomotor version of PAS was developed to assess the plastic properties of early-stage visuomotor integration processes (Suppa, Li Voti, Rocchi, Papazachariadis, & Berardelli, 2015). In the visuomotor PAS, a pattern-reversal visual stimulus (a black and white checkboard) presented in participants' right visual hemifield is paired with a TMS pulse over left M1. A total of 600 stimuli is presented at 1 Hz. Suppa and colleagues tested different ISIs, chosen following participants' VEP P1 latency (Jeffrey, 1971), which in their experimental sample, peaked on average after 100 ms from the pattern-reversal onset. Results showed that visuomotor PAS affected corticospinal excitability: with ISIs reflecting

participants' P1 latency plus 100 ms or plus 120 ms – which resembled the conduction time of the superior longitudinal fasciculus, the protocol increased MEP amplitude, while shorter ISI, reflecting participants' P1 latency + 40 ms, caused MEPs inhibition respect to baseline values. This evidence confirmed the speculation that the visual information elaborated in extrastriate areas takes about 100 ms to reach frontal areas, hence requiring such delay to interact with TMS-induced M1 activation and induce LTP-like plasticity. A similar pattern of results was obtained when the protocol was delivered with the same number of trials (i.e., 600) but with a longer inter-stimuli frequency (i.e., 0.25 Hz between paired stimuli); conversely, reducing the number of stimuli (i.e., 150 trials) proved to be unsuccessful. This evidence indicates that visuomotor PAS effects are dose-dependent but not frequency-dependent, as documented also for classic M1-PAS (Stefan et al., 2000). Finally, Suppa and colleagues found that this protocol not only induces local effects in M1 but also modulates functional connectivity between M1 and pre-motor areas. In particular, premotor-to-motor connectivity, assessed using paired-pulse TMS, increased after the excitatory protocol (the one exploiting the ISI corresponding to P1 latency + 100 ms), in line with the speculation that this protocol affects motor cortex connectivity through the superior longitudinal fasciculus, which connects occipital areas with premotor ones (Suppa, Li Voti, et al., 2015). The same research group (Suppa, Rocchi, et al., 2015) assessed the effects of visuomotor PAS in patients suffering from intermittent photic stimulation-induced photo paroxysmal response (PPR), a condition that is usually associated with epileptic syndromes and abnormal visuomotor integration (Waltz, Christen, & Doose, 1992). In a series of experiments, excitatory visuomotor PAS (ISI corresponding to individual P1 latency +100 ms) was compared among (*a*) a group of PPR-positive epileptic and non-epileptic patients, (*b*) a group of PPR-negative epileptic patients and (*c*) a group of healthy controls. Results showed that the protocol increased MEP amplitude in all tested groups, with larger effects

in PPR-positive patients, suggesting a correlation between PPR and the presence of abnormal plasticity. In a series of subsequent experiments, inhibitory visuomotor PAS (ISI corresponding to individual P1 latency + 40 ms) and an alternative version where the ISI exploited corresponded to P1 latency plus 140 ms were compared between PPR-positive patients and the healthy control group. Inhibitory protocol, as expected, reduced MEP amplitude in both groups and, selectively in PPR-positive patients, the one with an ISI corresponding to P1 + 140 ms was able to induce LTP-like plasticity, mirroring the excitatory outcomes of the visuomotor PAS with the ISI corresponding to P1 + 100 ms and suggesting a wider temporal window for the induction of plasticity in these patients. Another important difference between PPR-positive patients and healthy controls was that premotor-to-motor connectivity (as assessed with ppTMS) decreased instead of increasing after the administration of excitatory visuomotor PAS, suggesting possible structural anomalies in patients' superior longitudinal fasciculus. Clinical outcomes of the different PAS versions were not assessed. Overall, these results, besides supporting the hypothesis that abnormal visuomotor integration plays a central role in the pathophysiology of PPR, provide the first clinical evidence that the visuomotor PAS can be a useful tool to modulate dysfunctional visuomotor plasticity (Suppa, Rocchi, et al., 2015).

1.3.3. Pain-motor PAS

Cross-systems PAS protocols targeting the nociceptive system had also been developed aiming to modulate pain processing. Indeed, the motor system plays a crucial role in pain perception and pain modulation (e.g., Dubé & Mercier, 2011; Farina, Tinazzi, Le Pera, & Valeriani, 2003; Urban et al., 2004; Valeriani et al., 1999), exerting inhibitory control over the areas of the so-called 'pain matrix' (Garcia-Larrea & Peyron, 2013; Iannetti & Mouraux, 2010).

Suppa and colleagues (2013) developed a first pain-motor PAS, the laser PAS, which pairs painful laser stimulations of the right hand with TMS pulses over the contralateral (left) M1. The protocol comprises 90 stimuli delivered at 0.1 Hz for a total duration of 15 min (Suppa et al., 2013). Considering that previous studies highlighted that corticospinal excitability is modulated only when TMS over M1 is delivered 50 ms after the first Laser-Evoked Potential (LEP) negative component, N1 – which peaked, on average, 160 ms from laser stimulation onset and its cortical source is localized in parietal-temporal regions (Valeriani et al., 1999) – such ISI was exploited in laser PAS. Results showed that the protocol successfully enhanced corticospinal excitability and this enhancement lasted up to 50 min after the end of PAS. Importantly, MEPs enhancement was specific for the targeted muscle of the laser stimulation (*abductor digiti minimi*) and protocol efficacy was abolished when participants took a drug antagonist for NMDA-receptors, which play a main role in STDP (Caporale & Dan, 2008). The proposal put forward was that laser PAS probably elicited NMDA-dependent STDP in M1 cortical layers II and III, whose pyramidal axons are directly activated by TMS over M1 (Suppa et al., 2013).

The laser PAS was also applied in the clinical population. In a first study (Naro, Leo, Russo, et al., 2015), the protocol (with an ISI corresponding to individual LEP N1 + 50 ms) was applied in DoC patients with unresponsive wakefulness syndrome, characterized by the absence of motor response to painful stimulations (de Tommaso et al., 2013). Results showed, at a group level, the absence of laser PAS effects: the protocol was ineffective on M1 excitability, in pain-motor integration markers, and on clinical scales evaluating pain perception. However, at a single-subject level, some patients showed a transient enhancement of M1 excitability and short-lasting reshaping of pain-motor integration, both in neurophysiological and clinical evaluation. This evidence suggests that laser-PAS can be used to assess the severity of DoC and possible residual pain processes at a cortical level,

which may be a sign of partially preserved consciousness (Naro, Leo, Russo, et al., 2015). A second study applied laser PAS in Parkinson's disease (PD), investigating whether the presence of chronic pain might influence the protocol efficacy (Suppa, Leone, et al., 2017). All PD patients, independently from the presence of chronic pain or from being on drug treatment, showed reduced MEPs enhancement after laser PAS compared to healthy individuals. To assess whether this reduced enhancement might be caused by specific abnormalities in pain-motor integration rather than by unspecific ones in the motor system, in a second experiment, also the standard excitatory M1-PAS (ISI of 25 ms) was exploited. The results showed that PD patients without chronic pain had similar, reduced, neurophysiological responses to both PAS protocols; conversely, PD patients with chronic pain showed a statistically significant impairment between the two protocols, and only M1-PAS turned out effective in enhancing MEP amplitude. This latter evidence suggests that chronic pain in PD might influence the response to laser PAS through abnormal pain-motor integration (Suppa, Leone, et al., 2017).

A second pain-motor version of PAS, the pain PAS, combines a nociceptive electric stimulation of the right hand with a TMS pulse over left M1 (Gavaret et al., 2018). Pain PAS is delivered for 15 min at a frequency of 0.1 Hz for a total of 90 stimuli. In the original work by Gavaret and colleagues, different ISIs were tested based on the individual latency of the N2 component of pain-related evoked potentials, which reflects bilateral insular and cingulate sources and peaks, on average, after 150-160 ms from the painful electric stimulus onset (Lefaucheur et al., 2012). Pain PAS was effective in decreasing MEP amplitude selectively when the ISI correspond to participants' N2 latency minus 40 ms. No excitatory effects were reported with longer ISIs. Unfortunately, the nociceptive electric stimulation, even if prevented the risks of skin habituation (which is typical, for instance, of painful laser stimulation), activated a larger number of sensory afferent fibers (A-delta nerve fibers and

large-diameter A-beta axons) making difficult to disentangle the specific contribution of a specific pathway (i.e., pain-motor *vs.* somatosensory-motor) for the success of the protocol (Gavaret et al., 2018).

1.4. *Cortico-cortical* PAS

Cortico-cortical PAS (cc-PAS) are modified PAS protocols where both paired stimulations are delivered at a cortical level, usually with TMS, allowing to directly activate the cortico-cortical pathway connecting two areas. Importantly, cc-PAS can be considered both *within-system* and *cross-systems* protocols.

Cc-PAS are very useful protocols to adopt when the connectivity between two cerebral areas are well-known, as they allow to causally investigate the plastic properties of these connections and their effectiveness. Then, it should not be surprising that cc-PAS protocols were mainly developed to study connectivity between areas of the motor networks, even if, recently, a cc-PAS has been developed to investigate plastic properties within the visual system (**Figure 1.4; Table 1.3**).

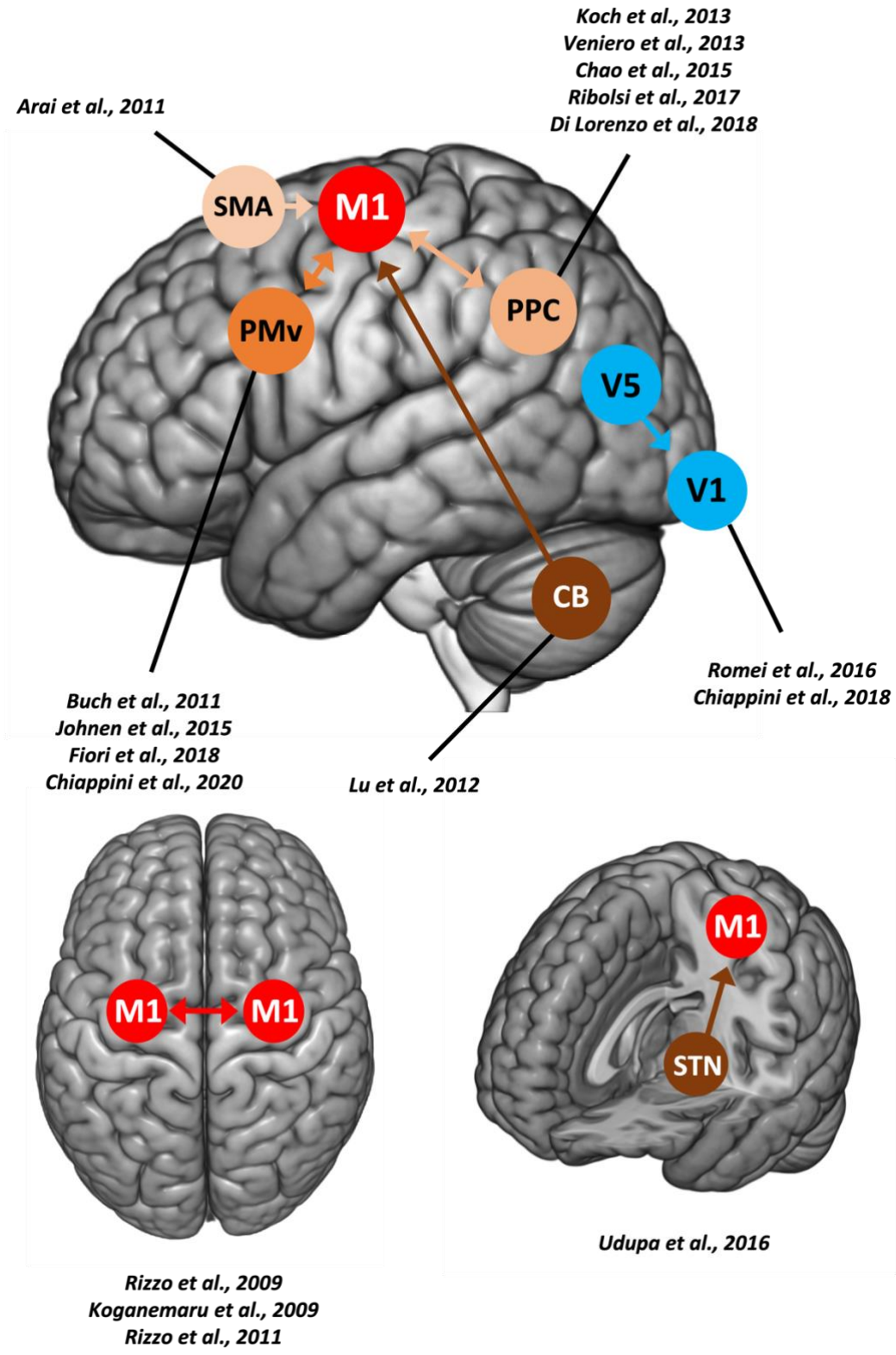

Figure 1.4. ***cortico-cortical*** **PAS**. Colored circles indicate sites of the cortical stimulation (i.e., TMS). Arrows indicate direction of *test* and *conditioning* pulses. Left hemisphere is depicted only for visualization purpose. *Legend*: SMA = supplementary motor area; PMv = ventral premotor cortex; M1 = primary motor cortex; PPC = parietal posterior cortex; CB = cerebellum; V5 = visual area 5; V1 = primary visual cortex.

cortico-cortical PAS	PAS parameters	conditioning pulse	test pulse	ISI	Effects
motor cc-PAS					
M1-M1 PAS	90 stimuli @ 0.05 Hz (30 min)	L / R M1	R / L M1	8 ms	↑
	180 stimuli @ 0.1 Hz (30 min)	L / R M1	R / L M1	15 ms	↑
				-25 / -15 / -5 / +5 / +25 ms	∅
PM-M1 PAS	90 stimuli @ 0.1 Hz (15 min)	ventral PM	M1	8 ms	↓ (at rest) / ↑ (during grasping)
				-8 ms	↓ (during grasping)
				40 ms	↑
				500 ms	∅
SMA-M1 PAS	150 stimuli @ 0.2 Hz (15 min)	SMA-proper	M1	6 ms	↑
				-15 ms	↓
				-15 / -10 / 3.2 ms	∅
PPC-M1 PAS	100 stimuli @ 0.2 Hz (8 min)	PPC	M1	5 / 20 ms	↓ (at rest) / ↑ (anterior-to-posterior TMS-induced current direction or active muscle contraction during cc-PAS)
				-5 / -20 ms	↑ (at rest) / ↓ (anterior-to-posterior TMS-induced current direction or active muscle contraction during cc-PAS)
				-50 / +50 ms	∅
	180 pulses @ 0.2 Hz (15 min)	PPC	M1	8 ms	↑
				100 ms	∅
cerebellum-M1 PAS	120 stimuli @ 0.25 Hz (8 min)	cerebellum	M1	2 ms	↑
				6 / 10 ms	↓
subcortical-M1 PAS	180 stimuli @ 0.1 Hz (30 min)	subthalamic nucleus	M1	3 / 23 ms	↑
				167 ms	∅
sensory cc-PAS					
V5-V1 PAS	90 pulses @ 0.1 Hz (15min)	V5	V1	20 ms	↑
				-20 / 0 ms	∅

Table 1.3. *cortico-cortical* PAS. Principal *cortico-cortical* PAS targeting motor and sensory systems. Effective protocols or replicated parameters are reported. For the ISIs, I reported all the tested ones with such protocol. ↑ = excitatory effects, ↓ = inhibitory effects, ∅ = ineffective ISI.

1.4.1. M1-M1 PAS

The first cc-PAS was developed by Rizzo and colleagues in 2009 and aimed to investigate the plastic mechanisms regulating the interhemispheric connectivity of M1 (Rizzo et al., 2009). M1 indeed, is widely interconnected with the homologs area of the opposite hemisphere through transcallosal connections (Carson, 2005) and this connectivity is fundamental for dexterity and movement coordination (e.g., Robert Chen, Gerloff, Hallett, & Cohen, 1997; Verstynen, Diedrichsen, Albert, Aparicio, & Ivry, 2005). The M1-M1 PAS pairs TMS pulses over left M1 (*conditioning pulse*) with ones over the homologous area of the right hemisphere (*test pulse*). A total of 90 paired stimulations is delivered at a frequency of 0.05 Hz. The ISI exploited is 8 ms, which follows the timing of interhemispheric inhibition (IHI) (e.g., Ferbert et al., 1992). The protocol, which can be considered *within-system*, was found effective in attenuating left-to-right IHI for at least 60 min and in increasing MEP amplitude obtained from the stimulation of right M1. Short-interval intracortical inhibition (SICI) and facilitation (ICF) remained unchanged. Reversing the direction of the paired stimulation (*conditioning pulse* over right M1 and *test pulse* over left M1), right-to-left IHI was attenuated but no effects were detected in left M1 excitability, pointing to a possible role of manual dexterity (all participants tested were right-handers) in the asymmetric modulation of corticospinal excitability. Importantly, the protocol was ineffective when delivered to a patient with callosal agenesis, suggesting that associative plasticity primarily relies on transcallosal circuits, rather than on local M1 stimulation (Rizzo et al., 2009). Behavioral facilitation of this protocol was detected in a motor task, showing a fastening of repetitive finger opposition movements and an increased duration of the thumb-index contact in the easier sequences of the task. These effects were restricted to the conditioned hand (i.e., right hand for left-to-right M1-M1 PAS; left hand for right-to-left M1-M1 PAS) and were not present in the more complex motor sequences of the task,

suggesting that the plastic effects of the protocol did not widespread to the surrounding areas of the motor system, e.g., the supplementary motor area (SMA), which is known to be involved in the programming and execution of more complex motor behavior (Rizzo et al., 2011).

By using a slightly different version of the protocol with an ISI of 15 ms and a frequency between the paired stimulations of 0.1 Hz, Koganemaru and colleagues (2009) found a significant change both at a behavioral and neurophysiological level. Behaviorally, this version of M1-M1 PAS improved participants' performance at the 9-hole peg test, a test that measures finger dexterity. Neurophysiologically, an enhancement of corticospinal excitability (MEPs enhancement) was observed both when the test pulse of cc-PAS was delivered over the right (left-to-right M1-M1 PAS) or the left M1 (right-to-left M1-M1 PAS). This result, in contrast with the previous work of Rizzo and co-workers (2009), may be related to the different ISI and frequency which might have activated different neuronal populations within and between M1 (Koganemaru et al., 2009).

1.4.2. PM-M1 & SMA-M1 PAS

First, cc-PAS protocols targeting two non-homologs brain regions were developed to study plastic properties between the principal nodes of the motor network. Indeed, M1 is widely interconnected with both the ventral premotor (PM) cortex (e.g., Buch, Mars, Boorman, & Rushworth, 2010; Quessy, Côté, Hamadjida, Deffeyes, & Dancause, 2016) and the caudal part of SMA, the so-called SMA-proper (e.g., Kim et al., 2010; Matsumoto et al., 2007). Thus, two different cc-PAS (i.e., PM-M1 PAS and SMA-M1 PAS) were introduced to study connectivity properties between M1 and these two areas (Arai et al., 2011; Buch, Johnen, Nelissen, O'Shea, & Rushworth, 2011).

In PM-M1 PAS (Buch et al., 2011), the *conditioning pulse* is delivered over left ventral PM and the *test pulse* over ipsilateral M1 with an ISI of 8 ms, a value based on previous paired-pulse TMS studies on the conduction time between these cerebral areas (e.g., Davare, Lemon, & Olivier, 2008). A total of 90 stimuli is administered at a frequency of 0.1 Hz. In the original study of Buch and co-workers, effects of the protocol were assessed, at rest, or during a reaching-and-grasping motor task, by measuring MEP amplitude induced by paired-pulse (over the same areas targeted by PAS) and single-pulse TMS. PM-M1 PAS led to different after-effects depending on whether they were assessed at rest or during the motor task. At rest, an inhibition of MEP amplitude was recorded during paired-pulse TMS assessment, with no effects on corticospinal excitability measured using single-pulse TMS; during the motor task, MEPs induced by both paired-pulse and single-pulse TMS increased. This facilitatory influence turned into an inhibitory one when the order of the paired stimulations was reversed – i.e., *conditioning pulse* over M1 and *test pulse* over ventral PM – suggesting that the direction of plasticity (LTP or LTD-like) depends on the direction of the paired stimulations. Finally, associative plasticity was anatomically specific: the effects emerged only when the conditioning pulse was delivered over the ventral PM, but not if it was delivered over the pre-SMA (Buch et al., 2011). The neurofunctional underpinnings of the protocol were deepened by using functional magnetic resonance imaging (Johnen et al., 2015). After PM-M1 PAS, functional connectivity between the stimulated areas, as well in dorsolateral circuits, increased, while decreased in the dorsal PM cortex, indicating that PAS modulatory effects spread to brain regions connected to the pathway targeted by the protocol. The effects were also timing-specific: when the ISI between the two paired stimulations was too long (500 ms), no connectivity changes were observed (Johnen et al., 2015). The PM-M1 PAS has also a behavioral outcome, improving finger dexterity in healthy participants (Fiori, Chiappini, & Avenanti, 2018). Recently, the PM-M1 PAS was found effective in

modulate MEP amplitude also exploiting the ISI of 40 ms, which is the timing of long-latency, likely indirect, inhibitory PM-to-M1 interactions (Fiori et al., 2017), suggesting that this cc-PAS can strengthen motor networks also through the modulation of indirect pathways (Chiappini et al., 2020).

SMA-M1 PAS (Arai et al., 2011) pairs *conditioning pulses* over SMA with near-simultaneous *test pulses* over bilateral M1 (TMS over both left and right M1 with a 0.8 ms inter-pulse interval) at a frequency of 0.2 Hz for a total of 150 stimuli. The protocol increased MEP amplitude when the ISI between the paired SMA-M1 stimulations was of 6 ms (namely, SMA-M1 conduction timing; Arai, Lu, Ugawa, & Ziemann, 2012) and decreased M1 excitability when the pulses over M1 preceded the ones over SMA of 15 ms. If the conditioning pulses were delivered over pre-SMA, which is not connected with M1 (Kim et al., 2010), no modulation of MEPs was recorded. Interestingly, a critical factor for the success of the protocol seemed bilateral M1 priming: this priming might induce metaplasticity in the SMA-M1 network, which would be necessary for the subsequent induction of plasticity during the PAS protocol (Arai et al., 2011).

1.4.3. PPC-M1 PAS

Another brain region that is connected, both directly and indirectly, with M1 is the posterior parietal cortex (PPC) and the first cc-PAS deploying such a cortico-cortical pathway was developed by Koch and co-workers (Koch, Ponzo, Di Lorenzo, Caltagirone, & Veniero, 2013). The PPC-M1 PAS repeatedly pairs a TMS pulse over the left PPC (*conditioning pulse*) with a TMS pulse over the ipsilateral M1 (*test pulse*). A total of 100 paired stimulations are delivered at a frequency of 0.2 Hz. The protocol proved to be effective in inhibiting corticospinal excitability when the ISI between conditioning and test stimulations was of 5 or 20 ms; conversely, the protocol enhanced MEP amplitude when the stimulation

of M1 was 5 or 20 ms before that of PPC (ISIs of -5 ms or -20 ms, thus reversing the order of the *conditioning* and *test pulse*, M1-PPC cc-PAS). These effects lasted for at least 20 min after the end of the protocol. The neurophysiological effects resembled the so-called 'anti-Hebbian' STDP: at a cellular level, for synapses more distant from the soma, the timing required for pre-pairing/post-pairing may shift such that the sign of synaptic modification can be opposite to the classic Hebbian STDP models (e.g., Froemke, Poo, & Dan, 2005). In other words, LTD may be induced when pre-synaptic cells (in PPC-M1 PAS: PPC) fire *before* the post-synaptic ones (in PPC-M1 PAS: M1) and LTP may be induced when pre-synaptic cells fire *after* post-synaptic ones. By (*a*) changing coil orientation and delivering anterior-to-posterior current flow or (*b*) administering the protocol while participants performed an active muscle contraction with the hand contralateral to TMS, Koch and colleagues found that PPC-M1 PAS induced classic Hebbian STDP (LTP induction with ISI of 5 ms and LTD induction with ISI of -5 ms). Thus, with PPC-M1 PAS is possible to induce antithetic forms of associative plasticity (Hebbian and anti-Hebbian) with the same temporal dependency, depending to (*a*) the stimulation of specific neuronal populations and (*b*) the activity state of the cortex during the protocol (Koch et al., 2013). The neurophysiological underpinnings of PPC-M1 PAS were further investigated by using EEG-TMS co-registration (Veniero, Ponzo, & Koch, 2013). TMS-evoked potentials (TEPs) were recorded from M1 and PPC after the administration of both the excitatory (ISI of -5 ms) and inhibitory (ISI of 5 ms) versions of PPC-M1 PAS. Results showed that, while TEPs over PPC was not modulated, M1 TEPs showed the opposite pattern of MEPs, namely, cortically evoked activity decreased when MEPs were enhanced and vice versa. Furthermore, the excitatory protocol increased alpha-band coherence between the two targeted areas, while the inhibitory one increased coherence only in the beta band. Since these bands reflect the activity of M1 and PPC (Rosanova et al., 2009; Salmelin & Hari, 1994), PPC-M1 PAS

seemed to increase phase coupling between these two areas and this increased coupling could, in turn, potentiate the efficacy of cortico-cortical communication in the parietal-motor pathway (Veniero et al., 2013). By using a positive ISI of 8 ms and increasing the number of paired stimulations, an alternative version of PPC-M1 PAS successfully enhanced corticospinal excitability, with maximum effects 60 min after the end of the protocol, indicating once more that the direction of plasticity strongly relies on the ISIs, which impact on different neuronal populations (Chao et al., 2015).

The parietal-motor PAS was also tested in schizophrenic patients to investigate possible asymmetrical hemispheric connectivity. Ribolsi and colleagues found that excitatory PPC-M1 PAS (ISI of -5 ms) targeting the left hemisphere was ineffective in modulating M1 excitability in such patients, while, when delivered over the right hemisphere, facilitated corticospinal excitability, even if this LTP-like enhancement became significant only 20 min after the end of the protocol. In a control group of healthy participants, PPC-M1 PAS induced comparable excitatory effects in both hemispheres. This evidence suggests the presence of asymmetrical hemispheric connectivity in schizophrenia, more evident for the left hemisphere (Ribolsi et al., 2017). The effects of PPC-M1 PAS were explored also in Alzheimer's disease (Di Lorenzo et al., 2018). Indeed, memory loss of such patients is related to early degenerative mechanisms in the hippocampus and associative cortices such as PPC and, in a broader perspective, to a disturbance in the brain's effective connectivity (Delbeuck, Van der Linden, & Collette, 2003). Applied in Alzheimer's patients, both LTP-inducing (ISI of -5 ms) and LTD-inducing (ISI of 5 ms) protocols were ineffective at least in modulating corticospinal excitability, providing support to the hypothesis of impaired cortico-cortical STDP in such form of dementia (Di Lorenzo et al., 2018).

1.4.4. Cerebellum-M1 & subcortical-M1 PAS

Two cc-PAS protocols were developed to target long-range M1 connectivity. In one, plastic properties of the cerebellar-dentato-thalamo-M1 pathway were investigated (Lu, Tsai, & Ziemann, 2012), while, in the other, the combination of deep brain stimulation of the subthalamic nucleus and TMS over M1 was tested on PD patients to induce associative plasticity in the motor system following a subcortical-cortical pathway (Udupa et al., 2016). The cerebellum-M1 PAS repeatedly pairs *conditioning pulses* over the right cerebellum with *test pulses* over the left M1. A total of 120 stimuli are delivered at a frequency of 0.25 Hz for a protocol length of 8 min. This cc-PAS was effective in decreasing MEP amplitude when the ISI was of 6 or 10 ms (i.e., the time of cerebellar-motor inhibition phenomena; Ugawa, Uesaka, Terao, Hanajima, & Kanazawa, 1995); conversely, with an ISI of 2 ms, MEP amplitude increased. At the same time, cerebellar-motor inhibition assessed with paired-pulse TMS was not modulated by the protocol, suggesting that associative plasticity is mainly induced at a cortical level, within M1, rather than in the cerebellar-cortical pathway (Lu et al., 2012).

The subcortical-M1 PAS was developed by Udupa and colleagues in 2016 to investigate the presence of abnormal plasticity in the connections between the basal ganglia and M1 in Parkinson's disease (PD) (Udupa et al., 2016). This protocol pairs deep brain stimulation of the subthalamic nucleus (*conditioning pulse*) with M1-TMS (*test pulse*). Over 30 min, a total of 180 stimuli is delivered at a frequency of 0.1 Hz. In PD, using deep brain stimulation, the best ISIs to enhance MEP amplitude is of ~3 ms (short-interval) and ~23 ms (medium-interval) (Kuriakose et al., 2010). Exploiting these ISIs in subcortical-M1 PAS, the stimulation increased M1 excitability for 45 min after the end of the protocol, while TMS markers of M1 intracortical facilitation and inhibition (ICF and SICI) did not change. At a

longer ISI (i.e., 167 ms), PAS was ineffective. Possible clinical outcomes of such PAS were not assessed (Udupa et al., 2016).

1.4.5. V5-V1 PAS

In recent years, a cc-PAS protocol had also been developed to target the visual system and, specifically, the connectivity between the visual motion area (V5) and V1 (V5-V1 PAS) (Romei, Chiappini, Hibbard, & Avenanti, 2016). Indeed, it was shown that back projections from extra-striate areas to V1 determine whether visual awareness will arise (e.g., Pascual-Leone & Walsh, 2001; Silvanto, Cowey, Lavie, & Walsh, 2005) and, more in details, that back projections from V5 to V1 may mediate motion awareness (Silvanto, Lavie, & Walsh, 2005). Thus, Romei and colleagues aimed to enhance visual motion sensitivity by increasing the synaptic efficacy within the visual system using the V5-V1 PAS. This protocol pairs *conditioning pulses* over left V5 with *test pulses* over V1, exploiting an ISI of 20 ms, namely the conduction time of V5-to-V1 projections (Silvanto, Lavie, et al., 2005). A total of 90 paired stimulations was delivered at 0.1 Hz (duration: 15 min). Using a motion coherence discrimination task, this cc-PAS protocol was found effective in boosting participants' performance and lowering the motion sensitivity threshold. Conversely, no effects were induced in participants' motion sensitivity when the two paired stimulation were synchronous (ISI of 0 ms) and when the direction of the stimulation was reversed (*conditioning pulse* over V1 and *test pulse* over V5) (Romei et al., 2016). Two years later, functional underpinnings of this protocol were deepened by Chiappini and colleagues by applying V5-V1 PAS in a state-dependent manner. During the protocol, to engage direction-specific V5 neurons, participants had to observe stimuli moving in a specific direction – i.e., leftwards or rightwards. The authors tested the hypothesis that the matching between the motion direction observed and the direction of the stimulation might selectively induce

plasticity in the neural pathway coding for that motion direction. Accordingly, V5-V1 PAS successfully enhanced motion sensitivity selectively when motion direction was congruent to the one used during the protocol, with no effects for the opposite condition. Interestingly, these effects were found only when the *conditioning pulse* intensity was below the phosphene threshold; conversely, when the intensity was set at the phosphene threshold, as tested by Romei in 2016, function-tuning V5-V1 PAS was ineffective. This evidence suggested that the administration of the protocol in a state-dependent manner, with the same parameters of the version 'at rest', blocked the induction of plasticity observed at rest, possibly due to an over-activation of V5 caused by the high intensity of TMS pulse over an area already activated. Hence, the activation state of the visual cortex is a key factor for the successful induction of cc-PAS associative plasticity (Chiappini, Silvanto, Hibbard, Avenanti, & Romei, 2018).

1.5. Modified PAS targeting frontal networks

Last but not least, in the last couple of years, modified PAS start to be developed outside the sensory-motor networks, trying to induce associative plasticity within frontal areas and related networks. In this final section, I will provide a brief overview of these very interesting (and recent) PAS protocols (**Figure 1.5**, **Table 1.4**).

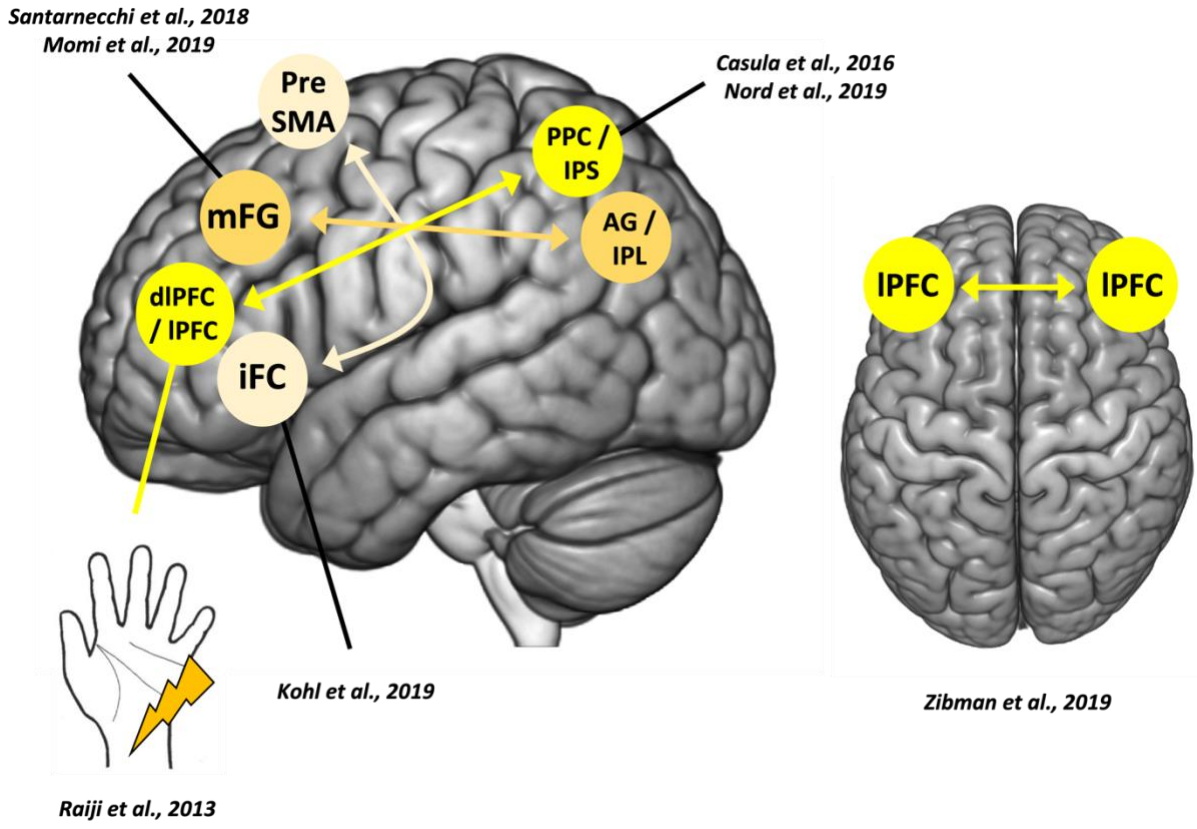

Figure 1.5. modified PAS outside sensory-motor areas. Colored circles indicate sites of the cortical stimulation (i.e., TMS). Arrows indicate direction of *test* and *conditioning* pulses. Left hemisphere is depicted only for visualization purpose. *Legend*: dlPFC = dorso-lateral prefrontal cortex; lPFC = lateral prefrontal cortex; mFG = middle frontal gyrus; iFC = inferior frontal cortex; PPC = posterior parietal cortex; IPS = inferior parietal sulcus; AG = angular gyrus; IPL = inferior parietal lobule.

