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**Unravelling the physiology of facial motor control:
voluntary and emotional pathways**

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Index

Summary.....	5
Introduction.....	6
Facial expressions production.....	7
Facial expressions recognition	12
<i>Research questions 1: Transcallosal connections of face and hand representation areas in the primary motor cortex: a TMS and TMS-EEG study.</i>	16
Introduction	17
Materials and methods	19
Ethical Approval	19
Electromyography	19
Transcranial magnetic stimulation	19
TMS-EEG	20
Experimental design	22
Experiment 1: interhemispheric inhibition in face and hand M1 representation areas.....	22
Experiment 2: interhemispheric signal propagation (ISP) in face and hand M1 representation areas.	23
Statistical analysis.....	23
Results.....	24
Discussion	30
Conclusions	35
<i>Research questions 2: Aging alters facial expressions processing and recognition: insights on possible neural mechanisms.....</i>	36
Introduction	37
Materials and Methods	40
Participants.....	40
Facial expressions stimuli (FES).....	40
Fixation patterns recordings	41
Electromyography (EMG)	42
Transcranial magnetic stimulation (TMS).....	42
Event Related Potentials (ERP)	42
Experimental design	43
Experiment 1: Gaze and fixation pattern during passive viewing of FES	44
Experiment 2: Influence exerted by FES on the P100 and N170 waves	44
Experiment 3: Influence exerted by FES on face M1 excitability.....	44
Experiment 4: Accuracy and response time to FES	44
Statistical analysis.....	45
Results.....	46
Discussion	54
Role of the gaze in the facial expressions processing	54
Role of the perceptive cortical areas in facial expressions recognition.....	55

The effect of facial expressions on M1 excitability	56
Facial expressions recognition in senior subjects.....	57
Conclusions	59
<i>Research questions 3: Sensorimotor integration and faces recognition in a model of altered face sensory information: neurophysiological study in patients with dentofacial deformities.</i>	<i>60</i>
Introduction	61
Materials and methods.....	63
Participants.....	63
Surgical treatment	64
Facial expressions stimuli	64
Electromyography	65
Transcranial magnetic stimulation	65
Electrical stimulation	66
Experimental design.....	67
Experiment 1: Study of brainstem circuits excitability in physiological conditions and in the presence of trigeminal nerve afferents alteration.....	67
Experiment 2: Study of excitability and SMI in DAO M1 representation area in physiological conditions and in the presence of trigeminal nerve afferents alteration.....	68
Experiment 3: Study of excitability and SMI in AD M1 representation area in physiological conditions and in the presence of trigeminal nerve afferents alteration.....	68
Experiment 4: Effect of the trigeminal nerve alteration on the recognition of facial expressions.....	69
Statistical analysis.....	69
Results.....	70
Discussion	76
Peripheral and subcortical excitability	77
Intracortical excitability and SMI interactions	77
Trigeminal nerve alteration and its influence on facial expressions recognition.....	80
Conclusions	82
<i>General Discussion</i>	<i>82</i>
References.....	85

Summary

Facial expressions are vital for social behaviour, especially for non-verbal communication since they convey human emotional states. Facial expressions are performed by the coordinated activity of facial muscles. These muscles have specific anatomical and histological features due to the wide range of functions they perform. However, the precise mechanisms of how these muscles are coordinated and influenced by emotional processes is still unclear.

Therefore, the scientific work done during this 3-year PhD program aimed at investigating the complex neural mechanisms involved in voluntary facial movements as well as the execution and recognition of facial expressions, focusing on the integration of sensory, motor, and cognitive components in facial expression recognition and examining how these processes are influenced by altered sensory information, aging.

Results provided the first demonstration of: 1) how interhemispheric connectivity in the face motor cortex is functionally distinct from the hand system, supporting a specialized and lightly coupled transcallosal organization for face motor control, 2) how aging influences facial expressions recognition, 3) how impaired sensorimotor integration leads to a reduction of facial expressions recognition ability.

Introduction

Facial muscles play a pivotal role in non-verbal communication, acting as a primary means of expressing emotional states and facilitating social interaction. These muscles are not only essential for facial expressions but also for their participation in vital functions such as mastication, speech production, and conveying affective responses (Cattaneo and Pavesi, 2014, Bress and Cascio, 2025). The uniqueness of the facial muscles lies in their structural and functional characteristics. Indeed, unlike other skeletal muscles, facial muscles are directly attached to the skin, enabling subtle and complex movements that allow for complex emotional expressions (Müri, 2016).

Facial muscles can be divided into two main groups: the masticatory muscles and the cutaneous muscles, each serving distinct functions and being innervated by different cranial nerves. The masticatory muscles group includes the masseter, temporalis, and pterygoid muscles. They are innervated by the trigeminal nerve (cranial nerve V) and are mainly involved in chewing, jaw movements, and positioning of the mandible (D'Andrea and Barbaix, 2006). Although these muscles play a critical role in mastication, they have a minimal role in face expressiveness.

In contrast, the cutaneous muscles are responsible for facial expressions and are mainly innervated by the facial nerve (cranial nerve VII) (Müri, 2016) (Figure 1). The cutaneous muscles can be further divided into two categories: the cranial muscles (temporoparietal and occipitofrontal regions) and the face muscles, which surround critical areas such as the nose, mouth, eyelids, and auricular region (Müri, 2016). The latter group includes: the orbicularis oculi, for eyelid movements; the zygomaticus major and the zygomaticus minor, which elevate the corners of the mouth and upper lip, facilitating smiling; the levator labii superioris, which lifts the upper lip; the levator anguli oris, which raises the mouth corners during smiling (Wachtel et al., 2008); the orbicularis oris, which encircles the mouth and enables lip closure, puckering, and articulation during speech (Franz and Eickhoff, 2013; Standring, 2016), the depressor anguli oris (DAO), which plays a crucial role in sad expressions by drawing the corners of the mouth downward (Ekman and Friesen, 1978; Hur et al., 2011); the depressor labii inferioris, which pulls the lower lip downward and laterally, often seen in expressions of doubt or sadness (Hur et al., 2011); and the mentalis, which protrudes and wrinkles the chin, producing a pouting expression (Chuang et al., 2004).

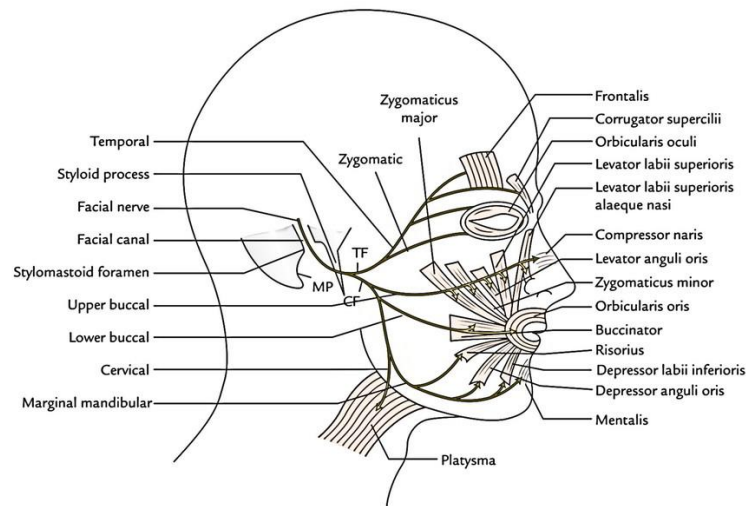


Figure 1: Schematic representation of face muscles. The facial nerve (CN VII) exits the skull through the stylomastoid foramen and forms the main trunk (MP) before entering the parotid gland, where it divides into the temporofacial (TF) and cervicofacial (CF) divisions. These two trunks give rise to the five terminal branches—temporal, zygomatic, buccal, marginal mandibular, and cervical—which radiate across the face to innervate the muscles of facial expression, including the frontalis, orbicularis oculi, zygomaticus, buccinator, orbicularis oris, and platysma. This branching network, known as the parotid plexus (pes anserinus major), ensures coordinated activation of upper and lower facial muscles during voluntary and emotional expression. (Modified from <https://www.earthslab.com/anatomy/face-anatomy/>)

The face muscles are characterized by a complex arrangement of muscular fascicles embedded in a variable connective tissue matrix beneath the skin. This forms a two-dimensional structure distinct from the three-dimensional muscle systems found in the limbs, where muscles are attached to bones and surrounded by connective tissue (D'Andrea and Barbaix, 2006). Notably, the muscles around the mouth, which are part of the superficial layer, overlap and function as a cohesive unit, effectively operating as a single morphological structure (D'Andrea and Barbaix, 2006). This unique organization underlies the complexity of face movements compared to other body regions, where muscle action is more isolated.

Facial expressions production

From a physiological point of view, the motor control of face muscles involves a sophisticated interaction between peripheral neuromuscular structures and central motor systems. At the peripheral level, the muscles involved in facial expressions are innervated by the facial nerve (cranial nerve VII), which arises from the facial nucleus in the pons and exits the skull through the stylomastoid foramen. This nerve divides into five terminal branches: temporal, zygomatic, buccal, mandibular, and cervical that innervate muscles across different regions (Standring, 2016; Purves et al., 2008). Face muscles also receive proprioceptive and tactile feedback from the trigeminal nerve (cranial nerve V), which supplies sensory

innervation to most of the face (Hannam et al., 2008). This sensory feedback is essential to refine motor output, coordinate symmetry, and regulate the intensity of expression in real time, ensuring adaptive and context-appropriate movements (Müri, 2016). Although face muscles lack classical muscle spindles, Ruffini-like receptors in the skin and muscles may contribute to proprioception, although this remains debated (Connor and Abbs, 1998; Nordin and Thomander, 1989). Regarding somatosensory afferent pathways, face muscles differ from other muscles of the body. Peripherally, sensory fibres transmit information to the posterior horns of the spinal cord, which then project to the thalamus and subsequently to the somatosensory cortex (S1) (Hall and Hall, 2020). In contrast, sensory information from the face travels through the trigeminal nerve and is relayed to three distinct nuclei in the brainstem: the mesencephalic nucleus, which processes proprioceptive signals from the masticatory and extraocular muscles; the principal nucleus, which handles epicritic sensory information from the face and oral mucosa; and the spinal trigeminal nucleus, which transmits nociceptive and thermal information (Hall and Hall, 2020).

The motor control of the face muscles is mediated by the facial nerve and its nucleus. The facial nucleus, located in the caudal portion of the ventrolateral pontine tegmentum, is the largest motor nucleus in the human brainstem (Cattaneo and Pavesi, 2014). It is organized in a musculotopic manner, where neurons innervating the same muscles are grouped together in longitudinal columns along a craniocaudal axis (Morecraft et al., 2001). This organization, as illustrated in (Figure 2), shows four main subnuclei: the dorsal subgroup, which innervates muscles around the eyes; the medial subgroup, which controls auricular muscles; and the intermediate and lateral subnuclei, which innervate the perioral region. The lateral subgroup, the largest, is further divided into a dorsal portion controlling the upper mouth and a ventral portion controlling the lower mouth (Holstege, 2002; Jenny and Saper, 1987).

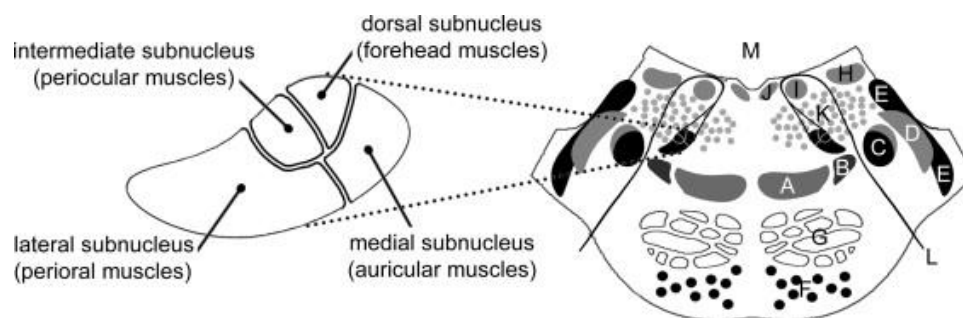


Figure 2: Anatomical representation of the motor nucleus of the facial nerve (VII cranial nerve). Right panel: Transverse section of the lower pons in humans showing the location of the facial nucleus and neighboring structures. (A) Medial lemniscus; (B) spinothalamic tract; (C) spinal trigeminal nucleus and tract; (D) inferior cerebellar peduncle; (E) cochlear nuclei; (F) basal nuclei of the

pons; (G) pyramidal tract; (H) vestibular nuclei; (I) nucleus of the abducens nerve; (J) medial longitudinal fascicle; (K) pontine reticular formation; (M) fourth ventricle. Left panel: Enlarged section of the facial nucleus indicating the division in subnuclei and the muscular groups innervated by motoneurons in each subnucleus. (Cattaneo & Pavesi, *Neuroscience & Biobehavioral Reviews*, 2014)

The facial nucleus receives motor projections from the face area representation in the primary motor cortex (M1). Research indicates that in humans, the face representations area of motor cortices is notably thicker and has more complex local circuitry compared to other species. Additionally, the face muscles have the highest degree of reliance on M1 for motor control, more so than other muscle groups represented in M1 (Cattaneo and Pavesi, 2014; Müri, 2016). It is important to note that the face representation in M1 sends bilateral projections, with a stronger influence on the contralateral side (Paradiso et al., 2005; Pilurzi et al., 2013). This pattern of cortical innervation of face muscles has been confirmed through studies in non-human primates (Morecraft et al., 2001) and in humans using transcranial magnetic stimulation (TMS) (Benecke et al., 1988; Meyer et al., 1994; Urban et al., 1997; Liščić and Zidar, 1998; Pilurzi et al., 2013) and profoundly differs from the motor control of limb muscles, where M1 projects exclusively contralaterally (Rothwell et al., 2021).

In non-human primates, several studies have shown that the facial nucleus receives input from various cortical regions beyond M1 (Morecraft et al., 1996, 2001). Notably, projections from both the dorsal (dPM) and ventral (vPM) premotor areas, located anterior to the M1 face representation, contralaterally innervate the lateral subnucleus of the facial nucleus, which controls muscles around the lower mouth (Morecraft et al., 2001). Furthermore, the caudal section of the anterior cingulate motor cortex (M4) also projects to the lateral subnucleus, specifically targeting the dorsolateral region that controls the upper mouth muscles (Morecraft et al., 2001). Bilateral projections to the medial subnucleus of the facial nucleus, responsible for the muscles around the eyes, originate from the supplementary motor area (SMA) and the rostral part of the anterior cingulate cortex (M3) (Morecraft et al., 1996, 2001) (Figure 3).

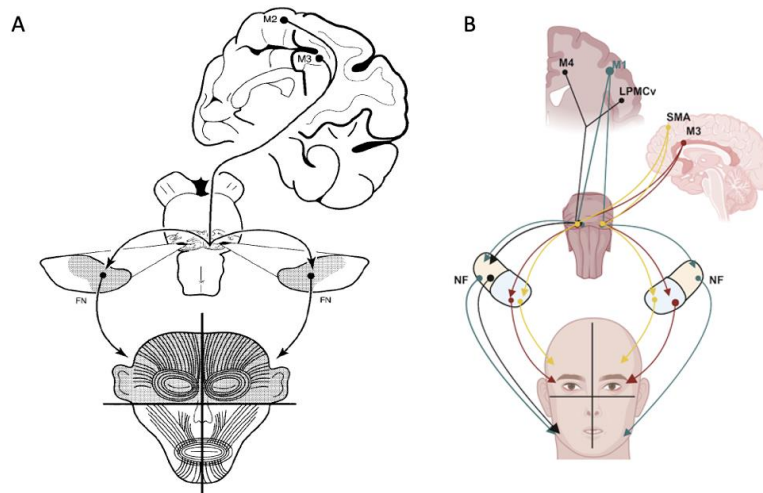


Figure 3: Schematic representation of cortical pathways controlling facial motor output. A) Summary diagram illustrating cortical face areas giving rise to bilateral innervation of the intermediate, dorsal and medial subnuclei. These subnuclei were all found to innervate upper facial musculature. This findings suggest that sparing of the upper facial musculature following middle cerebral artery infarction is due to sparing of the projection from M2 and M3, which reside in the territory of the anterior cerebral artery. The present findings and the fact that the rostral cingulate motor cortex (M3) receives widespread limbic and prefrontal inputs (Morecraft and Van Hoesen, 1993, 1998; Morecraft et al., 1998, 2000), and is itself considered one of the higher-order motor areas in the cortex (Shima et al., 1991; Shima and Tanji, 1998), supports the view that M3 may be involved in mediating upper facial expression of higher-order emotions (Damasio, 1994). FN, facial nucleus. B) The primary motor cortex (M1) and its premotor (LPMCv) and supplementary motor areas (SMA) project bilaterally to the facial nuclei (NF) in the brainstem, which innervate the facial muscles. These interhemispheric and corticobulbar projections enable symmetrical coordination of facial movements during expression and speech (Conti 2025).

Interestingly, the cingulate areas serve as critical relay points for limbic inputs into the face motor system, as demonstrated by connections between the limbic system and the prefrontal cortex to M3 (Morecraft and van Hoesen, 1993; 2007). Further studies have identified projections from the amygdala to M3 which in turn projects to the facial nucleus, suggesting the involvement of this pathway in regulating emotional facial expressions such as fear, anger, happiness, and sadness (Shima et al., 1991; Shima and Tanji, 1998; Morecraft et al., 2007). This role is supported by animal studies showing the activation of mirror neurons during the observation of face movements (Ferrari et al., 2003). These neurons, located in the lateral vPM and the opercular area, are strongly connected with the dPM, the insula, somatosensory cortices (I and II), intraparietal regions, the ventral prefrontal cortex, the striatum, amygdala, and thalamus. These networks are crucial for interpreting the facial expressions of others (Ferrari et al., 2017). This mirror neuron system for face movements differs from the hand, where visual information from the superior temporal sulcus is relayed to parietal areas before reaching the hand motor representation in vPM (Bonini et al., 2010; Gerbella et al., 2011). The differences in the development of the mirror neuron circuits related to emotional face movements can be attributed to the distinct developmental trajectories of hand and face muscle functions. During infancy, the development of hand

movement is largely supported by visual feedback from the child's own hand movements, integrating visuomotor information within the parietal-premotor networks (Del Giudice et al., 2009; Casile et al., 2011; Ferrari et al., 2013). In contrast, infants cannot observe their own facial expressions, and thus, caregivers, particularly the mother, play a crucial role in providing feedback on facial expressions and emotional states, often through imitation, reinforcing the emotional significance of the face's movements (Murray et al., 2016). This context likely facilitates the formation of face-related mirror neurons, which may emerge within the realms of communication and emotional exchange.

An additional difference between the control of limb and face muscles lies in the transcallosal interactions between the motor cortices of both hemispheres. For the upper limbs, the hand motor area in one hemisphere can inhibit the contralateral motor area to facilitate asymmetrical movements of the arms (Ferber et al., 1992). However, a previous TMS study found no evidence of such interaction between reciprocal face M1, suggesting either that this interaction does not occur or should be considerably weaker (Ginatempo et al., 2019a, Ginatempo et al., 2025).

Beyond interhemispheric connectivity, recent studies have highlighted further distinctions in motor control across effectors. Ginatempo et al. (2024) demonstrated that sensorimotor integration in cranial-cervical muscles is strongly shaped by the type of sensory afferent and by the functional role of the target muscle, with the *depressor angulis oris* (DAO) muscle showing distinct patterns of short afferent inhibition (SAI) and long-latency afferent inhibition (LAI) depending on trigeminal or facial input. In addition, Ginatempo et al. (2023) showed that intracortical inhibitory processes, such as short latency intracortical inhibition (SICI), are expressed differently in face compared to hand M1, pointing to a unique balance of excitatory and inhibitory circuits in the cortical control of face movements. Despite these functional distinctions, both face and hand motor areas receive comparable cerebellar projections, indicating that cerebellar output exerts a common modulatory influence on motor control across different muscle groups (Ginatempo et al., 2023; Ginatempo et al., 2024).

Furthermore, studies of patients with face movements impairments have highlighted a separation between emotional and voluntary face movements and their corresponding neural pathways. For instance, voluntary face paralysis which is defined as the inability to move the face intentionally on command, while spontaneous emotional expressions remain intact, can occur following lesions in regions such as the M1, the descending pyramidal tract, or the

brainstem (Hopf et al., 1992; Töpper et al., 1995; Trepel et al., 1996; Urban et al., 1998, 2001; Bouras et al., 2007; Kappos and Mehling, 2010). On the other hand, less common emotional face paralysis which is the converse condition in which spontaneous expressions (e.g., smiling when amused) are impaired, but voluntary movements are preserved, has been observed following damage to areas such as the contralateral anterolateral and posterior thalamus, the anterior striatocapsular region, or the medial frontal lobes (Bogousslavsky et al., 1987; Trosch et al., 1990; Hopf et al., 1992, 2000; Ross and Mathiesen, 1998; Michel et al., 2008). Additionally, emotional palsy can also arise after lesions in the brainstem, specifically in the medulla (Cerrato et al., 2003) or the ipsilateral pons (Hopf et al., 1992), supporting the idea that the neural pathways for voluntary and emotional facial expressions are independent up to the level of the facial nucleus.

Facial expressions recognition

Recognizing emotions expressed by others through their face is a crucial aspect of understanding non-verbal communication and enhancing social interactions (Blair et al., 2003, 2004). Facial expressions are thought to represent innate, automatic responses to stimuli that hold emotional significance. Ekman (1973) proposed that there are six basic emotions, which are universally recognized across cultures: happiness, sadness, fear, disgust, anger, and surprise (Ekman, 1994)(Figure 4).



Figure 4: Examples of the Ekman-Friesen pictures of facial emotions used in the computerized task. Sad, happy, angry, surprise, disgust and fear. (Ekman, 1994)

Research indicates that emotional information from facial expressions is processed based on the geometric features of the face, distinguishing emotional content through morphological categories (De Gelder et al., 1997; Calder et al., 2001). Moreover, face features not only convey emotional information but also provide cues about other characteristics such as

gender, identity, and age, which are processed separately at different stages (Adolphs, 2002). These various types of information may be handled by different subsystems in the brain, allowing for the discrimination of categorical perceptions (Adolphs, 2002) (figure 5).

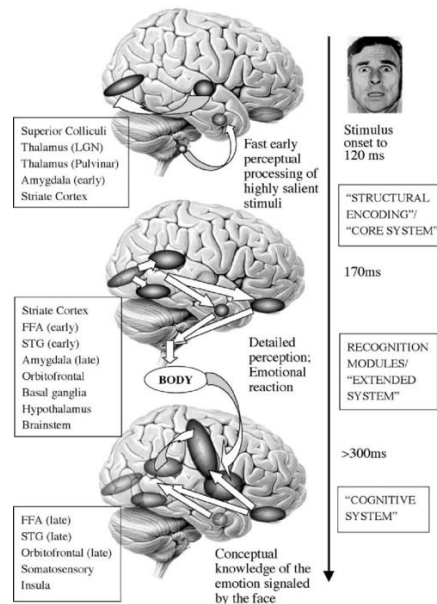


Figure 5: Processing of emotional face expressions. On the left are indicated some of the structures, shown in the middle, that are involved in the processing stages, indicated on the right portion. The figure begins with the onset of the stimulus, a face expressing an emotion at the top, and progresses through perception to final recognition of the emotion at the bottom. Certain brain structures are preferentially engaged in processing structural information of the stimulus (early perception), whereas others participate more in retrieving conceptual knowledge (detailed perception). LGN = lateral geniculate nucleus; FFA = fusiform face area; STG = superior temporal gyrus. (Adolphs, *Behavioral & Cognitive Neuroscience Reviews*, 2002)

Facial expression recognition relies on different neural pathways, primarily divided into subcortical and cortical systems. The subcortical pathway provides rapid and automatic processing of emotional stimuli, bypassing the primary visual cortex. This route originates in the retina, projects to the superior colliculus, and then relays information to the pulvinar nucleus of the thalamus before reaching the amygdala (Tamietto and de Gelder, 2010; Johnson, 2005). The amygdala plays a key role in detecting emotionally salient cues and modulating the response of other brain areas, including the motor system (Vuilleumier and Pourtois, 2007; Adolphs, 2008). Importantly, even in the absence of striate cortex input, residual emotional facial expression processing can still occur, reinforcing the role of subcortical mechanisms (Rodman et al., 1989; Morris et al., 1999). The cortical pathway, in contrast, involves higher-order perceptual analysis in the occipito-temporal cortex, particularly within the fusiform face area and superior temporal sulcus, which process configural and dynamic aspects of faces (Haxby et al., 2000; Duchaine and Yovel, 2015). Event-related potential (ERP) studies suggested that facial expressions discrimination occurs within 80–110 ms in the occipital cortex, followed by more refined processing in the

temporal lobe around 130–200 ms (Eimer and Holmes, 2002; Hinojosa et al., 2015). While some studies have reported early emotion-specific neural activity (Pizzagalli et al., 1999; Schyns et al., 2007), others have failed to identify clear expression-related effects in early visual processing stages, suggesting that emotional discrimination may occur later, beyond 200 ms (Luo et al., 2010; Vuilleumier and Pourtois, 2007).

Recently, researchers suggested an alternative and complementary hypothesis which is the simulation hypothesis, postulating that the recognition of others' expressions involves the internal simulation of corresponding motor representations (Goldman and Sripada, 2005; Niedenthal, 2007). This hypothesis was inspired by early primate studies showing that neurons in the premotor cortex fire both during the execution and observation of the same action (Gallese et al., 1996; Rizzolatti et al., 1999). Human neuroimaging studies confirmed that the vPM is activated during both the observation and execution of movements, suggesting the existence of an action observation–execution matching system (Sato et al., 2023). Support for this hypothesis comes from TMS studies demonstrating that observing facial expressions modulates corticospinal excitability, particularly the M1 (Carr et al., 2003; Balconi and Bortolotti, 2013; Paracampo et al., 2017; Ginatempo et al., 2020). For instance, it has been shown that the observation of happy expressions selectively enhances face M1 excitability, while negative expressions primarily modulate hand M1 activity (Ginatempo et al., 2020). Beyond M1, additional evidence indicates that a broader motor network, including premotor and supplementary motor areas, the insula, and the cerebellum, is also engaged during facial expressions processing (Hennenlotter et al., 2005; Ferri et al., 2016; Wood et al., 2016). These findings suggest that emotional face recognition depend on a distributed motor system, where both cortical and subcortical regions contribute to simulating and decoding the affective meaning of expressions.

Since recognizing facial expressions is a complex process involving perceptual and motor network activity, it is important to investigate how physiological and pathological factors influences facial expressions processing and face motor control.

In this context, although the neural basis of face motor control and facial expressions recognition has been widely investigated, several key issues remain unresolved. One concerns connectivity: while interhemispheric interactions of limb motor areas are well established, much less is known about how the two hemispheres coordinate activity in the face motor system, leaving the organization of transcallosal pathways largely unclear.

Another gap relates specifically to aging: although older adults show marked deficits in recognizing emotions, it is still uncertain whether these changes are confined to perceptual mechanisms or also extend to motor cortical areas such as M1. Finally, the role of sensorimotor integration itself in facial expressions recognition has been understudied, particularly under conditions of altered sensory input. In this perspective, clinical models such as dentofacial deformities provide a unique opportunity to examine how disrupted afferent feedback influences motor responses and emotion recognition.

Therefore, the aims of the present work were to investigate various aspects of face motor system function, face recognition, and the neural mechanisms involved in both motor and perceptual processing of facial expressions. These objectives were achieved through three studies, described below.

In the first study, the transcallosal connections between the face and hand motor representations in the M1 were explored to investigate the neural pathways that mediate the interaction between motor cortices and how these interactions influence face and hand movement control, with a specific focus on the bilateral connections in the face motor system.

The second study focused on the aging-related changes in the processing and recognition of facial expressions. This study aimed to uncover the neural mechanisms behind the decline in emotional recognition, emphasizing how age-related changes in brain activity might influence the face motor system and emotion processing pathways.

Finally, the third study investigates the SMI involved in facial expressions recognition using patients with dentofacial deformities as a model of altered SMI, focusing on how altered sensory input affects the neural mechanisms responsible for face processing and SMI coordination. This neurophysiological approach aimed to understand the complex connection between face motor responses and sensory processing in individuals with dentofacial deformities and how this impact facial expressions recognition.

Research questions 1: Transcallosal connections of face and hand representation areas in the primary motor cortex: a TMS and TMS-EEG study.

Introduction

The muscles of the lower half of the face receive bilateral projections from face primary motor cortex (fM1) with a contralateral predominance (Meyer et al., 1994; Benecke et al., 1988; Triggs et al., 2005; Urban et al., 1997, 2001; Werhahn et al., 1995; Cattaneo and Pavesi, 2014; Pilurzi et al., 2013). In particular, Pilurzi and colleagues (2013), in their transcranial magnetic stimulation (TMS) study, demonstrated that fM1 sends bilateral asymmetric projections to the lower face muscles, namely the DAO, and hypothesized that the ipsilateral response resulted from the direct corticobulbar projections, rather than the transcallosal connection. The corpus callosum is the largest white matter fiber bundle in the human brain (Ruddy et al., 2017) that plays a crucial role in connecting homologous cortical regions of the two hemispheres and represents an important pathway for interhemispheric communication. By playing an excitatory or inhibitory role, this structure is fundamental in limiting excessive crosstalk between hemispheres when cortical regions are asymmetrically activated, and in maintaining laterality and hemispheric specialization in motor and frontal regions (Voineskos et al., 2010).

Anatomical tracer studies in animals demonstrated that fM1 is connected with its homolog in the other hemisphere through callosal fibers at least in the owl monkey (Gould et al., 1986) and in the macaque monkey (Rouiller et al., 1994). In contrast with these findings, a neuroimaging study in humans, failed to identify transcallosal motor fibers connecting the lip areas in fM1 (Wahl et al., 2007). This difference may have an evolutionary explanation. In fact, face muscles are involved in the emotional expressiveness and their motor control in humans has differently changed from other animals to allow an evolutionary advantage in social behavior (Darwin, 1872). Thus, this peculiar feature of face motor system raised the doubt whether the transcallosal connection between fM1s is anatomically absent in humans (Wahl et al., 2007), as suggested by the above neuroimaging study, or just functionally inactive or poorly active, as suggested by neurophysiological studies (Ginatempo et al., 2019).

In humans, the transcallosal pathway can be functionally probed through paired-pulse TMS protocols which allow the assessment of interhemispheric inhibition (IHI). In this protocol, a conditioning stimulus applied over one M1 6-15 ms before the test stimulus over the other M1 produces a suppression of the motor evoked potentials (MEP) in the contralateral muscle (Ferbert et al., 1992). It has been demonstrated that this phenomenon is due to the activation of transcallosal outputs by the conditioning pulse since this effect was absent in patients with

agenesis of the corpus callosum (Meyer et al. 1995). IHI has been widely investigated and characterized in hand muscles, where it is considered fundamental in the production of voluntary asymmetrical movements (Ferber et al., 1992; Ziemann and Hallet, 2001). By contrast, in face muscles, IHI seems to be lacking, as demonstrated by a recent study (Ginatempo et al. 2019), which suggested that the corpus callosum is barely involved in the bilateral control of face muscles, probably because they are rarely activated asymmetrically (Cattaneo and Pavesi, 2014; Ginatempo et al., 2019). However, the same authors, proved the presence of IHI in fM1 of professional woodwind musicians, while failing to detect it in the fM1 of string-musicians and non-musicians (Ginatempo et al., 2021). These results emphasize the importance of IHI when fine and asymmetric movements are performed to control lower face muscles, as required by the complex task of playing woodwind instruments (Ginatempo et al., 2021). These findings suggest that fM1s are anatomically connected through the transcallosal pathway, but such connection is generally inactive, unless face muscles have developed special skills, as those required by the long-term learning and execution of fine movements, like playing woodwind instruments since childhood.

Based on the above background and given the contrasting results on the presence of transcallosal connection between fM1s in humans, with an anatomical-neuroimaging study (Wahl et al., 2007) suggesting its lack while the TMS-functional study (Ginatempo et al., 2021) suggesting its presence, in the present work it seemed a worth endeavor to adopt an approach which combines TMS with electroencephalographic (EEG) recordings. This approach is optimized to investigate effective connectivity from a stimulated cortical hub to interconnected areas, by reconstructing the spatiotemporal dynamics of TMS-evoked EEG activity. This approach has been previously used to investigate interhemispheric interactions by measuring the propagation of TMS-evoked activity from the stimulated hemisphere to the contralateral one, a phenomenon defined interhemispheric signal propagation (ISP) (Voineskos et al., 2010). This parameter has been standardized in the hand M1 (hM1), where it is considered to reflect the transcallosal-mediated interhemispheric inhibition (Voineskos et al., 2010). ISP is very relevant and significant from a clinical point of view since it can be computed even in populations where MEPs are not reliable or easily evocable, as demonstrated in stroke patients (Casula et al., 2021; Casula et al., 2020).

In the present work we used the TMS-EEG approach recording TMS-evoked potentials (TEP) to investigate, for the first time, the functionality of the transcallosal connections between the representation areas of face and hand M1 in healthy subjects.

Materials and methods

Ethical Approval

Eighteen healthy volunteers (25.25 ± 3.86 years old; 9 females) all right-handed according to the Oldfield inventory scale were enrolled for the study after giving written informed consent. All participants were tested for TMS exclusion criteria (Rossi et al., 2021). None of them had history or current signs/symptoms of neurological diseases. The experimental procedure was approved by the Local Ethical Committee (ID number 967/11/11/2021) and was in accordance with the Declaration of Helsinki.

The sample size was chosen based on an a priori analysis with G*Power 3.1 software assuming an expected effect size (Cohen's f) of 0.8 and a statistical power of 0.80 at a 0.05 alpha level. This calculation resulted in a total sample size of 18 subjects.

Electromyography

EMG was recorded from the DAO and first dorsal interosseus (FDI) muscles contralateral to the stimulation sites, using 9 mm diameter Ag-AgCl surface electrodes. For EMG recordings from the DAO, the active electrode was placed at the midpoint between the angle of the mouth and the lower border of the mandible, the reference electrode over the mandible border, 1 cm below the active electrode and the ground electrode over the right forehead (Pilurzi et al., 2013, 2020). For EMG recordings from the FDI, the active electrode was placed over the muscle belly, the reference electrode at the second finger metacarpophalangeal joint and the ground electrode over the forearm (Rossini et al., 2015). Unrectified EMG signals were recorded (D360 amplifier, Digitimer Ltd, Welwyn Garden City, UK), amplified ($\times 1000$), filtered (bandpass 3-3000 Hz), sampled (5 kHz per channel; window frame length: 250 ms) using a 1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and Signal 6 software on a computer and stored for off-line analysis.

Transcranial magnetic stimulation

TMS was performed using a figure-of-eight shaped coil with external loop diameter of 7 cm connected to a Magstim 200 stimulator (Magstim Co., Whitland, and Dyfed, UK). The optimal stimulation site, for the contralateral DAO and FDI muscles was carefully searched and then marked with a soft tip pen over the scalp, to maintain the same coil position throughout the experiments. For the DAO the handle of the coil pointed posteriorly and laterally, at approximately 30-45 deg to the interhemispheric line (Kujirai et al., 2006;

Ginatempo et al., 2022; 2023); for FDI the coil pointed backwards and laterally at 45 deg away from the midline. The resting motor threshold (RMT) was taken as the lowest TMS intensity that elicited, in the relaxed muscle, MEPs of 0.05 mV in at least 5 out of 10 consecutive trials and was expressed in percentage of the maximum stimulator output (MSO) (Groppa et al., 2012; Rossini et al., 2015).

TMS-EEG

Single-pulse TMS was combined with simultaneous registration of EEG. A TMS-compatible DC amplifier (BrainAmp, BrainProducts GmbH, Munich, Germany) was used to record EEG activity from the scalp. The EEG was continuously recorded from 64 scalp sites positioned according to the 10-20 International System, using TMS-compatible Ag/AgCl pellet electrodes mounted on an elastic cap. The ground electrode was positioned in AFz, while the reference was positioned on the tip of the nose. EEG signals were digitized at a sampling rate of 5 kHz. Skin/electrode impedance was maintained below 5 k Ω . During registration a strong tape on the EEG cap was used to identify, mark and keep the stimulation site. This allowed to isolate partially the electrodes from the TMS pulse.

TMS-EEG data were analyzed offline with Brain Vision Analyzer (Brain Products GmbH, Munich, Germany) and EEGLAB toolbox running in a MATLAB environment (MathWorks Inc., Natick, USA). As a first step, data were segmented into epochs starting 1 s before the TMS pulse and ending 1 s after it. We first removed and then replaced data, using a cubic interpolation, from 1 ms before to 10 ms after the TMS pulse from each trial. Afterwards, data were downsampled to 1000 Hz and band-pass filtered between 1 and 80 Hz (Butterworth zero phase filters). A 50 Hz notch filter was applied to reduce noise from electrical sources. Then, all the epochs were visually inspected and those with excessively noisy EEG were excluded from the analysis. On average we excluded 3.2 ± 4.6 epochs per dataset. Independent component analysis (INFOMAX-ICA) was applied to the EEG signal to identify and remove components reflecting muscle activity, eye movements, blink-related activity, and residual TMS-related artifacts based on previously established criteria (Casula et al., 2017; 2024). On average we removed 8.2 ± 6.7 components per dataset. Finally, the signal was re-referenced to the average signal of all the electrodes.