PAS	PAS parameters	peripheral stimulus / conditioning pulse	cortical stimulation / test pulse	ISI	Effects
dlPFC-PAS	90 stimuli @ 0.05 Hz (30 min)	electric stimulation of the MN	dlPFC	25 ms	↑
				100 ms	∅
Fronto-parietal cc-PAS	100 pulses @ 0.2 Hz (8 min)	dlPFC	PPC	10 ms	↑
				-10 ms	↓
	100 pulses @ 0.2 Hz (8 min)	lPFC	IPS	10 ms	∅
				-10 ms	↓
	180 stimuli @ 0.2 Hz (8 min)	MFG	AG/IPL	10 ms	↑
				-10 ms	↓
Frontal cc-PAS	100 stimuli @ 0.2 Hz (8 min)	IFC	preSMA	4 ms	↑
				-10 ms	↓
	180 stimuli @ 0.1 Hz (30 min)	R/L lPFC	L/R lPFC	8 ms	↑ / ↓

Table 1.4. Principal modified PAS targeting frontal networks. Effective protocols or replicated parameters are reported. For the ISIs, I reported all the tested ones with such protocol. ↑ = excitatory effects, ↓ = inhibitory effects, ∅ = ineffective ISI.

1.5.1. dlPFC-PAS

A modified *cross-systems* PAS protocol which induced plasticity in frontal areas – and, specifically, in the dorsolateral pre-frontal cortex (dlPFC) – was developed in 2013. Introduced by Rajji and colleagues, this PAS protocol repeatedly paired MN electric stimulations of the right wrist with TMS pulses over left dlPFC (Rajji et al., 2013). An ISI of 25 ms was deployed according to previous neurophysiological evidence that SEPs produced a negative peak in frontal areas after 25 ms, which amplitude were maximal over the electrode overlying dlPFC (i.e., F3) (Valeriani et al., 1998). To assess the effects of the protocol, TMS-EEG was used. Results showed that the cortical-evoked activity of dlPFC was enhanced after PAS administration, suggesting the induction of LTP within this area. Furthermore, it was found a potentiation in the coupling between theta and gamma band cortical oscillations – two bands related to dlPFC functioning and working memory (e.g., Canolty & Knight, 2010; Roux & Uhlhaas, 2014). Conversely, when a longer ISI was used (i.e., 100 ms), no effects were detected, proving timing dependency. In conclusion, this study showed for the first time that *cross-systems* PAS could induce timing-dependent associative plasticity also in non-sensory-motor regions (Rajji et al., 2013).

1.5.2. Fronto-parietal cc-PAS

A recent class of cc-PAS protocols moved away from the stimulation of motor regions and related cortico-cortical connections and focused on fronto-parietal networks, regulating executive and attentional systems. The first fronto-parietal cc-PAS was introduced by Casula and colleagues in 2016 and consisted in the repeated pairing of TMS pulses over left dlPFC with ones over PPC (Casula, Pellicciari, Picazio, Caltagirone, & Koch, 2016). Two versions were tested, according to the order of the paired stimulations: in one, the *conditioning pulse* was delivered over dlPFC and the *test pulse* over PPC (dlPFC-PPC cc-PAS), in the other the

order was reversed (i.e., PPC-dlPFC cc-PAS). Both versions exploited an ISI of 10 ms, accordingly to the transmission time of the fronto-parietal pathway. Using EEG-TMS, Casula and co-workers found that both protocols were effective in inducing STDP-like effects in TEPs. In details, dlPFC-PPC cc-PAS induced an enhancement of dlPFC response, reassembling LTP plasticity, while PPC-dlPFC cc-PAS induced LTD-like effects by decreasing dlPFC-evoked activity. This pattern suggested the induction of anti-Hebbian plasticity: LTP-like effects were found in dlPFC responses when its activation preceded TMS over PPC while the induction of LTD was found when PPC activation preceded TMS over dlPFC. In addition, bidirectional changes in high-frequency oscillatory activity of dlPFC were found: fronto-parietal cc-PAS enhanced dlPFC activity in beta and gamma bands while parieto-frontal cc-PAS decreased them, a result in line with a previous study relating modification in cortical high-frequency oscillatory activity to STDP (Azouz & Gray, 2003). Conversely, PPC response was not modulated by either of the two cc-PAS protocols (Casula et al., 2016).

Two years later, using fMRI, the functional connectivity underpinnings of fronto-parietal cc-PAS were deepened by Santarnecchi and colleagues through the use of a sustained attention task. The targeted frontal and parietal sites were selected according to participants' most negatively correlated nodes of the 'task positive network' – corresponding, on average, to the middle frontal gyrus (mFG) – and the 'default mode network' –corresponding, on average, to the angular gyrus (AG) – found during a preliminary fMRI baseline session. The functional connectivity at rest between AG and mFG tended to increase after parieto-frontal cc-PAS and an increased blood oxygenation level dependent (BOLD) response was found in pre-frontal areas during the attentional task. Conversely, after fronto-parietal cc-PAS, the increased BOLD response was found in parietal areas.

The success of fronto-parietal cc-PAS paved the way to modified versions that investigated its effects on cognitive functions mediated by frontal networks such as decision-making and fluid intelligence. In 2019, Nord and colleagues tested the influence of fronto-parietal cc-PAS on decision-making strategies (Nord et al., 2019). Targeting right lateral pre-frontal cortex (lPFC) as *conditioning pulse* and right intraparietal sulcus (IPS) as *test* one (i.e., lPFC-IPS cc-PAS), the effects of this protocol – and of its reversed version (i.e., IPS-lPFC cc-PAS) – were assessed using a 2-step reinforcement learning task. The 2-step task allowed to measure two different decision-making strategies, the model-free, habitual, learning strategy and the model-based, goal-directed, one, which cerebral underpinnings are slightly different (Daw, Gershman, Seymour, Dayan, & Dolan, 2011). Nord and colleagues found that IPS-lPFC cc-PAS was effective in shifting decision-making from a habitual to a more goal-directed strategy while lPFC-IPS cc-PAS did not induce any kind of modulation.

In the same year, cc-PAS efficacy was tested on fluid intelligence (*gf*), i.e., the ability to organize, filter and extrapolate new information (Gray, Chabris, & Braver, 2003). The stimulation protocol targeted mFG and the inferior parietal lobule (IPL), two areas considered key nodes of the *gf* network (Santarnecchi, Emmendorfer, & Pascual-Leone, 2017). A mFG-IPL cc-PAS and an IPL-mFG version of such cc-PAS were tested. To assess *gf* over time, an abstract reasoning task was administered: the Sandia matrices, which prevents over-learning effects; this task included both logical and relational trials, thus allowing to test two components of *gf* that activated the targeted regions of cc-PAS: pre-frontal areas for logical stimuli and parietal areas for relational ones (Matzen et al., 2010). Results showed an increased performance in the Sandia matrices after the administration of the protocol, specifically: mFG-IPL cc-PAS enhanced accuracy selectively in relational trials while IPL-mFG cc-PAS in logical ones, suggesting the induction of associative plasticity accordingly to the cortico-cortical direction of the protocol stimulations and the

role of the conditioned area in the *gf* network. Finally, when the two paired stimulations were delivered simultaneously (i.e., ISI of 0 ms) and when TMS was delivered only over mFG, no effects were found, proving the timing-dependency of the protocol and the importance of its 'associative' nature to modulate *gf* (Momi et al., 2019).

1.5.3. Frontal cc-PAS

Following the successful induction of STDP between pre-frontal areas and the parietal cortex, also plastic properties between regions of the frontal lobe were explored using cc-PAS.

The first cc-PAS targeting homologues frontal areas was developed in 2019 to deepen the interhemispheric connectivity in frontal regions regulating emotional and motivational processing, which are known to show asymmetrical activations in the two hemispheres (Kelley, Hortensius, Schutter, & Harmon-Jones, 2017). Zibman and colleagues introduced a version of cc-PAS (lPFC-lPFC cc-PAS) in which TMS pulses over lPFC (*conditioning pulse*) were repeatedly paired with ones over the homologous area of the opposite hemisphere (*test pulse*) with an ISI of 10 ms (Zibman, Daniel, Alyagon, Etkin, & Zangen, 2019). Outcomes of this protocol were assessed, behaviorally, using an emotional reactivity task and, neurophysiologically by recording TEPs over lPFC and measuring possible asymmetries in alpha-band power, a marker associated to emotional processing (e.g., Allen, Coan, & Nazarian, 2004). Results showed that the effects of lPFC-lPFC cc-PAS were dependent on the direction of the stimulation: indeed, left-to-right cc-PAS increased the attentional bias in the emotional reactivity task and led to a rightward change of alpha-band power, suggesting greater inactivation of targeted lPFC after the protocol. Conversely, right-to-left cc-PAS decreased attentional bias and led to a leftward change of alpha power. Furthermore, TEPs analysis showed that both cc-PAS increased interhemispheric signal

propagation in the direction of the paired stimulations. Overall, lPFC-lPFC cc-PAS successfully modulated emotional processing, changing the balance of hemispheric activation in the stimulated frontal cortices (Zibman et al., 2019).

In the same year, Kohl and colleagues introduced a second frontal cc-PAS protocol targeting the fronto-striatal network, involved in response-inhibition, by repeatedly pairing TMS pulses over the right inferior frontal cortex (iFC) with TMS pulses over pre-SMA (Kohl et al., 2019). In both conditions – i.e., iFC-preSMA cc-PAS and preSMA-iFC cc-PAS– two ISIs of 4 or 10 ms were tested. As the fronto-striatal network is involved in reactive-inhibition, the shorter ISI of 4 ms aimed to test the hypothesis that the induction of plasticity could be mediated by a cortical-subcortical pathway – i.e., the cortical activations caused by cc-PAS converged in the STN and associative plasticity occurred at this level. On the contrary, 10 ms-ISI was tested in order to understand if plasticity might be directly induced in the cortico-cortical pathway connecting iFC and preSMA. To assess the effects of the protocol on response-inhibition, a classic stop signal RT task was used (Logan, Cowan, & Davis, 1984). Results showed that the effects of frontal cc-PAS in the behavioral task varied as a function of participants' age: younger individuals showed a greater impairment following preSMA-iFC cc-PAS with the ISI of 10 ms while older individuals showed improvements after iFC-preSMA cc-PAS with 4 ms-ISI. These results suggested that the associative plasticity induced within the response-inhibition network might influence both cortico-subcortical and cortico-cortical pathways and, crucially, direction and plasticity of the targeted pathway strongly depended on the age of the participant (Kohl et al., 2019).

Chapter II

Mirror Neuron System

2.1. Mirror neurons

In 1992, the research group of Rizzolatti and colleagues made a discovery that would have revolutionized neuroscience: using single-cell recordings in the monkey's ventral premotor cortex (i.e., area F5), they found a novel class of neurons that discharged both when the monkey performed a given action and when the monkey observed the experimenter performing a motor act with a similar goal (di Pellegrino, Fadiga, Fogassi, Gallese, & Rizzolatti, 1992). Due to the 'mirroring' properties of these neurons, they were called 'mirror neurons' (Gallese, Fadiga, Fogassi, & Rizzolatti, 1996; Rizzolatti, Fadiga, Gallese, & Fogassi, 1996). The pioneering study of di Pellegrino and colleagues (1992) described three different types of mirror neurons, according to their firing properties. So-called '*strictly congruent*' mirror neurons discharge during execution and observation of the same action (e.g., when the monkey performed a grip and when it passively observes the same type of grip made by another agent), '*broadly congruent*' mirror neurons are instead activated not only during the observation of the same action performed (e.g., precision grip) but also during the observation of similar ones (e.g., power grip). Finally, '*logically-related*' mirror neurons respond to different actions in observing and execute conditions (e.g., they fire during the observation of an experimenter placing food in front of the monkey, and when the monkey grasps the food to eat it) (di Pellegrino et al., 1992).

First studies investigating the presence of such class of neurons in the human brain used neuroimaging techniques to provide correlational evidence that regions of the motor system similar to the ones found in monkeys are active both when people observe and execute movements. It was found that mirror activations are mediated by a large cortical network of areas including ventral PM, superior temporal sulcus (STS), inferior frontal gyrus, IPL, SMA, and the insula (e.g., Buccino et al., 2001; Decety et al., 1997; Grèzes & Decety, 2000). Nowadays, these areas are still considered the core regions of the Mirror Neuron System (MNS, **Figure 2.1**) (Jeon & Lee, 2018; Molenberghs, Cunnington, & Mattingley, 2012; Rizzolatti & Craighero, 2004). In the last twenty years, hundreds of studies are been published on the humans' MNS, exploiting different neuroscientific techniques, like non-invasive brain stimulations (e.g., Aglioti, Cesari, Romani, & Urgesi, 2008; Aziz-Zadeh, Maeda, Zaidel, Mazziotta, & Iacoboni, 2002; Fadiga, Fogassi, Pavesi, & Rizzolatti, 1995; Senot et al., 2011; Urgesi, Candidi, Fabbro, Romani, & Aglioti, 2006), EEG (e.g., Cheng et al., 2008; Fox et al., 2016; Muthukumaraswamy & Johnson, 2004; Oberman et al., 2005), or single-cell recording in pre-surgical patients (Mukamel, Ekstrom, Kaplan, Iacoboni, & Fried, 2010). All of them converged in considering mirror mechanisms a basic principle of brain functioning (Rizzolatti & Sinigaglia, 2016).

Besides visuo-motor mirror neurons, monkeys' and humans' brain is also endowed with mirror neurons with audio-motor properties (e.g., Aglioti & Pazzaglia, 2010; Galati et al., 2008; Gazzola, Aziz-Zadeh, & Keysers, 2006; Möttönen & Watkins, 2009; Pizzamiglio et al., 2005; Tettamanti et al., 2005; Watkins, Strafella, & Paus, 2003). For instance, Kohler and colleagues (2002) found that a population of monkey's neurons in area F5 (in details, in a region that is the homologs of humans Broca's area) discharge both when a specific action is performed or seen (e.g., dropping a stick, smashing a nut) and when the related sound is heard (Kohler et al., 2002). Again, in humans, using fMRI, Tettamanti et al. (2005) showed

that the precentral gyrus and the posterior part of IFG (i.e., Broca's area) are activated both during action observation and listening to action sentences. The activations in the pre-central gyrus, and especially during listening to hand-action sentences, corresponded to those found during the observation of the same actions. Conversely, the activation of IFG was particularly strong during the listening of mouth actions but was also during the listening of actions done with other effectors, suggesting that mirror neurons in IFG encode for a more general representation of action verbs than the ones in the pre-central gyrus (Tettamanti et al., 2005). Some authors has proposed that mirror neurons might be an evolutionary pre-curser for the development of speech (Rizzolatti & Arbib, 1998; Rizzolatti & Craighero, 2004).

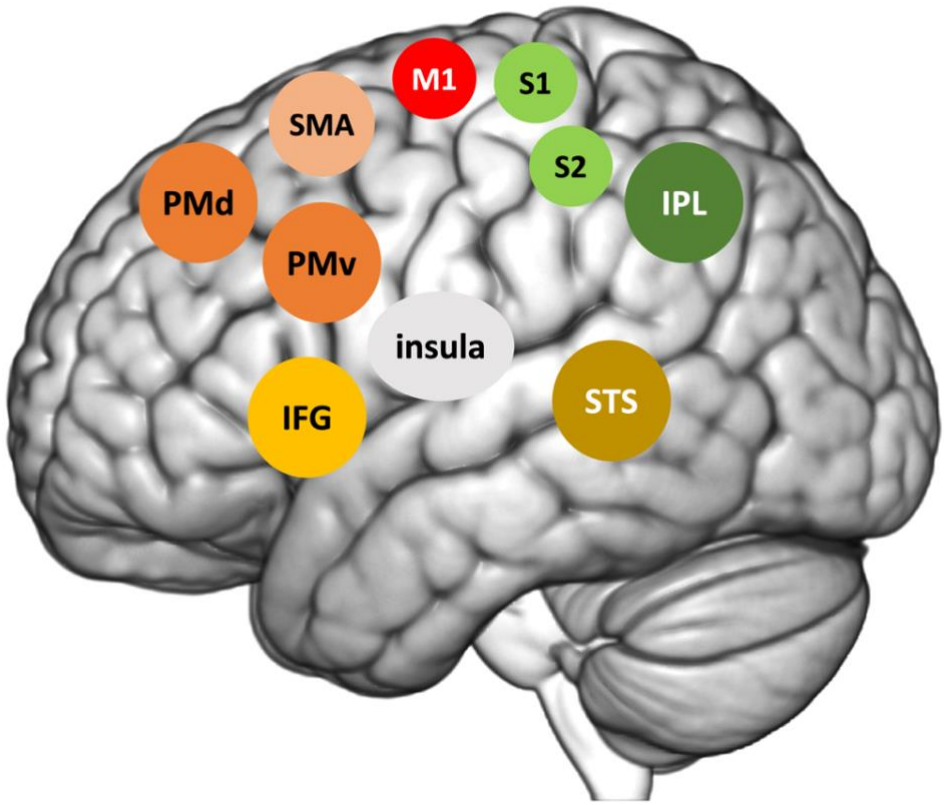

Figure 2.1. Principal areas of the human brain with mirror properties. *Legend*: PMd = dorsal premotor cortex; PMv = ventral premotor cortex; SMA = supplementary motor area; IFG = inferior frontal gyrus; M1: primary motor cortex; S1 = primary somatosensory cortex; S2 = secondary somatosensory cortex; IPL = inferior parietal lobule; STS = superior temporal sulcus.

Overall, all these findings have led to a widespread of the fields of cognition where mirror mechanisms, and thus mirror neurons, are involved. Indeed, mirror neurons not only play a fundamental role in mirroring visual- and audio-perceived actions (e.g., goal encoding of action, understanding an action), as already emerged from the pioneering studies on monkeys (e.g., di Pellegrino et al., 1992; Gallese et al., 1996; Kohler et al., 2002), but this mirroring occurs also for emotions (e.g., Enticott, Johnston, Herring, Hoy, & Fitzgerald, 2008; Kaplan & Iacoboni, 2006; Keysers & Gazzola, 2009; Schmidt, Sojer, Hass, Kirsch, & Mier, 2020, see **2.1.2**) and somatosensations (e.g., vicarious activation at the sight of a touch) (e.g., Blakemore, Bristow, Bird, Frith, & Ward, 2005; Keysers, Kaas, & Gazzola, 2010; Ward & Banissy, 2015, see **2.1.3**). Accordingly, abnormal mirror functioning was found in a vast range of neuropsychiatric disorders characterized by social and/or emphatic dysfunctions, like schizophrenia or autism (e.g., Dapretto et al., 2007; Enticott, Hoy, et al., 2008; Mehta et al., 2014; Oberman et al., 2005; Oberman & Ramachandran, 2007; Schunke et al., 2016).

Nowadays, it is established that mirror-based processing provides a fundamental route of knowledge of others, one which can be taken just by capitalizing on one's own motor (or visceromotor) representations and, thus, mirror neurons can be considered a fundamental substrate of our 'being human' (Rizzolatti & Sinigaglia, 2016). Furthermore, due to the complexity of the MNS, some authors have preferred to split it into different functional 'subsystems', according to their peculiar mirror properties (Cook, Bird, Catmur, Press, & Heyes, 2014; Keysers & Gazzola, 2009). Hence, in the following paragraphs, I am going to provide an overview of the principal MNS 'subsystems' discovered in recent years, with particular attention to those that have guided the experimental work during my doctorate: namely, the *action observation network* (**Chapter 4**) and the *tactile mirror system* (**Chapter 3**).

2.1.1. Mirroring actions: the *action observation network*

The *action observation network* is the subsystem of the MNS that is activated during the observation of both simple and complex actions like the movement of a finger or the grasping of a ball. This MNS network is fundamental for action understanding and execution, goal encoding, and imitation (Rizzolatti & Craighero, 2004). For example, a large number of brain-imaging and behavioral studies demonstrated that the activation of such a network correlates with the goal of the depicted action and with the own motor expertise in such an action (e.g., Abdollahi, Jastorff, & Orban, 2013; Calvo-Merino, Grèzes, Glaser, Passingham, & Haggard, 2006; Cattaneo et al., 2011; Cross, Hamilton, & Grafton, 2006; Iacoboni, Molnar-Szakacs, Gallese, Buccino, & Mazziotta, 2005; Senna, Bolognini, & Maravita, 2014). Namely, the observation of different actions with the same goal activated the same population of mirror neurons in the *action observation network* and the richer people's motor expertise is, the greater the sensitivity of this mirror mechanism to others' actions, allowing to better their ability to identify the outcome to which those actions are directed (Rizzolatti & Sinigaglia, 2016).

Historically, the *action observation network* was the first one to be deeply investigated in humans using TMS and fMRI. As a consequence, in the last two decades, a vast range of studies investigating its anatomic and functional substrates are been published (e.g., Aglioti et al., 2008; Avenanti, Bolognini, Maravita, & Aglioti, 2007; Aziz-Zadeh, Koski, Zaidel, Mazziotta, & Iacoboni, 2006; Aziz-Zadeh et al., 2002; Bach, Peelen, & Tipper, 2010; Buccino et al., 2001; Calvo-Merino et al., 2006; Caspers, Zilles, Laird, & Eickhoff, 2010; Catmur, Walsh, & Heyes, 2007; A. Engel, Burke, Fiehler, Bien, & Rösler, 2008; Fadiga, Craighero, & Olivier, 2005; Ge et al., 2018; Koch et al., 2010; Maranesi, Livi, Fogassi, Rizzolatti, & Bonini, 2014; Romani, Cesari, Urgesi, Facchini, & Aglioti, 2005; Urgesi et al., 2006). The first causal evidence that the humans' motor system is activated not only during

the execution but also during the observation of actions comes from a pioneering study of Fadiga and colleagues (1995) using TMS. Here, participants observed different visual stimuli (i.e., a grasping movement, a static 3-D object, an experimenter tracing geometrical figures, and a dimming light) while their M1 was stimulated with TMS and MEPs were recorded from the contralateral hand. Results showed that MEPs were enhanced selectively during the observation of the visual stimuli depicting actions (grasping movement and the experimenter tracing geometrical figures) while in the other two conditions they were not modulated. Furthermore, the MEP pattern in such conditions reflected the one of muscle activity recorded when the participant performed the same observed action (Fadiga et al., 1995). This evidence led the authors to suggest the existence, in the human brain, of a system matching action observation and execution, similar to the one found in monkeys a couple of years before (di Pellegrino et al., 1992). Hence, the recorded MEPs enhancement was mediated by the recruitment, within the motor system, of mirror neurons encoding the specific motor program used to produce the observed action. This phenomenon was called 'motor resonance' and, as said before, nowadays it is considered a *gold standard* neurophysiological measure to study the *action observation network* (and, in a wider perspective, the MNS) (Naish, Houston-Price, Bremner, & Holmes, 2014; Rizzolatti & Craighero, 2004). Due to the importance of motor resonance in the present thesis, I will now describe its main properties to provide the theoretical background to the experiments presented in **Chapter 4** exploiting such properties.

A vast range of experiments on motor resonance investigated whether TMS-induced MEP modulation occurs specifically in the pathway innervating the muscles involved in the observed action. Results of studies that used isolated, discrete movement of a single effector (e.g., adduction/abduction of a finger) to map motor resonance converged in stating that MEPs modulation (and thus the recruitment of M1 by the *action observation network*) is

widely mototopic, namely: it is detectable only in the muscles involved in the observed movement. For instance, if participants observed an abduction movement of the index finger – a simple movement which is known to recruit only the FDI muscle – and MEPs are simultaneously recorded from both FDI and ADM muscle, motor resonance (i.e., enhancement of MEP amplitude) would be detectable only in FDI (e.g., Avenanti et al., 2007; Catmur, Mars, Rushworth, & Heyes, 2011; Catmur et al., 2007; Loporto, Holmes, Wright, & McAllister, 2013; Romani et al., 2005; Urgesi et al., 2006). Things get trickier when the observed action is a complex one, like the grasping of a ball or a pinching movement, or when the action is made with the whole body. In this case, a larger number of muscles are recruited, and thus which muscles among the ones of the muscular district (e.g., hand and forearm for a grasping movement) involved in the action are 'facilitated' is more controversial (e.g., Aglioti et al., 2008; Cattaneo, Maule, Barchiesi, & Rizzolatti, 2013; Jola, Abedian-Amiri, Kuppuswamy, Pollick, & Grosbras, 2012; Lago & Fernandez-del-Olmo, 2011). Another important property of motor resonance is that, at least for discrete, unilateral movements, MEP modulation is also hemispheric specific. Namely, during the observation of a movement made with a single hand (e.g., the abduction movement of a right-hand index finger), only the contralateral (left) motor system is recruited by the *action observation network*. Furthermore, this lateralization of motor resonance seems to be easier to detect for the non-dominant hemisphere (e.g., the right hemisphere for right-handed people) (Aziz-Zadeh et al., 2002).

Other studies on the *action observation network* focused their attention on the properties that the observed visual stimulus must have to activate mirror neurons and, thus, gave rise to motor resonance. One of the first findings was that, differently from the monkey's mirror neurons, which are activated only when the movement had a goal (e.g., reaching food), in humans, also afinalistic movement (e.g., the mere abduction of a finger) recruited the *action*

observation network (Cattaneo & Rizzolatti, 2009). The perspective from which the movement is observed (i.e., egocentric or allocentric) is fundamental: different studies have highlighted how the observation of action in an egocentric perspective (corresponding thus to the orientation of the observer) maximize MEP modulation (e.g., Maeda, Kleiner-Fisman, & Pascual-Leone, 2002a). Furthermore, put the observer's body part with the same orientation of the observed one to maximize motor resonance (e.g., Urgesi et al., 2006). Also, the context in which an action is observed influences motor resonance: for example, observing a hand-object interaction (i.e., grasping a ball) facilitated cortico-spinal excitability to a greater extent compared to when there was no object in the scene to provide context to the observed movement (i.e., grasping alone) (e.g., Amoruso & Urgesi, 2016; Enticott, Kennedy, Bradshaw, Rinehart, & Fitzgerald, 2010; Riach, Holmes, Franklin, & Wright, 2018). Interestingly, also the observation of bio-mechanically impossible movements (e.g., extreme joint stretching) led to the emergence of motor resonance even if with different degrees of activation of *action observation network*-related sensory-motor areas (e.g., Avenanti et al., 2007; Romani et al., 2005). Similar findings were also obtained using fMRI (e.g., Bach et al., 2010; Buccino et al., 2001; Calvo-Merino et al., 2006; Costantini et al., 2005; Ge et al., 2018).

Finally, also the timing at which modulation of cortico-spinal excitability occurs, starting from the observation of a movement, was deeply investigated. Knowing the optimal timing between the visual processing of the action and the subsequent activation of the *action observation network* (and, thus, M1) is fundamental to understand how mirror neurons are recruited and whether this recruitment is automatic or mediated by a cognitive elaboration of the visual stimulus of movement. In this regard, convergent findings indicate that there are two 'activation levels': the first one is reflected by an early, mainly attentive, non-specific modulation of cortico-spinal excitability and the second one by a later, muscle-

specific modulation (Naish et al., 2014). In detail, different studies highlight that the earliest modulation of TMS-induced MEPs during action observation occurs about 80-100 ms after movement onset and it is not muscle-specific. It has been hypothesized that this early modulation reflects a fast, attention-based, response to whether or not the observed action is on the predicted side of space (e.g., Kilner, Vargas, Duval, Blakemore, & Sirigu, 2004; Lepage, Tremblay, & Théoret, 2010; Romani et al., 2005). A second, late modulation takes place about 200-300 ms from the onset of the observed action. This one reflects deeper processing of the action by the MNS, being influenced by factors specific to the observed movement such as the muscles involved (e.g., Cavallo, Heyes, Becchio, Bird, & Catmur, 2014), the correctness of the movement itself (e.g., Candidi, Sacheli, Mega, & Aglioti, 2014; Koelewijn, van Schie, Bekkering, Oostenveld, & Jensen, 2008) or the goal of the depicted action (e.g., Enticott et al., 2010).

Action observation network activation can also be investigated, behaviorally, through *automatic imitation* paradigms. *Automatic imitation* is a type of stimulus-response compatibility effect that occurs when the observation of an action involuntarily facilitates the performance of topographically similar action and/or interferes with the performance of a topographically dissimilar action (Heyes, 2011). For instance, using a simple reaction times (RT) task, Brass and coworkers (2001) found that tapping or lifting movements made with the index finger were initiated faster when they were visually cued by the onset of compatible rather than incompatible finger movement stimuli (i.e., faster RTs when the tapping movement was cued by a tapping visual stimulus rather than by a lifting one) (Brass, Bekkering, & Prinz, 2001). This difference in participant's RTs between compatible (congruent) and incompatible (incongruent) observed movements is considered the marker of *automatic imitation* and this effect was widely replicated by further studies using different body-related moving stimuli or different version of such a task (e.g., Bertenthal, Longo, &

Kosobud, 2006; Cracco & Brass, 2018; Craighero, Bello, Fadiga, & Rizzolatti, 2002; Heyes, Bird, Johnson, & Haggard, 2005; Kilner, Hamilton, & Blakemore, 2007; Longo, Kosobud, & Bertenthal, 2008). Furthermore, this phenomenon is particularly robust when movements made with hand, arm, foot, or mouth are observed and, thus, it represents the elective way, at a behavioral level, to investigate mirror functioning in humans (Cracco et al., 2018; Heyes, 2011).

2.1.2. Mirroring emotions

Mirror mechanisms operate also in the emotional domain and play a key part in processing and understanding others' emotions (Bastiaansen, Thioux, & Keysers, 2009; Rizzolatti & Sinigaglia, 2016). One of the basic emotions intensively studied both in monkeys and humans was the disgust and convergent findings highlighted that the anterior part of the insula contains mirror neurons that are activated both when such emotion is felt and when someone else being disgusted is observed (e.g., Carr, Iacoboni, Dubeau, Mazziotta, & Lenzi, 2003; Jabbi, Bastiaansen, & Keysers, 2008; Krolak-Salmon et al., 2003; Phillips et al., 1998; Wicker et al., 2003). For example, a study by Wicker and colleagues (2003) demonstrated, using fMRI, an overlap between insular activation elicited by one's own and others' disgust. During the experiment, in some blocks, participants were exposed to disgusting or pleasant odorants, whereas, in other blocks, they were presented with short movie clips of other individuals smelling the content of a glass and displaying a facial expression of disgust or pleasure. It was found that the observation of others' disgust activated the same neuronal substrates within the insula that were activated by the exposure to the disgusting odorants. This suggested a putative common insular ground for experiencing own disgust and for observing someone else being disgusted (Wicker et al., 2003).

In later years, similar mirror activations were also found for other basic emotions, like fear or pleasure, but also more complex emotions, like regret; suggesting that mirror neurons play a central role not only in understanding emotions but also in coding the emotional valence of salient stimuli (e.g., Canessa et al., 2009; Caruana et al., 2015; Enticott, Johnston, et al., 2008; Hennenlotter et al., 2005; Jabbi, Swart, & Keysers, 2007; Salvia, Süß, Tivadar, Harkness, & Grosbras, 2016; Schmidt et al., 2020). For instance, a study performed on patients who underwent surgery to implant intra-cerebral electrodes, showed that electrical stimulation of the anterior part of the cingulate cortex elicited a burst of laughter, often accompanied by a change in the emotional state, as indicated by a sudden appearance of mirth. Interestingly, seeing someone else laughing or exhibiting mirth activate the same site within the cingulate cortex, suggesting the presence of 'emotional' mirror neurons also in this area, likely specialized in the processing of positive emotions (Caruana et al., 2015). Other confirmations of the central role of the insula and the cingulate cortex for emotional mirroring processing come from studies on patients, showing that lesions in the aforementioned areas selectively impaired first-person and third-person experience of emotions (e.g., Adolphs, Tranel, & Damasio, 2003; Calder, Keane, Manes, Antoun, & Young, 2000). Also, dysfunctions in understanding other's emotions present in psychiatric syndromes like schizophrenia or autism, suggest that mirror mechanisms play a central role in the emotional domain (e.g., Dapretto et al., 2007; Haker & Rössler, 2009; Horan, Pineda, Wynn, Iacoboni, & Green, 2014; Marsh & Hamilton, 2011; Quintana, Davidson, Kovalik, Marder, & Mazziotta, 2001)

Hence, the aforementioned findings suggest that observing an emotion would elicit visceromotor processes and representations in the observer's brain that are similar to those processes and representations that would occur if the observers themselves were experiencing that emotion. This 'emotional' mirror mechanism could allow the observers to

identify others' emotions by capitalizing on their own visceromotor processes and representations concerning that emotion, being fundamental for the social and emphatic abilities typical of humans (Rizzolatti & Sinigaglia, 2016).

2.1.4. Mirroring somatosensations: the *tactile mirror system*

In the late 2000s, with the first evidence that social and emphatic abilities may be underpinned by mirror mechanisms, the speculation that also somatosensations could be 'mirrored' by our brain started to be explored. It was hypothesized that our brain, besides visuo-motor and audio-motor mirror neurons, could be endowed with mirror neurons with visuo-tactile properties which are mainly responsible for vicarious activations at the sight of touch (Keysers & Gazzola, 2009; Keysers et al., 2010).

In the pioneering study by Keysers and coworkers (2004), participants underwent an fMRI scan while they observed videoclips showing a leg being touched or experienced the same kind of touch on their leg. Results showed that the secondary somatosensory cortex (S2) was activated during both the observation and the experience of the touch, suggesting that this area might be fundamental for vicarious activations during the sight of stimuli conveying somatic information, like a touch (Keysers et al., 2004). In the following years, different fMRI studies replicated this finding, expanding the number of areas that are implicated in the so-called *tactile mirror system*. In fact, besides S2, also S1 (and especially Brodmann's area 2), STS, and PM are considered key nodes of this network, being active both during the perception and the observation of touch stimuli (e.g., Blakemore et al., 2005; Case et al., 2016; Ebisch et al., 2008, 2016; Kuehn, Mueller, Turner, & Schütz-Bosbach, 2014; Kuehn, Trampel, Mueller, Turner, & Schütz-Bosbach, 2013; Lee Masson, Van De Plas, Daniels, & Op de Beeck, 2018; Schaefer, Heinze, & Rotte, 2012). For example, Kuehn and colleagues (2014), using 7 T fMRI, showed that observing and physically experiencing touch elicit

overlapping activity changes in S1 that are also topographically organized. For instance, the observation of a touch to a finger activates the same region of S1 which encodes for that finger's somatosensations, in a similar way as happening in M1 during the observation of movements (Kuehn et al., 2014). Furthermore, the existence of visually-driven somatosensory maps in this primary cortical area corroborates its pivotal role within the *tactile mirror system* (Kuehn, Haggard, Villringer, Pleger, & Sereno, 2018).

Besides fMRI studies, also the ones using TMS showed the importance of somatosensory cortices, and especially S1, within the *tactile mirror system* (e.g., Bolognini et al., 2012; Bolognini, Rossetti, Fusaro, Vallar, & Miniussi, 2014; Bolognini, Rossetti, Maravita, & Miniussi, 2011; Bolognini, Rossetti, Convento, & Vallar, 2013; Case et al., 2017; Rossetti, Miniussi, Maravita, & Bolognini, 2012). For instance, Bolognini and colleagues (2011) showed that rTMS over S1, but not over S2, selectively reduced participants' performance during a visual task depicting hands being touched (Bolognini et al., 2011). The same research group found also that when participants underwent a go/no-go task where trials could depict touch and no-touch visual stimuli with a positive or a negative emotional value, rTMS over S1 disrupted performance only in trials where the emotional state is conveying by a tactile event. Interestingly, this modulation was found selectively after the stimulation of the right hemisphere S1. This evidence suggests that S1 also code for the affective valence of the observed touch (Bolognini, Rossetti, et al., 2013) and further studies confirmed the strict relationship between emotional and somatic mirror processing (e.g., Case et al., 2016, 2017; Morrison, 2016)

Other confirmations of the pivotal role of S1 for mirroring somatosensations come from studies exploiting EEG. Pisoni and colleagues (2018), using TMS-EEG co-registration, found that the sight of a touch to a human body part, but not to an object, triggered an early activation of S1 (after about 50-100 ms from the onset of the visual touch) as a real felt touch

did. This early modulation of S1 functioning is unconsciousness, being related to an effective connectivity network generated in the beta band; a band which is indeed associated with unconsciousness tactile processing, differently from conscious processing which occurs in the alpha band (Pisoni, Romero Lauro, Vergallito, Maddaluno, & Bolognini, 2018). At confirmation of the early modulation of S1 during the observation of touch stimuli, Rigato and coworkers (2019), recorded somatosensory event-related potentials during visual stimuli depicting a hand being touched in an egocentric (i.e., first-person) perspective or an allocentric (i.e., third-person) one. They found that vicarious touch affected early-stage somatosensory processing at different time points according to the perspective of the observed touch. Indeed, when the touch stimuli were presented in a first-person perspective, the modulation occurred at the level of the P45 component, a component in which cortical source is localized within S1. Conversely, when they were presented in a third-person perspective, the modulation occurred at a later component (P100), which instead it is localized within S2. This evidence suggests that the vicarious activation of S1 is modulated by the spatial characteristics of the observed visual stimuli of touch, occurring at a very early stage of somatosensory processing when such stimuli are referred at the own body (Rigato, Bremner, Gillmeister, & Banissy, 2019).

Altogether, this evidence highlights how S1 is a central node of the *tactile mirror system*, and that its mirror properties are likely mediated by the presence of visuo-tactile mirror neurons.

2.2. Origins of the human MNS

Even if the anatomo-functional properties of the MNS have been widely investigated in the last twenty years, less is known about how mirror neurons get their matching properties and which kind of plastic mechanism rules networks with mirror properties (Cook et al., 2014; Heyes & Catmur, 2020). Two different accounts are been theorized to explain the emergence of mirror neurons matching properties: the *adaptation* and the *associative* accounts. Importantly, both accounts assume that genetic information and experience contribute to the development of mirror neurons; crucially, they differ in the roles they assign to genetic evolution and (sensory-motor) learning in producing mirror neurons' matching properties (Catmur, Press, & Heyes, 2016; Heyes, 2014).

The *adaptation account* postulates that mirror neurons' matching properties are an 'adaptation' for action understanding and related social abilities; namely, a phenotypical characteristic that is genetically inherited and that was favored by natural selection to fulfill a particular function. Among common ancestors of monkeys and humans, some individuals had a stronger genetic predisposition to develop neurons with cross-modal matching properties and these individuals were more reproductively successful because these neurons enhanced their capacity to understand others' actions. In this account, motor (i.e., execute an action) and visual experience (i.e., observe an action) play a facilitative, 'triggering' role in the development of mirror neurons and related networks. In fact, sensory-motor matching properties of mirror neurons are, in the first place, due to a genetic predisposition. Consequently, this class of neurons is already present at birth, and cross-modal experience act as a mere trigger, in a sort of 'canalization' process (Gallese, Rochat, Cossu, &

Sinigaglia, 2009; Gallese & Sinigaglia, 2011; Giudice, Manera, & Keysers, 2009; Rizzolatti & Craighero, 2004).

Conversely, the *associative account* suggests that mirror neurons are a result of domain-general processes of associative learning instead of a product of a specific genetic predisposition. In detail, before associative learning, sensory neurons responsive to different high-level visual properties of an observed action are weakly connected to motor neurons in parietal and pre-motor cortices. However, with experience, the correlated excitation of sensory (visual) and motor neurons coding for similar action strength this connection and led to the emergence of mirror neurons' typical matching properties. Importantly, this correlated excitation must be both contingent (i.e., one event must reliably predict the other) and contiguous (i.e., events must occur close together in space and time) to be effective. Hence, imitation, observation of self-produced actions, or synchronous activities are all sources of correlated excitation of sensory and motor neurons encoding the same action, and, potentially, these are all experiences that could give rise to novel mirror matching. In these terms, the *associative account* implies that the characteristic matching properties of mirror neurons result from a genetically evolved process (i.e., associative learning) but that this process was not designed by genetic evolution to specifically produce mirror neurons. Indeed, it produces mirror neurons with matching properties when the developing system receives the correlated experience of observing and executing similar actions. Conversely, when, by instance, the system receives the correlated experience of observing objects and executing actions, the same associative process produces canonical neurons (Catmur et al., 2016; Heyes, 2010). Even if this account (as the *adaptation* one) has been theorized within the classical frame of visuo-motor mirror neurons (and the *action observation network*), the same reasoning is also valid for the visuo-tactile mirror neurons (and thus the *tactile mirror system*) by simply replacing motor experience with tactile one (Keysers & Gazzola, 2009).

The *adaptation account* represents somehow the classical theory on mirror neurons' origin and its experimental evidence comes mainly from studies investigating the 'goal encoding' properties of mirror neurons, which are speculated to be innate (Rizzolatti & Sinigaglia, 2016). Nevertheless, in the last decade, the *associative account* is been corroborated by different studies which showed how mirror neurons' matching properties can be successfully reshaped through different kind of sensory-motor learning paradigms (e.g., Bardi, Bundt, Notebaert, & Brass, 2015; Brunsdon, Bradford, Smith, & Ferguson, 2020; Catmur & Heyes, 2011, 2017; Catmur et al., 2007; Furukawa, Uehara, & Furuya, 2017; Kriváneková et al., 2011; Press et al., 2012). For example, in a pioneering study, Catmur and colleagues (2007) trained participants, for about one hour and a half, to make an abduction movement with the index finger every time they observed on a PC screen a similar movement made with the little finger and vice versa (the so-called counter-mirror paradigm). Before this training, as expected, participants showed muscle-specific motor resonance (i.e., FDI-MEPs enhancement during the observation of index finger movements and ADM-MEPs enhancement during the observation of little finger ones). After the training session, motor resonance was found to be 'reversed': the observation of little finger movements selectively enhanced MEPs recorded from FDI and the one of index finger movement selectively enhanced MEPs recorded from ADM, suggesting mirror neurons within the motor system had transiently learned the new visuo-motor associations presented during the training phase. Hence, the efficacy of this behavioral paradigm in shape motor resonance, which was replicated in further studies (e.g., Catmur et al., 2008, 2011; Cavallo et al., 2014), strongly suggests that MNS properties are neither innate nor fixed once acquired; instead, associative sensory-motor learning plays a fundamental role in their development, as postulated by the *associative account* (Catmur et al., 2007).

Even if nowadays is crystal clear that mirror neurons' properties can be shaped through sensory-motor learning, a still debated aspect of the *associative account* (and, in a wider perspective, of MNS literature) concerns which kind of plasticity rules such sensory-motor learning at a neurophysiological level and, thus, the emergence and the shaping of mirror neurons' matching properties. One of the hypotheses put forward suggests that Hebbian associative plasticity, and, in details, spike-timing-dependent plasticity (STDP), which is the neurophysiological basis of Hebbian learning (see **Chapter 1**), can be the better form of synaptic plasticity to explain the emergence of the matching properties of mirror neurons and related networks (Keysers & Gazzola, 2014; Keysers & Perrett, 2004). Especially during childhood, when an individual performs a new hand action, or feel a tactile sensation, he sees himself perform this action and this sensory input resulting from one's action is called 're-afference'. Considering the execution/observation of movement, at a neuronal level, activity in premotor neurons triggering a specific action, and activity in parieto-temporal neurons responding to the vision of this specific action would consistently and repeatedly overlap in time thanks to such cross-modal re-afferences. Thus, the synapses connecting representation of the same action within pre-motor (i.e., PM) and parieto-temporal areas (i.e., STS) are potentiated following Hebbian learning rules of contingency and contiguity (Keysers, Perrett, & Gazzola, 2014). Over time, this repeated, time-locked sensory-motor activation gives rise to mirror neurons responding to that association and allows them to shape their properties following STDP. The same phenomenon occurs within the visuo-tactile domain: seeing a touch on one's own body (which activated parieto-temporal areas) is generally associated with the feeling of a tactile sensation (activation of somatosensory cortices) and this repeated, contingent activation led to the emergence of cross-modal neurons with visuo-tactile matching properties (Keysers & Gazzola, 2014).

The possibility that a similar associative mechanism could mediate the emergence of neurons with mirror properties found confirmation in studies conducted on infants. Infants have indeed an early preference for body-related visual stimuli like faces or hands (e.g., Cassia, Turati, & Simion, 2004; Johnson, Dziurawiec, Ellis, & Morton, 1991; Van Der Meer, Van Der Weel, & Lee, 1995; Von Hofsten, 2004) and the ability to detect spatial and temporal contingency is present shortly after birth (Tarabulsy, Tessier, & Kappas, 1996). Furthermore, until 3 months of age, infants show a preference for perfectly contingent events; namely, for events whose sensory timing perfectly matches the infant's proprioceptive sensation, a condition that is optimal for Hebbian learning (Gergely & Watson, 1999; Giudice et al., 2009). At about 3-4 months a developmental shift occurs, with infants showing a preference for spatially and temporally non-contingent movements, which usually are not self-produced (e.g., Rochat & Striano, 2000; Schmuckler & Fairhall, 2001). This shift in preference has two consequences. First, it directs the infant's attention to actions that his developing brain does not yet fully predict, allowing to extent visuo-motor mirror activations also in a non-fully predictable context. Second, it focuses the infant's attention on the hand-actions of other people, potentially promoting learning by observation. This evidence on early visuo-motor development suggests that, until 3 months, infants are particularly attracted by self-produced movements, especially by their reaching and grasping attempts. These perceptual preferences ensure that infants have access to a large amount of contingent visuo-motor input for Hebbian learning, likely promoting the emergence of mirror neurons and, in a wider perspective, of the MNS (Giudice et al., 2009; Keysers & Gazzola, 2014). Indeed, different studies highlighted how mirror responses similar to the ones of the adult brain are present in infants since the first months of life (see, for a review: Lepage & Théoret, 2007; Quadrelli & Turati, 2016).

However, all the evidence carried on by the theoretical accounts that suggest the mediation of Hebbian learning in the shaping of mirror neurons properties in humans are not yet corroborated by any *in vivo* neurophysiological findings. Hence, considering the lack of knowledge about the precise plastic mechanism that rules the MNS, during my doctorate, I have tried to investigate this issue exploiting two ad-hoc developed PAS protocols to modulate (cross-modal PAS, see **Chapter 3**) and reshape (mirror PAS, see **Chapter 4**) mirror neurons' matching properties, following the induction of Hebbian associative plasticity.

Chapter III

Cross-modal PAS [1]

3.1. Aims of the study

In the four experiments presented in this chapter, I have tested the hypothesis that visuo-tactile mirroring properties of the somatosensory system might be ruled by Hebbian associative plasticity (see **2.2**) by developing a novel *cross-systems* PAS protocol. I named this new protocol 'cross-modal PAS' (cm-PAS) because it aimed at influence somatosensory cortical activity through the visual modality (and the recruitment of the *tactile mirror system*).

As said in the previous chapter, the observation of tactile events (e.g., seeing someone being touched) activates a cortical network – the *tactile mirror system* – largely overlapping the one implicated in tactile perception (Gallese, Keysers, & Rizzolatti, 2004; Gallese & Sinigaglia, 2011; Keysers & Gazzola, 2009; Keysers, Kaas, & Gazzola, 2010; see **Chapter**

[1] This chapter contains experiments already published in:

- Zazio, A.*, Guidali, G.*, Maddaluno, O., Miniussi, C., & Bolognini, N. (2019). Hebbian associative plasticity in the visuo-tactile domain: A cross-modal paired associative stimulation protocol. *NeuroImage*, *201*, 116025. [*Experiments 1-3*]

- Maddaluno, O.*, Guidali, G.*, Zazio, A., Miniussi, C., & Bolognini, N. (2020). Touch anticipation mediates cross-modal Hebbian plasticity in the primary somatosensory cortex. *Cortex*, *126*, 173–181. [*Experiment 4*]

* *shared first authorship*

2). In the cm-PAS, trying to modulate S1 functioning by exploiting such visuo-tactile mirroring mechanisms, I replaced the somatosensory MN electrical stimulation of the standard *within-system* S1-PAS (e.g., Litvak et al., 2007; Pellicciari, Miniussi, Rossini, & De Gennaro, 2009; Wolters et al., 2005; see **1.2.1**) with a single-frame videoclip showing a left hand being touched (acting as the peripheral stimulation). The paired cortical stimulation consisted of TMS pulses delivered over the right S1. Hence, my starting hypothesis was that, thanks to the visuo-tactile mirroring properties of S1, the repeated, time-locked, coupling of these two stimulations (the visual-touch stimulus and S1-TMS) should be able to induce timing-dependent Hebbian associative plasticity (i.e., LTP and/or LTD) within S1, in a similar way to what happens in *within-system* S1-PAS.

The effectiveness of the novel cm-PAS in inducing Hebbian plasticity was investigated in a series of four experiments by (*a*) varying the main variables of the protocol (i.e., timing between the two paired stimuli – *Experiment 1*; cortical site of TMS – *Experiment 2*; visual properties of the peripheral stimulus – *Experiment 3* and frequency between paired stimulations – *Experiment 4*) and (*b*) using behavioral and neurophysiological measures commonly adopted in standard S1-PAS protocols (see **Chapter 1**) and related to S1 functioning: namely, tactile acuity (*Experiments 1, 2, 3* and *4*) and SEPs (*Experiment 3*) (Litvak et al., 2007; Pellicciari et al., 2009; Wolters et al., 2005).

3.2. *Experiment 1*: Efficacy and timing dependency of cm-PAS

3.2.1. Aim

The first experiment of the study aims at assessing possible timing-dependent changes in tactile sensitivity following the cm-PAS as markers of Hebbian learning. As said in **Chapter 1**, timing dependency is a key property of Hebbian plasticity and, thus, of PAS protocols. Hence, I decided to test it in first place. For this purpose, I varied the cm-PAS ISI between the onset of the visual-touch stimulus (i.e., the sight of the hand being touched) and the S1-TMS pulse.

In the three within-subject sessions of the experiment, I tested the following ISIs: 20, 60, and 100 ms. The value of 20 ms was chosen by considering the ISI of traditional S1-PAS protocols (Wolters et al., 2005, see **1.2.1**): here, the ISI of 20 ms reflects the time-course of S1 activation by tactile afferences (Cohen, Bandinehi, Sato, Kufta, & Hallett, 1991; Macerollo et al., 2018). This value is optimal for inducing LTP in S1, matching the latency of SEPs N20, the first cortical component of such evoked potentials. However, since in the cm-PAS the somatosensory stimulation was replaced with a visual stimulus, such a short ISI should not be effective, unless a direct, very fast, sub-cortical pathway between thalamus visual nuclei and somatosensory areas is implied (e.g., Céline Cappe & Barone, 2005). Rather, the activation of S1 by touch observation should occur later, within a larger time window (Bolognini et al., 2014; Kuehn, Doehler, & Pleger, 2017; Martínez-Jauand et al., 2012; Pihko, Nangini, Jousmaki, & Hari, 2010; Pisoni et al., 2018). Hence, I assessed the engagement of cross-modal, mirror-like, mechanisms by the protocol adopting also the longer ISIs of 60 and 100 ms.

3.2.2. Methods and materials

3.2.2.1. Participants

Eighteen healthy volunteers took part in *Experiment 1* (9 males, mean age ± standard deviation, S.D.: 23.5 ± 3 years; mean education ± S.D.: 14.5 ± 2.1 years). All participants were right-handed, according to the Edinburgh Handedness Inventory (Oldfield, 1971), and had no contraindication to TMS, according to TMS safety guidelines (Rossi, Hallett, Rossini, & Pascual-Leone, 2009). The sample size was selected according to previous studies using PAS protocols (e.g., Litvak et al., 2007; Pellicciari et al., 2009; Stefan et al., 2000). The experiment was performed in accordance with the ethical standards of the Declaration of Helsinki and it was approved by the Ethical Committee of the IRCCS San Giovanni di Dio Fatebenefratelli (Brescia). Before taking part in the study, participants gave their written informed consent. The experiment, as well as *Experiments 2* and *3*, was conducted at the Cognitive Neuroscience Section of the IRCCS San Giovanni di Dio Fatebenefratelli (Brescia).

3.2.2.2. cm-PAS

The cm-PAS consisted in a modified version of the standard *within-system* S1-PAS protocol (Wolters et al., 2005), in which the electrical peripheral MN stimulation was replaced with a (cross-modal) visual stimulus depicting a touch (i.e., visual-touch).

During the cm-PAS, participants sat comfortably with their head on a chinrest to minimize possible movements and they were asked to fixate a PC monitor placed at a distance of 57 cm, where the visual stimuli were presented on a black background. Participants were presented with a videoclip consisting of two frames showing a left hand being touched on the palm. Each trial of the cm-PAS (total duration = 10 s) started with a fixation frame depicting the palm of a left hand (10 X 18° of visual angle), viewed from an egocentric

perspective. Participants were asked to fixate a red asterisk located in the center of the palm of the left hand, requiring to pay attention to the visual-touch events.

The fixation frame was presented for 9.7 s. As soon as the fixation frame ended, the visual-touch stimulus (7.5 X 8.5° of visual angle) appeared. The visual-touch stimulus consisted of a single frame showing the index finger of a right hand (seen from an allocentric perspective) touching the left hand on the palm (namely, the same hand used as fixation). The visual-touch frame (duration: 300 ms) started immediately (0 ms of delay) after the fixation frame, in turn giving rise to an apparent motion, namely, that the index finger moved and then touched the palm of the hand (the touching finger stopped on the red asterisk positioned on the hand used as fixation). The TMS pulse over right S1 (contralateral to the hand being touched depicted in the visual stimulus), and thus the ISI, was timed with respect to the contact between the index finger and the hand (i.e., the onset of the visual-touch frame). Three different ISIs were tested in three different sessions: 20, 60 and 100 ms (**Figure 3.1**). The actual timing of visual-touch stimuli was checked using a photodiode. Consistently with *within system* S1-PAS protocols (Wolters et al., 2005), the TMS pulses over S1 were delivered at 150% of individual resting motor threshold (rMT – see next paragraph). According to their rMT, participants were stimulated at a mean TMS intensity (± S.D.) of 61.8 ± 8.7% in the ISI-20 session, 61 ± 7.6% in the ISI-60 session and 61.4 ± 8.7% in the ISI-100 session (with no difference between sessions, $p > .48$). For masking the sound of the TMS, participants heard a white noise in a pair of headphones during the stimulation sessions.

Overall, the cm-PAS comprised a total of 150 paired stimulations, delivered at a fixed frequency of 0.1 Hz, for a total duration of 25 min. During the entire cm-PAS, participants were told to keep their left hand on the table in a position as similar as possible at the one shown on screen during the protocol (Medina & DePasquale, 2017). Since in PAS protocols

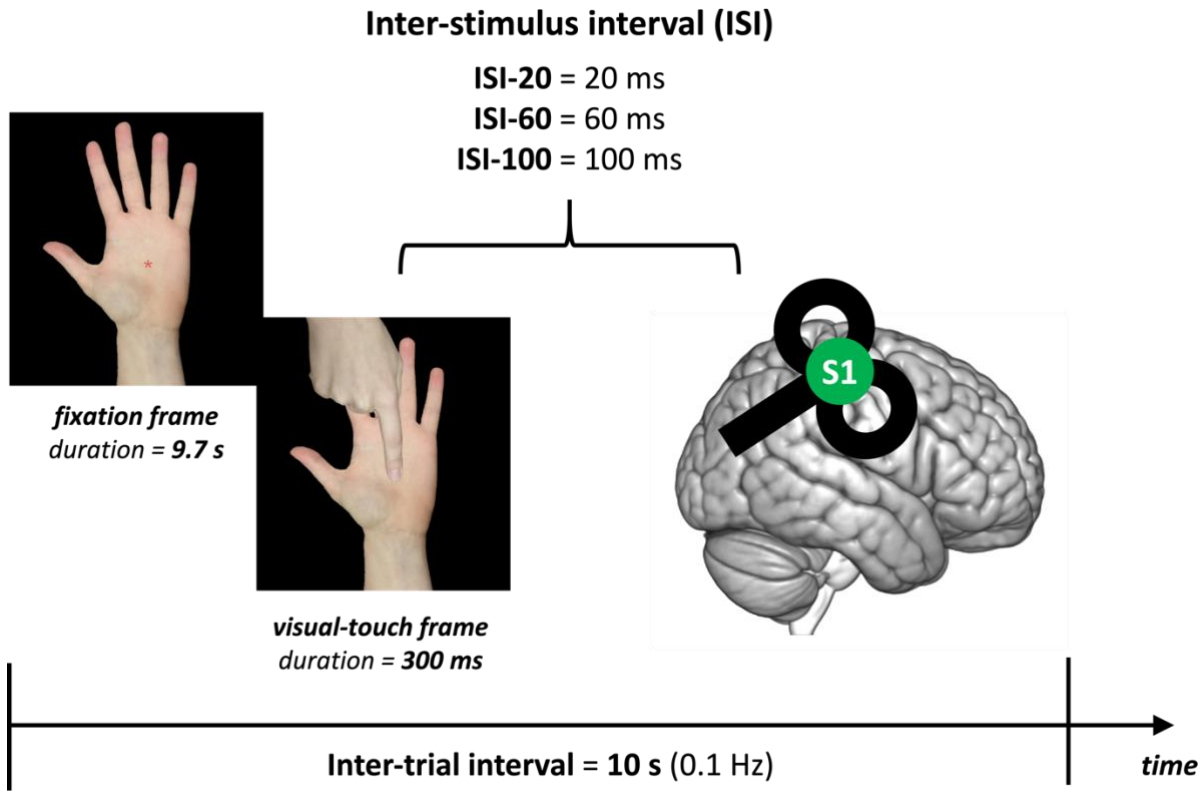

Figure 3.1. cm-PAS protocols (and related parameters) tested in *Experiment 1*.

paying attention to the stimuli is critical for the success of the protocol itself (Stefan et al., 2004), during the cm-PAS we ensured that participants were observing the visual-touch stimulus by asking them to detect rare events (presented in 15 out of 150 trials). These rare events consisted in double visual-touch trials in which the visual-touch frame was presented twice sequentially: a first visual-touch frame presented for 180 ms, followed by a second visual-touch frame lasting 300 ms (inter-trails intervals of 180 ms). In this condition, the TMS pulse was applied on the second visual-touch frame, with the same parameters described above. Participants were instructed to press the PC-mouse with their right index finger every time the double visual-touch trial was presented; performance in such attentional task was not analysed.

Trials randomization, timing of the stimuli and recording of the participant's responses were under computer control (E-Prime 2.0, Psychology Software Tool, Inc.).

3.2.2.3. TMS

TMS was delivered using a figure-of-eight coil (diameter = 70 mm) connected to a monophasic Magstim 200^2 stimulator (Magstim, Whitland, UK). At the beginning of every session the participants' rMT was assessed. The individual rMT was defined as the minimum TMS intensity (expressed as percentage of maximum stimulator output) able to elicit a MEP of at least 50 μV in the left hand's *Abductor Pollicis Brevis* (APB) muscle 5 times out of 10 during the stimulation of the right M1 (Rossi et al., 2009). For MEPs recording, a pair of Ag/AgCl surface electrodes in a bipolar montage was placed over the belly of the target muscle: the active electrode was placed over APB and the reference electrode over the metacarpophalangeal joint of the thumb. MEPs were visualized using the software BrainAmp (Brain Products GmbH, Munich, Germany). Participants' mean rMT was (mean ± S.D.) 40.9 ± 5.6%. After the determination of the individual rMT, S1 of the right hemisphere was identified 2 cm posterior from APB hotspot, according to previous PAS paradigms and TMS studies stimulating S1 (Giurgola, Pisoni, Maravita, Vallar, & Bolognini, 2019; Gorgoni et al., 2015; Wolters et al., 2005). As a control measure of correct coil positioning, after S1 was located, a few pulses at rMT intensity were delivered to be sure that no muscle twitches were elicited in the contralateral hand. For both M1 and S1 stimulation, the coil was 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 (Orth & Rothwell, 2004). TMS procedures were assisted with SofTaxic 2.0 neuronavigation system (E.M.S., Bologna, Italy, www.softaxic.com).

3.2.2.4. 2-Point Discrimination Task – 2-PDT

To assess the behavioral effect of the cm-PAS, tactile acuity was measured using a classical 2-point discrimination task (2-PDT), adopting a similar version to the one used by Case and

co-workers (2016, 2017). During the task, participants were blindfolded and comfortably seated in an armchair while an experimenter touched them with an aesthesiometer (North Coast Medical, Morgan Hill, USA) on the thenar eminence of the left-hand palm (i.e., the same part of the hand touched during the visual-touch trials) with 1 or 2 plastic tips. In a 2-alternative forced-choice task, participants were asked to verbally report whether they felt 1 or 2 tips; a second experimenter recorded the response on the PC.

Thirteen different distances (range: 3-15 mm) were tested in descendent blocks of 10 randomized trials comprising 5 trials with 1 tip, and 5 trials with 2 tips for each distance. This procedure was repeated 3 times, with a brief break of 1 minute at the end of each repetition, for a total of 30 trials for each distance (i.e., 15 with a single tip, 15 with two tips; for a total of 390 trials). The experimenter, which was the same throughout the three sessions of a single participant, was trained and instructed to touch always the same point on the participant's left-hand palm – point that was previously marked with a soft tip, in order to be consistent in the location throughout the duration of the 2-PDT and in the assessment after the cm-PAS – and to apply the same pressure. Participants were informed in advance about the occurrence of tactile stimuli, and they were asked to provide a quick response as soon as they felt the touch. Number of trials, as well as the location on the hand, were selected according to a pilot study on 15 healthy participants, in order to estimate the individual psychometric function and account for inter-participants variability.

3.2.2.5. Experimental procedure

Experiment 1 comprised 3 sessions during which the ISI between the onset of the visual-touch frame and the S1-TMS was varied, being of 20 ms (ISI-20), 60 ms (ISI-60) or 100 ms (ISI-100). Each experimental session started with the administration of the 2-PDT, followed by the determination of the individual rMT and the TMS hotspot. Then, the cm-PAS was

administered. Immediately after its end, tactile acuity was measured again. The order of the experimental sessions was counter-balanced among participants, with an inter-session interval of at least 72 hours, and each participant was tested at the same moment of the day throughout the sessions (in the morning or in the afternoon), thus to control the possible influence of circadian rhythm on PAS effectiveness (Sale, Ridding, & Nordstrom, 2007). On average, each session lasted about 1 h and 40 min.

3.2.2.6. Statistical analysis

Cm-PAS effects at the 2-PDT were assessed following Signal Detection theory (Green & Swets, 1966). Signal detection measures have the advantage of allowing for the separation of perceptual level and decision level by assessing the contribution of stimulus-related (i.e., perceptual sensitivity, *d'*) and participant-related (i.e., response bias, *c*) influences on performance: the *d'* parameter reflects the participant's accuracy to discern a sensory event from its background (perceptual sensitivity), while the *c* parameter reflects the participant's decision criterion of response (response bias). For sensory threshold estimation, *d'* data were linearly transformed in order to fit in a range between 0 and 1, and submitted to a logistic function fitting. Sensory threshold was then defined as the distance in mm at which performance was 50% (R, version 3.3.1 – R Core Team, 2016). In two participants, threshold estimation revealed negative values; they were therefore excluded from subsequent analyses, leaving the final analysed sample to 16 participants. We also considered as dependent variables the global performance, consisting in mean *d'* sensitivity and *c* regardless of the mm-distance (i.e., all distances collapsed).

Sensory threshold, response criterion and global performance were then separately analysed through a 2 X 3 within-subjects repeated-measures analysis of variance (rmANOVA), with factors Time (pre and post cm-PAS) and ISI (ISI-20, ISI-60, ISI-100). In every analysis, the

Kolmogorov-Smirnov test confirmed the normality of the distributions, and when appropriate, data sphericity was confirmed by Mauchly's test. As effect size index, partial eta-squared (η_p^2) values are reported. Multiple comparisons in post-hoc analyses were corrected by applying Tukey honest significant difference. Statistical significance was set at $p < .05$. If not otherwise specified, mean ± standard error (S.E.) is reported. Statistical analyses were performed using the software Statistica (version 10, Statsoft).

3.2.3 Results

The rmANOVA on sensory threshold (*d'* values) at the 2-PDT showed a significant ISI by Time interaction ($F_{2,30} = 6.55$, $p = .004$, $\eta_p^2 = .3$): post-hoc comparisons revealed a trend for a difference between pre and post cm-PAS only when the ISI was of 20 ms ($p = .078$), which reached significance when further explored with 2-tailed Student's paired t-test ($Pre_{ISI\text{-}20}$ = 9.35 ± .52 mm, vs. $Post_{ISI\text{-}20}$ = 8.3 ± .59 mm, t = 2.35, $p = .033$). The post-hoc comparisons also showed that sensory threshold after the cm-PAS with 20 ms of ISI was significantly lower than after cm-PAS with an ISI of 60 ms ($Post_{ISI\text{-}60}$ = 9.71 ± .53 mm, t = -2.37, p = .008) or of 100 ms ($Post_{ISI\text{-}100}$ = 9.65 ± .59 mm, t = -1.78, $p = .012$; **Figure 3.2**). Importantly, threshold before cm-PAS did not differed among ISIs ($p > .946$). The main effects of ISI ($F_{2,30} = .8$, $p = .458$, $\eta p^2 = .05$) and Time ($F_{1,5} = .02$, $p = .882$, $\eta_p^2 < .01$) were not significant. The analysis of the response criterion revealed a main effect of Time ($F_{1,15} = 10.04$, $p = .006$, $\eta_p^2 = .4$), indicating that response bias was significantly less conservative after every cm-PAS session ($Pre_{cm\text{-}PAS}$: c = -.73 ± .07, $Post_{cm\text{-}PAS}$: c = -.62 ± .07), independently from the ISI. Main effect of ISI was not significant ($F_{2,30} = .45$, $p = .64$, $\eta_p^2 = .03$), as well as the ISI X Time interaction ($F_{2,30} = .96$, $p = .39$, $\eta_p^2 = .06$).

Finally, results on global performance mirrored the previous ones on sensory threshold: the significant ISI by Time interaction ($F_{2,30}$ = 8.38, p = .001) showed that performance significantly improved only after cm-PAS with 20 ms of ISI ($Pre_{ISI\text{-}20}$: d' = 1.72 ± .12; $Post_{ISI\text{-}20}$: d' = 1.94 ± .11; t = -2.86, p = .03; $Pre_{ISI\text{-}60}$: d' = 1.78 ± .1, $Post_{ISI\text{-}60}$: d' = 1.64 ± .13, t = 1.98, p = .32; $Pre_{ISI\text{-}100}$: d' = 1.77 ±.15, $Post_{ISI\text{-}100}$: d' = 1.68 ± .12, t = 1.19, p = .77). Furthermore, performance after cm-PAS with 20 ms ISI was significantly higher as

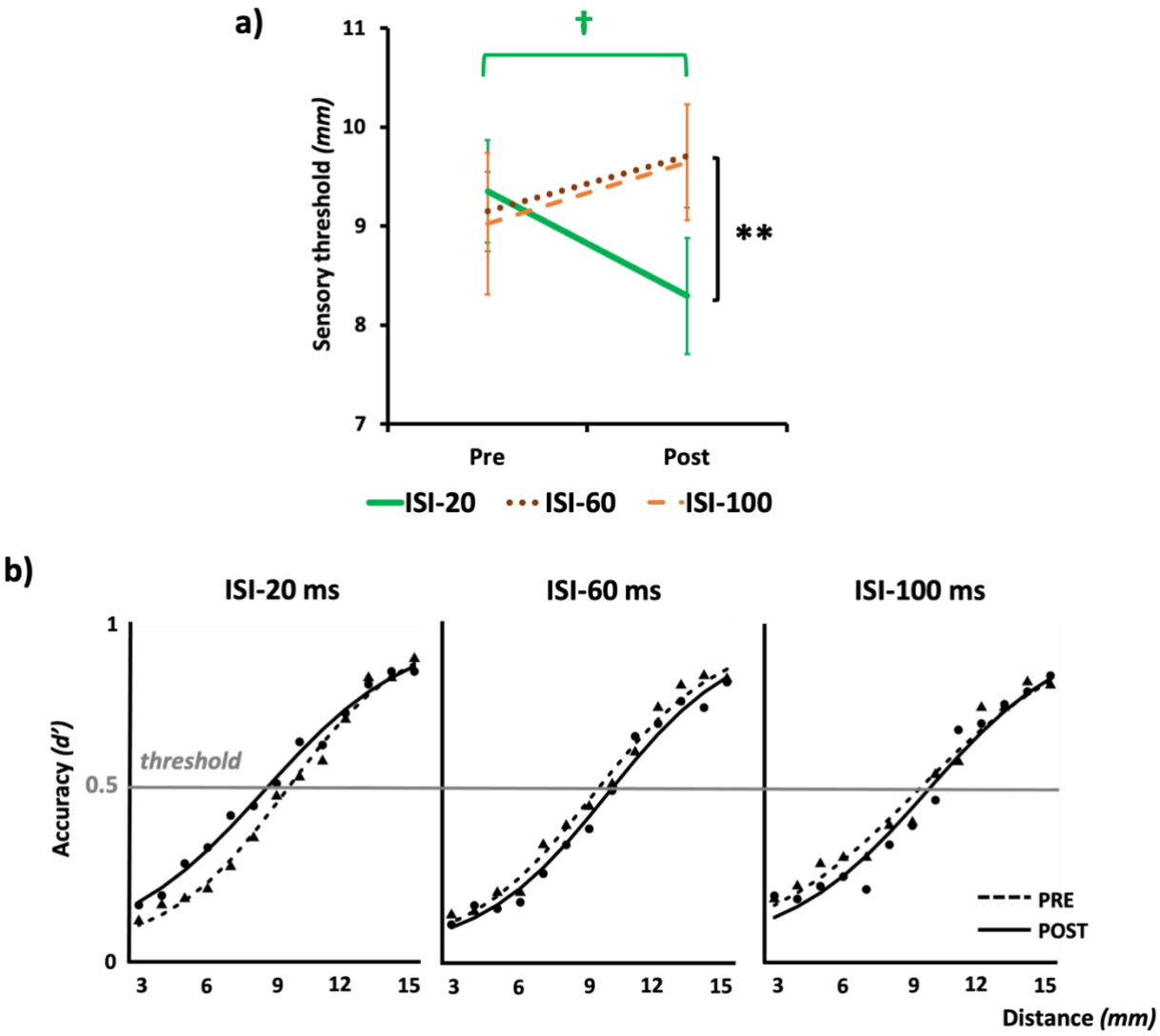

Figure 3.2. Results of *Experiment 1*. a) Effects of the different tested versions of the cm-PAS on tactile acuity at the 2-PDT: significantly lower threshold after the cm-PAS with the ISI of 20 ms (green straight line) compared to the protocols with the ISIs of 60 ms (brown pointed line) and 100 ms (amber straight-dotted line). ** = p < .01; error bars = S.E. **b)** Psychometric functions obtained from logistic fitting to raw data, before (dotted line, triangles) and after (black line, dots) the three tested protocols.

compared to that after cm-PAS at ISIs of 60 ms (t = 2.59; p = .001) and 100 ms (t = 1.81, p = .008). The main effects of ISI ($F_{2,30}$ = .78, p = .46, ηp^2 = .05) and Time ($F_{1,5}$ < .01, p = .94, ηp^2 < .01) were not significant.

3.2.4. Conclusions

The results of *Experiment 1* show a timing-dependent improvement in tactile acuity after cm-PAS, only when the ISI of 20 ms is applied, reflecting the possible induction of LTP-like plasticity by the PAS protocol. The ISIs of 60 and 100 ms are ineffective. Importantly, the enhancement of performance in the 2-PDT is not drive by a different way in which the participants approach the task after protocol administration, as demonstrated by the absence of statistically significant differences in the response criterion. As will be discussed in the general discussion of the present chapter (**par. 3.6.1**), considering my starting hypothesis, the efficacy of such short ISI is quite unexpected and the possible underpinned functional mechanisms will be deepened in *Experiment 4*.

3.3. *Experiment 2*: Cortical specificity of cm-PAS

3.3.1. Aim

The second experiment explores the cortical specificity of the cm-PAS by comparing the effect of pairing a visual-touch stimulus with TMS over S1 (namely, the protocol found effective in the previous experiment) to that induced by the pairing of the same visual stimulus with the cortical stimulation of the primary visual cortex (V1). This last (control) version of the protocol can be considered a sort of *within-system* PAS (i.e., both peripheral and cortical stimulations interact within the visual system), which should not induce cross-modal plasticity within S1, at least with the same temporal profile of the protocol where the cortical stimulation is delivered over S1 (i.e., 20 ms of ISI) (Foxe & Simpson, 2002; Pihko et al., 2010). However, considering that the aim of the present experiment was not to test the effectiveness of the PAS with the TMS delivered over V1, I did not measure possible plastic phenomena induced at the visual level by such protocol.