TMS-evoked activity data were analyzed with the following procedure according to previous studies (Casula et al., 2020, 2021; Voineskos et al., 2010). First, we rectified the TMS-evoked activity recorded over six electrodes surrounding the FDI and DAO hotspots, *i.e.* FC1, FC3,

C1, C3, CP1, CP3 for the left FDI and FC2, FC4, C2, C4, CP2, CP4 for the right FDI; FC3, FC5, C3, C5, F3, F5 for left DAO; FC4, FC6, C4, C6, F4, F6 for right DAO. These electrodes were chosen based on the average hotspot location and on the visual inspection of TEP amplitude. We then averaged the amplitude of the rectified TMS-evoked activity from 10 to 150 ms after the TMS pulse for the stimulated M1 and from 20 to 160 ms for the M1 contralateral to the stimulation. These time windows were chosen based on (1) the mean duration of the GABA-receptor-mediated inhibitory neurotransmission, *i.e.*, ~150 ms and (2) on the transcallosal interhemispheric latency, *i.e.*, ~10 ms (Casula et al., 2020, 2021; Ferbert et al., 1992; Jarczok et al., 2016; Voineskos et al., 2010). Finally, we computed the ISP both from the left hemisphere (LH) (ISP_{LH}) and from the right hemisphere (RH) (ISP_{RH}) with the following formula:

$$ISP = \frac{TMS \text{ evoked activity (non - stimulated M1)}}{TMS \text{ evoked activity (stimulated M1)}}$$

To assess the ISP balance between the two hemispheres, we computed the interhemispheric balance (IHB) as follows:

$$IHB = \frac{ISP_{LH}}{ISP_{RH}}$$

These same indexes were then computed from signals estimated in the source space by the two anatomical regions of interest, *i.e.*, FDI and DAO M1 hotspots. We chose to repeat the analysis at source level since our interhemispheric indexes could be biased by volume conduction effects (Casula et al., 2020), which can be avoided by calculating source-space connectivity with the minimum norm estimate method (Brunner et al., 2016; Stenroos and Hauk, 2013). To estimate the spatial source of cortical activity, we fitted a distributed source model consisting of 15'000,00 elementary current dipoles. These dipole sources were distributed at each vertex of a tessellated cortical mesh template surface derived from a standard 1 mm resolution brain (Colin27) in the Montreal Neurological Institute (MNI)

space, as provided by Brainstorm toolbox. First, the head model for source imaging was implemented according to the OpenMEEG boundary element method (Gramfort et al., 2010). Based on this head model, the inverse problem was solved using current density maps. Noise covariance for source reconstruction was obtained separately for each subject from a baseline window ranging from -850 to -350 ms in respect to TMS. The estimated source distribution was averaged across all the participants and four regions of interest (ROIs), corresponding to left/right hM1 hotspots and left/right fM1 hotspots, were chosen a priori to analyze source activity. The four ROIs were centered on each stimulated hotspot of the 18 participants and had an area of 40 vertices, corresponding to an average area of $8.14 \pm 0.7 \text{ cm}^2$. Source activity was analyzed in terms of z-scores using a baseline of -100 to -1 ms in respect to the TMS pulse.

Experimental design

Each participant underwent two experimental sessions: one to evaluate IHI and one to evaluate ISP and IHB.

Experiment 1: interhemispheric inhibition in face and hand M1 representation areas

Paired-pulse TMS was carried out with two Magstim 200 stimulators connected to two 70 mm figure-of-eight coils. To test IHI, a conditioning stimulus (CS) was delivered at 120% RMT intensity over one M1, which preceded by 4 and 10 ms a test stimulus (TS) delivered at 120% RMT over the contralateral M1. DAO MEPs were evaluated after 10 ms from the TS while FDI MEP after 20 ms, since previous studies highlighted that DAO MEP latency is around 10 ms (Pilurzi et al., 2013, 2020; Ginatempo et al., 2019), while FDI MEP latency is around 20 ms (Rossini et al., 2015). The experiment was divided up into four blocks: IHI in DAO and IHI in FDI from both hemispheres. In each block, ten unconditioned MEPs and ten conditioned responses for each ISI were recorded in randomized order using 1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK). To minimize the muscle contraction bias effect, an on-line rejection was made by the researcher when pre-stimulus EMG activity overcame a mean peak-to-peak amplitude of 0.05 mV. If the subject showed such condition, to ensure that at least 10 MEPs were collected in a resting condition, a number of 15 trials, for each condition, were recorded. During the off-line analysis, traces with pre-stimulus activity reaching a mean peak-to-peak amplitude of 0.05 mV were removed. This procedure ensured that a minimum number of 10 MEPs were included in the average MEP calculation. IHI was expressed as the ratio between the conditioned MEP and the unconditioned MEP amplitudes.

Experiment 2: interhemispheric signal propagation (ISP) in face and hand M1 representation areas.

Single-pulse TMS was delivered over the hotspot of the FDI and DAO in two different sessions during simultaneous EEG recordings; intensity of stimulation was set at 90% of the RMT. Each session consisted of two blocks of 120 single-pulse TMS applied at a random ISI of 4-5 s over FDI or DAO hotspots of the LH and RH. The order of stimulation of the two hemispheres was counterbalanced across subjects.

During TMS, participants were seated on a comfortable armchair in front of a monitor at 80 cm distance. They were asked to fixate on a black cross (6×6 cm) in the middle of a white screen and to keep their arms in a relaxed position. During TMS-EEG, participants wore in-ear plugs which continuously played a white noise that reproduced the specific time-varying frequencies of the TMS click, to mask it and avoid possible auditory event-related potentials responses (Rocchi et al., 2021). The intensity of the white noise was adjusted for each subject by increasing the volume (always below 90 dB SPL) until the participant was sure that s/he could no longer hear the click (Casula et al., 2022).

Statistical analysis

All data were analyzed using SPSS version 22 (SPSS Inc., Chicago, USA). Prior to undergoing ANOVA procedures, normal distribution of neurophysiological data was assessed by means of Shapiro-Wilks' test. Level of significance was set at $\alpha=0.05$. Sphericity of the data was tested with Mauchly's test; when sphericity was violated (*i.e.*, Mauchly's test < 0.05) the Greenhouse-Geisser correction was used. Pairwise comparisons were corrected by the Bonferroni method.

A first set of analyses was performed to assess the experimental sessions, hotspot, and hemisphere effects on TMS-EMG measures, *i.e.*, RMT and IHI. RMT was analyzed using a three-way repeated-measures (RM)-ANOVA with experimental session (TMS and TMS-EEG), area (FDI and DAO) and hemisphere (left and right, according to the side of TS delivery) as within-subject factors. Since the amount of IHI depends on MEP amplitude (Rossini et al., 2015) we performed a preliminary analysis to probe whether an inhibition occurred in each muscle representation area in M1. In order to compare the IHI between the two representation areas, we normalized the IHI effect for the test MEP amplitude, using the ratio as a variable. To evaluate whether an inhibition occurred in the IHI protocol, MEP raw

amplitude was analyzed using a two-way RM-ANOVA with protocol (unconditioned and conditioned MEP) and hemisphere (left and right, according to the side of TS delivery) as within-subjects factor separately for FDI and DAO.

If a significant effect of protocol on raw MEP amplitude was detected for both areas, a further two-way RM-ANOVA for IHI, was performed using MEP amplitude ratio as variable with area (FDI and DAO) and hemisphere (left and right as for TS) as within-subjects factors. If significant effect of protocol was found only for one of the two M1 areas, IHI analysis with MEP amplitude ratio was performed using paired Student's t-test to evaluate hemispheric differences.

A second set of analysis was performed to analyze TMS-EEG interhemispheric indexes at electrode level. TMS-evoked cortical activity was analyzed by means of a three-way RM-ANOVA with area (FDI, DAO), hemisphere (left, right) and stimulation (stimulated vs. non-stimulated) as within-subject factors. ISP was analyzed by means of a two-way RM-ANOVA with area and hemisphere as within-subject factors. IHB was analyzed by means of paired t-tests comparing the measures obtained from the two areas, *i.e.*, FDI and DAO hotspots. Analysis of ISP and IHB computed at source level were performed in the same way.

A third set of analyses was performed to verify the correspondence of TMS-EEG interhemispheric index computed at electrode level and at source level, to ensure that our results were not biased by volume conduction (Brunner et al., 2016; Stenroos and Hauk, 2013). To this aim we computed the Pearson's or Spearman's correlation coefficient (depending on data distribution) between the electrode level and source level ISPs recorded in the four conditions: left/right FDI and left/right DAO.

Finally, a fourth set of analysis was performed to assess whether interhemispheric measures, *i.e.*, IHI, and TMS-EEG interhemispheric measures computed at source level (ISP), reflected the same phenomenon. This analysis was performed by computing Spearman's or Pearson's correlation coefficient between TMS-evoked activity, ISP and IHI.

Results

The entire procedure was well tolerated by all participants with no significant side effects referred. RMT values are shown in Table 1. Three-way RM-ANOVA analysis of RMT showed a significant effect of the experimental session [$F_{(1,17)}=228.64$; $p<0.001$; $\eta^2=0.923$], revealing higher RMT in the TMS-EEG session than the TMS one; a significant effect of area

[$F_{(1,17)}=158.06$; $p<0.001$; $\eta^2=0.893$], with DAO having a higher RMT than FDI; a trending effect of hemisphere [$F_{(1,17)}=4.38$; $p=0.050$; $\eta^2=0.187$], where the right one seems to have greater RMT compared to the left hemisphere. Finally, significant effect was found for the interaction between session and area [$F_{(1,17)}=6.35$; $p=0.021$; $\eta^2=0.250$]. Post-hoc analysis of the interaction detected higher RMT for TMS-EEG session, compared to TMS alone, when assessing both FDI and DAO M1 areas ($p<0.001$); and greater RMT for DAO area than FDI during both sessions ($p<0.001$).

Table 1. Mean values of RMT observed for right and left hemispheres of FDI and DAO muscles representations in the M1 in both TMS and TMS-EEG sessions.

RMT (%MSO)	Left hemisphere FDI	Right hemisphere FDI	Left hemisphere DAO	Right hemisphere DAO
TMS session (n=20 MEPs)	37.65±5.98	38.70±5.93	52.05±7.75	52.75±6.67
TMS-EEG session (n=20 MEPs)	45.75±4.79	48.25±7.94	63.10±7.26	66.50±8.31

RMT, resting motor threshold; M1, primary motor cortex; TMS, transcranial magnetic stimulation; EEG, electroencephalography; MSO, maximum stimulator output; MEP, motor evoked potential; FDI, first dorsal interosseous muscle; DAO, depressor anguli oris muscle. The table represents means ± standard deviation.

Experiment 1: interhemispheric inhibition in face and hand M1 representation areas.

Analysis on IHI demonstrated that inhibition was clearly present in the FDI area of hM1 (Figure 6). In fact, RM-ANOVA showed an effect of protocol [$F_{(1,17)}=28.020$; $p<0.001$; $\eta^2=0.596$], with no effect of hemisphere [$F_{(1,17)}=0.207$; $p=0.654$; $\eta^2=0.011$] neither interaction between factors [$F_{(1,17)}=2.632$; $p=0.121$; $\eta^2=0.122$]. In the DAO area of fM1, IHI was not present, a facilitatory effect was rather observed, as shown in Figure 6. An effect of protocol was detected but conditioned MEP was larger than the unconditioned one [$F_{(1,17)}=8.464$; $p<0.010$; $\eta^2=0.332$], with no effect of hemisphere [$F_{(1,17)}=0.003$; $p=0.956$; $\eta^2<0.001$] nor interaction between factors [$F_{(1,17)}=1.515$; $p=0.235$; $\eta^2=0.082$]. Following these results, analysis on MEP amplitude ratios was conducted only for hM1, failing to detect any significant effect of hemisphere [$t_{(1,17)}=0.78$; $p=0.444$; $r=0.003$] (Bonferroni corrected alpha: 0.05).

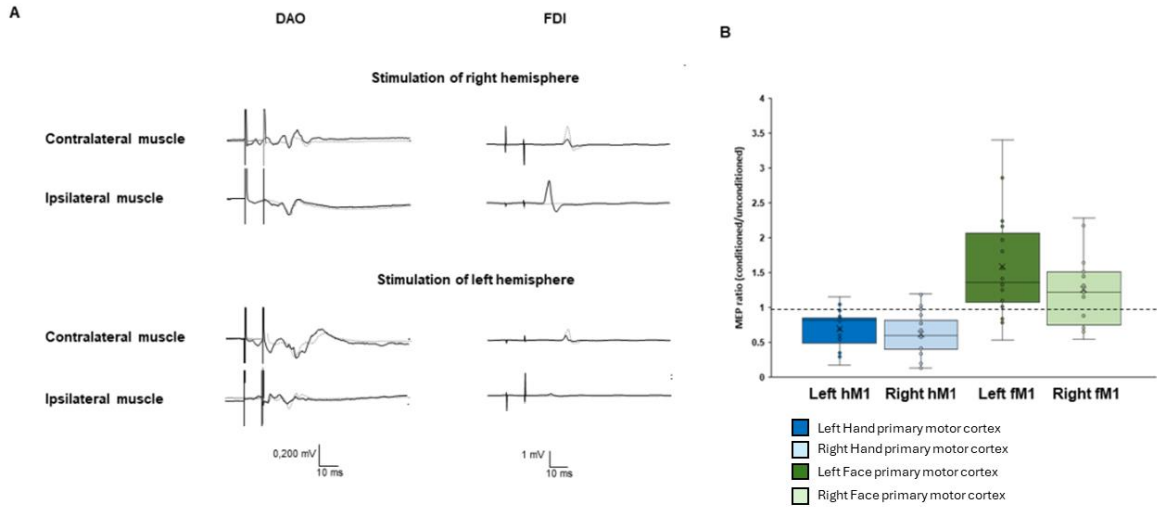


Figure 6. Effect of the Interhemispheric inhibition (IHI) protocol on the face and hand M1 representation areas (fM1 and hM1, respectively) following TMS stimulation of the left and right hemispheres. Panel A shows averaged data of a representative subject of interhemispheric inhibition (IHI). The black line represents the motor evoked potentials (MEPs) obtained following paired pulse TMS, while the grey and dot line represent MEP obtained following single pulse stimulation. In panel B graph reports MEP amplitudes, which are expressed as a percentage of the unconditioned MEP induced by the test stimulus alone. IHI was clearly detected in both left and right hM1s (dark blue and light blue, respectively), while it was absent in the left and right fM1s (dark green and light green respectively). The continuous line in the boxplot represents the median value, while the ‘x’ symbol represents the mean value of the group. Dots represent individual data. * $p < 0.05$.

Experiment 2: interhemispheric signal propagation (ISP) in face and hand M1 representation areas

TEPs resulted in a well-known sequence of positive and negative deflections lasting around 250 ms from the TMS pulse. The spatiotemporal distribution of TEPs is depicted in Figure 7, whereas the average amplitude for each experimental condition is presented in Table 2. Analysis of TEP showed a significant main effect of the stimulated area (FDI in hM1 or DAO in fM1) [$F_{(1,17)}=19.08$; $p<0.001$; $\eta^2=0.529$] and stimulation factor [$F_{(1,17)}=16.65$; $p=0.001$; $\eta^2=0.495$], revealing a general smaller TEP amplitude in the non-stimulated than stimulated hemisphere (mean difference: $0.349 \pm 0.85 \mu V$; Bonferroni corrected alpha: 0.025) regardless the laterality (left or right stimulation) [$F_{(1,17)}=1.702$; $p=0.209$; $\eta^2=0.091$]. The analysis did not detect any significant interaction among factors (all $p>0.05$).

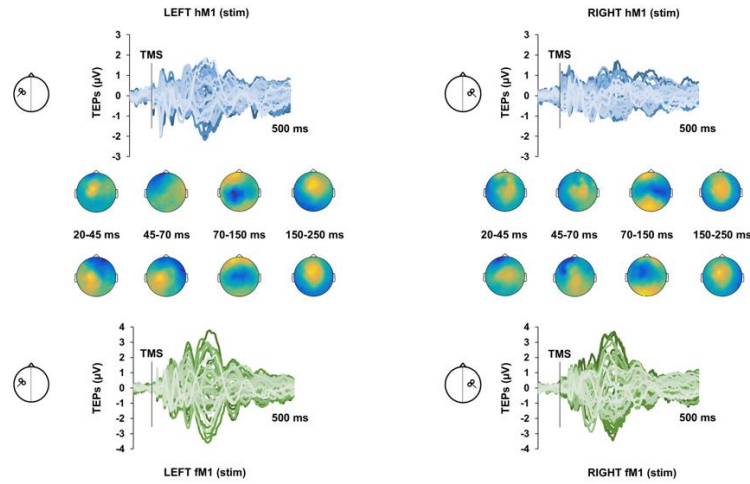


Figure 7. Spatiotemporal distribution of TEPs for each experimental condition. Blue lines depict TEP at single-electrode level after left (left upper panel) and right hM1stimulation (right upper panel). Green lines depict TEP at single-electrode level after left (left below panel) and right fM1stimulation (right below panel). Scalp maps depict the voltage distribution at specific time window chosen based on the TEP peaks.

Analysis of ISP did not reveal significant differences among the ISPs evoked from the left/right hM1/fM1 [area main effect: $F_{(1,17)}=0.026$; $p=0.873$; $\eta^2=0.002$; laterality main effect: $F_{(1,17)}=0.428$; $p=0.522$; $\eta^2=0.025$] and interaction between factors (all $p>0.05$). Indeed, all the ISP showed a similar suppression of activity in the non-stimulated hemisphere (*i.e.*, $ISP<1$) when stimulating both left hM1 (0.792 ± 7.015) and right hM1 (0.825 ± 7.03), and both left fM1 (0.869 ± 6.452) and right fM1 (0.851 ± 6.246) (Figure 8A).

Both the IHB, for hM1 and fM1, showed a stable balance (*i.e.*, $IHB\approx 1$) between the dynamics of the two hemispheres (hM1-IHB: 1.062 ± 0.131 ; fM1-IHB: 1.061 ± 0.078). Analysis of IHB confirmed the absence of significant differences between the ISP evoked from hM1 and fM1 [$t_{(17)}=0.014$; $p=0.989$; $r=0.003$] (Figure 9B).