Importantly, by targeting another functionally relevant area as V1, my experiment provides stronger clues on the functional selectivity of S1 in cm-PAS efficacy, rather than simply controlling for sensory and placebo rTMS confounds, as it would have happened if I had used a sham stimulation or a functionally 'neutral' area (such as the vertex) (Duecker & Sack, 2015).

3.3.2. Methods and materials

3.3.2.1. Participants

Ten participants, all right-handed, took part in *Experiment 2* (5 males, mean age ± S.D.: 23.7 ± 4.2 years; mean education ± S.D.: 14.3 ± 2.8 years). They were recruited by using the same criteria of *Experiment 1*.

3.3.2.2. Experimental procedure and statistical analyses

Materials, methods and statistical analyses were identical to those of the first experiment. The only difference pertained to the cm-PAS: here, only the ISI of 20 ms, which proved to be effective in the previous experiment (see results above), was used but I added a control condition during which the right V1 was stimulated to assess the cortical specificity of the cm-PAS (**Figure 3.3**).

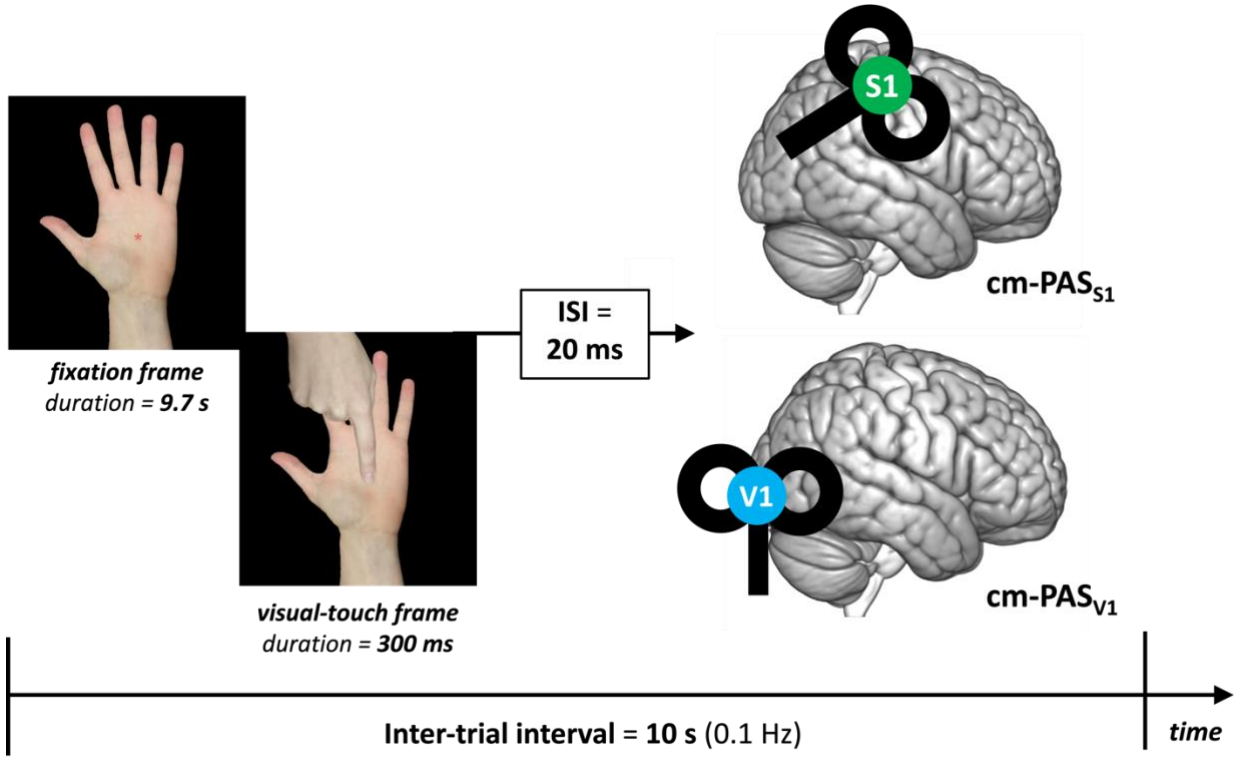

Figure 3.3. cm-PAS protocols (and related parameters) tested in *Experiment 2*.

Hence, *Experiment 2* comprises two experimental sessions (i.e., cm-PAS$_{S1}$; cm-PAS$_{V1}$). TMS intensity during the cm-PAS was, on average ± S.D., of 60.8 ± 6.1% for S1 stimulation and 59.8 ± 6.8% for V1 stimulation (not statistically different, $p = .168$). Participants' mean rMT (± S.D.) was of 40.2 ± 4.3%. The right V1 was identified 2 cm dorsal and 0.5 cm lateral from the inion, according to previous literature (Silvanto, Cowey, et al., 2005). For V1 stimulation, the coil was placed tangentially to the scalp with the handle hold horizontally to the right, thus to induce a lateral from medial current flown (Kammer, Beck, Erb, & Grodd, 2001). Neuronavigation procedures were carried on using the Softaxic 3.0 nauronavigator (EMS, Bologna, Italy).

Data from the 2-PDT was statistically analysed by performing a 2 X 2 rmANOVA, with the within-subjects factors Time (pre, post) and Area (S1, V1).

3.3.3. Results

With respect to the sensory threshold at the 2-PDT, the rmANOVA showed a significant Area X Time interaction ($F_{1,9} = 11.03$, $p = .009$, $\eta_p^2 = .55$): sensory threshold significantly decreased only after the cm-PAS stimulating the right S1 (Pre$_{S1}$ = 10.56 ± .42 mm, Post$_{S1}$ = 9.02 ±.31 mm, t = 4.53, $p = .019$), but not when the right V1 was targeted (Pre$_{V1}$ = 10.45 ± .58 mm; Post$_{V1}$ = 10.82 ± .53 mm; t = .39, $p = .8$); moreover, sensory threshold after cm-PAS over S1 was significantly lower than that after V1 stimulation (t = -2.8, $p = .007$; **Figure 3.4**). Threshold values before cm-PAS were comparable between sessions (t = .21, $p = .993$). Main effects of Area ($F_{1,9} = 2.78$, $p = .13$, $\eta_p^2 = .24$) and Time ($F_{1,9} = 4.69$, $p = .058$, $\eta_p^2 = .34$) were not significant.

Response criterion significantly increased after cm-PAS regardless of the targeted cortical areas (main effect of Time, $F_{1,9} = 58.24$, $p < .001$, $\eta_p^2 = .87$: Pre$_{cm\text{-}PAS}$: c = -.63 ± .08 vs.

$Post_{cm\text{-}PAS}$: c = -.43 ± .08). The main effect of Area was not significant ($F_{1,9}$ < .01, p = .98, η_p^2 < .01), as well as the Area X Time interaction ($F_{1,9}$ < .01, p = .95, η_p^2 < .01).

Results on global performance confirmed the pattern on sensory threshold. The Area by Time interaction was significant ($F_{1,9}$ =10.98, p = .009, η_p^2 = .55), and post-hoc analysis showed that performance significantly increased after cm-PAS only when TMS was

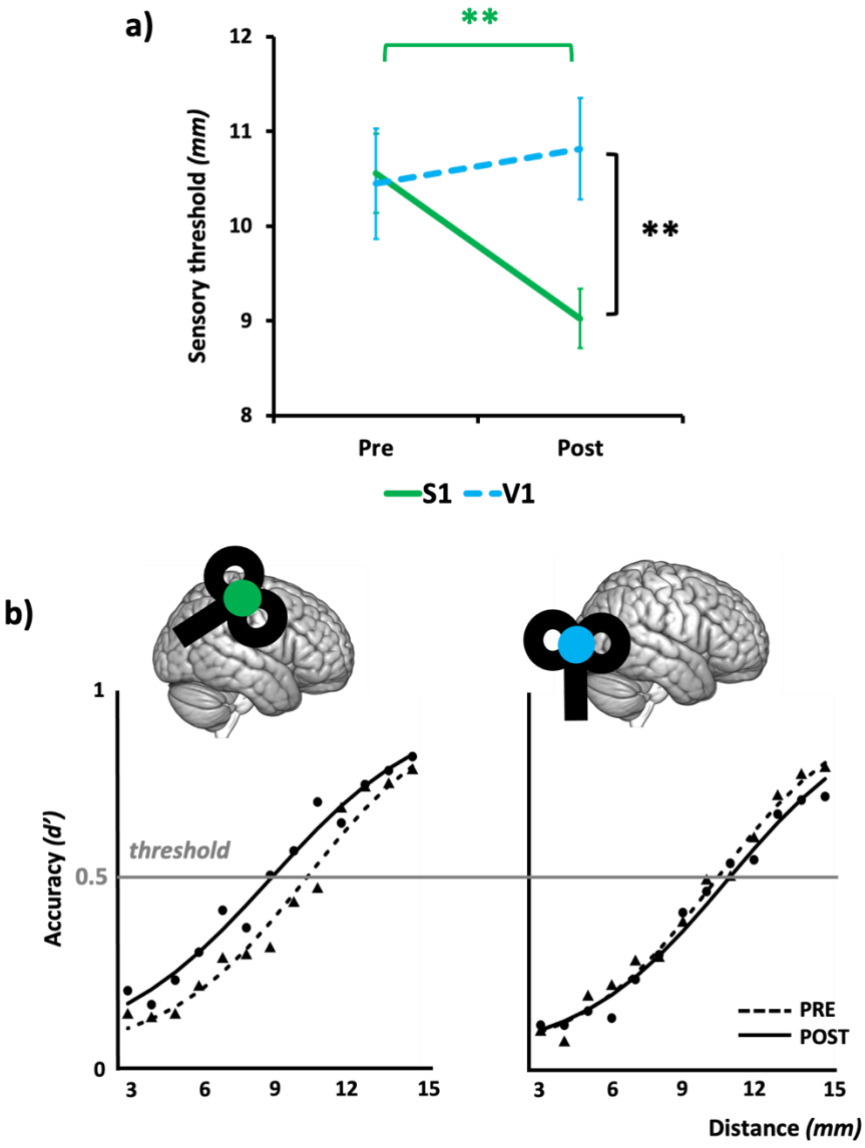

Figure 3.4. Results of *Experiment 2*. a) Effects of the different versions of the cm-PAS on tactile acuity at the 2-PDT: significantly lower threshold after the cm-PAS targeting S1 (green straight line) compared to the protocol targeting V1 (light blue dotted line). ** = p < .01; error bars = S.E. **b)** Psychometric functions obtained from logistic fitting to raw data, before (dotted line, triangles) and after (black line, dots) the tested protocols.

delivered over S1 (Pre_{S1}: *d'* = 1.50 ± .1, $Post_{S1}$: *d'* = 1.83 ± .07, t = -4.23, *p* = .023), but not after V1 stimulation (Pre_{V1}: *d'* = 1.52 ± .13, $Post_{V1}$: *d'* = 1.43 ± .12, t = 1.04, *p* = .735). Global performance after cm-PAS with S1 stimulation was significantly higher compared to cm-PAS over V1 (t = 2.92, *p* = .008). The main effects of Area ($F_{1,9}$ = 3.11, *p* = .111, η_p^2 = .26) and Time ($F_{1,9}$ = 4.66, *p* = .059, η_p^2 = .34) did not reach the significance level.

3.3.4. Conclusions

This second experiment highlights the cortical specificity of the cm-PAS: indeed, using the timing found effective in the previous experiment (ISI of 20 ms), only when the visual-touch stimulus is paired with a TMS pulse over S1, the protocol successfully enhances tactile acuity, bettering global performance in the 2-PDT. Conversely, pairing the visual touch with a TMS pulse over V1 led to no behavioural modification (i.e. 2-PDT).

3.4. *Experiment 3*: Visual specificity and neurophysiological correlates of cm-PAS

3.4.1. Aim

In the third experiment, I assess the neurophysiological effect of cm-PAS at the level of SEPs, searching for evidence that behavioral changes found in the previous two experiments are mirrored by cortical ones. Furthermore, the peripheral stimulation (i.e. the visual stimulus of touch) was modulated, investigating the visual stimulus specificity of the protocol. Here, I introduce a 'no-touch condition': namely, in the control version of the cm-PAS, a visual stimulus not depicting a tactile event (i.e., the sight of an approaching hand, not delivering any tactile stimulation) is presented. In fact, I hypothesize that a visual stimulus not depicting a touch should not recruit the visuo-tactile properties of S1 (see for example: Bolognini et al., 2011).

3.4.2. Methods and materials

3.4.2.1. Participants

Twenty participants, all right-handed (Oldfield, 1971), were recruited for *Experiment 3* following the same criteria of the previous experiments. One participant dropped out and two were excluded due to EEG artifacts, so that the final sample considered in the analyses comprised 17 participants (8 males, mean age ± S.D.: 23.6 ± 2.1 years; mean education ± S.D.: 14.5 ± 1.9 years).

3.4.2.2. SEPs

SEPs were induced by electric stimulation of the left MN, while EEG was continuously recorded. During SEP recording, participants were comfortably seated in an armchair with their left arm lying relaxed on a desk, and they were asked to fixate a cross on a PC-screen to minimize eye-movements.

MN stimulation was performed by using a battery-driven constant current electrical stimulator (STM140, High Technology Laboratory, Udine, Italy), using the same parameters and stimulator device of Pellicciari and co-workers (2009). Specifically, the anode was placed at the level of the wrist with cathode proximal, and 500 pulses were delivered with a pulse width of 200 μs at a frequency of 3.3 Hz, for a total duration of about 3 min. Stimulation intensity was set at 200% of the individual perceptual threshold (Cruccu et al., 2008). At this stimulation intensity, none of the participants had visible muscle twitches elicited by the MN stimulation. Before the cm-PAS, the stimulator position was marked on the skin, allowing its repositioning in the same exact location after the administration of the cm-PAS. The same parameters were applied for SEP recording after the cm-PAS.

EEG was recorded from 32 channels (FP1, FP2, F3, Fz, F4, FC5, FC1, FC2, FC6, T7, T8, C3, C1, Cz, C2, C4, CP5, CP3, CP1, CP2, CP4, CP6, P7, P3, Pz, P4, P8, PO7, PO3, PO4, PO8, Oz; BrainAmp, 32MR plus, BrainVision Recorder, Brain Products GmbH, Munich, Germany). The ground was placed on FPz and signal from all electrodes was referenced online to the right mastoid. Four additional electrodes in a bipolar montage were applied for vertical and horizontal electrooculogram. Skin/electrode impedance was maintained below 5 kΩ.

Analysis of SEPs was performed using BrainVision Analyzer 2 (Brain Products GmbH, Munich, Germany). EEG data was re-referenced offline to the average of the two mastoids and a low cut-off filter at 1 Hz (Butterworth zero phase filter; 12 db/oct) was applied. The

artefact induced by MN stimulation was removed by interpolating the signal in the first 4 ms after the electrical pulse, while artefacts related to eye movements were identified and corrected by means of Independent Component Analysis (ICA; algorithm: infomax). Continuous data was then segmented into epochs from 50 ms before to 100 ms after the electrical pulse, applying a baseline correction for the 20 ms preceding the stimulation. Signal recorded from corrupted channels was interpolated (not more than 1 for each participant; mean ± S.D.: .13 ± .34). Epochs were visually inspected, rejected when the signal exceeded ± 70 μV and/or if muscular artefacts were detected (mean ± S.D.: 3.09 ± 5.75%), and then averaged. SEP amplitude was measured at the peak of each component from a pooling of channels C4 and CP4 (Buchsbaum, Davis, & Bunney, 1977; Litvak et al., 2007), whose latency was identified from grand-average collapsing all conditions.

3.4.2.3. Experimental procedure and statistical analysis

Materials, methods and analyses of *Experiment 3* were the same of the previous experiments, except for the use of a control condition concerning the visual stimulus paired to TMS, and the additional recording of SEPs before and after the cm-PAS, just before the 2-PDT administration. According to the results of the two previous experiments, only the cm-PAS with 20 ms of ISI and TMS delivered over S1 was used. Moreover, to ensure the appropriate TMS intensity during the cm-PAS, the rMT was determined after the EEG cap for SEP recording was mounted, since EEG electrodes increase the distance between TMS coil and the scalp (Farzan et al., 2016).

Experiment 3 comprised two sessions, which differed for the visual stimuli displayed during the cm-PAS. In one session, the visual-touch stimuli were presented, as those used in the previous experiments; in the other session, visual-no-touch stimuli (300 ms of duration, as the visual-touch stimulus, with same dimensions) were presented: now, participants viewed,

from an allocentric perspective, the right index approaching the red asterisk which was also used as fixation point (**Figure 3.5**).

The two sessions were randomized across participants; each session lasted about 2 hours and 30 min. TMS intensity during the cm-PAS was, on average ± S.D., of 72.3 ± 9.4% for the visual-touch session, and of 71.7 ± 8.5% for the no-touch condition (p = .43). Participants' mean rMT ± S.D. was of 48 ± 6%.

Performance at the 2-PDT was analysed separately via a 2 X 2 rmANOVA, with the within-subjects factors Visual stimulus (visual-touch, visual-no-touch) and Time (pre, post). Statistical analysis on SEPs was performed on each SEP component separately by means of a 2 (Visual stimulus) X 2 (Time) rmANOVA.

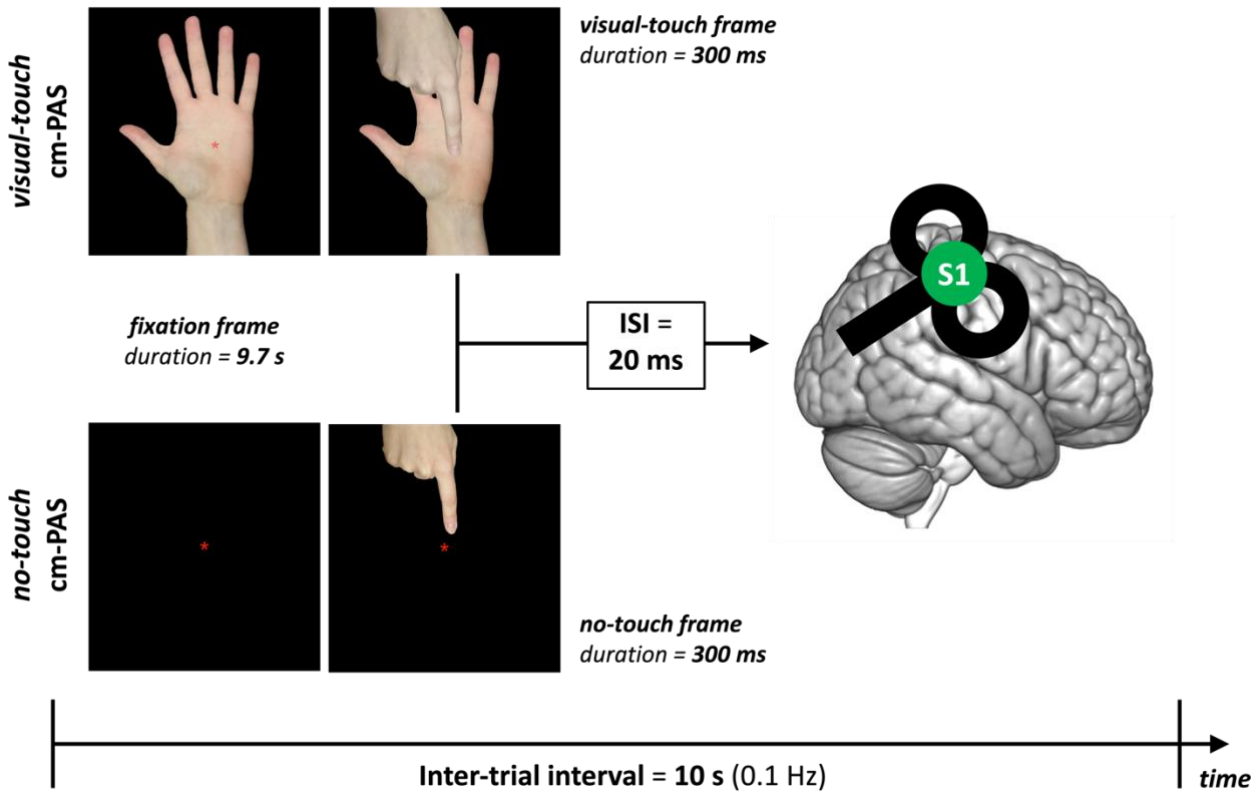

Figure 3.5. cm-PAS protocols (and related parameters) tested in *Experiment 3*.

3.4.3. Results

3.4.3.1. 2-PDT

The rmANOVA showed a significant Stimulus X Time interaction ($F_{1,16}$ = 33.53, p < .001, η_p^2 = .68), showing that the tactile threshold significantly decreased only in the visual-touch cm-PAS: $Pre_{visual\text{-}touch}$ = 11.99 ± .59 mm *vs.* $Post_{visual\text{-}touch}$ = 10.35 ± .52 mm, t = 4.45, p < .001. Conversely, after the cm-PAS with visual-no-touch stimuli, sensory threshold significantly increased ($Pre_{visual\text{-}no\text{-}touch}$ = 11.26 ± .52 mm, *vs.* $Post_{visual\text{-}no\text{-}touch}$ = 12.22 ± .61 mm, t = -2.67, p = .038). Participants' performance in the two sessions did not differ before the protocol (t = 1.33, p = .141), while it differed after the two cm-PAS (t = -2.85, p < .001; **Figure 3.6a-b**). The main effect of Time ($F_{1,16}$ = 1.47, p = .243, η_p^2 = .08) and Stimulus ($F_{1,16}$ = 1.04, p = .323, η_p^2 = .06) were not significant.

A significant main effect of Time was found for the response criterion ($F_{1,16}$ = 39.37, p < .001, η_p^2 = .71), with increased values after cm-PAS but independently of visual stimulus type ($Pre_{cm\text{-}PAS}$: c = -.54 ± .07, $Post_{cm\text{-}PAS}$: c = -.3 ± .08). The main effect of Stimulus ($F_{1,16}$ = .29, p = .597, η_p^2 = .02), as well as the Stimulus by Time interaction ($F_{1,16}$ = 2.56, p = .129, η_p^2 = .14), were not significant.

Results on global performance were consistent with the ones on sensory threshold: the significant Stimulus X Time interaction ($F_{1,16}$ = 28.18, p < .001, η_p^2 = .64) showed that the performance improved after the cm-PAS with visual-touch stimuli ($Pre_{visual\text{-}touch}$: d' = 1.21 ± .11 *vs.* $Post_{visual\text{-}touch}$: d' = 1.54 ± .11, t = -4.58, p < .001), while it remained unchanged in the visual-no-touch ($Pre_{visual\text{-}no\text{-}touch}$: d' = 1.3 ± .1 *vs.* $Post_{visual\text{-}no\text{-}touch}$: d' = 1.14 ± .13, t = 2.4, p = .102). Global performance before cm-PAS was comparable in the two sessions (t = -.91, p = .484), whereas after cm-PAS it was higher for the visual-touch session, as compared to the visual-no-touch session (t = 2.91, p < .001). The main effects of Time ($F_{1,16}$ = 2.53, p = .131, η_p^2 = .14) and Stimulus ($F_{1,16}$ = 1.8, p = .199, η_p^2 = .1) were not significant.

3.4.3.2. SEPs

Consistently with the literature on SEPs (Desmedt, Nguyen Tran Huy, & Bourguet, 1983; Macerollo et al., 2018; Mauguière et al., 1999), from grand-average collapsing all conditions we observed 5 main peaks (mean latency in parentheses): P14 (14 ms), N20 (19 ms), P25 (25 ms), N30 (30 ms), P40 (42 ms). Significant effects were found for P40 only: indeed, the rmANOVA showed a significant Stimulus by Time interaction ($F_{1,16}$ = 5.67, p = .03, η_p^2 = .26), and post-hoc comparisons revealed a significant increase in P40 after cm-PAS ($Pre_{visual\text{-}touch}$ = .80 ± .25 µV *vs.* $Post_{visual\text{-}touch}$ = 1.57 ± .29 µV, t = -2.81, p = .038) in the visual-touch condition only ($Pre_{visual\text{-}no\text{-}touch}$ = 1.34 ± .22 µV *vs.* $Post_{visual\text{-}no\text{-}touch}$ = 1.24 ± .27 µV, t = 0.4, p = .983; **Figure 3.6c**). P40 in the two sessions did not differ neither before (t = -2.13, p = .203), nor after (t = 1.4, p = .581) the cm-PAS. Main effects of Stimulus ($F_{1,16}$ = .39, p = .543, η_p^2 = .02) and Time ($F_{1,16}$ = 3.59, p = .076, η_p^2 = .18) were not significant. No significant main effects nor interactions emerged from analysis on other SEP components (see **Table 3.1**).

	Stimulus	***Time***	***Stimulus* X *Time***
P14	$F_{1, 16}$ = 1.62, p = .22, η_p^2 = .09	$F_{1, 16}$ = 1.53, p = .235, η_p^2 = .09	$F_{1, 16}$ = .9, p = .357, η_p^2 = .05
N20	$F_{1, 16}$ = 2.23, p = .155, η_p^2 = .12	$F_{1, 16}$ = .18, p = .673, η_p^2 = .01	$F_{1, 16}$ = 0.02, p = .883, η_p^2 < .01
P25	$F_{1, 16}$ = .26, p = .615, η_p^2 = .02	$F_{1, 16}$ = .81, p = .382, η_p^2 = .05	$F_{1, 16}$ = .49, p = .495, η_p^2 = .03
N30	$F_{1, 16}$ = .02, p = .88, η_p^2 < .01	$F_{1, 16}$ = 4.09, p = .06, η_p^2 = .2	$F_{1, 16}$ = 1.2, p = .291, η_p^2 = .07
P40	$F_{1, 16}$ = .39, p = .543, η_p^2 = .02	$F_{1, 16}$ = 3.59, p = .076, η_p^2 = .18	**$F_{1, 16}$ = 5.67, p = .03, η_p^2 = .26**

Table 3.1. Results from the analysis of SEP components.

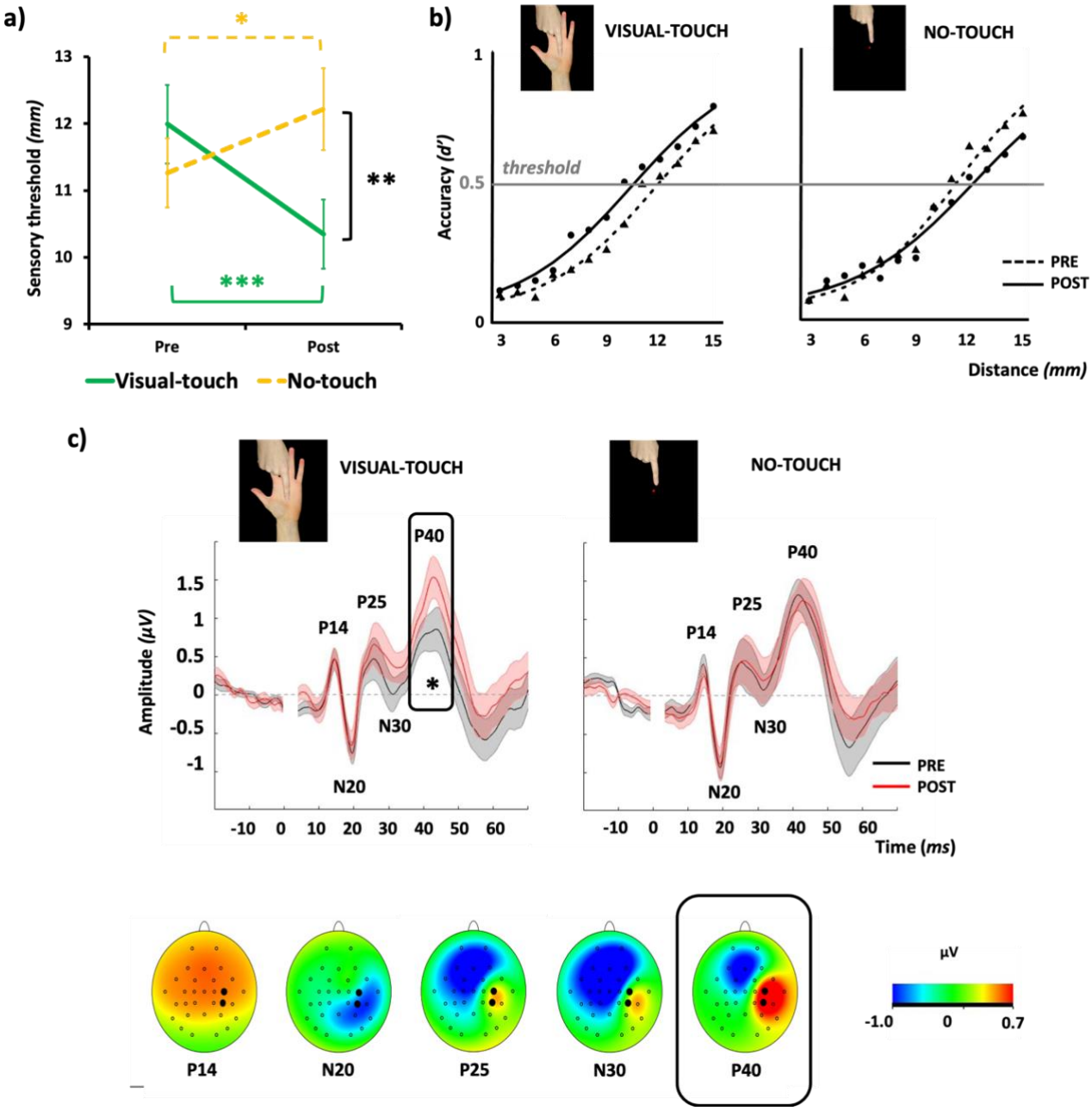

Figure 3.6. Results of *Experiment 3*. a) Effects of cm-PAS on tactile acuity at the 2-PDT: significant decrease of sensory threshold only in the Visual-touch (continuous green line) compared to No-touch (dotted yellow line) condition; * = $p < .05$, ** = $p < .01$, *** = $p < .001$. Error bars = S.E. **b)** Psychometric functions obtained from logistic fitting to raw data, before (dotted line, triangles) and after (continuous line, dots) cm-PAS in the visual-touch and no-touch condition. **c)** Effects of cm-PAS on SEPs. *Top*: SEPs as recorded from C4-CP4 pooling, in the visual-touch and in the no-touch condition, before (black) and after (red) cm-PAS. S.E. in shaded bars. The asterisk indicates the significant increase of P40 after cm-PAS ($p = .038$) in the visual-touch condition only. *Bottom:* topographies of main SEP components observed, taken from all conditions collapsed; amplitude range (μV) as shown in colorbar. Filled dots indicate C4-CP4 electrodes considered in the pooling.

3.4.4. Conclusions

Overall, behavioral results from *Experiment 3* replicated those from the first two experiments, showing an improvement in tactile acuity after cm-PAS. In this third experiment, I demonstrate that the behavioral enhancement brought about by the cm-PAS was visual-specific because it occurs only when participants observed a visual stimulus depicting a tactile event. Interestingly, when participants observed a mere approaching hand, without any tactile content, the opposite effect is found: namely, tactile acuity decreased instead of increasing.

Experiment 3 also provided evidence for a neurophysiological effect of cm-PAS, consisting of an enhancement of the P40 SEP component, which again emerged only after the cm-PAS with visual-touch stimuli. Indeed, the protocol not conveying any tactile content had no effect on any SEP component.

3.5. *Experiment 4*: Involvement of anticipatory mechanisms during cm-PAS

3.5.1. Aim

In the final experiment of my first study, I have manipulated participants' expectations about the upcoming visual-touch stimulus, trying to deepen the functional mechanisms underpinning the efficacy of the ISI of 20 ms.

Besides hypothesize that the cm-PAS exploits a fast, sub-cortical pathway, to induce plastic effects in S1 with the effective ISI of 20 ms found in the previous experiments (see **3.6.1** for the in-depth discussion of cm-PAS timing dependency), it can also be speculated that such efficacy may be mediated by an anticipatory, predictive-like, activation of S1, although these two hypotheses are not mutually exclusive. Namely, due to the repetitiveness of the cm-PAS, in which the paired stimuli occurred every 10 sec for 25 min, during the protocol, learned contingencies between the cm-PAS events (the visual-touch stimulus and the S1 activation by the TMS pulse) could be rapidly developed (Den Ouden, Friston, Daw, McIntosh, & Stephan, 2009; Kok, Jehee, & de Lange, 2012), so that after few trials the participant may anticipate tactile mirroring, consequently allowing the early, at an ISI of 20 ms, interaction between the S1 activation induced by the observed touch and that induced by the TMS pulse.

Hence, a cm-PAS where the frequency between trials is not fixed should avoid such anticipatory, expectation-based, activation of S1 by the visual-touch stimulus. If this is the case, a longer ISI, matching more closely the mirror recruitment of S1 by touch observation

(150 ms) (Bolognini et al., 2014; Pisoni et al., 2018), would become effective for the induction of Hebbian plasticity in S1. Conversely, if the effects of the cm-PAS are not mediated by sensory predictions, even an unpredictable cm-PAS would be effective with the ISI of 20 ms, in the same way of the standard *fixed-frequency* cm-PAS.

To prevent the anticipation of the upcoming visual-touch stimulus, in this last experiment, I have introduced a jittered version of the cm-PAS (*jittered* cm-PAS) by varying the inter-trials frequency, thus, avoiding that participants could make predictions about the time-course of the next trial (hence, about the arrival of the paired stimulations).

Participants underwent the original version of the cm-PAS tested in the previous three experiments, with a fixed frequency of 0.1 Hz (i.e., paired stimuli occur regularly every 10 s) and an ISI of 20 ms between the visual-touch stimulus and the TMS pulse. They also underwent two versions of the *jittered* cm-PAS, both featured by the paired stimuli occurring at varying intervals (5, 10 or 15 s), but differing with respect to the ISI between the onset of the visual-touch stimulus and the TMS pulse over S1: in one version, as in the *fixed-frequency* cm-PAS, the ISI was of 20 ms (*jittered* cm-PAS$_{20ms}$), in the other version, the ISI was of 150 ms (*jittered* cm-PAS$_{150ms}$), hence matching a timing that may reassemble the mirror activation of S1 by the sight of tactile stimuli (e.g., Bolognini et al., 2014).

3.5.2. Methods and materials [2]

3.5.2.1 Participants

Eighteen healthy volunteers took part in this fourth experiment (5 males, mean age ± S.D.: 25.4 ± 2.9 years; mean education ± S.D.: 16.8 ± 1.4 years). Two participants dropped out during the experimental sessions, leaving the final analyzed sample to 16 participants. This

[2] Stimuli, task, dataset and analysis of this experiment are freely accessible at *Open Science Framework*: https://osf.io/5vtkz/

latter experiment was conducted at the NeuroStimulation Laboratory of the University of Milano-Bicocca. The study was approved by the local Ethics Committee of Milano-Bicocca University.

3.5.2.2. cm-PAS protocols

To investigate the contribution of predictive-like mechanisms during the cm-PAS, we employed both the original version of the cm-PAS protocol developed in the previous three experiments (here called, for clarity, *fixed-frequency* cm-PAS) and a modified version in which the time interval between paired stimulations was not fixed (*jittered* cm-PAS), but it could vary in a random fashion among three different values being of 5, 10 and 15 s.

In the *fixed-frequency* cm-PAS, according to the previous experiments, the fixation frame lasted for 9.7 s and the visual-touch one lasted 300 ms, thus the frequency of the stimulation was fixed at 0.1 Hz (i.e., 1 trial – visual stimulus *plus* S1-TMS pulse - every 10 s). After 20 ms from the onset of the visual-touch frame, the TMS pulse was delivered.

Conversely, in the *jittered* cm-PAS, the fixation frame could last 4.7, 9.7 or 14.7 s (50 trials for each duration, given in random order) while the visual-touch frame still lasted 300 ms. Therefore, during this protocol, PAS frequency varied among 0.2 Hz (1 trial every 5 s), 0.1 Hz (1 trial every 10 s) and 0.067 Hz (1 trial every 15 s). To assess the timing-dependency of this modified version of the cm-PAS, two different *jittered* cm-PAS were tested: in one session, the TMS pulse over right S1 was delivered after 20 ms (*jittered* cm-PAS$_{20ms}$), as in the *fixed-frequency* cm-PAS, while in another session it was delivered after 150 ms (*jittered* cm-PAS$_{150ms}$) (**Figure 3.7**).

Both the *fixed-frequency* cm-PAS and the *jittered* cm-PAS consisted of 150 trials and lasted 25 min. As in the previous experiments, during every protocol, participants were engaged in a task to catch rare visual events (i.e., 15 trials of the cm-PAS depicted a double touch).

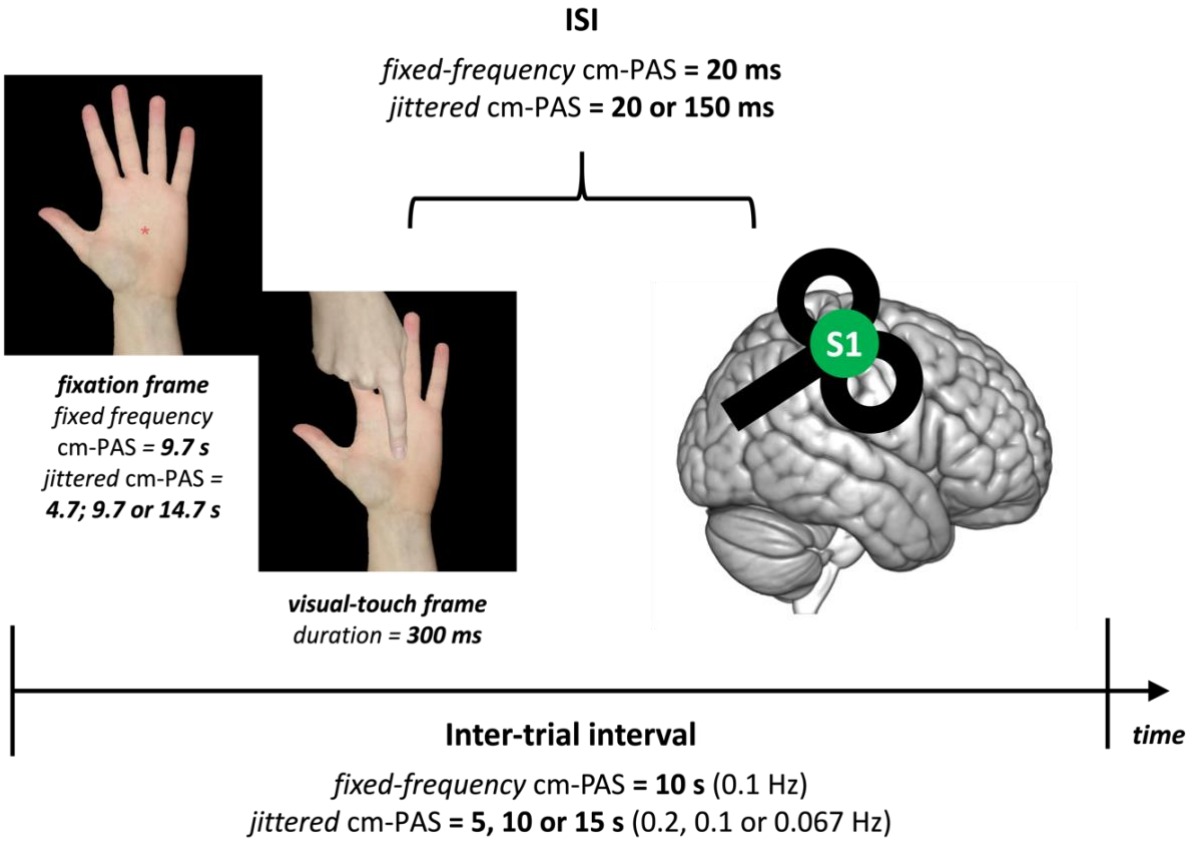

Figure 3.7. cm-PAS protocols (and related parameters) tested in *Experiment 4*.

Performance in such task was not analyzed. During the protocol, participants heard white noise in a pair of headphones to mask the sound made by the TMS. Trial randomization and timing of the TMS pulses were controlled using the software E-prime 2.0 (Psychology Software Tool, Inc.).

3.5.2.3. TMS

TMS was delivered using a figure-of-eight coil (diameter = 70 mm) connected to a Magstim Super Rapid^2 biphasic stimulator (Magstim Company, Whitland, UK). rMT of each participant was assessed at the beginning of every session. rMT was defined as the minimum TMS intensity that elicited a visually detectable muscle twitch in the left thumb in 5 out of 10 TMS pulses (Bolognini, Rossetti, et al., 2013; Rossi et al., 2009). The mean rMT was 52.2 ± 4.7 % (mean ± S.D.) of the maximal output stimulator. TMS positioning was assisted

with the SofTaxic 2.0 neuronavigation system (E.M.S., Bologna, Italy, www.softaxic.com) and S1 was found with the same methodology reported for the previous experiments.

3.5.2.4. Experimental procedure and statistical analysis

This last experiment comprised three sessions that differed only for the cm-PAS used: *fixed-frequency* cm-PAS; *jittered* cm-PAS$_{20ms}$ and *jittered* cm-PAS$_{150ms}$. The order of the three sessions was counterbalanced among participants. The experimental procedure was the same in every session and was similar to the one of the previous three experiments: each session started with the administration of the 2-PDT and the assessment of the individual rMT. The 2-PDT used in this experiment was the same version exploited in the previous three experiments. Then, the cm-PAS was administered and, immediately after the stimulation, a second assessment of participants' tactile acuity was performed. On average, a session lasted 1 h and 30 min. The three sessions were held at the same moment of the day (in the morning or in the afternoon) and at least 72 h passed between them, thus to prevent an overlapping of stimulation effects (Sale et al., 2007).

As in the previous experiments, performance at the 2-PDT (i.e., *d'*, *c* and sensory threshold) was analysed separately via a 3 X 2 rmANOVA, with the within-subjects factors Session (*fixed-frequency* cm-PAS, *jittered* cm-PAS$_{20ms}$, *jittered* cm-PAS$_{150ms}$) and Time (pre, post). Whenever data were not normally distributed, nonparametric tests were used (i.e. Friedman's ANOVA)

3.5.3. Results

The rmANOVA on the sensory threshold showed a significant main effect of Time ($F_{1,15}$ = 14.63, p = .002, η_p^2 = .49). Moreover, we found a significant Session X Time interaction ($F_{2,30}$ = 7.07, p = .003, η_p^2 = .32). Post-hoc analyses highlighted a decrease in the sensory

threshold after the *fixed* cm-PAS (Pre *fixed-frequency* cm-PAS= 9.59 ± 1.14 mm, Post *fixed-frequency* cm-PAS= 8.33 ± 1.40 mm, t = 4.13, p = .002). Interestingly, a change in the same direction was found after the *jittered* cm-PAS_{150ms} (Pre *jittered* cm-PAS_{150ms} = 9.55 ± 1.36 mm, Post *jittered* cm-PAS_{150ms} = 8.48 ± 1.12 mm, t = 3.52, p = .012). Post-hoc also revealed a difference in the sensory threshold after the *fixed-frequency* cm-PAS and the threshold after the *j*cm-PAS_{20ms} (Post *fixed-frequency* cm-PAS = 8.33 ± 1.40 mm *vs.* Post *jittered* cm-PAS_{20ms} = 9.50 ± 1.46 mm, t= -3.40, p = .005) and between the *jittered* cm-PAS_{20ms} and the *jittered* cm-PAS_{150ms} (Post *jittered* cm-PAS_{150ms} = 8.48 ± 1.12 mm *vs.* Post *jittered* cm-PAS_{20ms} = 9.50 ± 1.46 mm, t= 2.98, p = .017; **Figure 3.8**). Noteworthy, no difference in sensory threshold were observed before each stimulation session (p > .05). No other significant effects were found (Session: $F_{2,30}$ = 1.63, p = .213, η_p^2 = .09).

Regarding the response criterion, I performed a Friedman ANOVA because data in one condition (Post *fixed-frequency* cm-PAS) were not normally distributed. In line with previous experiments, no significant effects were found (χ^2_5 = 10.9, p = .053).

Results on global performance showed a significant main effect of Time ($F_{1,15}$ = 13.55, p = .002, η_p^2 = .48) and of the interaction Session by Time ($F_{2,30}$ = 7.11, p = .003, η_p^2 = .32). Post-hoc showed an improvement in participants' global performance after the *fixed-frequency* cm-PAS (Pre *fixed-frequency* cm-PAS: *d'* = 1.68 ± .28; Post *fixed-frequency* cm-PAS: *d'* = 2.00 ± .32; t = -4.41, p = .001). A significant improvement was found also after the *jittered* cm-PAS_{150ms} (*jittered* cm-PAS_{150ms}: *d'* = 1.68 ± .36; Post *jittered* cm-PAS_{150ms}: *d'* = 1.89 ± .34; t = -2.98, p = .045). No difference was found after the *jittered* cm-PAS_{20ms} (Pre *jittered* cm-PAS_{20ms}: *d'* = 1.74 ± .35; Post *jittered* cm-PAS_{20ms}: *d'* = 1.69 ± .38; t = .59, p = .99). No other significant effects were found (Session: $F_{2,30}$ = 1.40, p = .262, η_p^2 = .09).

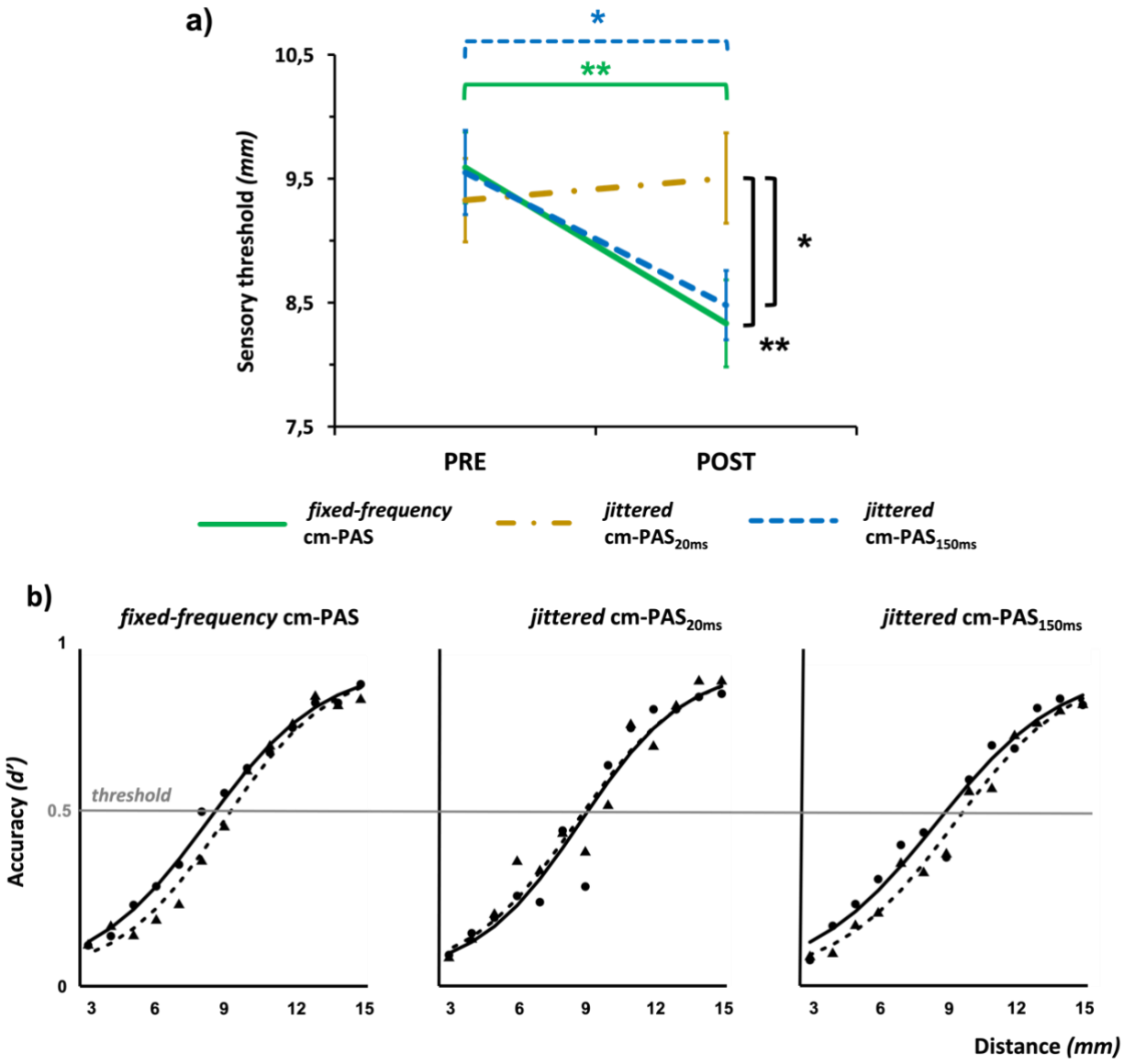

Figure 3.8. Results of *Experiment 4*. a) Effects of the different tested versions of the cm-PAS on tactile acuity at the 2-PDT: significantly lower threshold after the *fixed-frequency* cm-PAS (green straight line) and the *jittered* cm-PAS$_{150ms}$ (blue dotted line) compared to *jittered* cm-PAS$_{20ms}$ (amber straight-dot line). * = $p < .05$, ** = $p < .01$; error bars = S.E. **b)** Psychometric functions obtained from logistic fitting to raw data, before (dotted line, triangles) and after (black line, dots) the three tested protocols.

3.5.4. Conclusions

Taken together, the results of *Experiment 4* show, in line with the previous experiments, an enhancement of tactile acuity after the standard *fixed-frequency* cm-PAS with an ISI of 20 ms. Importantly, a similar behavioral effect is found also with the *jittered* cm-PAS exploiting the longer ISI of 150 ms. Conversely, jittering the frequency of the cm-PAS but exploiting the same ISI of the fixed-frequency protocol (20 ms) make the protocol ineffective, at least at a behavioral level.

3.6. General discussion

In the present chapter, I developed a novel *cross-systems* PAS protocol, the cm-PAS, targeting the somatosensory system and exploiting a cross-modal stimulation: namely, the observation of a hand being touched combined with a TMS-driven activation of S1.

Overall, the results found show that tactile acuity improves after the cm-PAS protocol only when *(a)* the ISI between the visual-touch stimulus and the TMS pulse is of 20 ms (*Experiment 1*), *(b)* TMS is delivered over S1 (*Experiment 2*), and *(c)* the visual stimulus depicts a hand being touched (*Experiment 3*). Interestingly, when the protocol is made less predictable (i.e., by jittering the frequency between one paired stimulation and the other), timing dependency needed between the two paired stimuli is longer (150 ms), suggesting that predictive-like activation of S1 may contribute to the success of the protocol (*Experiment 4*). Furthermore, the cm-PAS also affects SEPs, increasing the amplitude of the P40 component (*Experiment 3*). Hence, such novel *cross-systems* PAS protocol is effective in improving tactile acuity and in modulating SEPs. According to my starting hypothesis, these effects can be interpreted in terms of Hebbian associative LTP-like plasticity induced within S1 exploiting a visuo-tactile pathway of the *tactile mirror system*.

Although it is well known that Hebbian associative plasticity can be induced in S1 through classical *within-system* S1-PAS protocols, as well as by tactile coactivation paradigms (i.e., the repeated application of weak tactile stimuli to a body part leading to a significant modulation of tactile acuity in the stimulated skin area), much less is known about the possibility to induce similar plastic effects in a cross-modal way, for instance by pairing a tactile stimulus with a visual stimulus (Godde, Spengler, & Dinse, 1996; Godde,

Stauffenberg, Spengler, & Dinse, 2000; Hodzic, Veit, Karim, Erb, & Godde, 2004; Pleger et al., 2001; Sellien & Ebner, 2007). A first attempt in this direction was made three years ago by Kuehn and coworkers (2017), who presented a visual-tactile stimulation consisting of a classic tactile coactivation paradigm paired with the repeated presentation of visual stimuli showing a right index finger being touched. However, the authors did not find any significant modulation of the tactile acuity compared to the unimodal, tactile or visual, version of the paradigm (Kuehn et al., 2017). Conversely, the cm-PAS, by pairing a visual stimulus with the direct cortical stimulation of S1, was effective in modulating tactile acuity.

I speculate that the effectiveness of the cm-PAS compared to the study by Kuehn and colleagues (2017) may be due to the characteristics of the paradigm itself, combining a cortical somatosensory stimulation with touch observation in a time-specific way and exploiting TMS focality to activate the target area/system.

In the following sections, I will discuss more in detail cm-PAS properties and neurofunctional underpinned mechanisms that emerged from the results of the four experiments previously presented.

3.6.1. The intriguing case of cm-PAS timing dependency

Firstly, the ISI required for the interaction between the S1-TMS pulse and the visual-touch stimulus (i.e., 20 ms) is unexpected and, thus, thought-provoking. In fact, as stated in **3.2.1**, this value was initially selected as a control one, being too short to speculate that the visual stimulus can be already processed in S1 when the TMS occurred.

As said in **Chapter 1**, it is well known that the temporal relationship between two events is fundamental to give rise to Hebbian association effects: to induce synaptic plasticity, two neural events have to take place within a critical time range of a few tens of milliseconds (Caporale & Dan, 2008; Markram et al., 2011). In line with *within-system* S1-PAS protocols

(Wolters et al., 2005), the modulation of tactile acuity by the cm-PAS is time-dependent, emerging, at least in the fixed frequency version of the protocol, only with an ISI of 20 ms between the visual-touch stimulus and the TMS pulse, while being absent with longer ISIs of 60 and 100 ms. As said in the aims of *Experiment 1*, the ISI of 20 ms matches the arrival time of the afferent input S1 (Allison et al., 1989; Macerollo et al., 2018), and it is the same ISI effective in classical S1-PAS protocols for LTP induction (e.g., Pellicciari et al., 2009; Wolters et al., 2005).

The fact that the same ISI is also effective in the cm-PAS, where the MN stimulation is substituted by a visual, complex, stimulus, is then thought-provoking. Indeed, an interval of 20 ms seems quite short for cross-modal, visual, recruitment of S1. At date, there are not yet a consensus about the exact timing S1 is activated following touch observation. For instance, as described in **Chapter 2**, paired-pulse TMS and event-related potential studies have shown that S1 activation by touch observation occurs between 50 and 300 ms (Bolognini et al., 2014; Pihko et al., 2010; Pisoni et al., 2018).

A first hypothesis, corroborated by the results of my fourth experiment, is that such a short time course reflects an 'anticipatory' tactile effect. Considering that during the (fixed frequency) cm-PAS participants observed for 25 min a hand being touched repetitively every 10 sec (0.1 Hz), it is possible that after a few trials, they may start to anticipate the touch stimulus before its actual occurrence (i.e., extracting the regularities between trials) (Carlsson, Petrovic, Skare, Petersson, & Ingvar, 2000; Kimura & Katayama, 2015, 2018). This, in turn, could have anticipated the mirror-like activation of S1, which then occurred rhythmically in the brain every time the new trial started. Such anticipation of the visual-touch stimulus would allow a more rapid interaction with the cortical TMS pulse occurring as soon as at 20 ms, a time that reflects the typical latency of S1 activation by direct somatosensory afference (Cohen et al., 1991). Accordingly, the timing of 20 ms does not

reflect, from a temporal perspective, the real interaction between the mirror activation of S1 by touch observation and its cortical stimulation by TMS. Rather, it would reflect the interaction between an anticipated (before its actual visual occurrence) tactile mirroring and the TMS pulse in S1.

From this perspective, mechanisms of prediction may also be involved. In particular, within the MNS, theoretical (Friston, Mattout, & Kilner, 2011; Kilner, 2011; Kilner, Friston, & Frith, 2007; Wolpert, Doya, & Kawato, 2003) and empirical works (Aglioti et al., 2008; Avenanti, Annella, Candidi, Urgesi, & Aglioti, 2013; Kilner et al., 2004; Maranesi et al., 2014; Schippers & Keysers, 2011; Southgate, Johnson, Osborne, & Csibra, 2009) have proposed that the *action observation network* generates predictions of the observed action. For instance, Maranesi and colleagues (2014) showed that when the context is highly predictable, also in term of when an event will occur, mirror neurons of monkeys' ventral premotor area are activated a long time in advance before the beginning of the observed action (Maranesi et al., 2014). Such a generative model starts with a prior expectation (prediction) about the goal of an observed action; given this prior, a prediction of the sensory consequences of the action is generated. Contextual information in which the action is embedded serves to build up a prior and offers guidance to the perceiver's expectations (Kilner, Friston, et al., 2007; Maranesi et al., 2014). Thus, it is possible that similar anticipatory activations occur also in a visuo-tactile network with mirror properties like the *tactile mirror system* (Blakemore et al., 2005), as suggested by the cm-PAS. Based on such predictive-like mechanisms, our protocol may act by generating a reafference 'tactile' prediction signal from the observed action, anticipating the time-course of interaction between the observed touch and the TMS-induced somatosensory activity.

This hypothesis seems to be supported by the results of *Experiment 4*: the jittered version of the cm-PAS, by preventing the anticipation of the visual-touch stimulus, and in turn the pre-

activation of S1, allows the interaction of the TMS pulse and the visual-touch stimuli in S1 at a longer latency (150 ms). Such latency is more in line with the mirror activity of S1, at least as documented by single-pulse TMS investigations (Bolognini et al., 2014). Due to the less predictability of the upcoming paired stimulations given by the jittered frequency of the PAS protocol, the cortical network mediating the induction of Hebbian associative plasticity cannot be activated in an anticipatory manner, and consequently, the ISI of 20 ms between the visual-touch stimulus and the TMS pulse becomes too short to allow their neural interaction. However, it has to be noted that, in a predictive account, prediction accuracy has to be in the order of milliseconds to induce Hebbian plasticity (Friston, 2005; Friston et al., 2011) and from my results there is no direct evidence (i.e., neurophysiological or neurofunctional measures, but also behavioral ones as reaction times) that this happens during the *fixed-frequency* cm-PAS. Future studies should deepen this issue.

Another account to explain the short ISI of the cm-PAS is suggested by works on cross-modal interactions in primary sensory areas (Bieler, Sieben, Schildt, Brigitte, & Hanganu-opatz, 2017; Convento, Vallar, Galantini, & Bolognini, 2013; Ghazanfar & Schroeder, 2006; Henschke, Noesselt, Scheich, & Budinger, 2015; Iurilli et al., 2012; Murray et al., 2016). In details, early visuo-tactile interactions are supported by either direct (feed-forward) connections between S1 and V1, as well as by subcortical feed-forward projections from the thalamus (Céline Cappe & Barone, 2005; Driver & Noesselt, 2008; Foxe & Simpson, 2002; Sieben, Roder, & Hanganu-Opatz, 2013). In particular, thalamic nuclei offer a fast pathway for information transfer between different cortical sensory areas, rapidly relaying this integrated information to the cortex by their multiple thalamo-cortical connections (Cappe et al., 2009; Driver and Noesselt, 2008; Tyll et al., 2011). Through thalamo-cortical routes, the visual information can reach S1, even bypassing V1 (Bieler, Sieben, Cichon, et al., 2017;

Sieben et al., 2013). A similar route may be invoked to explain the early latency of visuo-tactile/TMS interactions driven by the cm-PAS.

It has to be noted that this second hypothesis is not mutually exclusive to the previous one: namely, it is possible that the anticipatory, predictive-like, activation of S1 during the cm-PAS have exert an influence on these feed-forward thalamus-cortical connections. However, it is important to consider that short-latency cross-modal interactions typically affect lower processing stages, insensible to the nature of the stimuli (Cappe et al., 2009; Driver and Noesselt, 2008). The fact that the efficacy of the cm-PAS depends on the type of the paired visual stimulus, being present only when the visual stimulus conveys tactile information (*Experiment 3*), suggests further involvement of higher-order association cortical areas, such as posterior parietal and premotor areas containing visual-tactile neurons (e.g., Duhamel, Colby, & Goldberg, 1998; Fogassi et al., 1999) and showing vicarious activation by the sight of touch (for a review: Keysers et al., 2010). These high-level association cortices would be responsible for top-down influences on S1 activation involving feedback pathways, which made the cm-PAS effective only with visual stimuli conveying a tactile content (see next paragraph and *Experiment 3*), selectively activating mirror-touch cortical networks (Bolognini et al., 2014).

3.6.2. Cortical and visual specificity of cm-PAS

Results of *Experiment 2* showed that the efficacy of the cm-PAS is area-specific, not occurring when TMS is applied to V1; this selectivity rules out possible interpretations of cm-PAS effects as due to unspecific modulation of the participant's arousal level (Foerster, Schmitz, Nouri, & Claus, 1997). However, it has to be noted that the inefficacy of the cm-PAS targeting the occipital cortex on tactile performance does not exclude that the same protocol could be able to modulate unimodal visual processing (e.g., visual acuity or VEP)

or its potential efficacy on tactile processing if different ISIs are used, fit the time course of the functional interplay between V1 and S1 as discussed before (rapid V1-S1 feedforward connections and/or feedback influences from multisensory regions to primary cortices; Driver and Noesselt, 2008).

As showed by *Experiment 3*, the feature of the visual stimulation is also fundamental for the cm-PAS effectiveness. The visual stimulus must depict a tactile event to improve tactile perception, while the mere view of an approaching hand does not have any facilitatory effect: this result further supports the specific involvement of the *tactile mirror system* – and thus of the visuo-tactile mirroring properties of S1 (e.g., Blakemore et al., 2005; Bolognini et al., 2014, 2011; Ebisch et al., 2008; Rossetti et al., 2012). Interestingly, the observation of a moving hand, without any tactile component, combined with S1-TMS at the same ISI of 20 ms tends to reverse the cm-PAS effects, impairing tactile sensitivity (see **Figure 6a**), suggesting an influence of such a protocol version also on the *action observation network*. I hypothesize that this effect is reminiscent of sensory attenuation, a phenomenon associated with mechanisms of sensory feedback prediction by which the intensity of somatosensation caused by self-generated movement is reduced (e.g., Blakemore, Wolpert, & Frith, 1998; Waszak, Cardoso-leite, & Hughes, 2012). Recently, it was shown that tactile attenuation may also occur during action observation (Rossi et al., 2002; Vastano et al., 2016): in this account, decreased tactile sensitivity induced by the cm-PAS with action observation stimuli could be due to the reinforcement of motor resonance mechanism, which negatively impacts somatosensory processing (Avenanti et al., 2007; Urgesi et al., 2010). However, it has to be noted that this hypothesis remains a speculation because in *Experiment 3* we did not control for the time- and area-specificity of this sort of inhibitory effect, at variance with the facilitatory side of cm-PAS, which was deeply explored throughout all the study.

3.6.3. Neurophysiological correlates

Last but not least, at the neurophysiological level (*Experiment 3*), the improvement in tactile acuity after the cm-PAS was accompanied by an increase in SEP amplitude, consistent with classical S1-PAS studies describing LTP-like changes (Wolters et al., 2005). Nonetheless, my results diverged from previous findings in terms of latency: while S1-PAS studies reported a modulation of SEPs earliest components between 20 and 30 ms after MN stimulation (Litvak et al., 2007; Pellicciari et al., 2009; Wolters et al., 2005), I observed a modulation of a later SEP component, namely, the P40. Such a difference in latency is likely due to the distinct neural pathways involved in the cm-PAS compared to classical S1-PAS (see **3.6.1**; Lacey & Sathian, 2016).

Although most of the studies on SEPs in humans focused on earlier components evoked by MN electrical stimulation (i.e., P14-N20-P25-N30, also detected in our experiment, but not affected by cm-PAS, see **Figure 6c**; e.g., Buchner et al., 1995; Macerollo et al., 2018; Mauguière et al., 1999), there is evidence suggesting that P40 originates at the cortical level, specifically in S1 (Allison et al., 1989; Allison, Wood, McCarthy, & Spencer, 1991; Gorgoni et al., 2014; Matsunaga, Nitsche, Tsuji, & Rothwell, 2004) and that it could be associated with a first cognitive processing of the tactile stimulus (Desmedt et al., 1983). Interestingly, the cortical origin of P40 seems to be localized in Broadman's areas (BA) 1 and 2, while earlier components, such as N20 and P25, in BA 3b (Allison et al., 1989, 1991; Gorgoni et al., 2014). Within the human S1, BA 3b is considered the primary stage for tactile processing, while BA 1 and, especially, BA 2 are involved in a secondary stage related to the integration of uni- and cross-modal stimuli (along with other brain areas such as the secondary somatosensory cortex and the insula; Cardini et al., 2010; Keysers et al., 2010; Kuehn et al., 2018, 2014, 2013; Meehan et al., 2009; Meftah et al., 2009). Importantly, fMRI studies showed that both BAs 1 and 2 are also activated by touch observation (Blakemore et

al., 2005; Schaefer, Xu, Flor, & Cohen, 2009), while the mirror properties of BA 3b are more controversial (Keysers et al., 2010; Kuehn et al., 2018). In my experiment, the increased P40 amplitude is present only after the cm-PAS involving touch observation and is absent during action observation; therefore, this electrophysiological effect might further support my proposal of a reinforcement of the specific mirror activity of S1 induced by the cm-PAS.

In conclusion, these findings show the efficacy of the cm-PAS in modulating tactile sensitivity and an early component of SEPs (i.e., P40), likely through the induction of associative, LTP-like, plasticity mechanisms in S1, mediated by anticipatory mechanisms driven by the repetitiveness of the protocol itself. This evidence suggests that it is possible to modulate visuo-tactile mirror properties following Hebbian learning.

Chapter IV

Mirror PAS [3]

4.1. Aims of the study

In the three experiments presented in this chapter, I have introduced a second *cross-systems* PAS, the mirror PAS (m-PAS). As the name suggests, this novel protocol target the MNS and, in detail, the *action observation network* (Rizzolatti & Craighero, 2004; see **Chapter 2)**. Differently from the cm-PAS described in the previous chapter, which induces Hebbian plasticity within the somatosensory system, the m-PAS aims at inducing a novel, atypical, visuo-motor association within the motor system by repeatedly pairing *(a)* TMS pulses over right M1 with *(b)* the observation of lateralized (i.e., made with a single hand and involving a single muscle) right-hand movements (the abduction of the right-hand index finger), hence ipsilateral to the stimulated hemisphere.

As said in **2.1.1**, under normal conditions, action observation induces an increase of MEP amplitude (i.e., the motor resonance phenomenon) that is specific for the muscle involved in the actual execution of the observed action (Fadiga, Fogassi, Pavesi, & Rizzolatti, 1995). Crucially, this effect is also hemispheric-specific: the observation of unilateral hand movements recruits the contralateral motor system, as in the case of their execution (Aziz-

[3] This chapter contains experiments already published in:

- Guidali G., Carneiro S. I. M. & Bolognini N. (2020) Paired Associative Stimulation drives the emergence of motor resonance. *Brain Stimulation*, 13(*3*), 627-636 [*Experiments 1-2*]

Zadeh et al., 2002; Garry, Loftus, & Summers, 2005). In a Hebbian learning perspective (see **2.2**), 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 (e.g., Catmur et al., 2016; Hanuschkin, Ganguli, & Hahnloser, 2013; Keysers & Gazzola, 2014). Thus, if the m-PAS is effective in inducing timing-dependent plastic phenomena in the MNS following Hebbian learning, motor resonance should emerge also for the ipsilateral movement conditioned during the protocol.

Similar to the cm-PAS experiments, the efficacy of the m-PAS was investigated modulating *(a)* the timing between the two paired stimuli (*Experiment 1*), *(b)* the content of the visual stimulus (*Experiment 2*), and *(c)* the site of cortical stimulation (*Experiment 3*). In all experiments, as a marker of motor resonance (Fadiga et al., 1995; Naish et al., 2014), MEPs amplitude was recorded during the observation of contralateral and ipsilateral (concerning the stimulated hemisphere) hands which could be static or perform an index finger movement (i.e., the same kind of movement presented during the m-PAS). To control the possible muscle specificity of the novel visuo-motor association induced by the m-PAS, MEPs were recorded from both a target muscle (FDI, i.e., the same muscle involved in the observed movement) and a control one (ADM). In *Experiment 3*, also the possible behavioral correlates of m-PAS-induced plasticity were deepened through the administration of a classical imitative compatibility task measuring *automatic imitation*, a behavioral marker of the *action observation network* activation (e.g., Boyer, Longo, & Bertenthal, 2012; Catmur & Heyes, 2011; Hétu, Taschereau-Dumouchel, Meziane, Jackson, & Mercier, 2016; Jiménez et al., 2012; see **2.1.1**)

4.2. *Experiment 1*: Efficacy and timing dependency of m-PAS

4.2.1. Aim

In the first experiment, the effectiveness of the m-PAS in reshaping motor resonance was assessed, also considering the timing dependency of the protocol by changing the timing of TMS pulse delivery with respect to the onset of the visual stimulus of movement. As already stated in *Experiment 1* of the cm-PAS (see **3.2.1**), timing dependency is the key signature of Hebbian learning (Caporale & Dan, 2008) and thus I investigated it in the first place also for the m-PAS. Two ISIs were selected: the first one was of 25 ms, hence reproducing the conduction time of the corticospinal tract, and then the temporal visuo-motor contingence that features motor control (Scott, 2016). The second one was of 250 ms, in line with the chronometry of MNS activation, which is known to recruit M1 after such timing from the onset of a visual movement (Cavallo, Heyes, Becchio, Bird, & Catmur, 2014, see **2.1.1**). Considering that the m-PAS aims at 'create' a novel visuo-motor association, I hypothesize that the shorter timing (25 ms) might be the more effective in inducing a novel motor resonance phenomenon because it closely reflects the timing of action execution and thus the contingency between make the action *and* observe the same action needed, in a Hebbian learning account, to create a novel visuo-motor association (Keysers & Gazzola, 2014). Conversely, the timing that reflects M1 recruitment by MNS (250 ms) implied that the stimulated M1 should be already activated by the presented visual stimulus to achieve 'contingency' between the two paired stimulations of the protocol. However, at this time point, during the observation of movements made with the right hand, like the ones presented during the protocol, only the contralateral, left M1 (which is not stimulated during the m-

PAS) are recruited by the MNS and thus, I hypothesize that such timing should not be effective, at least when the PAS aims to create a novel visuo-motor association.

4.2.2. Methods and materials [4]

4.2.2.1. Participants

Twenty healthy volunteers took part in the first experiment; two of them were excluded due to EMG artifacts leaving the final analyzed sample to 18 participants (6 males, mean age ± S.D. = 22.8 ± 1.8 years; mean education = 14.6 ± 1.7 years). They were all right-handed according to the Edinburgh Handedness Inventory (Oldfield, 1971); none of them had contraindications to TMS (Rossi et al., 2009). The sample size of this experiment was determined by means of an a-priori within-subjects rmANOVA (effect size F = .4; Alpha Error Level: p = .05; Statistical Power = .95, Actual Power = .95), using the software G*Power 3.1 (Faul, Erdfelder, Buchner, & Lang, 2009). The study was approved by the Ethical Committee of the University of Milano-Bicocca and it was performed following the ethical standards of the Declaration of Helsinki. All participants gave their written informed consent to the experiment.

4.2.2.2. m-PAS

The m-PAS protocol was a modified version of the classical M1-PAS (Stefan et al., 2000; Wolters et al., 2003) in which we substituted the electric stimulation of the median nerve with a video clip depicting a hand movement. During the protocol, participants sat comfortably on a chair in front of a PC monitor placed at a distance of 57 cm with their hands positioned out of view.

[4] Stimuli, task, dataset and analysis of this experiment are freely accessible at *Open Science Framework*: https://osf.io/3ujkv/

Each trial of the m-PAS began with the presentation of a frame depicting the dorsal view of a static right hand ('static frame', duration: 4250 ms). Immediately after its end (delay of 0 ms), 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 rMT. 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$_{250ms}$) (**Figure 4.1**). The 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 15 min (Conde et al., 2013).