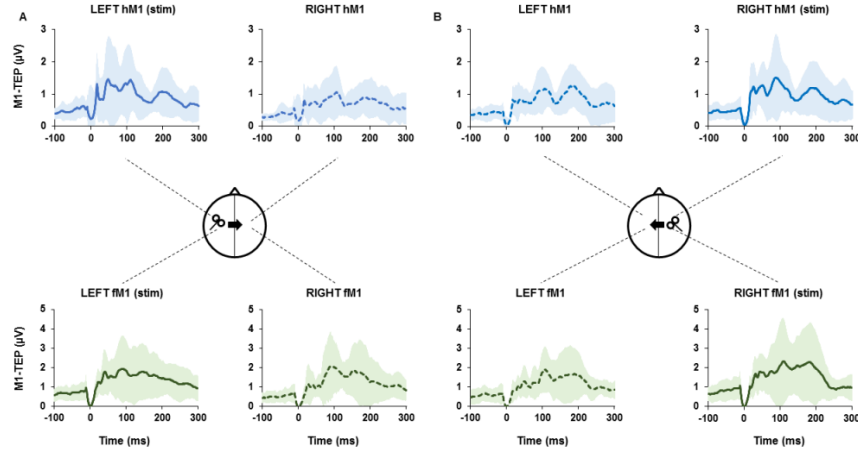


Figure 8. TMS evoked potential (TEP) following the stimulation of the left and right face and hand M1 representations. Rectified amplitude of TEP after stimulation (stim) of the left primary motor cortex (M1) (Panel A) and of the right M1 (Panel B) in the representation area of the first dorsal interosseus muscle (hM1, blue lines) and of the depressor anguli oris muscle (fM1, green lines). Dashed lines depict the activity in the non-stimulated contralateral hemisphere. Shaded lines depict standard error.

When computed at source level, ISP analysis revealed a consistent reduction of interhemispheric propagation (<1) over the four ISP with values similar to the ones computed at electrode level (left-hM1: 0.858 ± 0.331 ; right-hM1: 0.864 ± 0.236 ; left-fM1: 0.894 ± 0.256 ; right-fM1: 0.905 ± 0.368). This result was confirmed by the RM-ANOVA reporting no significant main effect of the area factor [$F_{(1,17)}=0.191$; $p=0.668$; $\eta^2=0.004$]; of the laterality factor [$F_{(1,17)}=0.100$; $p=0.922$; $\eta^2=0.001$] nor of the area*laterality interaction [$F_{(1,17)}=0.004$; $p=0.947$; $\eta^2=0.001$] (Figure 9A)

When computed at source level, both hM1-IHB and fM1-IHB showed a stable balance (i.e., $IHB \approx 1$) between the dynamics of the two hemispheres (hM1-IHB: 1.02 ± 0.094 ; fM1-IHB: 1.11 ± 0.111), this was confirmed by the paired t-test conducted between the two conditions [$t_{(17)}=-0.963$; $p=0.349$; $d=-0.227$] (Figure 9B).

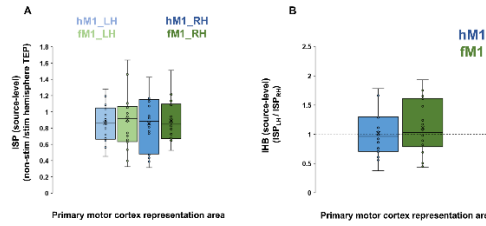


Figure 9. Interhemispheric signal propagation (ISP) and interhemispheric balance (IHB) computed at electrode level in the left and right hand (hM1) and face (fM1) representation areas of the primary motor cortex (M1). Graphs in panel A show ISP obtained after the stimulation of left (LH) and right (RH) hemispheres in hM1 and fM1. Graphs in panel B show IHB expressed as a ratio between left and right ISP in both hM1 and fM1. The continuous line in the boxplot represents the median value, while the ‘x’ symbol represents the mean value of the group. Dots represent individual data. * $p < 0.05$.

Table 2. TEP amplitude for each experimental condition.

	LEFT hM1	RIGHT hM1	LEFT fM1	RIGHT fM1
stimulated	1.062 (0.576)	1.225 (0.589)	1.451 (0.698)	1.497 (0.538)
non-stimulated	0.907 (0.294)	0.702 (0.211)	1.191 (0.422)	1.153 (0.453)

h, hand; M1, primary motor cortex; f, face. Data are expressed as mean $\pm$ SD

Correlation analysis

Correlation analysis showed a significant negative correlation between the TEP of the stimulated hemisphere and the relative ISP, regardless of the area (hM1 or fM1) and the laterality (left or right) of stimulation. Specifically, ISP was lower at higher levels of responses of the stimulated cortex (*i.e.*, higher TEP). This appeared when stimulating the left hM1 ($r=-0.452$, $p=0.045$); the right hM1 ($r=-0.66$, $p=0.002$); the left fM1 ($r=-0.601$, $p=0.005$) and the right fM1 ($r=-0.452$, $p=0.045$) (Figure 10A). Correlation analyses between ISP and IHI were significant ($r=0.563$, $p=0.015$) only when testing inhibition from the left hM1 (*i.e.*, ISP obtained stimulating the right hM1 and IHI elicited when delivering the test pulse to the left hM1). None of the other correlations were significant (all $p>0.05$) (Figure 10B). Analysis between the ISPs computed at electrode and source level confirmed ISP correlation showing a strong linear relation (*i.e.*, $r>0.5$) when stimulating the left hM1 ($r=0.597$, $p=0.009$); the right hM1 ($r=0.576$, $p=0.006$) and the right fM1 ($r=0.705$, $p<0.001$); a moderate direct relation was observed when stimulating the left fM1 ($r=0.473$, $p=0.024$).

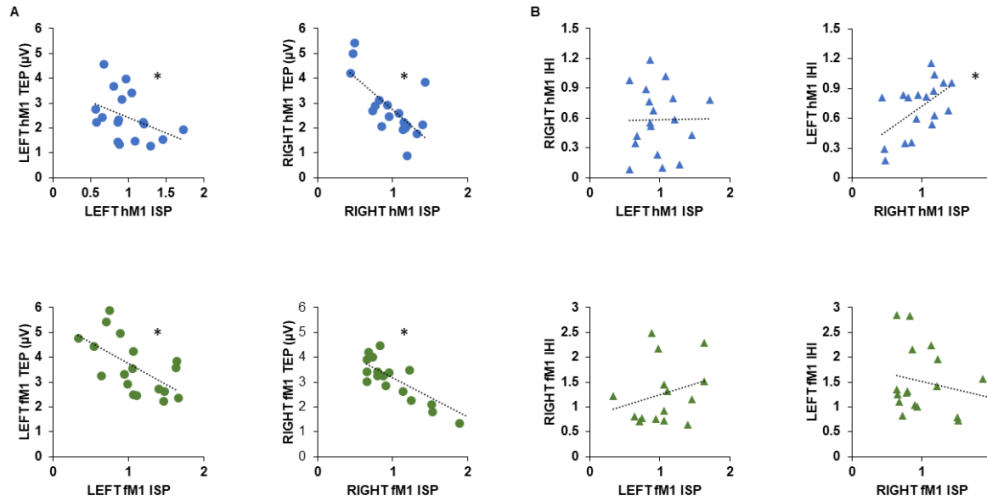


Figure 10. Correlation analysis between interhemispheric inhibition (IHI), TMS-evoked potentials (TEPs) and interhemispheric signal propagation (ISP). Correlation analyses between ISP and TEP amplitude elicited after the stimulation of the hand primary motor cortex (M1) (hM1, blue circles) and of the face M1 (fM1, green circles) (panel A). Panel B shows correlation analyses between ISP and IHI evoked after stimulation of the hM1 (blue triangles) and of the fM1 (green triangles). * $p < 0.05$.

Discussion

The present work reinvestigated the functionality of the transcallosal pathway connecting fM1 of the right and left hemispheres in healthy subjects, since previous neuroimaging (Wahl et al., 2007) and neurophysiological (Ginatempo et al. 2019) studies did not provide evidence of a transcallosal connection between the representation areas of the lower face muscles in M1, in contrast with the widely described interhemispheric connections between the hM1s (Febert et al., 1992; Ginatempo et al., 2019; Ziemann and Hallet, 2001). To do so, ISPs of TEPs were compared with MEPs obtained in IHI protocols, where fM1 and hM1 were stimulated in both hemispheres. In line with previous findings, data confirmed the presence of IHI in hM1 (Febert et al., 1992; Ziemann and Hallet, 2001), since the MEP of the FDI muscle was suppressed when the contralateral hemisphere was stimulated, as well as the lack of IHI in fM1 (Ginatempo et al., 2019). The facilitatory effect observed in face representation area of M1 during IHI protocol is in line with the results of the first study reporting this observation (Ginatempo et al., 2019). This study demonstrated for the first time the absence of IHI in the DAO muscle, even though it was clearly present in the FDI and, with slightly reduced effectiveness in the upper trapezius (UT) (Matthews et al., 2013). Also, in this study a clear facilitation, rather than inhibition, in the DAO was observed even at shorter ISIs (1-4 ms). Due to the short ISIs, it is unlikely that this facilitation is the result of “interhemispheric facilitation” (IHF) described in hand muscle, since IHF was only seen with subthreshold CS.

Secondly, it is unlikely that we activated the corpus callosum pathway required for interhemispheric interactions with an ISI less than 5–10 ms (Meyer et al., 1995). It has been suggested that IHI is absent in the DAO and that the early facilitation is the result of a convergence at the brainstem level of ipsilateral projections, activated by the CS, and contralateral projections activated from the TS (Ginatempo et al., 2019). On the contrary, TEPs in the non-stimulated hemisphere were significantly lower in amplitude, compared to the ones obtained in the stimulated hemisphere, regardless the laterality and the stimulated M1 area. In the same way, the ISP showed the lower propagation of activity in the non-stimulated hemisphere without significant differences among the four hotspots considered.

As previously described, ISP is the ratio between TEP evoked in the non-stimulated hemisphere and in the stimulated one (Casula et al., 2020; Voineskos et al., 2010). This index has been used in many works as a measure of the efficiency of interhemispheric transmission, given its relationship with IHI and callosal fractional anisotropy (Casula et al., 2020, 2021; Jarczok et al., 2016; Määttä et al., 2017; Voineskos et al., 2010). In this light, the ISP results of this study, while confirming data in hM1, also suggest the presence of a transcallosal connection between the two fM1s, in contrast with the neuroimaging study of Wahl et al. (2007) and the TMS study of Ginatempo et al. (2019). However, the ISP findings are in agreement with anatomical studies demonstrating the connection between fM1 and its homolog in the other hemisphere through callosal fibers in the owl monkey (Gould et al., 1986) and in the macaque monkey (Rouiller et al., 1994) and with data from a neurophysiological study demonstrating the presence of IHI in fM1s at rest of subjects undergoing a long-term training of face muscles, such as professional woodwind musicians (Ginatempo et al., 2021). Hence, the present study seems to explain and clarify this controversy, sustaining the presence of an anatomical pathway between the two fM1s, through the corpus callosum, which is, however, likely poorly active from the functional point of view, as shown by the fact that this connection does not affect the peripheral motor response (*i.e.* no IHI recorded from the DAO muscle). This hypothesis is supported by the absence of correlation between ISP (anatomical index) results and IHI (functional index) results in fM1. This explanation is also supported by a previous study, which demonstrated the presence of IHI for fM1 in woodwind musicians as opposite to string musicians and non-musicians (Ginatempo et al., 2021). This study emphasized the importance of fine practice and skill in inducing functional and plastic changes in the transcallosal connection between fM1s, resulting in its functional empowerment, which thus became detectable through IHI

protocols (Ginatempo et al. 2021). In this light, it is possible that we failed to detect any significant effect also due to the low activation of the transcallosal pathway connecting fM1s in healthy subjects, which further study aimed at investigating ISP and IHI correlation in woodwind musicians by using a TMS-EEG technique could clarify.

Overall, the results of the present study highlighted a significant correlation between IHI and ISP when stimulating the hM1 of the left dominant hemisphere (*i.e.*, right FDI). This result is in line with the hypothesis that ISP could be related, at least to some extent, to interhemispheric inhibition (Casula et al., 2020, 2021; Ishibashi et al., 2022; Voineskos et al., 2010). In particular, Casula and colleagues (2020) demonstrated that participants with a higher inhibition of MEP amplitude also showed less interhemispheric propagation of TMS-evoked activity. Ishibashi and colleagues (2022) highlighted that a reduced ISP reflects reduced transmission of activity from the stimulated hemisphere to the unstimulated one. However, it is important to consider that this hypothesis needs to be further tested by specific studies investigating the relation between ISP and IHI in larger samples. Notably, in the present study, we observed that higher levels of M1 excitability were correlated to lower propagation of contralateral TEPs, *i.e.* lower ISP, although the correlation between IHI and ISP was not significant when stimulating the right non-dominant hemisphere. This result could be due to the instability of MEPs (Daligadu et al., 2013) and TEPs (Casula et al., 2020) evoked in the non-dominant hemisphere, which leads a less impact on both IHI and ISP phenomena, as already observed in a previous study (Casula et al., 2020). In this regard, Ziemann and Hallett (2001) proposed a model to explain the motor dominance of the left hemisphere in sequential movements: the right and left hemispheres have identical motor capabilities in controlling the contralateral hands, but motor dominance occurs due to asymmetric inhibitory interactions between the left and right hemispheres. In addition, a neuroimaging study demonstrated that the left hemisphere has a more prominent role in regulating IHI between the left and right M1 during sequential movements (Hayashi et al., 2008). Ishibashi and colleagues (2022) demonstrated that the ISP from the left to the right hemisphere during right-hand rapid movement was higher than ISP from the right to the left hemisphere during left-hand rapid movement, concluding that the left M1 more strongly inhibits the right M1 during right-hand rapid movement relative to the inhibition from the right to left M1 during left-hand rapid movement. On the contrary, the correlation results between IHI and ISP for fM1, failed to detect any significant correlation for both sides. This result not only highlights that fM1s are anatomically connected by a transcallosal pathway,

which is functionally poorly active, but it also demonstrates, for the first time, an absence of a hemispheric dominance for face muscle district. This hypothesis could be supported by the fact that lower face muscles receive a bilateral projection from fM1, and it is likely that it has been observed, in TMS studies (Pilurzi et al., 2013; 2020) a contralateral predominance only due to technical reasons. In fact, the recording of MEP from ipsilateral DAO is biased from TMS-artifact leading to observe a lower amplitude response which probably did not reflect a clear hemispheric dominance. This observation could explain the importance to perform bilateral and symmetrical movements required for face expressiveness. On the other hand, it has to be noted that the absence of a significant correlation between IHI and ISP when stimulating the hM1 of the right hemisphere (*i.e.*, left FDI) could be explained by the different physiological origin of MEPs and TEPs. Indeed, MEPs reflect the excitability of the pyramidal tract, which is affected by a combination of spinal, cortical and subcortical mechanisms (Rossini et al., 1994). Differently, TEPs result from the activation of inhibitory and excitatory post-synaptic cortical potentials (Casula et al., 2020; Ilmoniemi et al., 1997). Due to their different nature of signal, this likely results as a different variability of these measures which could be higher in non-dominant hemisphere than dominant. These differences may underlie the absence of correlation in the right hemisphere for hM1. In addition, different TMS intensities were used to elicit ISP and IHI, 90% of RMT and 120% of RMT respectively. The intensities used for IHI and ISP protocol were chosen based on previous studies in which these protocols were standardized and used as models for the functional and spatial investigations of transcallosal pathway (Ferber et al., 1992; Voineskos et al., 2010; Jarczok et al., 2016; Casula et al., 2021; Ishibashi et al., 2022). However, it cannot be ruled out that different intensities could elicit different responses, which should be investigated in further studies using similar intensities in TMS-EEG and TMS-EMG sessions.

The computation of the ISP at source level led to replicate interhemispheric results. This is relevant since when a cortical area is stimulated with TMS, activity propagates to the surrounding areas, making it difficult to distinguish cortical activation from conduct volume. To rule out a possible conduct volume bias in our results we repeated our analysis computing interhemispheric measures from source activity computed with the minimum norm estimate method (Stenroos and Hauk, 2013; Brunner et al., 2016). Our analysis showed a strong overlapping between the electrode-level and source-level activity, a result that supports the reliability of ISP.

The findings of the present study have practical implications in the physiological and clinical domains. In fact, it demonstrates for the first time that it is possible to record TEPs following TMS over fM1. So far, in healthy subjects, fM1 activity and corticobulbar pathways innervating the lower face muscles were investigated only by evaluating the peripheral motor response (*i.e.*, MEPs). This is relevant since in lower face muscles, such as the DAO, MEPs are not always evocable due to their higher RMT, compared to other motor area representations, *e.g.*, the hand area. It is also important to consider that MEPs are not easily evocable in a wide range of clinical populations with cortical damages or corticobulbar lesions such as stroke, Parkinson's disease, epilepsy, and other neurological diseases (Assogna et al., 2020; Casula et al., 2021; Tremblay et al., 2019). In these patients, TMS-EEG seems to be a valid diagnostic and prognostic technique to deeply investigate the real damage caused by the disease and its extension. In this light, this study paves the way to investigate and to compare M1 and transcallosal pathway excitability in different motor area representations using TMS-EEG measures which require lower stimulation intensity than TMS protocols, making these measures more accessible and tolerable for patients with neuropsychiatric disorders.

Limitations of the study

We acknowledge that this study has some limitation. First, it has been enrolled only right-handed subjects which did not allow to study hemispheric dominance in fM1. Second, we did not record ipsilateral MEP from DAO muscle which could help to compare ipsilateral TEP and MEP correlations which could clarify the role of hemispheric dominance in fM1 in the lack of significant correlation between IHI and ISP. In addition, only ten pulses for each conditioned stimulation for IHI protocol were delivered, which could lead a variability in the results even if this does not explain the interhemispheric difference, which is probably due to the hemispheric dominance as documented by several papers (Daligadu et al., 2013; Casula et al., 2020). Moreover, further study using combined TMS-EEG approach should take well-established measures to minimize artifacts caused by TMS stimulation, such as a foam layer and online visualization of TEPs. In addition, it is important to underline that the investigation of interhemispheric dynamics with TMS-EEG is a relatively new topic, thus many questions regarding the exact physiological mechanisms underlying the TMS-EEG measures are still unsolved. TMS-EEG is a powerful technique that can offer different

approaches and measure to investigate interhemispheric dynamics, such as the analysis of very early TEP components (Bartoletto et al., 2021; Zazio et al., 2022). A systematic comparison among the different TMS-EEG and TMS-EMG measures is still lacking and further investigation on this topic are needed. Finally, in the present study the analysis of p15 was not performed due to the type of pre-processing analysis (*i.e.* the cubic interpolation until 10 ms from the TMS pulse) which should be included in future studies.