As in the cm-PAS, to ensure that participants were looking with attention to the visual stimuli (Stefan et al., 2004), 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

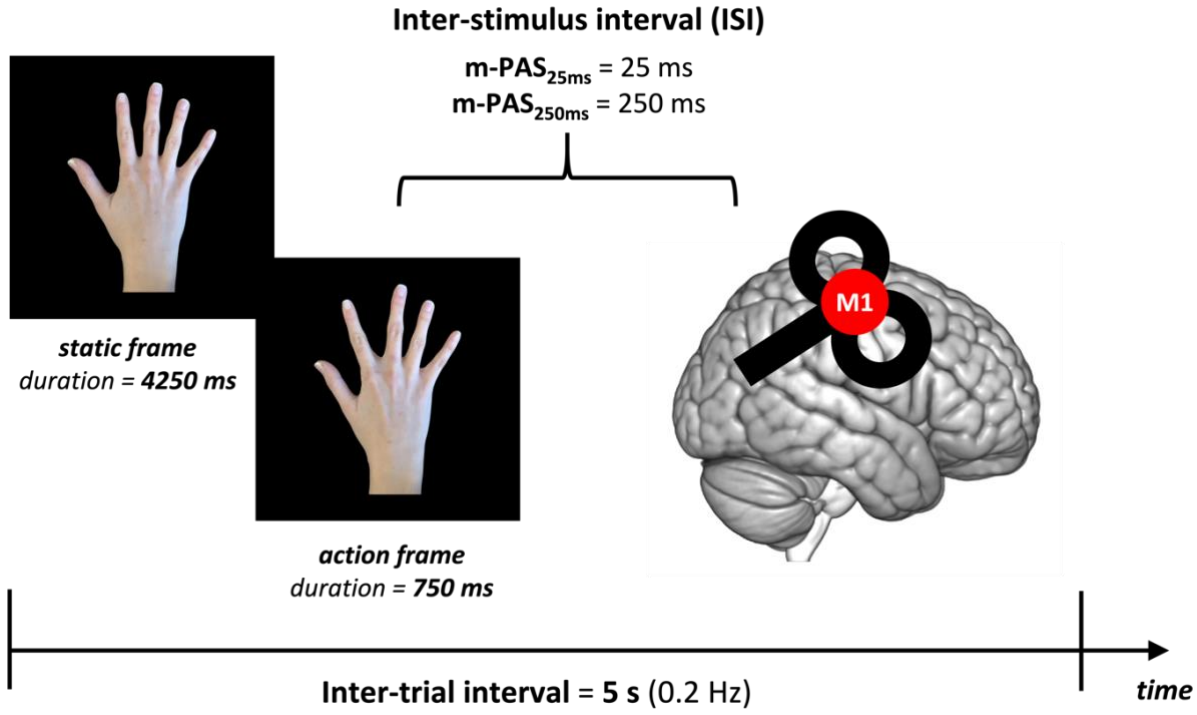

Figure 4.1. m-PAS protocols (and related parameters) tested in *Experiment 1*.

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 96.8% (S.D. = ± 2.33%). Trials randomization and timing of the stimuli were presented under computer control using the software E-Prime (3.0, Psychology Software Tool, Inc.).

4.2.2.3. 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 video clips showing static or moving hand stimuli (i.e., action-observation task) (for a similar procedure, see: Avenanti et al., 2007; Aziz-Zadeh et al., 2002; Catmur et al., 2011; Cavallo et al., 2014). Participants were seated in a chair in front of a PC-screen distant approximately 57 cm from their faces. 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 video clip was presented (duration = 2 s). In 'movement trials', the video clip 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 video clip, a TMS pulse was delivered over the right M1, with an intensity of 120% of the participant's rMT (Avenanti et al., 2007; Cavallo et al., 2014; Naish et al., 2014). The inter-trial interval was jittered between 8 and 10 s (R Chen et al., 1997; Rossi et al., 2009).

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

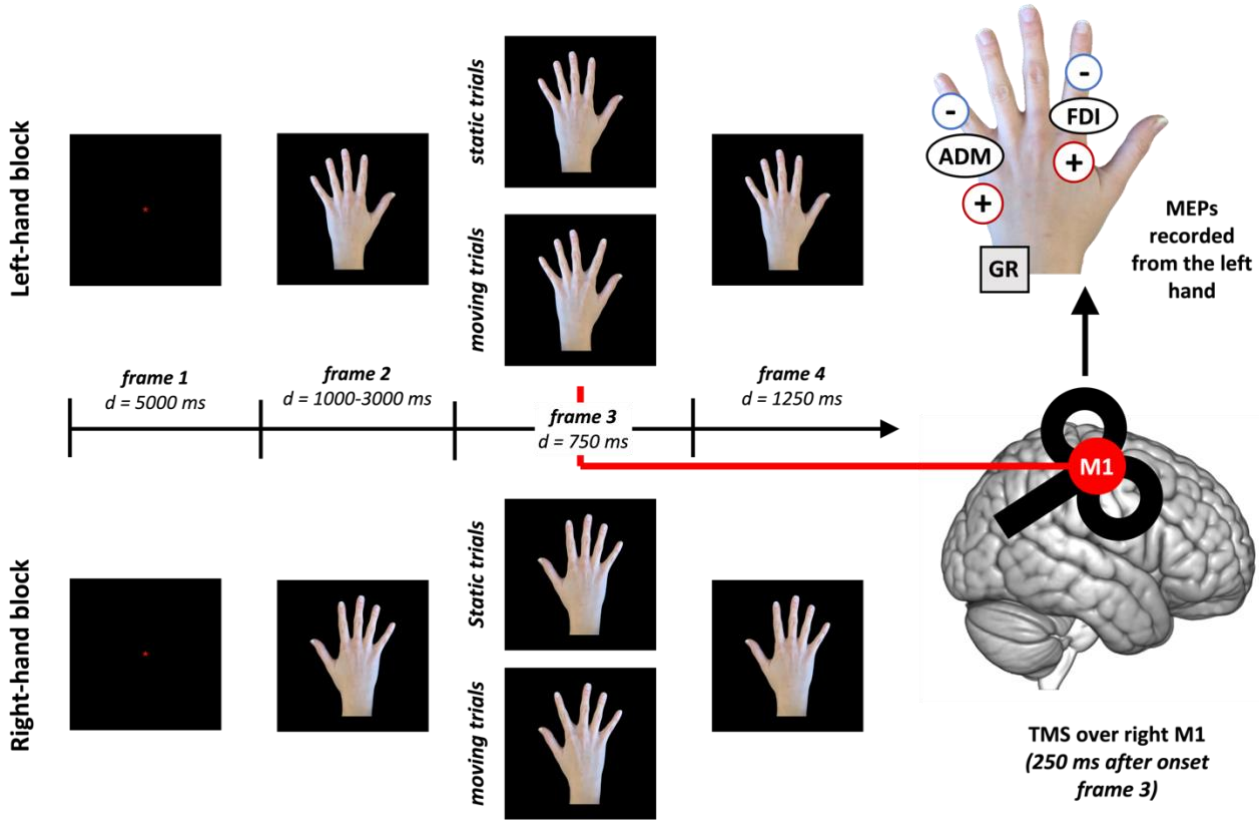

Figure 4.2. Action-observation task adopted to detect motor resonance. TMS was always delivered over right M1 and MEPs were recorded from FDI and ADM muscles of the left hand.

in a randomized order: half (20) of the trials showed the static hand and the other half (20) the moving index finger (**Figure 4.2**).

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 attentive task was 98.5% (S.D. = ± 1.45%).

Trials randomization and timing of the stimuli were presented under computer control using the software E-Prime 3.0 (Psychology Software Tool, Inc.).

4.2.2.4. TMS and EMG recording

TMS pulses were delivered during the m-PAS, and during MEP recording, by using a figure-of-eight coil (diameter = 70 mm) and 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 an MEP of at least 50 μV in the left hand's FDI 5 times out of 10 during the stimulation of right M1 (Rossi et al., 2009). On average, during the m-PAS$_{25ms}$ session, participants presented an rMT of 60.1% (S.D. = ± 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 ≈1.5 mV in the contralateral FDI muscle. During the experiment, 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 (Avenanti et al., 2007; Fadiga et al., 1995; Orth & Rothwell, 2004). 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).

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 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 (Avenanti et al., 2007). The ground electrode was placed over the

left wrist. Before 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).

4.2.2.5. 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 counterbalanced 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. Followed this 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 the afternoon) and at least 48 h passed between them, thus to prevent an overlapping of stimulation effects (Sale et al., 2007).

4.2.2.6. Statistical analysis

MEPs were analyzed off-line using the Signal software (version 3.13, Cambridge Electronic Devices, 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 the 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 S.D. from the mean of each condition (i.e., static hand trials, movement trials) were considered outliers and thus, excluded from the analysis. On average, the 4.76% (S.D. = ± 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., Alaerts, Heremans, Swinnen, & Wenderoth, 2009; Novaes et al., 2018): for both the left and the right-hand trials, and 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 an 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 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 (η_p^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 1.6, www.jamovi.org).

4.2.3 Results

4.2.3.1. Motor resonance before 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, η_p^2 = .401) and a significant Muscle X viewed Hand interaction ($F_{1,17}$ = 22.15, p < .001, η_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 (179.0 ± 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 = -.19, 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.

4.2.3.2. m-PAS effects

Results from the rmANOVA showed a significant Condition X viewed Hand X Muscle interaction ($F_{2,34}$ = 4.31, p = .021, η_p^2 = .202), as well as main effects of Condition ($F_{2,34}$ = 6.67, p = .004, η_p^2 = .282) and Muscle ($F_{1,17}$ = 4.59, p = .047, η_p^2 = .213). No other significant effect was found (all *Fs* < 3.09, all *ps* > .06, see **Table 4.1** for all main effects and interactions).

The Muscle X Condition X viewed Hand interaction was further explored with two separate rmANOVA, one for each muscle. For the FDI muscle, this analysis showed significant main effects of the factors Condition ($F_{2,34}$ = 6.25, p = .005, η_p^2 = .269) and viewed Hand ($F_{1,17}$ =

6.23, p = .023, η_p^2 = .268), and of the Condition X viewed Hand interaction ($F_{2,34}$ = 6.35, p = .005, η_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 = -3.6, 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$_{left\text{-}hand}$ = 179.0 ± 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 = -3.82, p = .006) (**Figure 4.3a,** see **Figure 4.4** for single participants' distribution).

For the ADM muscle, the rmANOVA showed no significant effects of factors Condition ($F_{2,34}$ = 2.82, p = .074, η_p^2 = .142), viewed Hand ($F_{1,17}$ < .01, p = .994, η_p^2 < .001) and their interaction viewed Hand X Condition ($F_{2,34}$ < .01, p = .991, η_p^2 = .001) (**Figure 4.3b**).

Factor/Interaction	*F*	*p*	η_p^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**

Table 4.1. m-PAS effects in *Experiment 1*: results from the rmANOVA conducted on the ΔMEPs.

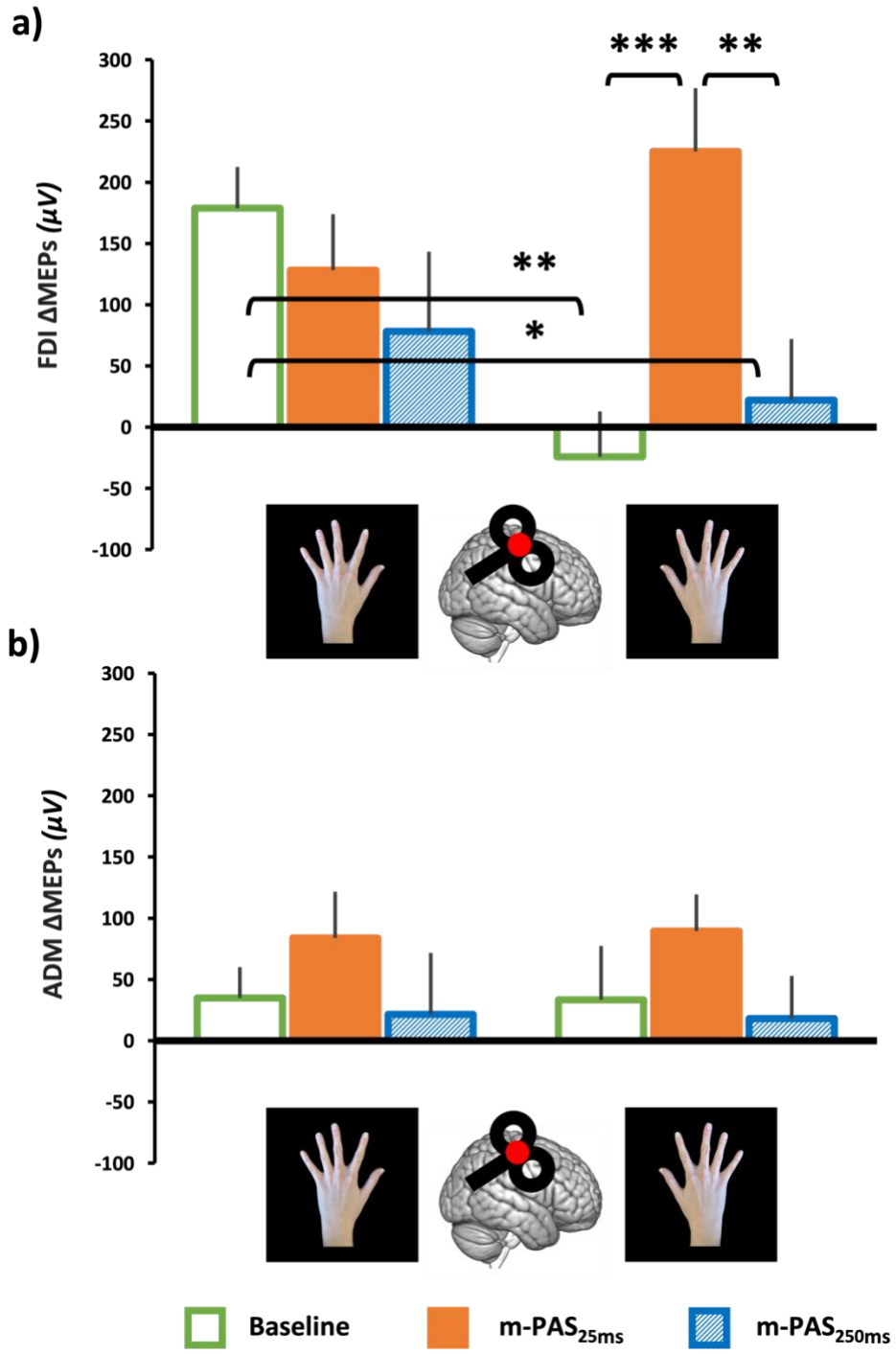

Figure 4.3. Results of *Experiment 1*. Mean ΔMEPs from FDI **(a)** and ADM **(b)** before (green blank bars) and after m-PAS (orange bars: m-PAS$_{25ms}$; blue striped bars and circles: m-PAS$_{250ms}$). After m-PAS$_{25ms}$, observation of index-finger movements of the right hand brought about a facilitation of MEPs recorded from the left FDI, induced by TMS of the right M1 (ipsilateral with respect to the viewed hand). * = $p < .5$; ** = $p < .01$; *** = $p < .001$; error bars = S.E.

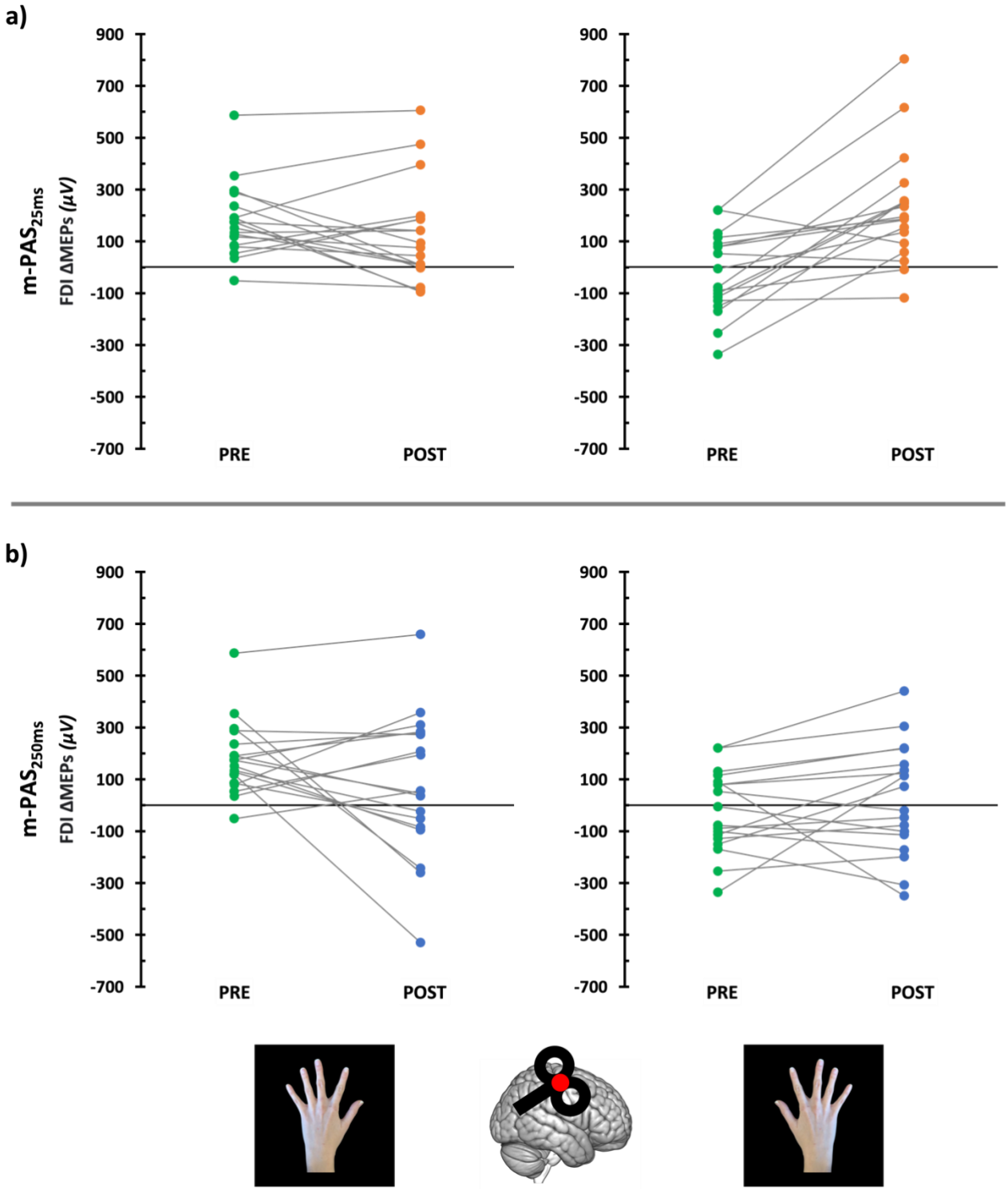

Figure 4.4. Individual ΔMEPs during the observation of contralateral (left panels) and ipsilateral movements (right panels) before (green dots) and after (orange and blue dots) m-PAS protocols in *Experiment 1*. **a)** m-PAS$_{25ms}$ session. **b)** m-PAS$_{250ms}$ session.

4.2.4. Conclusions

The results of *Experiment 1* show that the m-PAS is effective in inducing novel visuo-motor association (i.e., the emergence of motor resonance during the observation of the ipsilateral – right – movement conditioned during the protocol, which is not present at baseline). Atypical motor resonance is induced only when the timing between the paired stimulations is of 25 ms while no effects are induced when it was of 250 ms. Importantly, this atypical motor resonance follows somatotopic rules: indeed, the m-PAS induces no effect on MEPs recorded from the muscle not involved in the observed movement (ADM).

4.3. *Experiment 2*: Visual specificity of m-PAS

4.3.1. Aim

In the second experiment, I investigated the visual specificity of the m-PAS, hence the actual recruitment of the *action observation network* (and thus the MNS). Indeed, if the m-PAS relies on the recruitment of this system, the pairing of the TMS pulse with a visual stimulus showing a non-biological movement, hence not processed by the human MNS (Rizzolatti & Craighero, 2004; Tai, Scherfler, Brooks, Sawamoto, & Castiello, 2004), should be able to affect motor resonance neither for human actions nor the non-biological movements.

To verified this hypothesis, I introduced a modified version of the m-PAS, called *scissors*-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 even if it is kinematically similar to an abduction movement of a finger). The effects of this protocol on motor resonance were compared to those of the m-PAS found effective in the previous experiment, by using the same action-observation tasks of *Experiment 1*, as well as a scissors version of it. Based on findings from the first experiment, in both PAS protocols, an ISI of 25 ms was used between the two paired stimulations.

4.3.2. Methods and materials [5]

4.3.2.1. 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 ± S.D. = 22.4 ± 3.5 years; mean education ± S.D. = 14.6 ± 1.7 years). They were all right-handed according to the Edinburgh Handedness Inventory (Oldfield, 1971); none of them had contraindications to TMS (Rossi et al., 2009).

4.3.2.2. Experimental procedure

Materials, methods, TMS paradigms, and MEPs recording procedures of *Experiment 2* were the same as in the first experiment. This second experiment comprised two within-subjects experimental sessions: in one session, the participants underwent the same m-PAS of *Experiment 1*, with visual stimuli of movements (.e., the right hand performing an abduction movement of the index finger) paired with TMS pulses over M1 (i.e., m-PAS); in another session, the visual stimulus showed a pair of scissors making an opening/closing movement (*scissors*-PAS, **Figure 4.5**). 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, the hand action-observation tasks were administered, which showed the left or the right hand. Instead, before and after the scissors-PAS, only the action-observation task showing the left hand was presented, along with a new version of it depicting scissors. In this last task, trials could depict static or moving scissors (same type of trials of the hand action-observation task used in the first experiment; **Figure 4.6**).

[5] Stimuli, task, dataset and analysis of this experiment are freely accessible at *Open Science Framework*: https://osf.io/3ujkv/

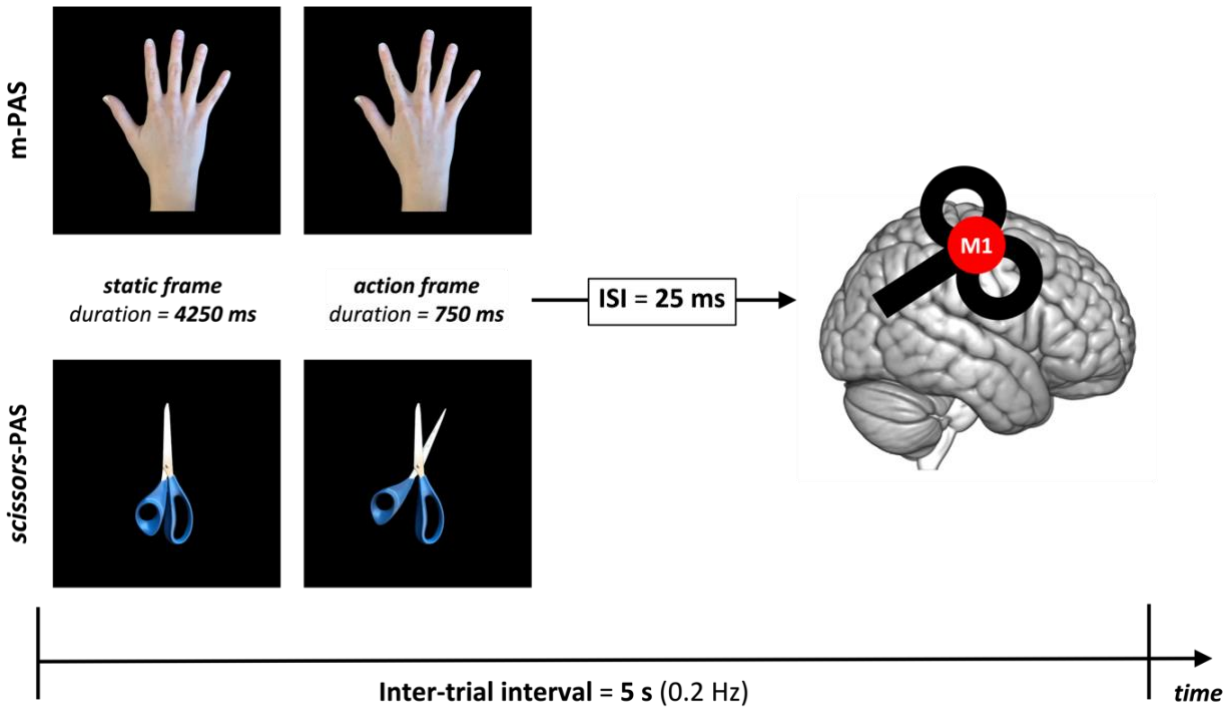

Figure 4.5. PAS protocols (and related parameters) tested in *Experiment 2*.

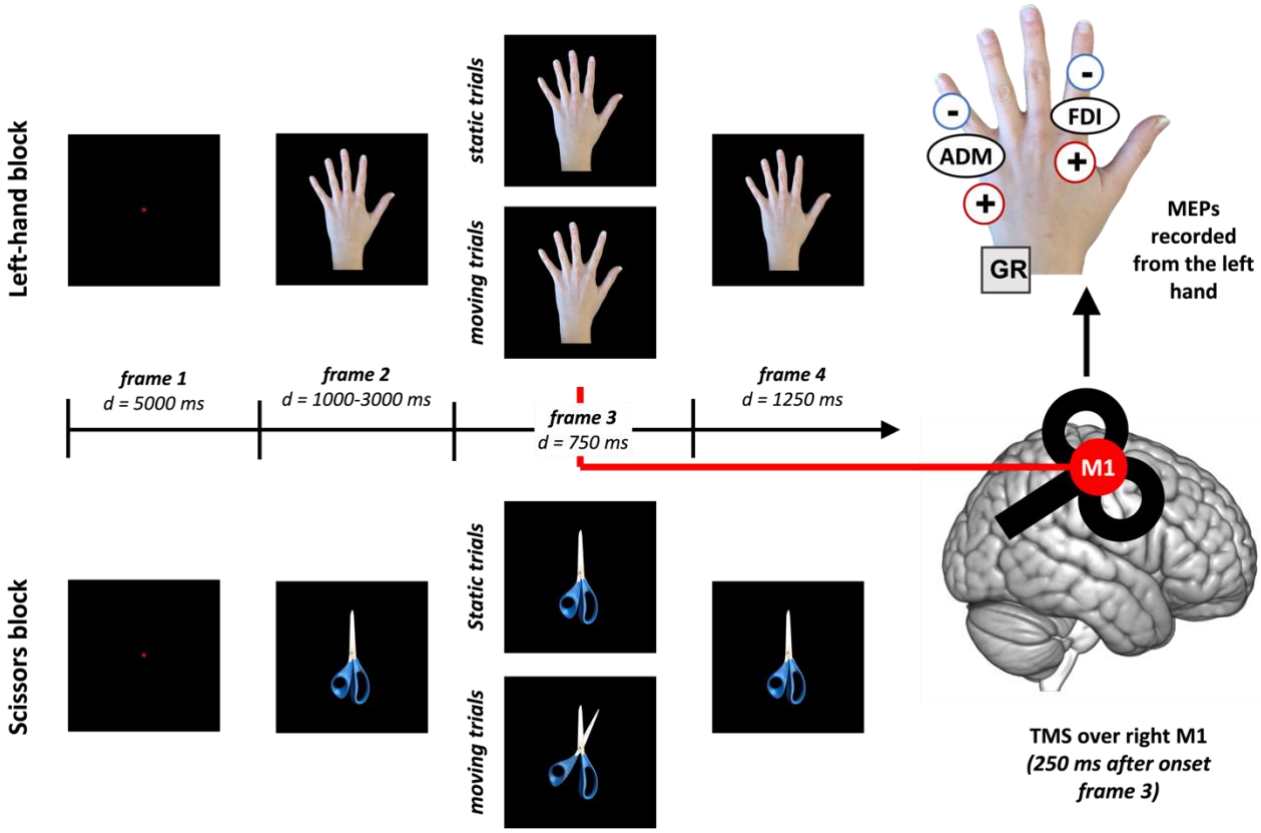

Figure 4.6. Action-observation task adopted during *scissors*-PAS session in *Experiment 2*. TMS was always delivered over right M1 and MEPs were recorded from FDI and ADM muscles of the left hand.

The order of the two sessions (m-PAS, *scissors*-PAS) was counterbalanced among participants and they were held at the same moment of the day (in the morning or the afternoon). At least 48 h passed between them (Sale et al., 2007). On average, during the m-PAS session, TMS was delivered with a mean intensity of 47.1% (S.D. = ± 7.3%) of the maximum stimulator output while, during the *scissors*-PAS session, the mean TMS intensity was of 47.4% (± 7.7 %, *vs.* the TMS intensity of m-PAS, $p = .99$).

4.3.2.3. Statistical analysis

MEPs were analyzed off-line using the same procedure of *Experiment 1*. On average, in the action-observation tasks, outliers analysis led to discarding 4.52% (S.D. = ± 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 detail, a preliminary Muscle (FDI, ADM) X viewed Stimulus (left hand$_{\text{m-PAS}}$, right hand, left hand$_{\textit{scissors}\text{-PAS}}$, scissors) rmANOVA was performed to verify, before each PAS protocol, the presence of motor resonance in the action-observation tasks. Then, m-PAS effects were assessed through a Session (m-PAS, *scissors*-PAS) X viewed Stimulus (left and right hands for the m-PAS; left hand and scissors for the scissors-PAS) X Time (pre-PAS, post-PAS) X Muscle (FDI, ADM) rmANOVA. The Lilliefors-corrected Kolmogorov-Smirnov test confirmed the normality of the distributions. Partial eta-squared (η_p^2) was reported as an effect size value for all the rmANOVAs conducted. Significant main effects were further explored by applying the Bonferroni correction. For each variable, mean ± standard error (S.E.) is reported.

4.3.3. Results

4.3.3.1. Motor resonance before PAS

The preliminary rmANOVA to investigate motor resonance in the baseline sessions showed a significant Muscle X viewed Stimulus interaction ($F_{3,57}$ = 3.49, p = .021, η_p^2 = .155), as well as a main effect of both factors Muscle ($F_{1,19}$ = 5.17, p = .035, η_p^2 = .214) and viewed Stimulus ($F_{3,57}$ = 7.9, p < .001, η_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: left hand movements = 235.4 ± 39.2 μV, vs. right hand movements = -30.1 ± 54.2 μV; t = 4.37, p < .001; *scissors*-PAS: left hand movements = 234.7 ± 71.8 μV vs. scissors movements = 21.4 ± 49.6 μV; t = 3.51, p = .018).

4.3.3.2. PAS effects

Results from the rmANOVA showed a significant Session X viewed Stimulus X Time X Muscle interaction ($F_{1,19}$ = 5.33, p = .032, η_p^2 = .219; see **Table 4.2** 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, η_p^2 = .303), viewed Stimulus X Time interaction ($F_{1,19}$ = 20.52, p < .001, η_p^2 = .519) and viewed Stimulus ($F_{1,19}$ = 10.22, p = .005, η_p^2 = .35). No other statistically significant effect was found (all Fs < 1.76, all ps > .2).

The significant Session X viewed Stimulus X Time interaction was then split in two separate rmANOVAs, one for each PAS session. With respect to the m-PAS session, it showed a significant viewed Stimulus X Time interaction ($F_{1,19}$ = 31.32, p < .001, η_p^2 = .622): as in

Experiment 1, a motor resonance effect induced by the observation of right hand movements emerged after the m-PAS (Pre-PAS = -30.1 ± 54.2 μV *vs.* Post-PAS = 193.8 ± 34.1; t = -3.73, p = .004; **Figure 4.7a**, left panel); 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). The effect of factors viewed Stimulus ($F_{1,19}$ = 3.64, p = .072, η_p^2 = .161) and Time ($F_{1,19}$ = .59, p = .454, η_p^2 = .03) was not statistically significant.

The rmANOVA conducted for the *scissors*-PAS showed only a main effect of viewed Stimulus ($F_{1,19}$ = 7.65, p = .012, η_p^2 = .287), but neither of the factor Time ($F_{1,19}$ = .21, p = .656, η_p^2 = .011) nor of the viewed Stimulus X Time interaction ($F_{1,19}$ = 1, p = .33, η_p^2 = .05). Thus, the *scissors*-PAS was unable either to affect motor resonance for human actions, or to induce a facilitation effect for non-biological movements (**Figure 4.7a**, right panel; see **Figure 4.8** for single participants' distribution).

Factor/Interaction	***F***	***p***	η_p^2
Session	1.51	.234	.074
viewed Stimulus	**9.50**	**.006**	**.333**
Time	.02	.905	.001
Muscle	**9.95**	**.005**	**.344**
Session X viewed Stimulus	1.25	.276	.062
Session X Time	.39	.543	.02
viewed Stimulus X Time	**16.08**	**< .001**	**.458**
Session X Muscle	1.21	.285	.06
viewed Stimulus X Muscle	**7.37**	**.014**	**.279**
Time X Muscle	.05	.827	.003
Session X viewed Stimulus X Time	**6.25**	**.022**	**.247**
Session X viewed Stimulus X Muscle	.99	.33	.05
Session X Time X Muscle	.84	.37	.043
viewed Stimulus X Time X Muscle	**10.19**	**.005**	**.349**
Session X viewed Stimulus X Time X Muscle	**5.33**	**.032**	**.219**

Table 4.2. PAS effects in *Experiment 2*: results from the rmANOVA conducted on the ΔMEPs.

For the ADM muscle, the rmANOVA did not show any significant main effect or interactions (all *Fs* < 4.25, all *ps* > .053, **Figure 4.7b**).

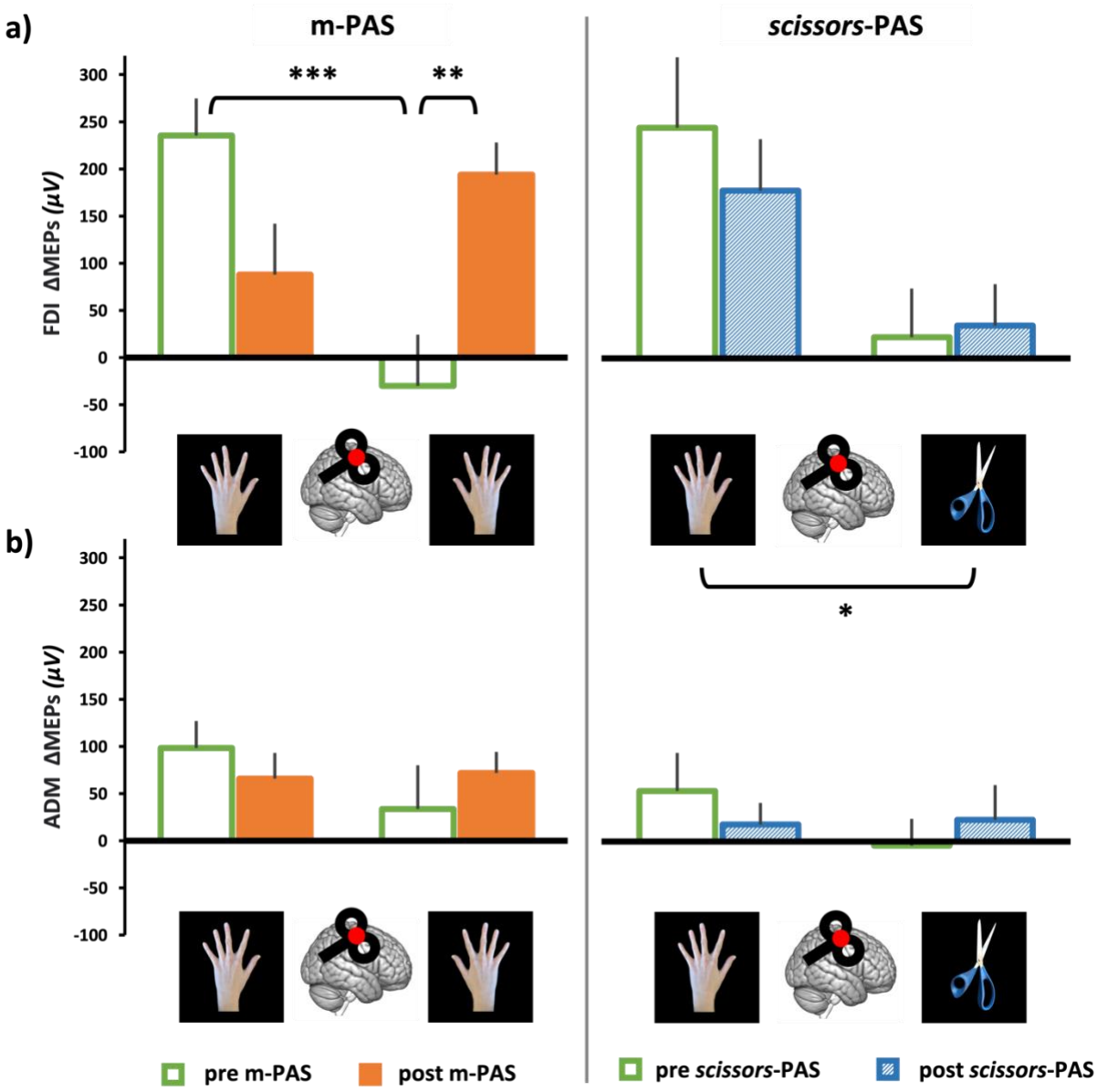

Figure 4.7. Results of *Experiment 2*. Mean ΔMEPs from FDI **(a)** and ADM **(b)** before (green blank bars) and after m-PAS (orange bars, left panel) and *scissors*-PAS (blue stripped bars, right panel). Before both PAS protocols, only the view of index-finger movements of the left hand induces a motor resonance effect on MEPs recorded from the left FDI. The m-PAS induces a facilitation of MEPs recorded from the left FDI by the view of right, ipsilateral to TMS, index-finger movements. The *scissors*-PAS did not affect motor resonance. * = $p < .5$; ** = $p < .01$; *** = $p < .001$; error bars = S.E.