Conclusions

The present study aimed at investigating the physiological nature of the connection between fM1s through a TMS and TMS-EEG approaches in healthy human subjects. TMS-EEG results demonstrated the presence of a connection between face M1s, which was not detected with TMS only, suggesting this transcallosal pathway is functionally poorly active.

***Research questions 2: Aging alters facial expressions processing
and recognition: insights on possible neural mechanisms***

Introduction

Facial expressions are crucial for human non-verbal communication and behaviour (Müri, 2016). Indeed, the capability to recognize and react to facial expressions is considered to be one of the most important social skills (Blair, 2003, 2004).

Several neurobiological models suggest that the recognition of facial expressions involves the coordinated participation of multiple cortical areas such as the inferior occipital gyrus and the superior temporal sulcus, (Calder and Young, 2005; Haxby et al., 2000; Rossion et al., 2003) but also some motor areas including the premotor and M1 cortices (Carr et al., 2003). In particular, the role of M1 in the processing of facial expressions has been demonstrated by TMS studies, which detected an increase of the corticospinal tract excitability in response to either pleasant or unpleasant emotional stimuli, that represent actions of the whole human body (Baumgartner et al., 2007; Coombes et al., 2009; Ginatempo et al., 2021; Hajcak et al., 2007; Hortensius et al., 2016; Schutter et al., 2008; Williams et al., 2006). Moreover, several studies found evidence for increased corticospinal excitability in response to simultaneously presented emotional pictures and congruent music (Baumgartner et al., 2007) or to pleasant and unpleasant scenes (Hajcak et al., 2007). Oliveri and colleagues (2003) provided evidence that the supplementary motor cortex (SMA) may act as a neural gateway in conveying emotion-related information from the limbic system to M1 for initiating action programs.

Interestingly, a TMS-study demonstrated also an increase following the passive viewing of facial expressions in the corticospinal motor tract excitability, with selective increases to fearful facial expressions (Schutter et al., 2008). All of these works, demonstrating a direct connection between emotion and motor control systems, used the hand motor system as a general model, since this system is easier to study through TMS. However, a previous study showed that activation of the face and hand representation areas within M1, during the passive viewing of facial expressions, differs depending on the face emotion observed (Ginatempo et al., 2020). Specifically, the view of happy faces reduced face M1 intracortical inhibition, whereas the view of sad faces reduced hand M1 intracortical inhibition as demonstrated by the reduced short-latency intracortical inhibition TMS-protocol (Ginatempo et al., 2020). This distinction was attributed to the diverse functions of these muscle groups: face muscles are associated with non-verbal communication, while hand muscles play a role in protecting the body (Ginatempo et al., 2020).

On the other hand, as revealed by event-related potentials (ERP) studies (Adolph et al., 2002), the passive viewing of facial expressions is able to activate several cortical areas at different times. In fact, ERP allows to investigate the time-course of the information-processing cascade from early to late processing stages (Rossion and Jacques, 2012). The analysis of facial expressions involves distinct stages, each accompanied by specific ERP components: the P100 (Campanella et al., 2006; Jung et al., 2012; Lee et al., 2010; Turetsky et al., 2007) and the N170 (Campanella et al., 2006; Jung et al., 2012; Lee et al., 2010; Turetsky et al., 2007; Wynn et al., 2008). The P100 is believed to reflect early-stage of the visual information processing and it is considered as an index of automatic attention allocation (Luck et al., 1994). In this light, several studies have reported larger P100 amplitude following the viewing of faces than rather objects, with a specific modulation by facial expressions (Lee et al., 2010; Pizzagalli et al., 2002; Turetsky et al., 2007; Utama et al., 2009). The N170 is a posterior temporal negative peak (Bentin et al., 1996; Dundas et al., 2014; Kovács et al., 2013; Luo et al., 2010; Rossion et al., 2000; Rossion and Jacques, 2012), which seems to reflect the earliest stage of facial structure encoding and/or face identification (Cauquil et al., 2000; Eimer and Holmes, 2002; Vuilleumier and Pourtois, 2007). In the literature, contrasting results exist on the effect of facial expressions on N170 amplitude, since several studies suggest that the N170 amplitude and latency are modulated by facial expressions (Ashley et al., 2004; Eimer et al., 2003; Holmes et al., 2003) while other studies found the opposite result (Eimer and Holmes, 2002; Holmes et al., 2005; Obayashi et al., 2009). ERP has been widely used to understand how aging can influence the perception of facial expressions; while most of these studies found altered N170 in senior compared to the young subjects for both amplitude (Daniel and Bentin, 2012; Gao et al., 2009; Goncalves et al., 2018; Liao et al., 2017) and latency (Loi et al., 2021), there are contrasting results regarding the effect of aging on P100 amplitude. In particular, some studies found an aging effect on the amplitude of P100 (Boutet et al., 2020; Loi et al., 2021) while others failed to detect any significant effect (Fernandes et al., 2023; Goncalves et al., 2018; Liao et al., 2017). Even if these contrasting results were probably due to methodological differences, it is likely that the occipital and temporal cortical areas can play a role in the reduced facial expressions recognition skill in senior subjects with specific regards to negative facial expressions which probably depend by an altered attention, particularly for negative expressions (Loi et al., 2021). In fact, several studies demonstrated that facial expressions processing and recognition is impaired with aging (Calder et al., 2003; Hilimire et al., 2014; Liao et al., 2017; Ruffman et al., 2008; Sullivan and Ruffman, 2004; Sullivan et al., 2007, 2017) with specific regards to

negative facial expressions (Keightley et al., 2007; Loi et al., 2021; Ruffman et al., 2008; Sullivan and Ruffman, 2004; Sullivan et al., 2007, 2017), while the perception of positive expressions seems to remain preserved with age (Loi et al., 2021) or, at least worse, but the magnitude of this difficulty is substantially smaller (Gunning-Dixon et al., 2003; Ruffman et al., 2008). These discrepancies could be due to differences in the activity of several cortical and subcortical regions such as the prefrontal cortex (Gunning-Dixon et al., 2003; Keightley et al., 2007; Kim et al., 2019; Murray et al., 2022; Williams et al., 2006), premotor cortex (Keightley et al., 2007), cingulate cortex (Keightley et al., 2007; Murray et al., 2022), occipito-temporal areas (Keightley et al., 2007; Fernandes et al., 2023; Murray et al., 2022); amygdala (Adolphs et al., 1994, 1995); Gunning-Dixon et al., 2003; Ruffman et al., 2008; Vuilleumier and Pourtois, 2007), basal ganglia (Calder et al., 2003; Williams et al., 2006; Ruffman et al., 2008) and insula (Calder et al., 2001; Keightley et al., 2007; Murray et al., 2022) .

Although the role of age in the perception and recognition of facial expressions has been thoroughly investigated, the mechanism underling these age-related deficiencies is still unclear. Several studies show that the identification of negative emotions is more accurate when visually scanning the upper face, while disgust and happiness are better identified when watching the lower part of the face (Bassili, 1979; Calder et al., 2001; Ebner et al., 2012; Eisenbarth and Alpers, 2011; Sullivan et al., 2007; Wong et al., 2005). Interestingly, studies show that neonates prefer looking at the eyes rather than other face features (Batki et al., 2000; Hoehl et al., 2008; Hood et al., 1996; Vecera and Johnson, 1995). Such arguments suggest that a preference for looking at the eyes over other face features develops early in infancy (Langten et al., 2000). On the contrary, senior adults prefer to look at the lower part rather than at the upper part of the face (Sullivan et al., 2007, 2017; Wong et al., 2005). These findings suggest that differences in facial expressions processing and recognition between senior and young adults might depend on differences in their gaze patterns. However, the role of gaze and fixation patterns in facial expressions recognition is still unclear. It could be possible that such variation with age might lead to change the way to perceive, to allocate attention, to process and eventually to discriminate facial expressions. However, this comprehensive perspective on the recognition of facial expressions process has been not demonstrated since all the previous studies separately investigated fixation patterns, ERPs, motor response with TMS and facial expressions recognition demonstrating a difference between senior and young adults which was limited to their respective domains.

Therefore, the aim of the present work was to systematically investigate the gaze and fixation patterns in senior and young subjects, and to correlate them with neurophysiological parameters: occipito-temporal cortical areas excitability, recognition, and excitability of the face representation area in M1 (face M1) when passively viewing different facial expressions. To this aim, gaze and fixation patterns were investigated through the recording of ocular movements in senior and young subjects during passive viewing of faces expressing emotions. In the same experimental conditions, the two groups underwent investigation of the perception process in occipital and temporal cortical areas using ERP recordings of the P100 and N170 waves; face M1 excitability was assessed with TMS protocols and, eventually, the ability to distinguish face emotional expressions was evaluated through a recognition task.

Materials and Methods

Participants

Thirty-four healthy subjects, right-handed according to the Oldfield inventory scale, were enrolled in the study. The subjects were divided in two age groups: young adults (mean age 24.28 ± 0.85 ; range 21-32 years old; 7 males and 10 females) and senior adults (mean age 64.06 ± 1.10 ; range 57-73 years old; 6 males and 11 females). For both groups, values are reported as mean $\pm$ standard error of the mean (SEM). The sample size was based on an a priori analysis with G*Power 3.1 software assuming an expected effect size $d=0.5$ and a statistical power of 0.80 at a 0.05 alpha level which resulted in a total sample size of 34 subjects. The effect size for the power analysis was chosen based on previous studies investigating fixation patterns in senior and young subjects showing different pattern in facial expressions process (Mantantzis et al., 2020; Murphy and Isaacowitz, 2010; Sullivan et al., 2007). An informed written consent was obtained from all subjects before experimental sessions. The experimental procedure was approved by the local ethical committee and conducted in accordance with the Helsinki Declaration. Exclusion criteria for TMS protocol were represented by certified hemisphericity, tinnitus, presence of intracranial devices and history and/or current signs/symptoms of neurological and/or psychiatric diseases (Rossi et al., 2021). Recordings were carried out in a quiet room while subjects were seating in a comfortable chair and were asked to stay relaxed but alert during the experiment.

Facial expressions stimuli (FES)

In all experiments, images from the Karolinska Directed Emotional Faces (KDEF) database were presented to participants (Lundqvist et al., 1998). The KDEF database is a set of 4900

pictures of human faces from 70 individuals, 35 females and 35 males. There are seven distinct emotional expressions, each of which is photographed twice from five different angles. For our stimuli set, the frontal images of the expressions "neutral", "happy", and "sad" of 5 females and 5 males subjects were selected. Each of the selected image was replicated 5 times and the 150 images were randomly arranged in a sequence, which made up our stimuli set. Visual stimuli for TMS and EEG recordings were shown on a 17" LCD display, with a 1280x1024 resolution and a 70 Hz refresh rate, by using the PsychToolbox software (Brainard, 1997), running in MATLAB (Version 2015b, MathWorks, Inc., Natick, MA, United States). For all the experimental sessions the duration of the visual stimulus was set at 300 ms. This choice was based on previous studies, which reported 300 ms as an adequate time window for the stimulus to reach M1 (Cuthbert et al., 2000; Ginatempo et al., 2020; Loi et al., 2021; Schutter et al., 2008; Smith and Smith, 2019).

Fixation patterns recordings

The gaze data were acquired at the University of Sassari, in a dedicated 8 x 6 x 3.10 m (l x w x h) room equipped with a chair and a desk, a Tobii Eye Tracker connected to a PC running the Tobii Pro Studio Eye Tracking software. The acquisition environment followed ITU-T Recommendation P.911 (Radiocommunication Bureau, 2020). Participants were asked to freely observe the images. Each participant sat down in front of the Tobii monitor, which was calibrated before each acquisition. All participants reported normal or corrected-to-normal vision. The distance between the participant head and the monitor was 2.5 times the height of the Tobii monitor. Each capture session took approximately 20 minutes.

To discriminate fixation parameters between groups, each FES was divided into three ROI (mouth, forehead, eyes and nose). These ROIs were chosen to maintain an equal spatial range for each ROI. Ocular movements analysis was focused on the period in which FES were presented. The parameters assessed for the analysis were number of fixations and time of fixation. Considering the very small number of fixations per image, we chose to evaluate not the absolute number but the number of trials in which each subject looked to a particular ROI, expressed as a fixation frequency. In addition, through the Tobii software analysis, the pupil size diameter was measured from the moment the image appeared on the screen to assess autonomic response to the faces.

Electromyography (EMG)

Surface EMG was recorded from the right DAO muscle using a 9-mm diameter Ag-AgCl surface electrodes. The active electrode was placed at the midpoint between the angle of the mouth and the lower border of the mandible, the reference electrode over the mandible border, 1 cm below the active electrode, and the ground electrode over the right part of the forehead (15, 43). EMG signals were recorded (D360 amplifier, Digitimer Ltd, Welwyn Garden City, UK), amplified (x1000), filtered (bandpass 3-3000 Hz) and sampled at 5 KHz using a 1401 power analog-to-digital converter and Signal 6 software (Cambridge Electronic Design, Cambridge, UK).

Transcranial magnetic stimulation (TMS)

TMS was performed using a 70-mm figure-of-eight shaped coil connected to a Magstim 200 stimulator through a Bistim module (Magstim Co., Whitland, and Dyfed, UK). The recording of MEP was performed on the right DAO muscle, stimulating the contralateral face M1 (Loi et al., 2021). The optimal stimulation site for the contralateral DAO, defined as the cortical spot where a greater MEP was observed, was carefully searched and marked with a soft tip pen over the scalp to keep the same coil position throughout the experimental session. The handle of the coil pointed posteriorly and laterally at about 30-45 degree from the interhemispheric line (Ginatempo et al., 2020, 2022; Pilurzi et al., 2020). The RMT was defined as the lowest TMS intensity that evoked, in the resting muscle condition, MEP of at least 0.05 mV in 5 out of 10 consecutive trials, expressed in percentage of the maximum stimulator output (MSO) (Pilurzi et al., 2013, 2020; Rossini et al., 2015). The paired-pulse TMS protocols were administered with the same coil to assess SICI and ICF. The stimuli consisted of a subthreshold conditioning stimulus (CS) preceding by a suprathreshold test stimulus (TS) with an interstimulus interval (ISI) of 3 ms for SICI and 10 ms for ICF. In both SICI and ICF, the CS intensity was set at 80% RMT and the TS intensity at 120% RMT. Signal 6.05 (Cambridge Electronic Design, Cambridge, UK) software was used for the MEPs assessment. SICI and ICF measures were expressed as the ratio between the conditioned MEP amplitude, elicited by the CS, and the unconditioned MEP, evoked by the TS alone.

Event Related Potentials (ERP)

The early perceptive stage of the face processing was assessed through ERP analysis. Two components of the ERP were chosen for face processing assessment: i) the P100 wave (i.e., the positive wave occurring 100 ms after the face presentation onset), ii) the N170 wave (i.e.,

a negative wave that appears at about 170 ms after the face presentation onset). EEG was recorded using a Neuroscan Synamps system (Compumedics, Charlotte, CN, USA) connected to a 32 channels EEG cap with Ag/AgCl electrodes (Compumedics, Charlotte, CN, USA). EEG signals were streamed to a PC and monitored online through Neuroscan software. Recording electrodes were sited according to the 10–20 international EEG system including Fp1, Fp2, F7, F3, Fz, F4, F8, Ft7, Fc3, Fcz, Fc4, Ft8, T3, C3, Cz, C4, T4, Tp7, Cp3, Cpz, Cp4, Tp8, T5, P3, Pz, P8, T6, O1, Oz, O2. Recordings were referenced offline to the mean reference and the ground electrode was positioned on Fpz. In the offline analysis, the average reference was used. Skin impedances were kept below 5 k Ω and the sampling frequency during recording was set at 1000 Hz. Off-line EEG processing was performed with EEGLAB 14.1.1 (Delorme and Makeig, 2004) running in MATLAB (Version 2015b, MathWorks, Inc., Natick, MA, United States). The EEG signals were epoched (-1 to +1 s) and demeaned using a baseline from -500 to -10 ms. The signal was filtered using a band-pass (1–100 Hz) and a band-stop (48–52 Hz) of fourth order Butterworth filter. Epochs were visually inspected and traces with an excessively noisy signal were excluded (less than 10% for each participant). Residual artifacts were identified using an Independent Component Analysis algorithm (INFOMAX ICA) and eliminated after visual inspection, based on time, frequency, scalp and trials distribution, and amplitude criteria (Rogasch et al., 2013, 2014). Latency and amplitude were chosen as parameters to analyze the P100 and N170 waves, in every single subject for each condition from channels T6 (right N170), T5 (left N170), and Oz (P100). Wave amplitude was measured as baseline to peak amplitude. The analyzed data were separately averaged for each FES. P100 peak was identified as a positive peak with latency between 80 and 100 ms (Adolphs, 2002), while the N170 wave as a negative peak with latency between 130 and 200 ms (Hinojosa et al., 2015).

Experimental design

The study was composed of four experimental sessions, in which the effect of the passive view of facial expressions was assessed on: i) the gaze and fixation patterns, ii) the excitability of perceptive cortical areas, iii) the motor cortex excitability iv) the ability to recognize. All sessions were performed in random order in each subject and separated by at least one week from one another.

In each session, the stimulation trial started with a dark background and then FES were shown to the subjects for 300 ms on the display with a black background followed by inter-trial intervals varying randomly between 4800 and 5200 ms.

Experiment 1: Gaze and fixation pattern during passive viewing of FES

The experiment investigated the time of fixation on each ROIs of the subjects during the passive viewing of the FES. The ocular movement recordings were performed continuously over 150 trials (50 FES per each image (neutral, happy and sad faces)).

Experiment 2: Influence exerted by FES on the P100 and N170 waves

Experiment 2 consisted of an ERP session. EEG activity was continuously recorded over 300 trials (each image was randomly repeated twice, to ensure a higher mediation for the evaluated waves): 100 neutral, 100 happy and 100 sad FES. ERP analysis was focused on the period in which FES were shown.

Experiment 3: Influence exerted by FES on face M1 excitability

SICI and ICF were tested in face M1. Twenty unconditioned and twenty paired MEPs for each ISI (3 ms for SICI or 10 ms for ICF) and each condition (neutral, happy, and sad faces) were delivered to the subjects. To ensure an adequate number of trials each image was randomly repeated twice (for a total of 180 trials). RMT, raw MEP amplitudes and amplitude ratios of paired to unconditioned MEP were used as variables. To minimize the contraction bias in the amplitude of MEP, an online rejection of those trials in which pre-stimulus EMG activity was detected during the recording was performed. This allowed to record at least 20 trials in a clearly resting condition. When needed, offline rejection of active traces was also performed during data measurements and analysis. In all the recordings, discarded offline-MEPs were at maximum 10% of the total 20 (2 at maximum).

Experiment 4: Accuracy and response time to FES

A response time task was run by the software “Stim2” (Compumedics, Charlotte, CN, USA), which presented the different FES and recorded the subjects’ responses. Subjects were instructed to keep the fixation on the screen, visualize each picture, and discriminate, as quickly and as accurately as possible, whether the target face expressed happiness or sadness, while they were instructed to ignore the neutral ones, through the pression of one of two possible response buttons located in a response box, with their right-hand fingers. 150 FES showing neutral, happy, and sad facial expressions were shown. The number of correct

responses (%) for each FES and the average time taken for each response were used as variables for the statistical analysis.

Statistical analysis

Statistical analysis was performed with SPSS 26 software (SPSS Inc, Chicago, IL, USA). Mixed and repeated measures (RM) analysis of variance (ANOVA) and planned post-hoc t-tests with Bonferroni correction for multiple comparisons were used. Compound symmetry was evaluated with the Mauchly's test, and the Greenhouse-Geisser correction was used when required. Results were considered statistically significant when p-value was < 0.05 . Effect size was also evaluated with partial eta squared (η^2p).

Experiment 1: For the analysis of both fixation frequency and fixation duration, a three-way mixed-ANOVA was performed with FES (neutral, happy, and sad) and ROI (mouth, forehead, and eyes and nose) as within-subjects factors and group (young and senior) as between-subjects. If no effect of FES was detected, two-way mixed-ANOVA was performed pulling together FES data, using ROI as within-subjects factor and group as between-subjects factor. For the pupil size analysis, a three-way mixed-ANOVAs was performed with side (left and right eyes) and FES (neutral, happy, and sad) as within-subjects factors and group (young and senior) as between-subjects factor.

Experiment 2: A two-way mixed-ANOVA was performed on the P100 waves by using the amplitude and the latency separately as variables with FES (neutral, happy, and sad) as within-subjects factor and group (young and senior) as between-subjects factor. A three-way mixed-ANOVA on N170 waves, using the amplitude and the latency separately as variables, with side (T5 and T6) and FES (neutral, happy, and sad) as within-subjects factor and group (young and senior) as between-subjects factor was performed.

Experiment 3: A one-way ANOVA was used to determine group differences in RMT. A preliminary mixed-ANOVA on raw TS MEP amplitude was performed with FES (neutral, happy, and sad) as within-subjects factor and group (young and senior) as between-subjects factor. In case of no significant effect of FES on MEP amplitude, a two-way mixed-ANOVA separately for SICI and ICF, was performed using amplitude ratio as variable with FES (neutral, happy, and sad) as within-subjects factor and group (young and senior) as between-subjects factor.

Experiment 4: A two-way mixed-ANOVA on the percentage of correct responses with FES (happy and sad) as within-subjects and group (young and senior) as between-subjects factor was performed. A two-way mixed-ANOVA on the response time parameters with FES (happy

and sad) as within-subjects and group (young and senior) as between-subjects factor was performed.

Finally, Spearman bivariate correlation analysis was performed to explore the link between neurophysiological parameters (fixation frequency, fixation duration and pupil size; P100 and N170 amplitudes and latencies; SICI and ICF ratios) and recognition task parameters (accuracies and response times) that showed any significant difference for group and emotions (Table 2). To evaluate any correlation regardless of age, a correlation analysis was performed pulling groups together. A multiple comparison correction was performed, and only correlations with p values ≤ 0.016 have been considered significant.

Results

Experiment 1: Gaze and fixation pattern during passive viewing of face emotional expressions stimuli (FES)

Fixation frequency - The analysis showed that there was not any significant effect of emotions on fixation frequency which was instead influenced by the observed region of interest (ROI). Indeed, mixed-ANOVA showed a significant effect of ROI ($F_{2,31}=22.641$; $p<0.001$; $\eta^2p=0.414$) but a non-significant effect of FES ($F_{2,31}=2.684$; $p=0.07$; $\eta^2p=0.077$), group ($F_{1,32}=1.643$; $p=0.209$; $\eta^2p=0.049$) nor interaction among factors. Post-hoc analysis on the ROI factor detected higher fixation rate in the eyes and nose-ROIs compared to the mouth ($p<0.001$) and forehead-ROIs ($p=0.049$), with a significant difference between the forehead-ROI and the mouth-ROI ($p=0.001$).

Since no-significant FES effect was found, data from different emotions were pulled together and a two-way-mixed ANOVA was performed. Mixed-ANOVA showed a non-significant effect of group ($F_{1,100}=4.052$; $p=0.05$; $\eta^2p=0.039$) but a significant effect of ROI ($F_{2,99}=71.575$; $p<0.001$; $\eta^2p=0.417$) and interaction between factors ($F_{2,99}=3.229$; $p=0.042$; $\eta^2p=0.031$). Post-hoc analysis on the ROI factor revealed higher fixation frequency in the eyes and nose-ROI than mouth-ROI ($p<0.001$) and forehead-ROI ($p<0.001$), and a significant difference between forehead-ROI and mouth-ROI ($p<0.001$). Bonferroni test on the interaction between group and ROI revealed a reduced fixation frequency for the young compared to the senior subjects only in the mouth-ROI ($p<0.001$) (Figure 11).

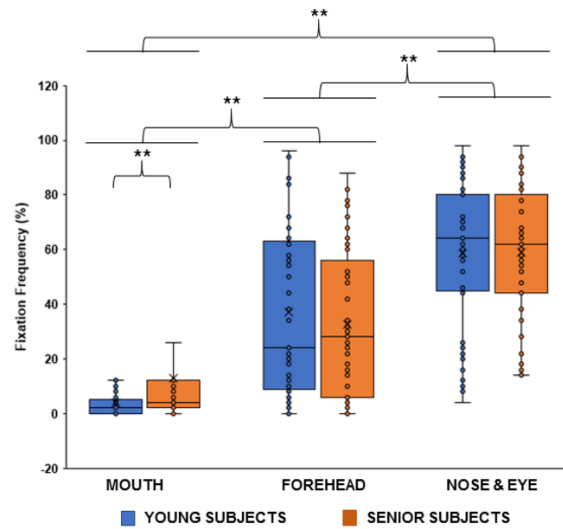


Figure 11. Fixation frequency of the gaze patterns during the passive viewing of FES The graph reports fixation frequency for the young ($n=17$) and senior ($n=17$) groups in the different regions of interest (mouth, forehead, and eyes/nose) during the passive viewing of faces expressing neutral, happy, or sad emotions. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. * $p<0.05$; ** $p<0.01$.

Fixation duration - Three-way ANOVA on fixation duration detected a significant effect of ROI ($F_{2,31}=19.275$; $p<0.001$; $\eta^2p=0.376$), but a non-significant effect of FES ($F_{2,31}=3.011$; $p=0.058$; $\eta^2p=0.086$), group ($F_{1,32}=0.047$; $p=0.829$; $\eta^2p=0.001$) and interaction among factors. Post-hoc analysis on ROI factor highlighted a reduced fixation duration in the mouth-ROI than in the eyes and nose-ROIs ($p<0.001$) and forehead-ROI ($p=0.001$) (Figure 12).

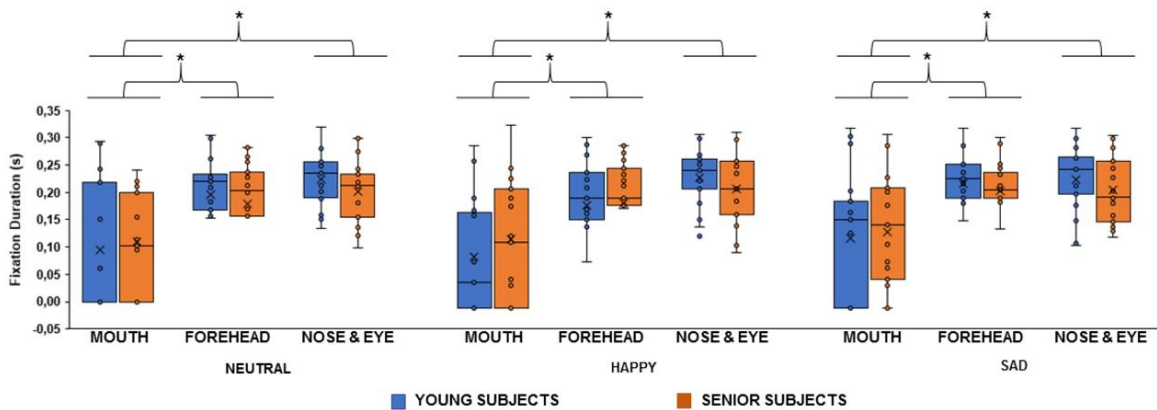


Figure 12. Fixation time of the gaze patterns during the passive viewing of FES. The boxplots show fixation time for the young ($n=17$) and senior ($n=17$) groups in the different regions of interest (mouth, forehead and eyes/nose) during the passive viewing of faces expressing neutral, happy or sad emotions. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. * $p<0.05$.

Pupil size - The pupil size was reduced in senior compared to young subjects. Mixed-ANOVA on pupil size showed a significant effect of group ($F_{1,32}=12.354$; $p=0.001$; $\eta^2p=0.279$), but a non-significant effect of FES ($F_{2,31}=1.950$; $p=0.172$; $\eta^2p=0.057$), side ($F_{1,32}=0.683$; $p=0.415$; $\eta^2p=0.021$) and interaction among factors. Bonferroni test showed a reduced pupil size in senior subjects compared to the young subjects, with no effect of emotions (Figure 13).

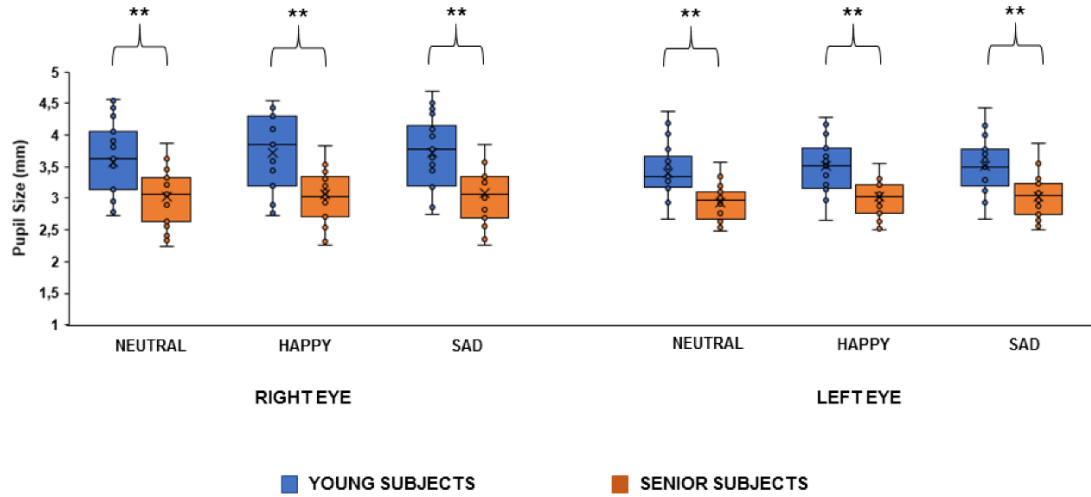


Figure 13. Pupil size during the viewing of FES. The boxplots report the right and left eye pupil size for the young ($n=17$) and senior ($n=17$) groups during the passive viewing of faces expressing neutral, happy, or sad emotions. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. $**p<0.01$.

Experiment 2: Influence exerted by FES on the P100 and N170 waves.

The P100-N170 complex was detected in all subjects in all conditions.

P100 wave – P100 latency was neither influenced by group or FES. Senior subjects compared to young subjects showed reduced P100 amplitude after viewing sad expressions compared to neutral ones.

A non-significant effect of FES ($F_{2,31}=0.96$; $p=0.386$; $\eta^2p=0.029$), group ($F_{1,32}=0.132$; $p=0.718$; $\eta^2p=0.004$) nor interaction between factors was observed by the two-way mixed-ANOVA on P100 wave latency (Figure 14a).

A non-significant effect of FES ($F_{2,31}=1.19$; $p=0.306$; $\eta^2p=0.036$) and group ($F_{1,32}=1.289$; $p=0.265$; $\eta^2p=0.039$) but a significant interaction between the factors ($F_{2,31}=3.71$; $p=0.040$; $\eta^2p=0.104$) was detected by the two-way mixed-ANOVA on P100 amplitude. Post-hoc analysis on the interaction revealed a reduced P100 wave following sad expressions compared to neutral expressions in senior subjects ($p=0.04$), who also had decreased P100 wave compared with young subjects, only for sad expressions ($p=0.033$) (Figure 14b).

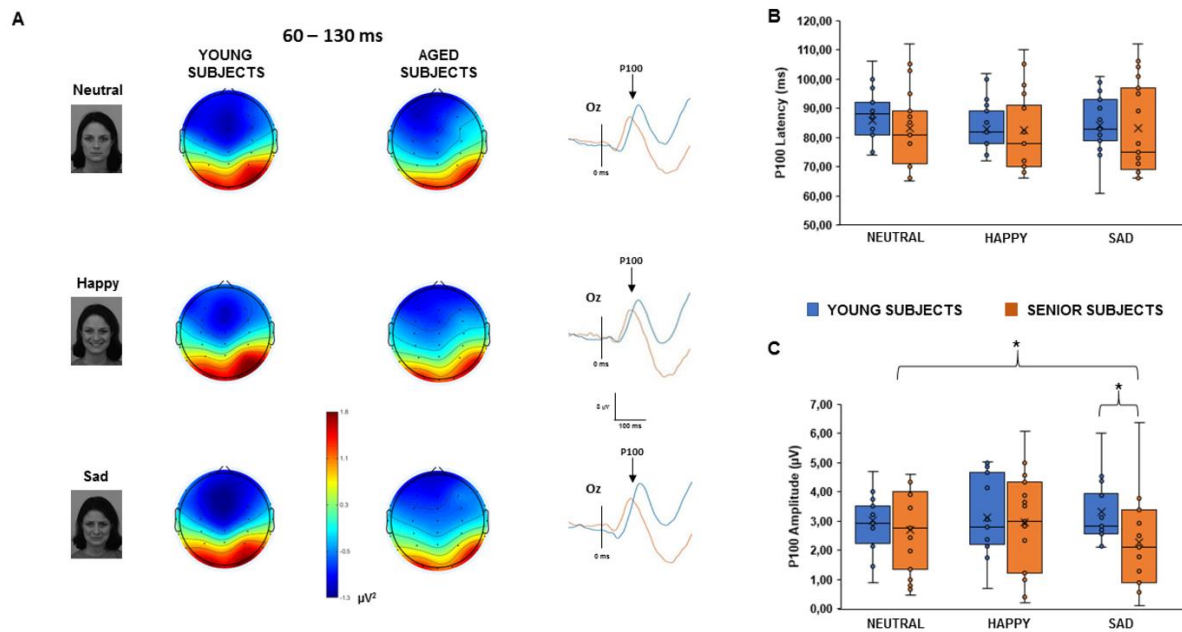


Figure 14. Influence exerted by FES on P100 latency and amplitude. The figure reports the effect of the passive viewing of faces expressing neutral, happy or sad emotions on the amplitude of P100 wave recorded from the young ($n=17$) and senior ($n=17$) groups. Panel A shows scalp distribution and averaged data of the P100 wave. Panel B reports boxplots of the P100 latency, while panel C reports P100 amplitude. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. * $p<0.05$.

N170 wave - A clear right-lateralization effect on N170 wave amplitude was detected regardless of the group but senior subjects showed delayed N170 latency regardless the FES. Three-way mixed-ANOVA on the N170 latency showed a significant effect of group ($F_{1,32}=8.067$; $p=0.008$; $\eta^2p=0.201$) and side ($F_{1,32}=4.536$; $p=0.04$; $\eta^2p=0.124$), but a non-significant effect of FES ($F_{2,31}=1.520$; $p=0.227$; $\eta^2p=0.045$) and interaction between factors. Bonferroni test showed a delayed N170 wave latency in senior subjects compared to the young subjects ($p=0.008$) (Figure 15a).

The statistical analysis on the N170 amplitude showed a significant effect of side ($F_{1,32}=6.058$; $p=0.02$; $\eta^2p=0.159$) but a non-significant effect of group ($F_{1,32}=2.189$; $p=0.149$; $\eta^2p=0.064$), FES ($F_{2,31}=1.071$; $p=0.341$; $\eta^2p=0.032$), nor interaction between the factors (Figure 15b).

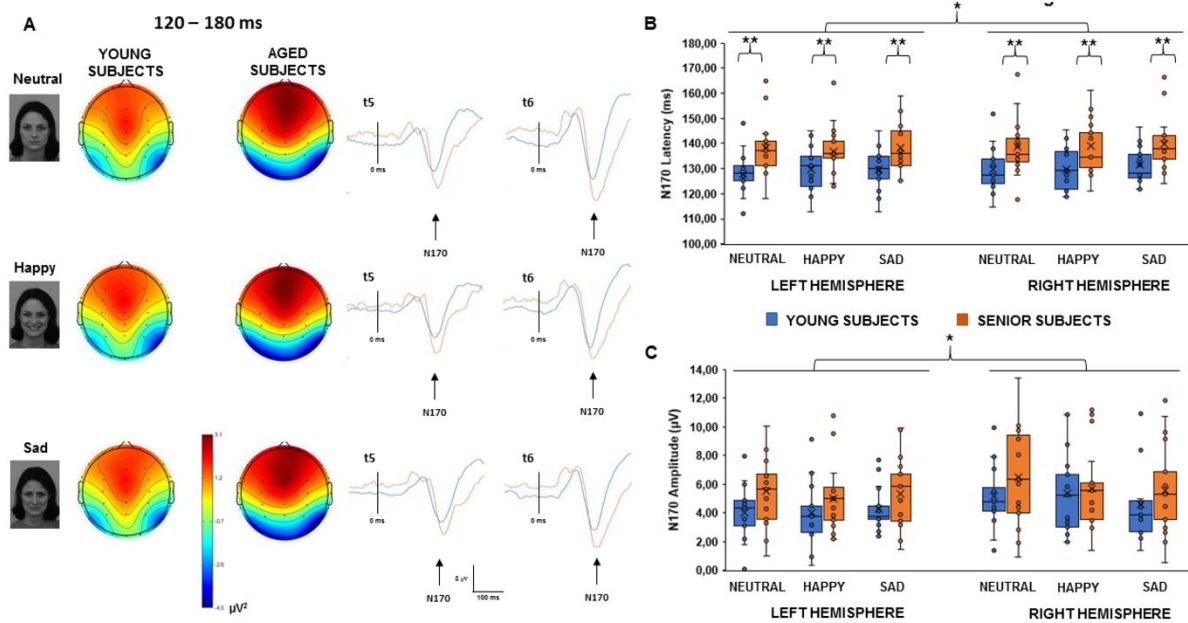


Figure 15. Influence exerted by FES on the N170 latency and amplitude. The figure reports the effect of the passive viewing of faces expressing neutral, happy, or sad emotions on N170 waves recorded from young ($n = 17$) and senior ($n = 17$) groups in both hemispheres. Panel A shows scalp distribution and averaged data of N170. Panel B reports boxplots showing N170 latency, while panel C shows N170 amplitude. The continuous line in the boxplots represents the median value, while the 'x' symbol represents the mean value of the group.; * $p < 0.05$. ** $p < 0.01$.