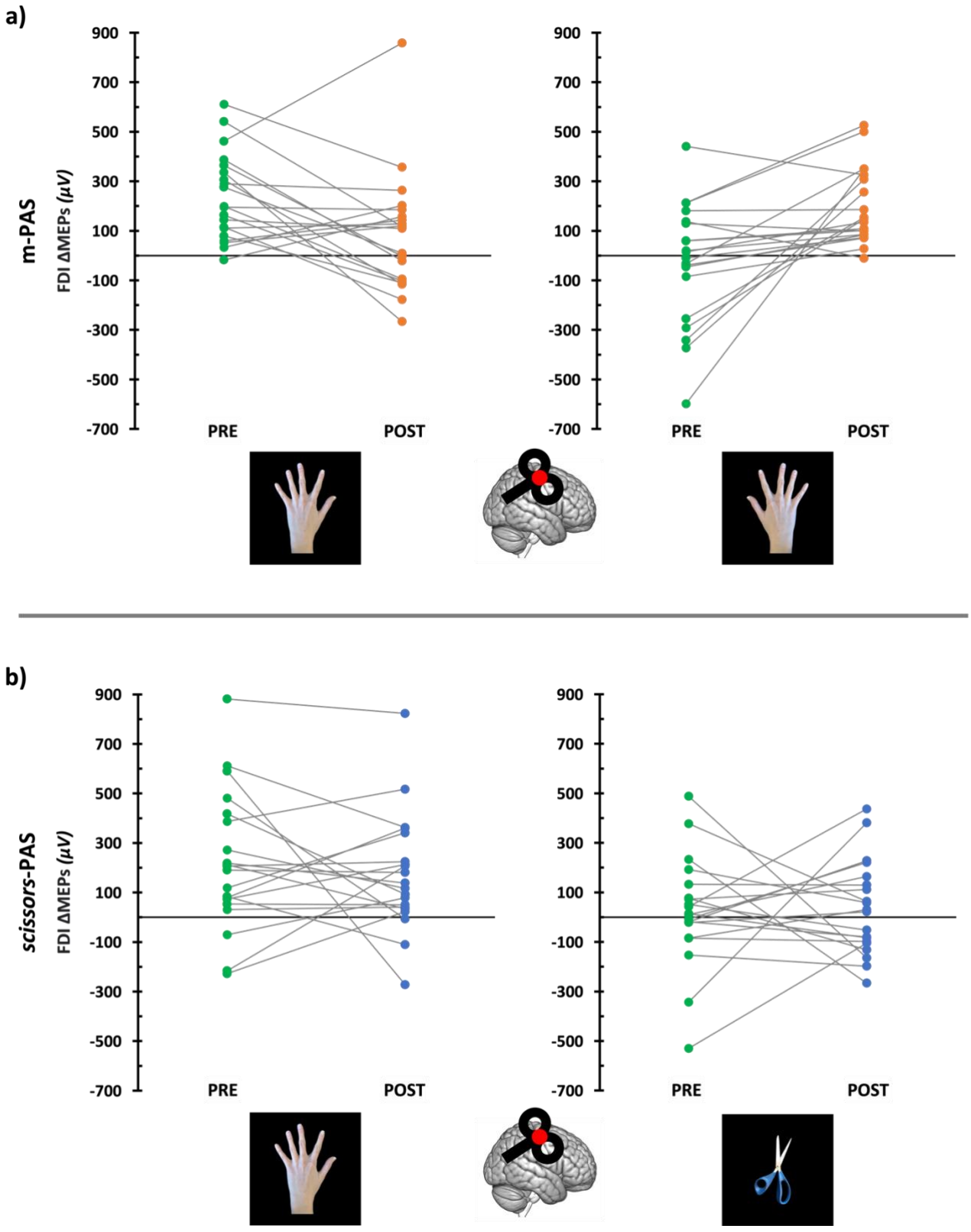

Figure 4.8. Individual ΔMEPs during the observation of contralateral biological movements (left panels), ipsilateral biological movements (upward right panel) and non-biological movement (downward right panel) before (green dots) and after (orange and blue dots) PAS protocols in *Experiment 2*. **a)** m-PAS session. **b)** *scissors*-PAS session.

4.3.4. Conclusions

Experiment 2 shows that the m-PAS is effective in shaping motor resonance only when the visual stimulus of the protocol depicts a moving hand (i.e., a biological movement). Conversely, when a moving pair of scissors (i.e., a non-biological movement) is repeatedly presented during the protocol, no shaping of motor resonance is induced after the PAS administration. These results strongly corroborate the hypothesis that m-PAS effects are mediated by the MNS rather than a more general visuo-motor integration network.

4.4. *Experiment 3*: Cortical specificity and behavioral correlates of m-PAS

4.4.1. Aim

In the final experiment, I explored the cortical specificity and the possible behavioral outcomes of the m-PAS. Considering cortical specificity, I tested whether a version of the m-PAS in which the TMS pulses target the left M1 instead of the right one (*left-M1* m-PAS) might influence the shaping of motor resonance in the same way as the standard m-PAS did (in this experiment called '*right-M1* m-PAS' for clarity). Importantly, in the *left-M1* m-PAS, the visual stimulus always depicted a right-hand movement and so, in this m-PAS version, the protocol acted at the level of a typical visuo-motor association (i.e., activation of contralateral M1 during the observation of unilateral contralateral movement), and therefore it might be unable to induce a motor resonance effect if MEPs are always recorded from left-hand muscles (with TMS was delivered over the right M1).

The second aim was to explore the effect of the m-PAS at a behavioral level. To this aim, I administered to participants, before and after the protocol, an imitative compatibility task. As said in **Chapter 2**, this task is usually used to assess the *automatic imitation* phenomenon, a behavioral marker of the *action observation network* activation (Heyes, 2011). Here, I used a version adapted from previous literature (e.g., Boyer et al., 2012; Catmur & Heyes, 2011; Catmur, Walsh, & Heyes, 2009; Hétu et al., 2016) where participants respond with their left hand while observing a left (ipsilateral to response hand) or a right hand (contralateral) lifting the index or the middle finger. Participants had to press a key with the same (congruent trials) or the opposite finger (incongruent trials) that that observed moving on the screen. My starting hypothesis was that the administration of the m-PAS (in detail, the *right-M1*

protocol) should influence participants' performance in such a task, likely with a different modulation in congruent and incongruent trials and/or according to the observed hand (left or right).

4.4.2. Methods and materials [6]

4.4.2.1. Participants

Nineteen healthy volunteers took part in *Experiment 3*; four of them did not complete the final session of the experiment due to the Covid-19 outbreak, leaving the final analyzed sample to 15 participants (6 males, mean age ± S.D. = 25 ± 3.3 years; mean education ± S.D. = 16.5 ± 1.4 years). They were all right-handed according to the Edinburgh Handedness Inventory (Oldfield, 1971) and none of them had contraindications to TMS (Rossi et al., 2009).

4.4.2.2. Imitative compatibility task

The imitative compatibility task was adapted from previous literature (Boyer et al., 2012; Catmur & Heyes, 2011; Catmur et al., 2009). Participants sat comfortably on a chair in front of a PC monitor placed at a distance of 57 cm with their hands positioned out of view.

The task consisted of 4 blocks in which the instruction gave to the participant change according to the way they had to respond to the viewed stimuli of movements. In the congruent (imitative) blocks, they had to respond with the same finger which was seen moving on the screen. Conversely, in the incongruent (non-imitative) blocks, they had to respond with the opposite finger which was seen moving on the screen (**Figure 4.9a**). In detail, considering congruent blocks, the precise instruction gave to participants was: '*if you*

[6] Stimuli, task, dataset and analysis of this experiment are freely accessible at *Open Science Framework*: https://osf.io/vsqan/

see the index finger moving, you have to press the mouse button with your index finger. Conversely, if you see the middle finger moving, you have to press the mouse button with your middle finger'. In the incongruent blocks, the instruction gave was: '*if you see the index finger moving, you have to press the mouse button with your middle finger. Conversely, if you see the middle finger moving, you have to press the mouse button with your index finger*'. Every block was presented twice and the same block was never presented sequentially. The order of the blocks was kept fixed throughout all the experimental sessions.

Regardless of the block, each trial of the task started with a black frame (duration: 1000 ms). Then, a second frame depicting a left or a right hand at rest seen in an egocentric perspective appeared ('static frame'). This frame could have a duration of 400, 600, or 800 ms to prevent that participants expected the start of the following frame. After this, the 'movement frame' appeared (duration: 400 ms), depicting a lifting right-/left-hand index finger or middle finger and giving the illusion that the hand made a lifting movement with such finger. These two kinds of movements were merely introduced to give a double choice of response to participants, hence making the task more difficult (Catmur et al., 2009). Here, participants have to respond as fast as possible by pressing one of the two buttons of the mouse (left button with the middle finger, right button with the index finger) with their left hand and according to the block instructions (i.e., in the congruent blocks, with the same finger which has moved on the screen or, in the incongruent ones, with the opposite finger, see above). Participants' response could be recorded for a maximum of 1500 ms from the onset of the 'movement frame'; if no response was recorded in such a time window, the trial was considered 'missed'. Finally, a 'static frame' re-appeared (duration: 400 ms; **Figure 4.9b**), and the trial ended. In each block, a total of 60 trials were presented in a randomized order (for a total number of 240 trials per task): half of them (30) depicted the left hand, and the other half the right one. Of these, half (15) depicted a lifting index finger and the other half

a lifting middle finger. The task lasted about 10 min (2 min and 30 s each block).

At the start of every session, a practice version of the task (with two blocks – one imitative and one non-imitative – of 10 trials) were administered. Participants received visual feedback about the correctness of their answers. No analysis was further conducted on this practice version of the task.

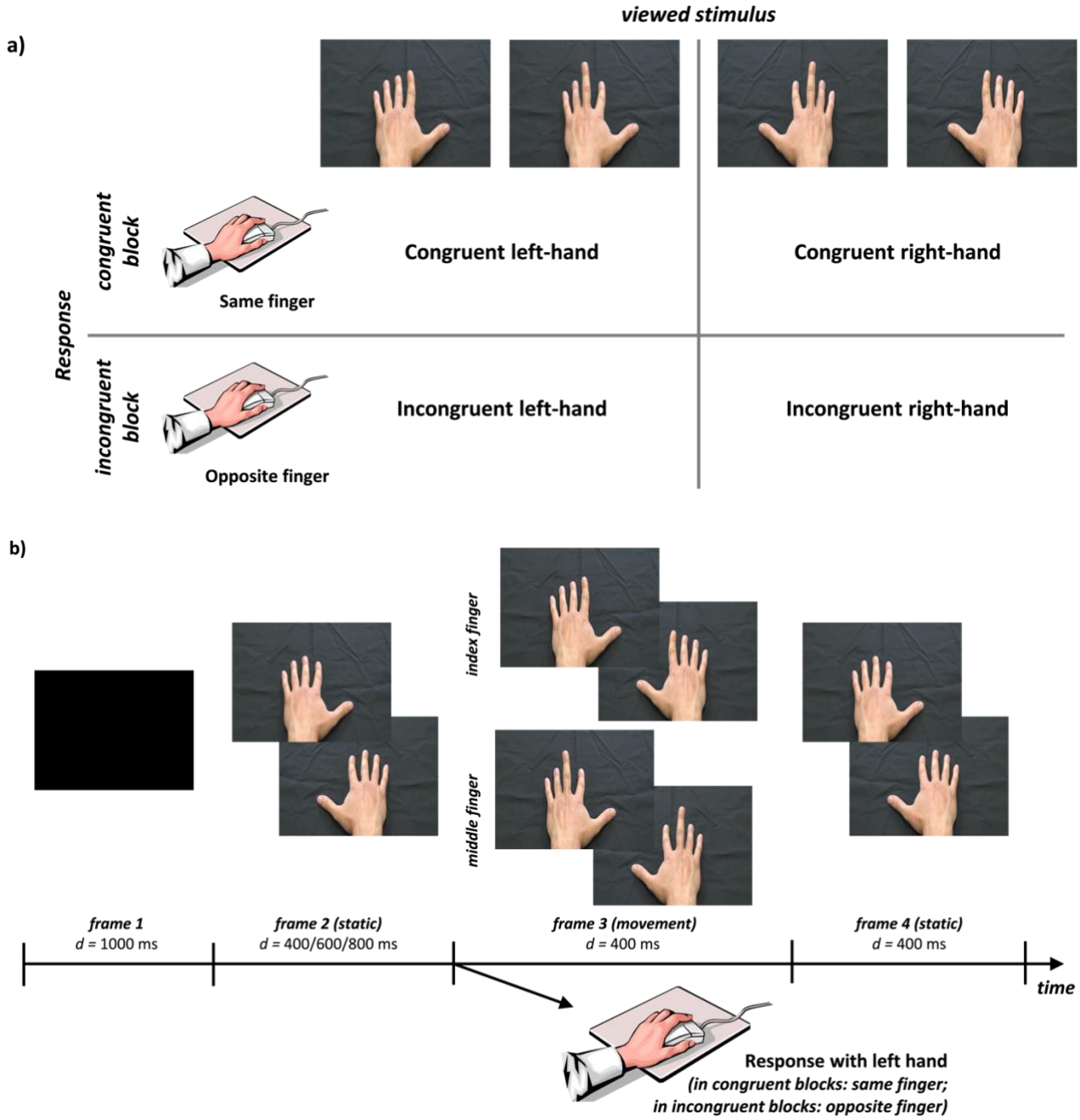

Figure 4.9. Imitative compatibility task. a) The four typologies of trials presented to participants during the task according to the block (congruent, incongruent) and the observed hand (left, right). Participants always respond with their left hand. **b)** Example of a task trial.

Trials randomization and timing of the stimuli were presented under computer control using the software E-Prime (3.0, Psychology Software Tool, Inc.).

4.4.2.3. Experimental procedure

Materials, methods, TMS paradigms, and MEPs recording procedures of *Experiment 3* were the same as in the two previous experiments. TMS was delivered using a biphasic figure-of-eight coil (diameter = 70 mm) connected to a Nexstim Eximia stimulator (Nexstim, Helsinki, Finland). This third experiment comprised two within-subjects experimental sessions: in one session, the participants underwent the m-PAS found effective in Experiments *1* and *2*, where TMS was delivered over right M1 (here called *right-M1* m-PAS); in another session, TMS during the m-PAS was delivered over left M1 (*left-M1* m-PAS, **Figure 4.10**). In both PAS protocols, the ISI between the visual stimulus and the TMS pulse was 25 ms and the visual stimulus depicted a right hand performing an abduction movement of the index finger.

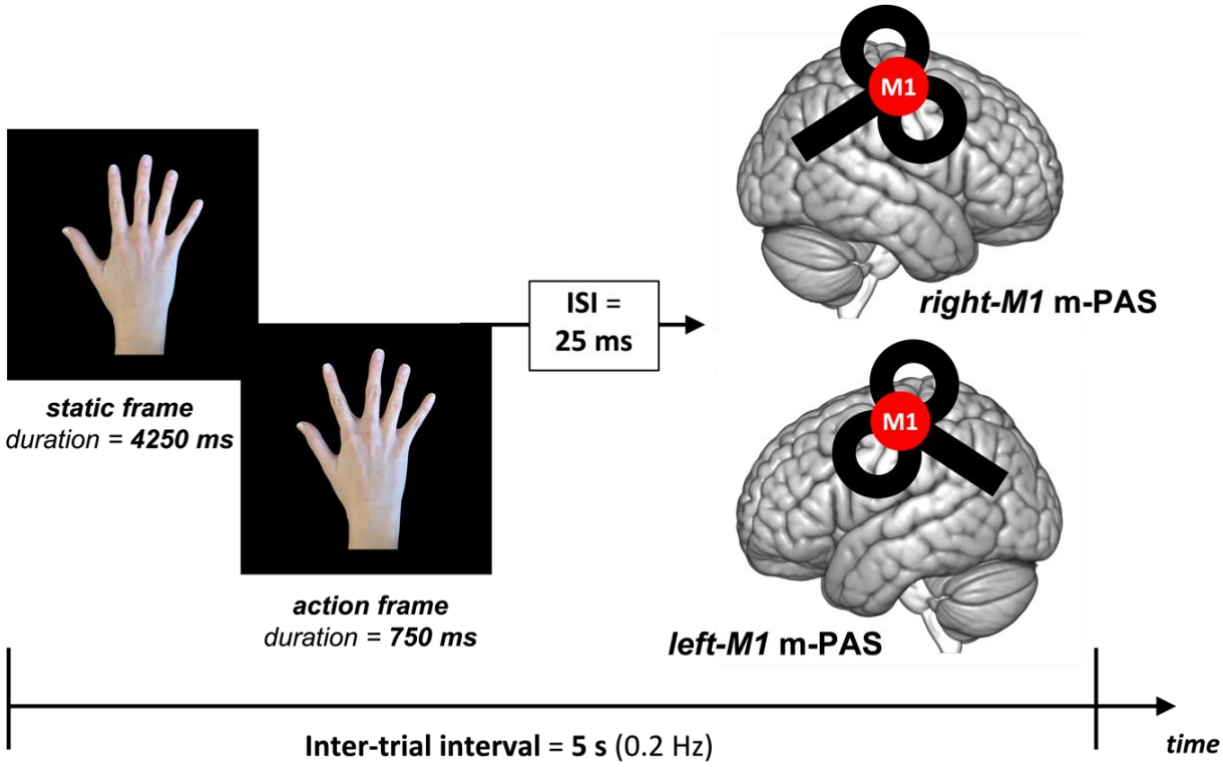

Figure 4.10. m-PAS protocols (and related parameters) tested in *Experiment 3*.

The imitative compatibility task and the action-observation task were administered before and after the m-PAS. As in the previous experiments, in this latter task, TMS was always delivered over the right M1 (**Figure 4.2**). Considering that two tasks had to be performed before and after the m-PAS, I decided to reduce the number of trials in each block of the action-observation task to 30 (instead of 40, see **4.2.2.3**) to avoid that it lasts too long, likely missing the time window of m-PAS after-effects. The order of the two tasks was kept fixed within a participant but counterbalanced between participants. The order of the two sessions (*right-M1* m-PAS, *left-M1* m-PAS) was counterbalanced among participants and they were held at the same moment of the day (in the morning or the afternoon). At least 48 h passed between them (Sale et al., 2007). On average, during the *right-M1* m-PAS session, TMS was delivered with a mean intensity of 48.3% (S.D. = ± 8.5%) of the maximum stimulator output while, during the *left-M1* m-PAS session, the mean TMS intensity was of 48% (± 7.7%, *vs.* the TMS intensity of *right-M1* m-PAS, $p = .99$).

4.4.2.4. Statistical analysis

4.4.2.4.1. Neurophysiological analysis

MEPs were analyzed off-line using the same procedure of the previous experiments. On average, in the action-observation tasks, outliers analysis led to discarding 3.5% (S.D. = ± 1.6%) of MEPs recorded (mean number of discarded trials = 8.4 ± 3.8, out of 240). Statistical analyses for the action-observation task were conducted with a series of rmANOVAs, following the same statistical approach of *Experiments 1 and 2*. In detail, a preliminary Muscle (FDI, ADM) X Session (*right-M1* m-PAS, *left-M1* m-PAS) X viewed Hand [left (contralateral to M1-TMS in the action-observation tasks) hand, right (ipsilateral) hand] was performed to verify, before each PAS protocol, the presence of motor resonance in the action-observation tasks. Then, m-PAS effects were assessed

through a Session (*right-M1* m-PAS, *left-M1* m-PAS) X Time (pre, post) X viewed Hand [left (contralateral) hand, right (ipsilateral) hand] X Muscle (FDI, ADM) rmANOVA.

4.4.2.4.1. Behavioral analysis

In the imitative compatibility task, RTs were the dependent variable (e.g., Boyer et al., 2012; Catmur & Heyes, 2011; Mengotti, Ticini, Waszak, Schütz-Bosbach, & Rumiati, 2013). Participants made few errors; hence accuracy was at the ceiling in almost every condition (mean accuracy in the baseline assessment for congruent trials = 98.3 ± .4%, incongruent trials = 94 ± 1%, but see **4.4.3.2.3**). For this reason, as already done in previous literature using this task, I decided to focus my investigation on RTs. Trials with RTs higher or lower than 2 S.D. from the mean of the task, as well as trials marked as 'missing', or with an error, were excluded from subsequent analyses. On average, for each participant, a total of 42.7 (S.D. = ± 20.8) trials out of 960 were excluded (4.44 ± 2.17% of total trials presented). Considering that trials depicting the lifting index finger or the lifting middle one were merely introduced to give a double choice of response to participants, such trials were mediated and not further differentiated in the analysis (e.g., Catmur et al., 2009).

A preliminary Session (*right-M1* m-PAS, *left-M1* m-PAS) X Block (congruent, incongruent) X viewed Hand (left, right) rmANOVA was performed on raw RTs to verify that the two baseline sessions of the task did not differ and that the RTs distribution was the one expected from previous literature (e.g., Bertenthal et al., 2006; Boyer et al., 2012). Then, as a dependent variable accounting for *automatic imitation*, according to the depicted hand, RTs in the congruent trials were subtracted to RTs in the incongruent ones ($\Delta RT_{Incongr\text{-}Congr}$ – the greater is such index, the greater is the difference in RTs between non-imitative and imitative conditions; for a similar procedure, see: Catmur et al., 2009; Mengotti et al., 2013) and a Session (*right-M1* m-PAS, *left-M1* m-PAS) X Time (pre, post)

X viewed Hand (left, right) rmANOVA was conducted to explore possible effects of the m-PAS.

For the sake of completeness, I also reported the results of the analysis conducted on accuracy. Here, a preliminary Session (*right-M1* m-PAS, *left-M1* m-PAS) X Block (congruent, incongruent) X viewed Hand (left, right) rmANOVA was performed to verify that the two baseline sessions of the task did not differ and that the accuracy distribution was the one expected from previous literature (Bertenthal et al., 2006; Boyer et al., 2012). Then, to explore m-PAS effects, a 2 X 2 X 2 X 2 rmANOVA with factors Session (*right-M1* PAS, *left-M1* PAS), Time (pre, post), Block (congruent, incongruent) and viewed Hand (left, right) was performed.

In all the analyses, the Lilliefors-corrected Kolmogorov-Smirnov test confirmed the normality of the distributions. As an effect size value, partial eta-squared (η_p^2) was reported for all the rmANOVAs conducted. Significant main effects were further explored by applying the Bonferroni correction. For each variable, mean ± standard error (S.E.) is reported.

4.4.3. Results

4.4.3.1. Neurophysiological effects

4.4.3.1.1. Motor resonance before m-PAS

Results from the rmANOVA conducted on the baseline sessions to detect motor facilitation showed a main effect of factor viewed Hand ($F_{1,14}$ = 13.23, p = .003, η_p^2 = .49) and a significant Muscle X viewed Hand interaction ($F_{1,14}$ = 12.47, p = .003, η_p^2 = .47). As in the previous experiments, post-hoc analysis highlighted the classical, muscle-specific (FDI), motor facilitation effect at baseline induced by the observation of the index-finger movement of the left hand only (196.8 ± 43.4 μV; all *ps* < .02), with no difference between

the two baseline sessions (*right-M1* m-PAS$_{\text{left hand}}$ = 222.8 ± 56.1 μV *vs. left-M1* m-PAS$_{\text{left hand}}$ = 170.7 ± 48.1 μV; t = .789, p = .99). No other significant main effects or interactions were found (all Fs < 3.1, all ps > .1).

4.4.3.1.2. m-PAS effects

Results from the rmANOVA conducted to investigate m-PAS effects showed a significant Session X viewed Hand X Time X Muscle interaction ($F_{1,14}$ = 4.83, p = .045, η_p^2 = .257; see **Table 4.3** for all main effects and interactions). This quadruple interaction was further explored with two separate rmANOVAs, separated for each muscle.

For the FDI muscle, Session X viewed Hand X Time ($F_{1,14}$ = 6.18, p = .026, η_p^2 = .306), session X viewed Hand ($F_{1,14}$ = 6.79, p = .021, η_p^2 = .327), viewed Hand X Time

Factor/Interaction	***F***	***p***	η_p^2
Session	.92	.355	.061
Time	.93	.352	.062
Viewed Hand	4.07	.063	.225
Muscle	**10.14**	**.007**	**.42**
Session X viewed Hand	.89	.36	.06
Session X Time	.04	.852	.003
Viewed Hand X Time	4.45	.052	.243
Session X Muscle	1.69	.214	.108
viewed Hand X Muscle	3.14	.098	.183
Time X Muscle	.58	.459	.04
Session X viewed Hand X Time	2.205	.16	.136
Session X viewed Hand X Muscle	**9.33**	**.009**	**.4**
Session X Time X Muscle	3.76	.073	.212
viewed Hand X Time X Muscle	2.01	.178	.126
Session X viewed Hand X Time X Muscle	**4.83**	**.045**	**.257**

Table 4.3. m-PAS effects in *Experiment 3*: results from the rmANOVA conducted on the ΔMEPs.

interactions ($F_{1,14}$ = 5.63, p = .033, η_p^2 = .287) and viewed Hand ($F_{1,14}$ = 6.24, p = .026, η_p^2 = .308) reached the significance level. Other main factors and interactions were not statistically significant (all *Fs* < 2.54, all *ps* > .133).

The significant Session X viewed Hand X Time interaction was then split in two separate rmANOVAs, one for each m-PAS session. With respect to the *right-M1* m-PAS session, it showed a significant viewed Hand X Time interaction ($F_{1,19}$ = 11.28, p = .005, η_p^2 = .446): as in *Experiment 1* and *2*, a motor resonance effect induced by the observation of right hand movements emerged after the *right-M1* m-PAS (Pre-PAS = -4.3 ± 32.6 μV *vs.* Post-PAS = 296.7 ± 71.9 μV; t = -3.72, p = .006; **Figure 4.11a**, left panel); crucially, the magnitude of this effect was comparable to that found for the left hand (Pre-PAS = 222.8 ± 56.1 μV; Post-PAS = 107.2 ± 61.6 μV; all *ps* > .207). The effect of factors viewed Hand ($F_{1,14}$ = .1, p = .753, η_p^2 = .007) and Time ($F_{1,14}$ = 3.18, p = .096, η_p^2 = .185) was not statistically significant.

The rmANOVA conducted for the *left-M1* m-PAS showed only a main effect of viewed Hand ($F_{1,14}$ = 14.5, p = .002, η_p^2 = .509), but neither of the factor Time ($F_{1,14}$ = .01, p = .918, η_p^2 = .001) nor of the viewed Hand X Time interaction ($F_{1,14}$ = .42, p = .53, η_p^2 = .029). Thus, the *left-M1* m-PAS was unable either to affect motor resonance for ipsilateral nor for contralateral hand movements (**Figure 4.11a**, right panel; see **Figure 4.12** for single participants' distribution).

For the ADM muscle, the rmANOVA did not show any significant main effect or interactions (all *Fs* < 3.38, all *ps* > .087, **Figure 4.11b**).

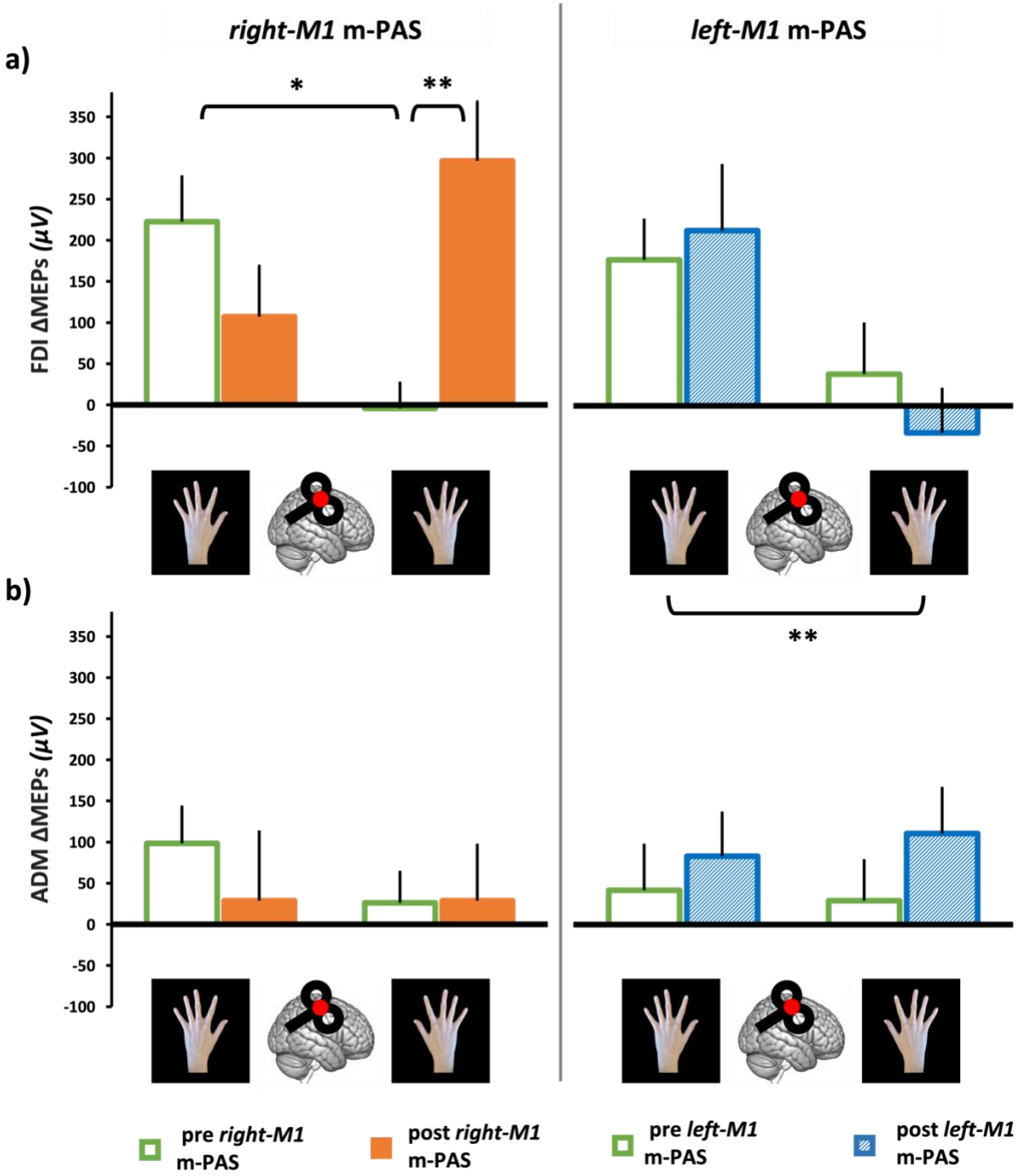

Figure 4.11. Results of *Experiment 3* – action-observation task. Mean ΔMEPs from FDI **(a)** and ADM **(b)** before (green blank bars) and after *right-M1* m-PAS (orange bars, left panel) and *left-M1* m-PAS (blue stripped bars, right panel). Before both PAS protocols, only the view of index-finger movements of the left hand induces a motor resonance effect on MEPs recorded from the left FDI. The *right-M1* m-PAS induces a facilitation of MEPs recorded from the left FDI by the view of right, ipsilateral to TMS, index-finger movements. The *left-M1* m-PAS did not affect motor resonance. * = $p < .5$; ** = $p < .01$; error bars = S.E.

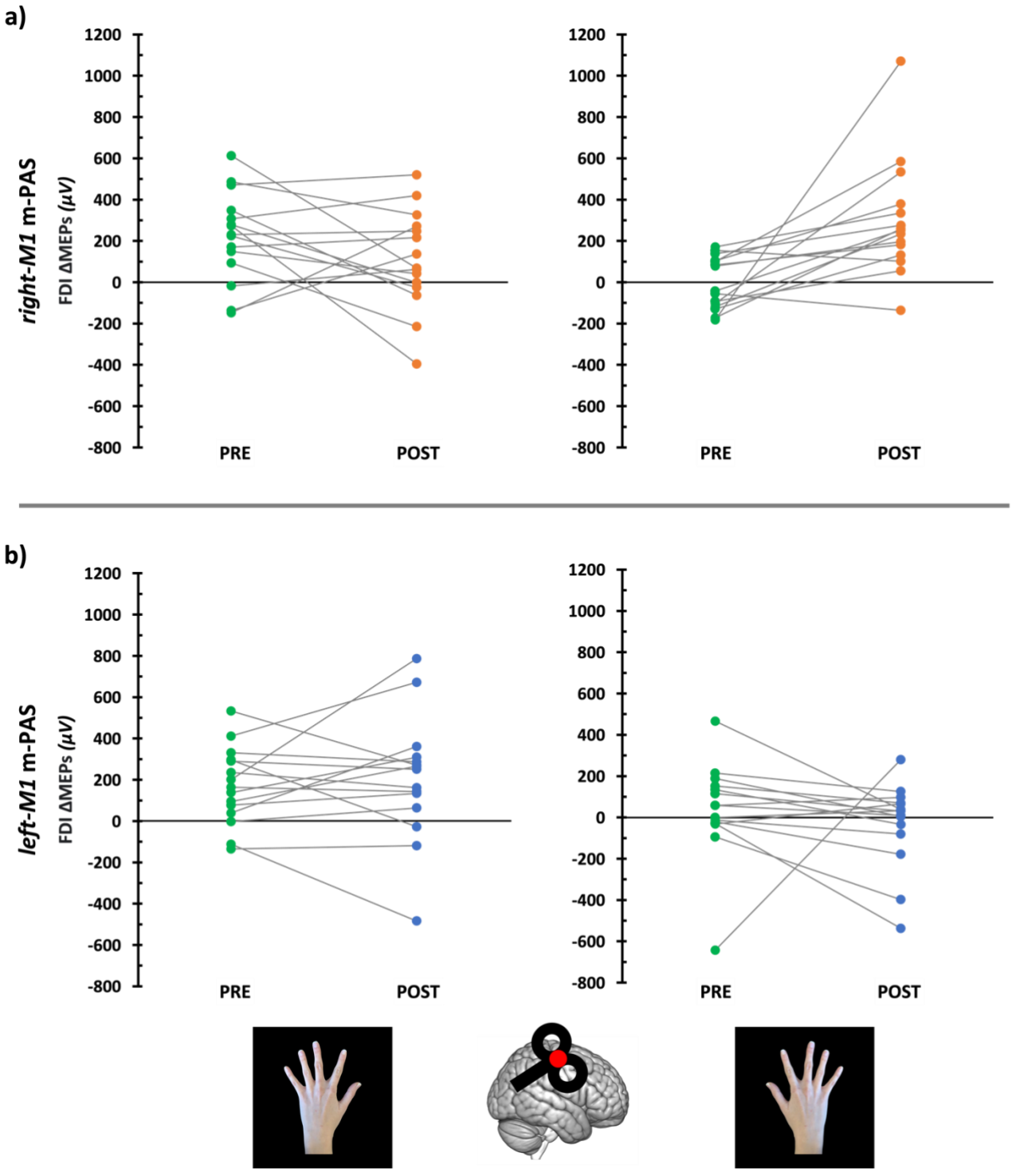

Figure 4.12. Individual ΔMEPs during the observation of contralateral (left panels) and ipsilateral movements (right panels) before (green dots) and after (orange and blue dots) m-PAS protocols in *Experiment 3*. **a)** *right-M1* m-PAS session. **b)** *left-M1* m-PAS session.