Experiment 3: Influence exerted by FES on face M1.

Three out of 34 subjects (2 young and one senior subjects) did not perform TMS experiments due to their high RMT, therefore 31 subjects were included in the TMS analysis. RMT and raw MEP amplitude values were not significantly different in the two groups, as reported in Table 3.

Table 3. Motor evoked potentials (MEP) recorded from the right depressor anguli oris following single stimulus (unconditioned MEP) and in paired stimulation to elicit short interval intracortical inhibition (SICI) and intracortical facilitation (ICF) in young and senior subjects following the passive viewing of neutral, happy and sad expressions.

Group	RMT (%MSO)	Unconditioned MEP amplitude (mV)			SICI conditioned MEP amplitude (mV)			ICF: Conditioned MEP amplitude (mV)		
		Neutral FES	Happy FES	Sad FES	Neutral FES	Happy FES	Sad FES	Neutral FES	Happy FES	Sad FES
Young Subjects	55.12±2.0	0.22±0.02	0.21±0.02	0.23±0.03	0.11±0.01	0.13±0.01	0.12±0.01	0.27±0.02	0.28±0.03	0.31±0.03
Senior Subjects	59.15±2.2	0.23±0.05	0.23±0.06	0.22±0.05	0.16±0.04	0.18±0.04	0.20±0.05	0.37±0.07	0.40±0.07	0.36±0.07

RMT, resting motor threshold; MSO, maximum stimulator output; MEP, motor-evoked potential; mV, millivolts; FES, facial expressions still represents means ±SEM

One-way ANOVA on RMT showed a non-significant effect of group ($F_{1,29}=1.765$, $p=0.194$). The analysis on the raw MEP amplitude showed a non-significant effect of group ($F_{1,29}=0.064$, $p=0.801$; $\eta^2p=0.002$), FES ($F_{2,28}=1.418$, $p=0.25$; $\eta^2p=0.047$) nor interaction among the factors.

SICI – Senior subjects showed smaller SICI than young subjects. In addition, reduced SICI was detected following the viewing of happy faces, for the young group, and for both happy and sad expressions in the senior group. Mixed-ANOVA on MEP amplitude ratio showed a significant effect of FES ($F_{2,28}=11.793$, $p<0.001$; $\eta^2p=0.289$), group ($F_{1,29}=12.837$, $p=0.001$; $\eta^2p=0.307$) and interaction between the factors ($F_{2,28}=3.569$, $p=0.03$; $\eta^2p=0.110$). Post-hoc comparisons on FES factor revealed that SICI was significantly reduced following the view of happy ($p=0.02$) and sad ($p<0.001$) facial expressions compared with neutral faces. Post-hoc analysis for the interaction between factors showed reduced SICI for happy faces compared to neutral faces in the young group ($p=0.03$); while SICI was reduced when viewing sad ($p<0.001$) and happy faces ($p=0.04$) compared to neutral in the senior group (Figure 16a).

ICF – Statistical analysis showed an increased ICF in senior compared with young subjects. Mixed-ANOVA on MEP amplitude ratio showed a significant effect of group ($F_{1,29}=5.387$, $p=0.028$; $\eta^2p=0.157$) but a non-significant effect of FES ($F_{2,28}=1.819$, $p=0.179$; $\eta^2p=0.059$), and interaction between factors. The significant effect of group highlighted an increased ICF for the senior compared to the young group (Figure 16b).

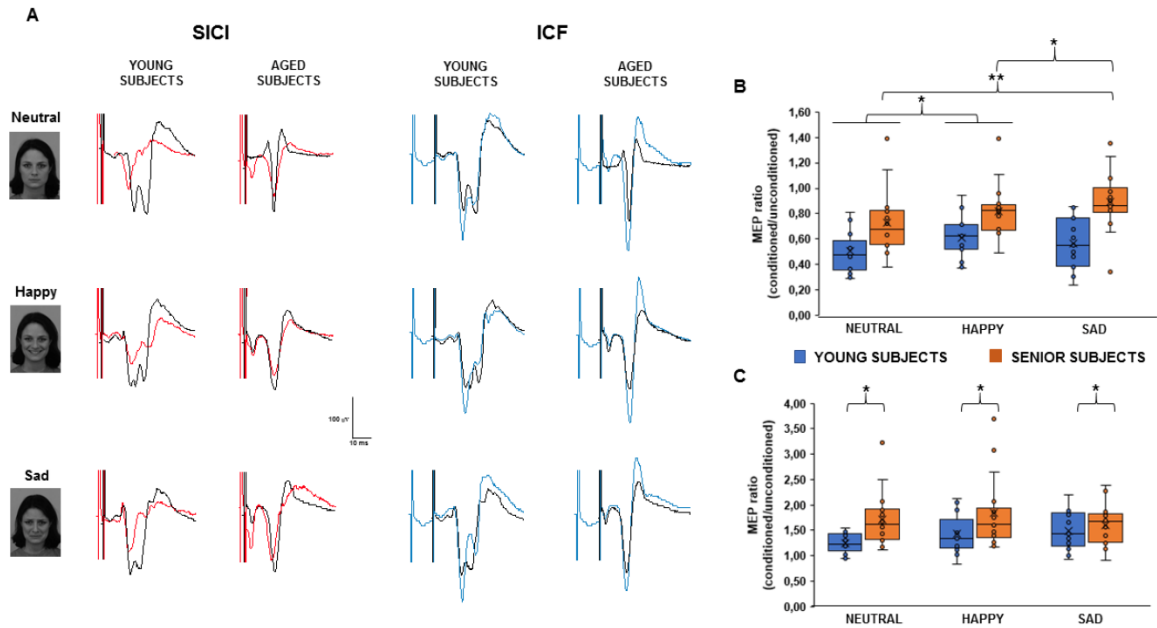


Figure 16. Influence exerted by FES on the excitability of M1 representation area of the right DAO muscle. The figure reports the effect of the passive viewing of faces expressing neutral, happy, or sad emotions on TMS protocols delivered to young ($n=15$) and senior ($n=16$) groups. Panel A shows averaged data of short intracortical inhibition (SICI) and intracortical facilitation (ICF). The black line represents the motor evoked potentials (MEP) obtained following single pulse TMS, while the red and blue line represent MEP obtained following paired stimulation in the SICI and ICF protocols, respectively. Panel B shows SICI boxplots, while panel C ICF. SICI and ICF are expressed as ratio between the paired and unconditioned MEP. MEPs were recorded from the right depressor anguli oris (DAO) muscle. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. The bars show the significant effect of the interaction between the factors (GROUP*EMOTIONS). In fact, Post-hoc analysis showed reduced SICI for happy faces compared to neutral faces in the young group; while SICI was reduced when viewing sad and happy faces compared to neutral in the senior group * $p<0.05$. ** $p<0.01$.

Experiment 4: Accuracy and response time to FES

Accuracy - Senior subjects showed reduced accuracy compared to the young subjects in the recognition of FES. Moreover, the former group took longer time to recognize FES, specifically when viewing sad facial expressions. The two-way mixed-ANOVA on the percentage of correct responses found a significant effect of group ($F_{1,32}=12.745$; $p=0.001$; $\eta^2p=0.285$) but a non-significant effect of FES ($F_{2,31}=2.147$; $p=0.153$; $\eta^2p=0.063$) nor interaction between the factors. Post-hoc analysis showed a reduced percentage of correct responses in senior compared to young subjects ($p=0.001$) (Figure 17a).

Response time - Two-way mixed-ANOVA on the response time detected a significant effect of FES ($F_{2,31}=55.695$, $p<0.001$; $\eta^2p=0.635$), group ($F_{1,32}=4.627$, $p=0.04$; $\eta^2p=0.126$) and interaction between the factors ($F_{2,31}=6.391$, $p=0.017$; $\eta^2p=0.166$). Post-hoc analysis on group factor showed delayed response time in the senior compared to the young group ($p=0.04$). Both groups took a longer time to recognize sad expressions than happy expressions ($p\leq 0.001$). However, post-hoc comparison on the interaction between factors revealed that the

differences between groups were significant only when recognizing sad expressions ($p=0.021$) (Figure 17b).

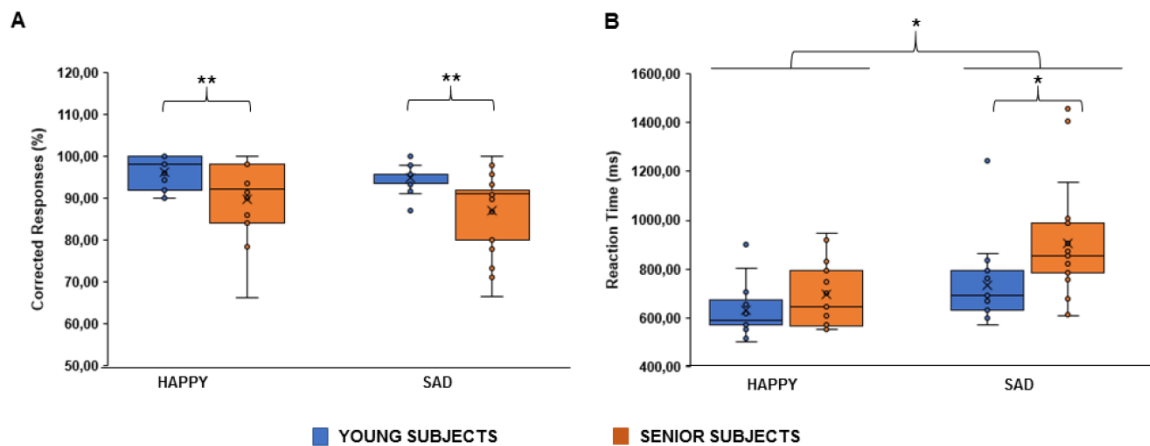


Figure 17. Accuracy and response time to FES. The graphs show the percentage of correct responses (A) and response time (B), of two different groups, young ($n=17$) and senior ($n=17$), during the passive viewing of faces expressing neutral, happy, or sad emotions. The continuous line in the boxplot represents the median value, while the 'x' symbol represents the mean value of the group. * $p<0.05$; ** $p<0.01$.

Correlation analysis

Correlation analysis was performed only for those variables that showed a significant effect of both emotion and group factors. Therefore, we performed correlation among SICI, p100 and reaction time accuracy results. Only SICI-sad was significantly and inversely correlated with the accuracy in the recognition of sad faces ($r=-0.440$; $p=0.015$) (Figure 18).

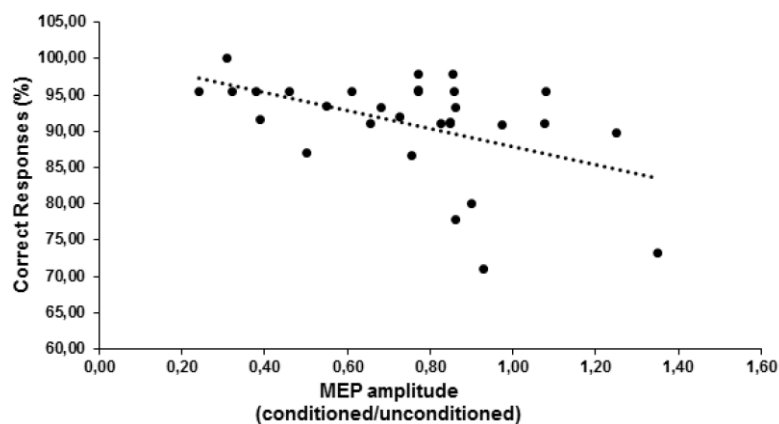


Figure 18. Correlation analysis. The charts refer to the SICI correlation results when all subjects viewed faces expressing sadness. Graphs show correlations between SICI and mean pupil size ($n=31$ subjects) (A), and with the percentage of corrected responses ($n=30$ subjects, 1 outlier removed from the analysis) (B). Graphs describe correlations between SICI and the latency of N170 ERP component of the right (C) and left (D) hemispheres ($n=31$ subjects). SICI is expressed as ratio between the paired and unconditioned MEP amplitude. Dotted lines express trends of the correlation.

Discussion

Overall, the results of the present study revealed that senior people prefer to look at the lower part of the face, accompanied by impaired processing of sad faces at the early perceptive stage, as evidenced by the analysis of the P100 and N170 ERP-components. In senior subjects, along with an altered perception of sad facial expressions, an increased excitability of face M1 was detected particularly when viewing sad faces, and this was negatively correlated with their recognition skills.

Role of the gaze in the facial expressions processing

Data showed larger pupil sizes in young subjects compared to senior subjects, in line with previous papers (Bitsios et al., 1996; Chen et al., 2022), which could be due to muscle features in senior subjects (Erdinest et al., 2021; Eriksson et al., 1965). However, other studies showed that pupil size reduction in senior subjects could be due to alterations of the sympathetic nervous system (Bitsios et al., 1996; Chen et al., 2022; Daneault et al., 2012; Eriksson et al., 1965). In particular, it is well known that the pupil size reflects the balance between the opposing parasympathetic cholinergic constrictor system and sympathetic noradrenergic dilatator system modulation (Bitsios et al., 1996). In addition, several studies demonstrated that the pupil size is strongly modulated by the activity of the locus coeruleus (LC) (Hayat et al., 2020; Joshi et al., 2016; Liu et al., 2017; P. R. Murphy et al., 2014). In fact, LC seems to play a critical role not only in the modulation of the pupil size but also in controlling arousal and attention (Sara and Bouret, 2012), tracking brain state, cognitive (Fazlali et al., 2016; Swift et al., 2018) and emotional processing (Oliva and Anikin, 2018). In this light, it is reasonable to hypothesize that the reduced pupil size in senior subjects could underlie a reduced activity of LC, with consequent effects on the attention and arousal systems which are at the bases of the emotional processing of facial expressions (Luo et al., 2010; Oliva and Anikin, 2018).

On the other hand, in line with previous papers, our cumulative analysis of the gaze patterns of all participants showed a preference to look at the upper areas of the face (eyes, nose and forehead) rather than at the mouth area (Sullivan et al., 2007; Wong et al., 2005) but when comparing the two groups, it emerged that senior subjects had a greater tendency to look at the mouth than young subjects (Sullivan et al., 2007, 2017; Wong et al., 2005). Since, several studies demonstrated that looking at the lower part of the face allows a better recognition of happy expressions, while sad expressions are better recognized when the subjects fix the

upper face (Ebner et al., 2012; Sullivan et al., 2007; Wong et al., 2005). It is likely that the different fixation pattern observed in the senior group could underlie a compensative mechanism, which may allow them to better recognize happy expressions at the expense of sad one's recognition in a similar way to young subjects (Wong et al., 2005). Gaze and pupil size results confirmed previous studies and our hypothesis suggesting that aging can influence gaze patterns which in turn could be due to the LC activity resulting in a reduced attention and fixation towards the sad faces which could lead to a reduced recognition for sad expressions.

Role of the perceptive cortical areas in facial expressions recognition

As described by the smaller P100 amplitude, occipital visual cortex excitability was reduced in senior subjects compared to the young group, but only when viewing sad faces. The P100 wave is associated to the processing of low-level visual features of a stimulus (i.e. contrast and luminance) (Herrmann et al., 2005; Itier and Taylor, 2002, 2004). However, it is also influenced by attention-related modulation of intrinsically salient stimuli (Hillyard and Anllo-Vento, 1998; Luck et al., 2000; Rossion and Jacques, 2012). Since in the present study color, contrast and luminance compositions of the images were consistent, it can be excluded that these basic parameters influenced our results. Therefore, it can be suggested that the differences observed in early visual attentional processes between young and senior subjects were due to a different processing of sad facial expressions. This is supported by previous studies reporting a reduced attention paid by senior people toward negative emotions, suggesting that, with aging, people tend to focus more on positive emotions (Carstensen and Mikels, 2005). It could be speculated that the visualization pattern of faces exhibited by senior people, i.e., an increased number of fixations to the mouth area of emotional faces, may reduce the occipital cortical excitability following the passive viewing of sad expressions, preserving the attention for the happy ones.

On the other hand, a clear age difference in early configural processing of the faces, regardless of the emotions observed, was found as a delayed N170 wave along with a clear right-lateralization effect of the N170 for both latency and amplitude. The lateralization effect is in agreement with several studies (Gainotti, 2019; Herrmann et al., 2005; Luo et al., 2010), supporting the theory of the right hemisphere as the most relevant in face processing for both groups. The N170 wave is influenced by configural characteristics of faces, which are processed in the temporal gyrus (Hinojosa et al., 2015; Rossion, 2014), and used for

subsequent expression categorization (Bentin et al., 1996, 2006; Calvo and Beltrán, 2014; Rossion, 2014; Rossion et al., 2000). In accordance with previous results, the N170 latency was delayed in senior subjects, regardless of the expression observed (Loi et al., 2021; Tsolaki et al., 2017). Researchers described a reduced perception of spatial configuration of faces with aging, regardless of expression (Daniel and Bentin, 2012; Gao et al., 2009), and a delayed N170 latency with unaffected amplitude was observed when configural relationship between the eyes and the rest of the face was altered (Itier et al., 2007), suggesting that the general delayed N170 observed in the present study may describe the altered configural processing performed by the senior subjects.