4.4.3.2. Behavioral effects

4.4.3.2.1. RTs before m-PAS

The rmANOVA conducted on the two baseline sessions of the imitative compatibility task showed a significant effect of main factors Block ($F_{1,14}$ = 105.51, p < .001, η_p^2 = .883) and viewed Hand ($F_{1,14}$ = 131, p < .001, η_p^2 = .903) but neither of main factor Session ($F_{1,14}$ = .74, p = .404, η_p^2 = .05), nor of the other interactions (all Fs < 4.33; all ps > .056). This pattern highlighted that RTs distribution differed only across the two blocks and according to the viewed hand but not across the two sessions. Indeed, participants responded faster to congruent blocks (416.6 ± 12 ms) at variance with incongruent ones (542.6 ± 17.2 ms; t = -10.3, p < .01) and they were faster when the left hand – ipsilateral to the hand used to respond – was depicted (458.1 ± 14.3 ms) compared to trials where the right one was showed (501.1 ± 14,9 ms; t = -11.4, p < .01). Importantly, this pattern is the one expected from previous literature on *automatic imitation* using such a task (e.g., Bertenthal et al., 2006; Boyer et al., 2012; Catmur & Heyes, 2011): participants instructed to imitate a movement (congruent blocks) respond faster when the position of the imperative stimulus and the response spatially correspond (left-hand trials) than when they do not (right-hand trials). Conversely, when participants are instructed to make the opposite movements to those observed (incongruent blocks), their RTs were significantly longer and RTs were faster in the spatially incompatible trials (left-hand trials) than in the compatible ones (right-hand trials).

4.4.3.2.2. m-PAS effects

Results from the rmANOVA conducted on $\Delta RT_{Incongr\text{-}Congr}$ to detect m-PAS effects on *automatic imitation* showed a significant Session X Time X viewed Hand interaction ($F_{1,14}$ = 6.15, p = .026, η_p^2 = .305), as well as main effects of Time ($F_{1,14}$ = 5.96, p = .029, η_p^2 =

.298) and viewed Hand ($F_{1,14}$ = 7.76, p = .015, η_p^2 = .357). No other significant effect was found (all Fs < 2.68, all ps > .161, see **Table 4.4** for all main effects and interactions). The triple interaction was further explored with two separate Time X viewed Hand rmANOVAs, one for each session.

For *right-M1* m-PAS session, main factor viewed Hand ($F_{1,14}$ = 14.6, p = .002, η_p^2 = .51) and, crucially, Time X viewed Hand interaction was found statistically significant ($F_{1,14}$ = 5.1, p = .04, η_p^2 = .267). Post-hoc analysis reveals that after the protocol, participants' $\Delta RT_{Incongr-Congr}$ differed when the right hand is depicted (133.6 ± 11.2 ms) compared to the left one (95.1 ± 12.4 ms; t = -4.4, p < .001) while in baseline this difference is not statistically significant (left-hand $\Delta RT_{Incongr-Congr}$ = 118.3 ± 15.6 ms, right-hand $\Delta RT_{Incongr-Congr}$ = 132.1 ± 16.2 ms; t = -1.58, p = .76, **Figure 4.13a**, left panel). Main factor Time did not reach statistically significance ($F_{1,14}$ = .45, p = .513, η_p^2 = .031)

For *left-M1* m-PAS session, only a significant main factor Time was found ($F_{1,14}$ = 9.76, p = .007, η_p^2 = .411): regardless of the viewed hand, $\Delta RT_{Incongr-Congr}$ in baseline (126.8 ± 18.4 ms) was greater than after the administration of the m-PAS (99.5 ± 13.8 ms). Main factor viewed Hand ($F_{1,14}$ = 1.22, p = .288, η_p^2 = .08) and Time X viewed Hand interaction ($F_{1,14}$ = .89, p = .362, η_p^2 = .06) were not statistically significant.

Factor/Interaction	*F*	*p*	η_p^2
Session	.22	.644	.016
Time	**5.96**	**.029**	**.298**
Viewed Hand	**7.76**	**.015**	**.357**
Session X Time	.63	.441	.043
Session X viewed Hand	2.68	.124	.161
Viewed Hand X Time	1.3	.275	.084
Session X Time X viewed Hand	**6.15**	**.026**	**.305**

Table 4.4. m-PAS effects in *Experiment 3*: results from the rmANOVA conducted on $\Delta RT_{Incongr-Congr}$

To further explore the behavioral outcomes of m-PAS, I run a correlational analysis between left-hand/right-hand $\Delta RT_{Incongr-Congr}$ before and after the administration of the two protocols. I decided to use Kendall's tau-b (τb) as correlational index because is more sensitive to small sample size (Bonett & Wright, 2000; Long & Cliff, 1997). This analysis showed that $\Delta RT_{Incongr-Congr}$ in baseline and after the administration of the *left-M1* m-PAS were positively correlated: namely, for both the depicted hands of the imitative compatibility task, participants tend to have the same response pattern (i.e., low/high $\Delta RT_{Incongr-Congr}$) before and after the protocol administration (left hand: τb = .64, $p < .001$; right hand: τb = .6, $p < .001$, **Figure 4.13b**). Interestingly, after *right-M1* m-PAS, and for both hands, this significant correlation is lost (left hand: τb = .09, $p = .697$; right hand: τb = .2, $p = .328$, **Figure 4.13b**).

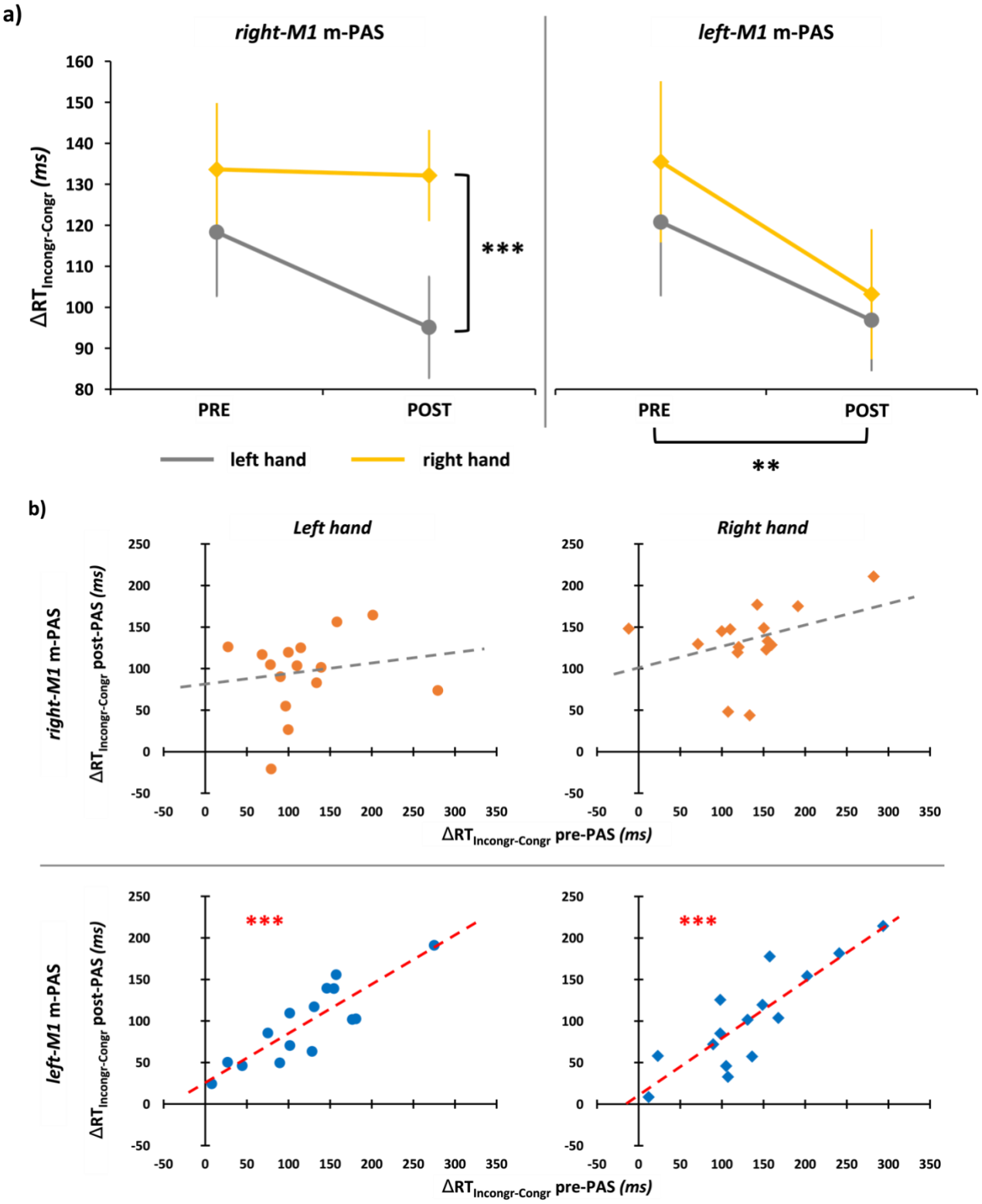

Figure 4.13. Results of *Experiment 3* – imitative compatibility task. a) $\Delta RT_{Incongr\text{-}Congr}$ before and after the *right-M1* (left panel) and the *left-M1* m-PAS (right panel). A significant difference between left-hand (grey line) and right-hand (yellow line) $\Delta RT_{Incongr\text{-}Congr}$ is found only after the *right-M1* m-PAS. **b)** Correlation between $\Delta RT_{Incongr\text{-}Congr}$ before (*x* axis) and after (*y* axis) the m-PAS. Upper panels: *right-M1* m-PAS. Lower panels: *left-M1* m-PAS. Left panels: trials depicting left hands. Right panels: trials depicting right hands. Significant correlations are found only in the *left-M1* m-PAS session, for both trails depicting left and right hands. ** = p < .01; *** = p < .001; error bars = S.E.

4.4.3.2.3. Accuracy in the imitative compatibility task

The preliminary rmANOVA performed on the baseline accuracy showed that the two baselines did not differed (main effect of Session: $F_{1,14} = 2.47$, $p = .139$, $\eta_p^2 = .15$; Session X Block X viewed Hand: $F_{1,14} = .02$, $p = .891$, $\eta_p^2 = .001$) and that participants made a greater number of error during incongruent trials depicting right hands (Block X viewed Hand: $F_{1,14} = 13.85$, $p = .002$, $\eta_p^2 = .497$; incongruent right-hand trials: 90.7 ± 1.4% *vs.* congruent left-hand trials: 99 ± .3%, t = 9.15; $p < .001$; *vs.* congruent right-hand trials: 97.4 ± .5%, t = 6.66; $p < .001$; *vs.* incongruent left-hand: 97.6 ± .5%, t = 7.59; $p < .001$). This result is expected from literature, which described that participants are usually less accurate in such kind of trial compared to the other three typologies (Catmur & Heyes, 2011).

Factor/Interaction	***F***	***p***	η_p^2
Session	.84	.374	.057
Time	**9.5**	**.008**	**.404**
Block	**37.63**	**< .001**	**.729**
Viewed Hand	**47.41**	**< .001**	**.772**
Session X Time	1.16	.224	.103
Session X Block	.52	.482	.036
Time X Block	.19	.668	.014
Session X viewed Hand	.03	.856	.002
Block X viewed Hand	**20.81**	**< .001**	**.598**
Time X viewed Hand	1.23	.286	.081
Session X Time X Block	.42	.527	.029
Session X Block X viewed Hand	.07	.789	.005
Session X Time X viewed hand	3.76	.073	.212
Time X Block X viewed Hand	< .01	.975	< .001
Session X Time X Block X viewed Hand	.02	.894	.001

Table 4.5. m-PAS effects in *Experiment 3*: results from the rmANOVA conducted on participants' accuracy in the imitative compatibility task.

The subsequent Session X Time X Block X viewed Hand showed no significant effect of the quadruple interaction ($F_{1,14}$ = .02, p = .894, η_p^2 = .001; see **Figure 4.14** and **Table 4.5**), suggesting that, at variance with RTs, m-PAS had no specific effects on participants' accuracy.

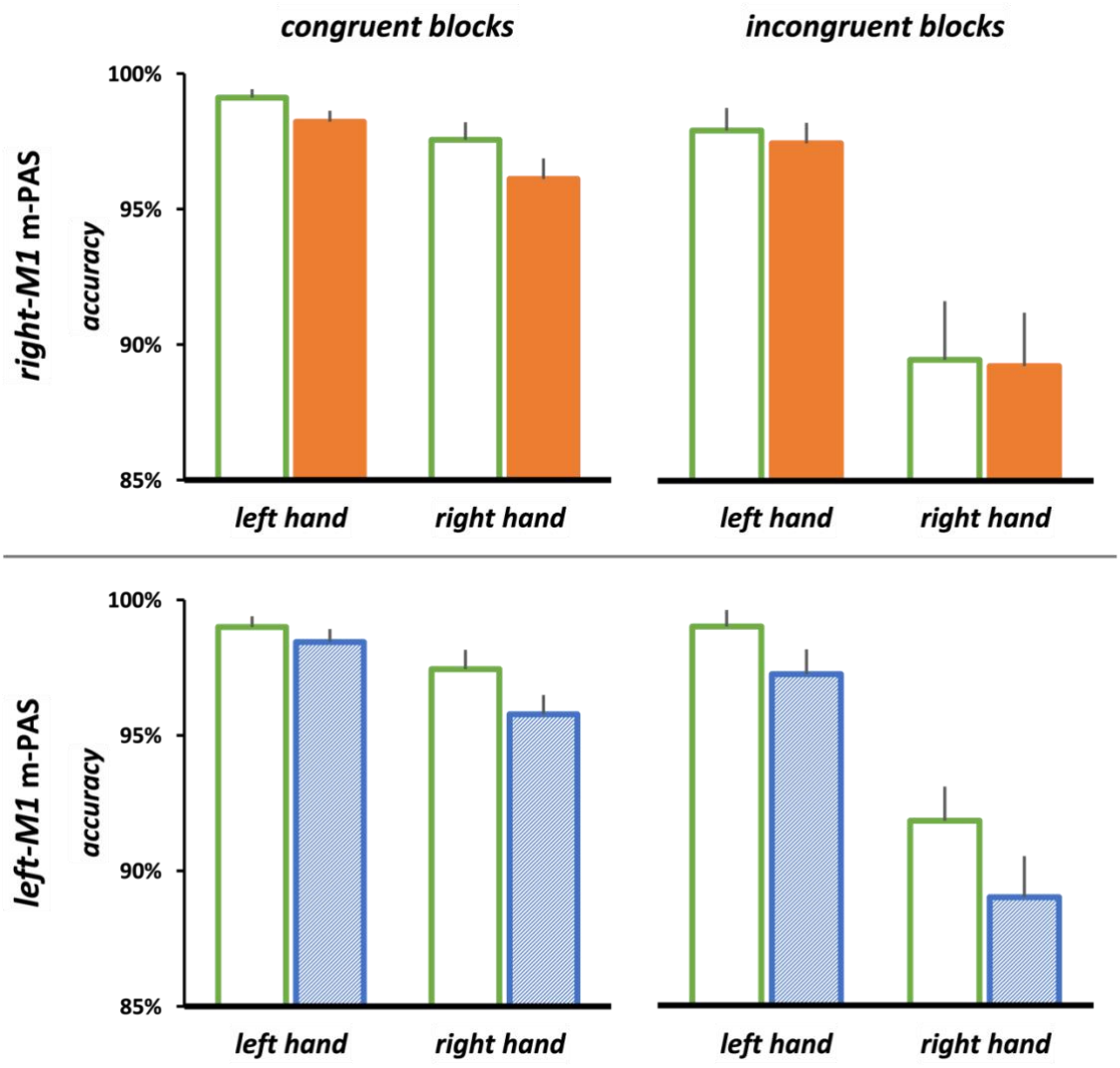

Figure 4.14. Results of *Experiment 3* - accuracy in the imitative compatibility task. Accuracy before (green blank bars) and after *right-M1* m-PAS (orange bars, upper panels) and *left-M1* m-PAS (blue stripped bars, lower panels) in congruent (left panels) and incongruent blocks (right panels). Independently from the condition, incongruent right-hand trials significantly differed from the other three kinds of trial. However, a strong ceiling effect can be observed in all the

4.4.4. Conclusions

Results of *Experiment 3* show once more the effectiveness of the m-PAS targeting right M1 in inducing atypical motor resonance phenomena during the observation of ipsilateral visual stimuli of movements. Conversely, when the protocol targets the left M1 (contralateral to the visual hand stimulus), no change in motor resonance is detected. The other key finding of *Experiment 3* is that the *right-M1* m-PAS is capable to induce a behavioral modification in an imitative compatibility task: indeed, after the protocol administration, participants' ΔRTs between incongruent and congruent trials (a marker of *automatic imitation*) does not differ to the baseline only in trials depicting right hands (i.e., the same hand conditioned during the m-PAS) while it decreases in trials depicting left ones. Crucially, after the *left-M1* m-PAS, such decrement occurs for both the depicted hands. Furthermore, after the *right-M1* m-PAS, participants' pattern of response (i.e., $\Delta RT_{Incongr\text{-}Congr}$) is no more correlated to the one followed before the protocol administration.

4.5. General 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 all experiments, I 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 (Aziz-Zadeh et al., 2002; Maeda, Kleiner-Fisman, & Pascual-Leone, 2002b), while it does not occurs when viewing ipsilateral (right) hand movements or the opening/closing movement of a pair of scissors (Rizzolatti & Craighero, 2004). The key finding is the emergence of motor facilitation contingent upon the observation of ipsilateral (right) hand movements selectively after the m-PAS with an ISI of 25 ms while no effects are induced (*a*) after the one with an ISI of 250 ms (*Experiment 1*), (*b*) after the *scissors*-PAS, pairing motor cortical stimulation with the view of non-biological movements (*Experiment 2*) and (*c*) after the *left-hemisphere* m-PAS, pairing TMS over left M1 with the view of contralateral (right) movements (i.e., the same visual stimuli of the standard m-PAS) (*Experiment 3*). Additionally, no effect is induced by the m-PAS on MEPs recorded from ADM, confirming that motor resonance follows somatotopic rules (Avenanti et al., 2007; Naish et al., 2014). Finally, in *Experiment 3*, modulation of RTs has been found in the imitative compatibility task, suggesting that m-PAS has an impact also at a behavioral level.

4.5.1. m-PAS effects on motor resonance

In *Experiment 1*, following the m-PAS with an ISI of 25 ms, the observation of index-finger movements of the right-hand causes motor facilitation, which was absent in the baseline, and still absent after the protocol exploiting a longer ISI of 250 ms. Noteworthy, the magnitude of m-PAS$_{25ms}$-induced motor facilitation is comparable to the 'normal' motor facilitation for contralateral hand movements detected in the baseline, which remains unaffected by the protocol. This evidence suggests that the hemispheric-specific motor resonance develops from the extraction of a statistical relationship between our actions and their sensory consequences (Keysers & Gazzola, 2014). The m-PAS is able to promote the emergence of a novel link between visual and motor representations, so that motor neurons to respond to the view of unusual (here ipsilateral) motor programs. Importantly, my results 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 (Scott, 2016). Conversely, if the transcranial activation of M1 is delivered with the chronometry of MNS activation (250 ms) (Cavallo et al., 2014; Naish et al., 2014), no visuo-motor association can be created.

It can be speculated that Hebbian learning driven by the m-PAS is, therefore, a bottom-up, plastic, process that starts with the induction of associative 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 (Keysers & Gazzola, 2014). In the superior temporal sulcus (STS), spiking of neurons representing the vision of action occurs later than that of the premotor neurons that trigger the same action (Keysers, Xiao, Földiák, & Perrett, 2001; Molenberghs, Brander, Mattingley, & Cunnington, 2010); 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 (Keysers & Gazzola, 2014; Kilner, Friston, et al., 2007).

The results of *Experiment 2* confirmed the speculation that the associative plasticity induced by the m-PAS likely occurs within the MNS. 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) does not promote the emergence of novel visuo-motor associations for the view of a tool (i.e., the pair of scissors) (e.g., A. Engel, Burke, Fiehler, Bien, & Rosler, 2008; Kessler et al., 2006). This evidence strongly supports the specific recruitment of the *action observation network* during m-PAS, and thus our conclusion that Hebbian associative plasticity within the MNS mediates the formation of visuo-motor associations.

Finally, the ineffectiveness of the m-PAS targeting the left M1 (the 'correct' hemisphere concerning the depicted visual stimulus of movement) found in *Experiment 3* allows me to better define the functional properties of the protocol. This result suggests that to induce associative plasticity within the MNS, and thus reshape motor resonance, the stimulation of the ipsilateral (with respect to the visual stimulus of movement) hemisphere is required for the emergence of the novel visuo-motor association, at least with the ISI of 25 ms. Importantly, in my experiment, the *left-M1* m-PAS acted as a mere control condition for the standard m-PAS targeting the right M1, and the possible neurophysiological outcomes of such control protocol were not tested.

4.5.2. Behavioral effects of m-PAS

Besides MEPs recorded during action observation, in *Experiment 3*, the m-PAS is found capable to modulate participants' RTs in an imitative compatibility task. As said in **Chapter**

2 such a task allows to measure *automatic imitation*, a behavioral marker of MNS activation (Heyes, 2011), in the present study computed as the difference between RTs in incongruent and congruent trials ($\Delta RT_{Incongr-Congr}$) (e.g., Catmur et al., 2009; Mengotti et al., 2013). My findings show that such difference is reduced after the administration of the two m-PAS compared to the baselines, even if with a different pattern between the two protocols. The fact that the reduction in ΔRT seems to be present independently from the session or the viewed hand (i.e., the main effect of factor Time found in the Session X Time X viewed Hand rmANOVA) suggests that such modulation may be explained as a 'learning effect' of the task. Namely, the interference of *automatic imitation* is reduced when participants made the imitative compatibility task after the protocol likely because they already knew the task demand. Incongruent trials, at variance with congruent ones, required also a spatial re-mapping of the stimulus-response association, hence making them more difficult (see) and potentially more sensible to learning processes across a session (Catmur & Heyes, 2011).

Crucially, I found that after the *right-M1* m-PAS this decrement in ΔRTs concerned only the observation of left hands while trials depicting right hands were unaffected. This result suggests that selectively after the m-PAS targeting a novel visuo-motor association, the modulation of *automatic imitation* (i.e., decrement of $\Delta RT_{Incongr-Congr}$ value) is somehow 'blocked' when right hands are observed. These findings lead me to hypothesize that the emergence of motor resonance for right-hand movements found after the m-PAS may interfere with the behavioral performance (and thus with *automatic imitation*). As shown by the neurophysiological results, after the (*right-M1*) m-PAS, thanks to the induction of associative plasticity within the MNS, the observation of a moving right hand recruited also the ipsilateral (right) M1. This atypical (and somehow 'novel') activation may require additional computational resources (i.e., additional recruitment of visuo-motor mirror neurons) within the motor system (and the MNS). This additional recruitment occurred also

during the imitative compatibility task when the right hand is observed, likely interfering with *automatic imitation* processes and leading to the pattern of RTs found. However, from the present results, this hypothesis remains a speculation because I have no evidence of the neurophysiological substrates mediating the effects found in the imitative compatibility task. Nevertheless, a further clue that the *right-M1* m-PAS exerts an effect at a behavioral level comes from the finding that only in the control session (i.e., *left-M1* m-PAS) *automatic imitation* marker ($\Delta RT_{Incongr-Congr}$) is positively correlated before and after the administration of the protocol (namely, participants who have lower/higher $\Delta RT_{Incongr-Congr}$ values still have lower/higher ones after the m-PAS). Future studies should deepen all these findings.

The task that I used suffered from some limitation that has to be highlighted. Firstly, from my results, it cannot be fully ruled out in which extent the spatial processing of the motor response in trials depicting the right hand (i.e., the fact that participants have to respond with the opposite, left, hand) may have contributed to the modulation found in ΔRTs (e.g., Boyer et al., 2012; Cracco et al., 2018; Jansson, Wilson, Williams, & Mon-Williams, 2007; Jiménez et al., 2012). In the second place, the sample size of *Experiment 3* might be relatively small (n = 15) for a behavioral task like the imitative compatibility one, potentially being too sensible (or not enough sensible) to modulation across the different sessions, especially in a repetitive-measures experimental design as the one used here. In the third place, as state in **4.4.2.4.1**, I have 'merged' in the analysis index and middle finger trials, following previous literature (e.g., Catmur & Heyes, 2011). For the sake of completeness, I have also run an explorative analysis with a further 'viewed Finger' within-subjects factor, but no specific effects are detected (Session X Time X viewed Hand X viewed Finger interaction: $F_{1,14} = 1.19$, $p = .321$, $\eta_p^2 = .078$). In future, an imitative compatibility task depicting two movements where the hand muscles involved are different (i.e., observing the little finger instead that the middle one; indeed, this latter movement – used in the imitative

compatibility task – involved FDI as well as the index finger movement) may help to investigate the possible specific effect of the observed finger, which could mirror the 'muscle specificity' found in neurophysiological results. Finally, even if in line with previous literature (e.g., Catmur & Heyes, 2011; Mengotti et al., 2013), the evidence that the task is very simple, and thus that accuracy is almost at the ceiling in every condition (see **Figure 4.14**), may have limited the chance to detect an effect of the m-PAS also on participants' accuracy. Hence, the use of a more complex imitative compatibility task could be appropriate in future studies.

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.

Conclusions and future perspectives

The results of the series of experiments conducted during my doctorate show the efficacy of *cross-systems* PAS protocols targeting cross-modal and sensory-motor networks of the human brain. Both the cm-PAS (**Chapter 3**) and the m-PAS (**Chapter 4**) can induce behavioral and neurophysiological modification through the repeated pairings of peripheral, sensory (visual) stimuli (for the cm-PAS: a hand being touched; for the m-PAS: a moving index-finger) and TMS pulses over cortical areas that are recruited by such paired stimuli through mirroring mechanisms (in the cm-PAS: S1; in the m-PAS: M1). This evidence strongly suggests the induction of Hebbian associative LTP-like plasticity after the two protocols (Caporale & Dan, 2008); induction which is reflected, after the cm-PAS, by an enhancement of tactile acuity (see **3.2**; **3.3**; **3.4** and **3.5**) and a SEP component (P40, see **3.4**), and, after the m-PAS, by the emergence of atypical motor resonance during the observation of ipsilateral (to TMS) movements (see **4.2**; **4.3** and **4.4**) and the modulation of *automatic imitation* in an imitative compatibility task (see **4.4**).

The key variables for the effectiveness of a PAS protocol, i.e., timing dependency and specificity of the two (peripheral and cortical) paired stimulations (Suppa, Quartarone, et al., 2017), have been proven for both my protocols, allowing to state that these PAS induce Hebbian associative plasticity thanks to the specific characteristics of the depicted visual stimulus and the stimulated cortical area. Furthermore, both protocols are been replicated in

different experiments, suggesting that they are reliable tools to modulate S1 and M1 plasticity exploiting an indirect, visual, pathway and the mirror properties of the human brain.

Considering the current literature about PAS protocols (see **Chapter 1**), at the best of my knowledge, my protocols represent the first successful attempt to target high-order networks, like the *tactile mirror system* or the *action observation network,* by pairing peripheral visual stimuli that act through mirror activations (in fact, at date, humans' high-order networks are been studied with PAS only using *cortico-cortical* protocols, see **1.4** and **1.5**). This evidence provides a new frame to investigate the functional interplay of cross-modal and sensory-motor systems, demonstrating once more the usefulness of PAS protocols to study and modulate *in vivo* human brain plasticity, not only in sensory systems but also in complex networks.

Concerning MNS literature (see **Chapter 2**), these findings corroborate *associative* accounts on the origins of mirror neurons' matching properties postulating that these properties (and thus mirror neurons) are a byproduct of experience and they can be shaped through sensory-motor learning (Catmur et al., 2016; Keysers & Gazzola, 2014). Even if previous studies have already highlighted the possibility to influence visuo-tactile and visuo-motor mirror properties of the human brain using non-invasive brain stimulation techniques or behavioral paradigms (e.g., Bolognini, Miniussi, Gallo, & Vallar, 2013; Bolognini et al., 2014; Catmur et al., 2011, 2007; Press et al., 2012), the exploitation of a PAS protocol to achieve this modulation has allowed the testing of the specific hypothesis that Hebbian associative plasticity may be the neurophysiological underpinning. A hypothesis nowadays investigated only using computational models (e.g., Antunes, da Silva, & de Souza, 2018; Hanuschkin et al., 2013). Within the central nervous system, cross-modal integration and sensory-motor processing operate with temporal windows of few milliseconds and precise communication

between specific cortical areas is essential for the integration of different sensory modalities (e.g., Driver & Noesselt, 2008; Ferezou et al., 2007; Henschke et al., 2015; Luo, Kothari, & Moss, 2017; Palva & Palva, 2018). Hence, in my opinion, the high temporal and spatial resolution of the two PAS protocols that I developed represents a strong advantage and differentiates them from other neuro-modulatory paradigms targeting visuo-tactile or visuo-motor integration processes like visuo-tactile coactivation protocols (e.g., Kuehn et al., 2017) or sensory-motor training involving action observation, like *counter-mirror* ones (see **2.2**) (e.g., Bardi et al., 2015; Catmur et al., 2008, 2007; Cavallo et al., 2014).

As stated above, my results suggest the induction of Hebbian associative LTP-like plasticity within the MNS. However, to be able to hypothesize the involvement of STDP, as postulated by recent theorization within the *associative account* framework (Antunes et al., 2018; Keysers & Gazzola, 2014), the induction of LTD (likely mirrored by a decrement of tactile acuity, for the cm-PAS, and a loss of motor resonance, for the m-PAS) should be proved when a different timing between the two paired stimulations is exploited (Markram et al., 2011). Hence, further studies using a wider range of ISIs between the visual stimulus and the TMS pulse of the two PAS protocols may be conducted to better define the neurophysiological properties of the associative plasticity induced by my novel protocols.

Furthermore, from the current results, the precise locus of plasticity (i.e., which synapses within the MNS are the target of the two protocols – and thus which MNS areas mediate the induction of plasticity) can only be speculated. In fact, the neurophysiological pathways activated by the paired stimulations of the two protocols are complex and, hypothetically, both feedforward and feedback connections could play a central role in the induction of plasticity (as stated in the discussion at the end of each study). For instance, the m-PAS, differently from other *cross-systems* protocols acting at a visuo-motor integration level (i.e., the visuo-motor PAS, see **1.3**, Suppa et al., 2015), activated a complex 'cognitive' network

– the *action observation network* – where different cortico-cortical pathways and areas are involved at the same time (e.g., Molenberghs et al., 2012). Hence, I cannot exclude that, similarly to the cm-PAS (see **3.6.1**), predictive-like mechanisms driven by the repetitiveness of the protocol may have influenced the effectiveness of my results (e.g., Kilner et al., 2004). Overall, this neurophysiological complexity is typical of *cross-systems* PAS and only future studies exploiting my two protocols integrated with other neuroscientific techniques like EEG or fMRI would deepen the precise cortical pathway in which associative plasticity is induced or the exact neurofunctional substrates. In this regard, modified PAS protocols targeting frontal networks described in the first chapter (e.g., Momi et al., 2019; Santarnecchi et al., 2018; see **1.5**) offer a good benchmark of PAS potential when integrated with other neurophysiological techniques and, in future, similar investigations could be made also for the cm-PAS and/or the m-PAS. Future studies may also introduce, during the administration of the protocols, a second, conditioning TMS pulse over one of the nodes of the MNS (e.g., PM, IPL, or STS) (Rizzolatti & Craighero, 2004). This would create a sort of cortico-cortical PAS version of the cm-PAS and/or the m-PAS primed by the sensory stimulation (i.e., touch stimulus for the cm-PAS, moving hand for the m-PAS), allowing to better study the contribution of associative areas of the MNS in the induction of plasticity. For instance, such protocols might show their effectiveness only when the conditioning pulses are delivered over temporo-parietal areas like STS or IPL (and not when they target frontal areas like PM), suggesting the mediation of (and, thus, that associative plasticity is induced in) anterior areas of the MNS.

Besides using these protocols as tools to investigate the functional properties of the MNS in the healthy brain, they may be used also in the clinical setting and such exploitation could dramatically increase their potential. Indeed, my findings highlight that is possible to promote associative plasticity in primary sensory-motor areas thanks to a salient visual

stimulus, hence bypassing the afferent, spino-cortical pathway (at variance with the M1-PAS or the S1-PAS): this evidence might be of great importance when translated in a clinical population. For instance, the m-PAS can be tested in stroke patients with cortical damages at the motor system to induce adaptive plasticity in M1 exploiting the spared visual pathway and potentially restore motor functionality. Similarly, the cm-PAS can be used with deafferented patients or patients with phantom limb syndrome to promote plastic changes within S1, bypassing the damaged afferent spino-cortical pathway through the visual one. If such preliminary studies proved to be effective, these protocols can be integrated into long-term rehabilitation programs as a sort of novel 'mirror therapy', likely increasing the positive outcomes of these therapies based on action observation or imitation which effectiveness is still debated or controversial (e.g., Ertelt et al., 2007; Franceschini et al., 2012; Garrison, Winstein, & Aziz-Zadeh, 2010; Ramachandran & Altschuler, 2009; Tani et al., 2018; Zhang, Fong, Welage, & Liu, 2018). For example, in the future, the m-PAS could be a very promising rehabilitation tool due to the ease of administration, not requiring any active, voluntary movement from the patient and its relatively short length (i.e., 15 min).

Finally, another line of research could be focused on deepening the neurophysiological changes that can be induced by the two protocols in a specific clinical population compared to a healthy sample, to understand if a dysfunctional brain can be plastic similar to a healthy one and, thus, whether PAS protocols are still effective. For example, considering the key role of the MNS in psychiatric disorders like schizophrenia (e.g., McCormick et al., 2012; Meherwan et al., 2014) or borderline personality disorder (e.g., Beeney, Hallquist, Ellison, & Levy, 2016; Mier et al., 2013), future studies may deepen whether the cm-PAS and/or the m-PAS are still effective in such pathologies and which differences occur in such patients where the MNS – and thus the *action observation network* and the *tactile mirror system* – is known to be partially dysfunctional (Iacoboni & Dapretto, 2006).

In conclusion, the results of my thesis show the efficacy of the two PAS protocols I developed and shed more light on the plastic mechanisms that rule cross-modal and sensory-motor networks with mirror properties in the adult brain, suggesting that Hebbian associative plasticity plays a central role within these networks. Even if very promising, my findings – and hence the two *cross-systems* PAS developed during my doctorate – can be considered first steps towards a better understanding of the neural plastic mechanisms that rule high-order cross-modal and sensory-motor integration within our brain. Only the future will show whether these steps will successfully lead to a leap which could fill the aforementioned gap in the literature.

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[7] This part is written in Italian because it is likely the only part of the thesis which would be read by curious readers.

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