In fact, previous works assessing fixation-related potentials showed that at least N170 is modulated by the saccadic movements across faces (Buonocore et al., 2020; Huber-Huber et al., 2019). Previous works demonstrated that the saccadic movements can facilitate target categorization not only when it is represented by words (Dimigen et al., 2012), but also by facial expressions. In this light, it could be hypothesized that the different fixation patterns between senior and young subjects may depend on altered saccadic and fixation patterns, influencing ERP perceptive stages when observing faces. On the other hand, the modulation of the P100 and the N170 ERP-components might be explained by an impairment of attentional patterns which could be linked to the different noradrenergic tone in the senior subjects (Norbury et al., 2007; Terbeck et al., 2015). This consideration is supported by studies that found that the noradrenergic system can modulate the fusiform gyrus activation when processing faces (Norbury et al., 2007; Terbeck et al., 2015). This LC-occipito-temporal interaction may play, at least partially, a crucial role in the FES recognition skills in the senior group observed in the present study. These results suggested that probably the alteration of the gaze, due to the modulation of its pattern for sad faces, and the reduced pupil size, could result as reduced P100 and delayed N170 and then a reduced early perception of facial expressions.

The effect of facial expressions on M1 excitability

Results confirmed the findings of our previous study, showing that face M1 excitability was increased in the senior group, as demonstrated by a lower SICI and a higher ICF, than young subjects (Loi et al., 2021). A reduced SICI in hand M1 in senior subjects was previously observed (Peinemann et al., 2001), suggesting a reduction of the γ -aminobutyric acid type A (GABAA) system with aging, as also supported by previous animal studies which showed a

decline of GABA-mediated inhibition with aging (McGinley et al., 2010; Smith et al., 2009). However, other studies found no age differences in SICI (Hua et al., 2008; Ling et al., 2005; Schmidt et al., 2010; Yu et al., 2006). The discrepancies observed in human studies may be partially explained by a study of Hermans and colleagues (Hermans et al., 2018) which found reduced SICI in older subjects' M1 but not differences in GABA releasing compared to the young subjects. This study suggested that GABA levels are not altered in senior people, while post-synaptic GABAergic receptors may be impaired by aging (Hermans et al., 2018).

This is the first study showing a reduced SICI in the face M1 of senior subjects when passively viewing sad faces. Previous studies in young subjects demonstrated that, while hand M1 excitability increases when viewing negative FES (Borgomaneri et al., 2021; Ginatempo et al., 2020; Hortensius et al., 2016; Schutter et al., 2008), face M1 enhances its activation when viewing happy faces (Ginatempo et al., 2020; Loi et al., 2021). This result could underlie the activation of the cortical pathway which connects the temporal and fusiform areas to the motor cortex through an important integrative node that is represented by the ventral premotor cortex (vPM) and is responsible for the processing of others' face-mouth actions (Ferrari et al., 2017). The connection between vPM and face M1 is mediated by GABAergic circuits and could be responsible for the face M1 response to faces (Bäumer et al., 2009). In this light, the correlation between the N170 latency and the SICI of sad faces, describes that a delayed temporal activation when viewing sad facial expressions may induce a reduced GABAergic modulation in face M1, suggesting that, the temporal-vPM pathway may be differently modulated by the viewing of sad facial expressions.

On the other hand, the correlation between SICI and pupil size for sad expressions suggests the hypothesis that aging may modulate face M1 excitability through a direct or indirect noradrenergic-GABA_A interaction when selectively viewing sad faces.

Therefore, data suggest that an alteration of the attention and the early perception of facial expressions, as demonstrated by the alteration of gaze, pupil size and ERP results, could lead a modulation of inhibitory intracortical circuits.

Facial expressions recognition in senior subjects.

Data demonstrated a reduction of accuracy of facial expressions recognition, namely sadness, by senior subjects, as supported by the delayed reaction time in this group. Although previous

research has provided support to the hypothesis that the elderly have specific deficits in the ability to recognize FES (Isaacowitz and Stanley, 2011; Sullivan et al., 2007; Sullivan and Ruffman, 2004), other works are in contrast with this hypothesis (Calder et al., 2003; Wong et al., 2005). In the present study a reduced recognition of happy faces was found in the senior group, in contrast with previous works (Calder et al., 2003; Wong et al., 2005), but in agreement with others reporting a mild impairment in the recognition of happy expressions (Gunning-Dixon et al., 2003; Ruffman et al., 2008). The recognition might have been made more difficult by the very short duration of the visual stimuli, as also suggested by the delayed N170 in senior subjects. Moreover, the difficulty in distinguishing between neutral and sad faces may pose a greater challenge for senior subjects compared to their younger counterpart, as indicated by the P100 findings. This could significantly impact the recognition task. In addition, the reduced SICI in face M1 was linked to an impaired accuracy in the recognition of sad faces, suggesting that, regardless age, increased face M1 excitability when viewing sad expressions, may be disadvantageous to better discriminate sad faces. In this light, the altered GABAergic system observed in senior subjects could be an influence factor in the recognition of sad facial expressions. This finding supports, at least for sad expressions, the hypothesis of a link between excitability of motor cortical areas and facial expressions recognition, in accordance with previous studies (Adolphs, 2002; Balconi and Bortolotti, 2013; Carr et al., 2003; Goldman and Sripada, 2005) which state that understanding face emotions requires individuals to simulate the state of the face shown by estimating the motor representations that gave rise to the perceived emotion. It could be suggested that a shift of the attention due to the different gaze pattern and consequentially reduced early perception of facial expressions, as demonstrated by ERPs study, could induce a reduction of inhibitory intracortical circuits. This phenomenon could underlie a modulation of sensory-motor simulation that impact on facial expressions recognition.

Limitations of the study

We acknowledge that this study has some limitations. First, we did not have a baseline recording for pupil size before or without viewing facial expressions. Therefore, we cannot rule out a possible general effect of the passive viewing of the facial expressions on the pupil size. The reason for this choice is that the experimental procedures was quite demanding for the subjects, which did not allow a longer eye movement recording. In addition, the protocol used in the present study did not allow us to normalize the data for subjective eye features,

which would have helped to rule out a possible subjective bias. Second, the results of our experiment explored only the effect of neutral, happy, and sad facial expressions, therefore they cannot be generalized to other emotions. Third, we did not record any direct attentional measure to evaluate if any preexisting difference between groups could explain the results of the present study. In addition, future studies should compare baseline SICI/ICF measurement in young and senior subjects with those obtained after the visual stimuli to rule out a possible baseline bias.

Conclusions

The results of our study suggest that gaze patterns and early elaboration facial expressions process, observed in senior individuals, indicate that aging diminishes visual attention toward sad faces. This phenomenon seems to be associated with heightened excitability in the face M1 and decreased activity of GABA_A circuits mediating SICI in face M1; which, in turn, correlates with compromised recognition abilities, particularly when processing negative facial expressions.

Research questions 3: Sensorimotor integration and faces recognition in a model of altered face sensory information: neurophysiological study in patients with dentofacial deformities.

Introduction

The efficient execution of skilled movements relies on the harmonious interplay between the sensory and motor systems, where their precisely timed co-activation is used to converge into the central nervous system centres to promote plasticity (Asan et al., 2022). Sensorimotor integration (SMI) process, as delineated by Wolpert and colleagues (1998), is the process through which sensations about one's body part and the external environment are assimilated to shape movement. This complex process unfolds across diverse regions of the central nervous system, spanning the spinal cord, thalamus, basal ganglia, cerebellum, and various cortical areas (Wolpert and Kawato, 1998).

In humans, SMI can be tested with paired-pulse TMS protocols, where conditioning electrical stimuli given to a peripheral nerve are followed by TMS of the contralateral M1 at short intervals SAI (Classen et al., 2000; Pilurzi et al., 2020, 2013; Tokimura et al., 2000) or long intervals (long-afferent inhibition, LAI) (Chen et al., 1999; Classen et al., 2000; Pilurzi et al., 2020). It has been proved that SAI is due to the activation of intracortical inhibitory circuits within the M1 (Reis et al., 2008) and that it is modulated by cholinergic and GABA-aergic activity in the S1 (Tamburin et al., 2001; Tokimura et al., 2000; Turco et al., 2018). Furthermore, various studies have indicated that SAI is influenced by arousal, attentional, and emotional systems (Koizume et al., 2017; Mineo et al., 2018; Mirdamadi et al., 2017). On the other hand, LAI is significantly understudied compared to SAI and the neural circuitries underlying LAI is still unclear (Turco et al., 2017). Since, LAI occurs at long intervals between the nerve stimulus and TMS pulse, it has been hypothesized that LAI depends on a widespread activation of various somatosensory cortical areas, as it is also modulated by GABA-b receptors activity (Turco et al., 2018)

It has been demonstrated that the SMI process is influenced by the task and the muscle district involved especially in the cranial district (Ginatempo et al., 2023; Turco et al., 2018). In the cranial district, there are two different muscle systems, i.e., the masticatory and the facial muscles systems (Cattaneo and Pavesi, 2014). In the masticatory system, sensory inputs are not essential in basic masticatory movements cycle, but these inputs become indispensable for adapting movements to the food's hardness and compensating for unexpected perturbations (Morquette et al., 2012). The movement is greatest around the corners of the mouth, an area highly prone to skin distortion during jaw-movement (Lund, 1991) and cutaneous afferents from trigeminal sensory pathways in this area play a pivotal role in food manipulation within the oral cavity and the precision control of mouth

movements (Morquette et al., 2012). Consequently, disorders involving masticatory muscles and the trigeminal system, such as dentofacial deformities (DD), may lead to deficit in mastication due to alterations in sensory information (Lund, 1991).

On the other hand, face muscles participate in the mastication, but their main role is played in facial expressions execution (Cattaneo and Pavesi, 2014). Due to their functions, face muscles undergo specific motor control and SMI processes (Ginatempo et al., 2023, 2022). It has been recently hypothesized that face M1 plays a crucial role not only in motor control, but also in facial expressions recognition (Ginatempo et al., 2020; Loi et al., 2021). In fact, several studies showed that regions associated with somatosensory functions, play a pivotal role in representing the body state defining an emotion. Activation of these areas becomes prominent in instances where there is a substantial emotional reaction to a facial expressions, aiding in the comprehension of others' emotions through simulation (Halberstadt and Niedenthal, 2001; Pourtois et al., 2004). It has been suggested that in order to perform facial expressions recognition, individuals construct internal motor representations when they visually perceive others' facial expressions (Halberstadt and Niedenthal, 2001). Carr and colleagues (2003) expanded this understanding by demonstrating that both passive perception and intentional mimicry of facial expressions activate shared brain structures. These structures encompass the ventral premotor cortex, the insula, the amygdala, the basal ganglia, the left frontal operculum, and the right frontal cortices (Carr et al., 2003). Despite these revelations highlighting the importance of SMI in facial expressions recognition, the specific role of this integration and how it is re-established following a disruption remains elusive.

Among head and face disorders, DD is a common issue (Cenzato et al., 2021), characterized by an abnormal growth pattern of the maxilla and/or mandible resulting in misalignment that cannot be remedied solely through orthodontic interventions (Rühl et al., 1994). The DD disorder offers the most suitable model to study how the shift of the sensory information reaching the cortex can influence SMI, possibly resulting into an impairment in facial expressions recognition.

Therefore, aim of the present study was to evaluate SMI process in patients with DD and indication to surgery, taken as a model of altered sensory information from the craniofacial district, to investigate whether this deficit could lead to an impairment in facial expressions recognition. To achieve this aim, neurophysiological and behavioural assessments were performed in patients and, for comparison, in a group of sex-and age matched healthy

subjects (controls) to detect possible abnormalities in *i*) brainstem and M1 excitability as well as in SMI, and *ii*) the ability to recognize facial expressions.

Materials and methods

Participants

This prospective study was conducted at the Department of Biomedical Sciences and University Hospital of Sassari between December 2021 and September 2023. Approval for the study protocol was obtained from the independent Ethical Committee of the University Hospital of Cagliari (approval no. PG/2021/16477), and the research adhered to the principles outlined in the Helsinki Declaration. All subjects provided informed written consent before being engaged in the experimental sessions.

Eighteen controls (mean age 24.56 ± 1.02 years; range 21-38 years old; 5 males and 13 females) and eighteen patients (mean age 27.38 ± 1.39 years; range 19-40 years old; 4 males and 14 females) participated in the study. All participants were right-handed as determined by the Oldfield inventory scale (Oldfield., 1971) Patients presented a DD (Class II malocclusion in 9 patients and Class III malocclusion in the remaining 9 patients) requiring bimaxillary (Bimax) orthognathic surgery in 4 patients and Bimax plus genioplasty in 14 patients. The degree of malocclusion was evaluated using the Peer Assessment Rating (PAR) Index, a validated tool for assessing the severity of dental alignment and occlusion. PAR scores were recorded for all patients both before surgery and at 30 days post-surgery. Exclusion criteria were peripheral damage to the facial or trigeminal nerves, history of vestibular pathologies, major surgery, trauma, injuries, or radiation therapy targeting the head and neck regions. Individuals with metallic implants near the head, a history of seizures, past or current assumption of medications active on the central nervous system, past or present neurological or psychiatric disorders were excluded. All patients underwent a psychological evaluation at the onset of the preoperative orthodontic treatment to exclude any pre-existing or current psychiatric conditions, including attention-deficit/hyperactivity disorder, depression and other relevant psychological disorders. Additionally, to rule out obstructive sleep apnea, specific anamnesis was performed, and soft tissue volumetric studies of the upper airway were conducted using three-dimensional CT imaging. The posterior airway space was reconstructed in 3D during virtual surgical planning, and its volume was calculated to identify any significant reductions. Patients diagnosed with OSAS who required bimaxillary

advancement were excluded from this study. Additionally, participants must be able to provide informed consent (Rossi et al., 2021).

Enrolment in the study and preoperative neurophysiological evaluation were conducted for patients meeting the inclusion criteria. Patients were subsequently excluded from follow-up if they experienced iatrogenic sectioning of trigeminal nerve branches, required a second surgery, faced an early relapse of DD, or refused further evaluation.

Recordings were performed in a quiet environment, with subjects seated on a comfortable chair, instructed to remain relaxed but alert during the experiment.

Surgical treatment

Before surgery, all patients underwent a maxillo-facial computed tomography (CT) and scans of their dental arch models. Based on these examinations, virtual surgical planning was carried out, simulating jaw movements to produce surgical occlusal guides for the repositioning of the maxilla and mandible (De Riu et al., 2020, 2018). Surgical procedures were performed in all patients by the same team. The surgery was conducted under general anaesthesia. All patients underwent a Le Fort 1 osteotomy of the upper jaw and a sagittal split ramus according to Epker. Genioplasty was combined with the jaw osteotomies, if deemed necessary for aesthetic purposes. The osteotomy was executed using piezoelectric tools (BoneScalpel, Misonix, Farmingdale, New York, USA) and rigid fixation was achieved with titanium plates and screws (Stryker Corp., Kalamazoo, MI, USA), according to techniques and surgical approaches previously described in the literature (De Riu et al., 2020, 2018).

Following the surgery, all patients were subjected to the same antibiotic and corticosteroid therapeutic protocols.

Facial expressions stimuli

In the recognition task, images from the Karolinska Directed Emotional Faces (KDEF) database (Lundqvist et al., 1998) were presented to participants. The KDEF database is a set of 4900 pictures of human faces from 70 individuals, 35 females and 35 males. There are seven distinct emotional expressions, each of which is photographed twice from five different angles. For our stimuli set, the frontal images of the expressions "neutral", "happy", and "sad" of 5 females and 5 males subjects were selected. Each of the selected images was replicated 5 times and the 150 images were randomly arranged in a sequence, which made up our stimuli set. Visual stimuli were shown on a 17" LCD display, with a 1280x1024 resolution and a 70

Hz refresh rate, by using the PsychToolbox software (Brainard, 1997), running in MATLAB (Version 2015b, MathWorks, Inc., Natick, MA, United States). The duration of the visual stimulus was set at 300 ms. This choice was based on previous studies, which reported 300 ms as an adequate time window for the stimulus to reach frontal cortical areas (Cuthbert et al., 2000; Ginatempo et al., 2020; Loi et al., 2021; Smith and Smith, 2019).

Electromyography

Surface EMG signals were recorded in all participants using a 9-mm diameter Ag-AgCl surface electrodes. For the blink reflex recording, the EMG was recorded from the orbicularis oculi muscles (OO) positioning the active electrode over the lower lid at the centre of the eye and the reference electrode at the edge of the angle of the eye (Esteban, 1999). Recording electrodes were positioned identically at both eyes. EMG signals were recorded (D360 amplifier, Digitimer Ltd, Welwyn Garden City, UK), amplified (x5000), filtered (bandpass 5-5000 Hz) and sampled at 5 KHz using a 1401 power analog-to-digital converter and Signal 6 software (Cambridge Electronic Design, Cambridge, UK).

For the TMS experiments, EMG was recorded from the right DAO and from the right AD muscles. For DAO EMG, the active electrode was placed at the midpoint between the angle of the mouth and the lower border of the mandible, the reference electrode over the mandible border, 1 cm below the active electrode (Ginatempo et al., 2020; Loi et al., 2021). For AD EMG, the active electrode was placed over the muscle belly and the reference electrode about 2 cm anterior to it, adjacent to the inferior border of the mandible (Ginatempo et al., 2024). In both case the ground electrode was placed over the right part of the forehead. EMG signals were amplified (x1000), filtered (bandpass 3-3000 Hz) and sampled at 5 KHz.

Transcranial magnetic stimulation

TMS was performed using a 70-mm figure-of-eight shaped coil connected to a Magstim 200 stimulator through a Bistim module (Magstim Co., Whitland, and Dyfed, UK). The motor evoked potential (MEP) was recorded from the right DAO and AD muscles, stimulating the respective contralateral representation area of the M1 (Ginatempo et al., 2024; Loi et al., 2021). The representation areas for the DAO and AD muscles are located within the lateral aspect of the precentral gyrus, corresponding to the orofacial motor region of M1 (Cattaneo and Pavesi, 2014). The optimal stimulation site for the contralateral DAO and AD, defined as the cortical hotspot where a greater MEP was observed, was carefully searched, and marked with a soft tip pen over the scalp to keep the same coil position throughout the experimental

session. The handle of the coil pointed posteriorly and laterally at about 30-45 degrees from the interhemispheric line (Ginatempo et al., 2024; Pilurzi et al., 2020, 2013). The resting motor threshold (RMT) was defined as the lowest TMS intensity that evoked, in the resting muscle condition, MEP of at least 0.05 mV in 5 out of 10 consecutive trials, expressed in percentage of the maximum stimulator output (MSO) (Pilurzi et al., 2020, 2013; Rossini et al., 2015). Paired-pulse TMS protocols were administered with the same coil to assess short-interval cortical inhibition (SICI). Finally, SMI function was assessed through measurements of SAI and LAI.

Electrical stimulation

Electrical stimulation (ES) was performed using a 9-mm diameter Ag-AgCl surface electrodes. For the blink reflex recordings, ES over the right supraorbital nerve (SON) was delivered at the supraorbital notch, via cup electrodes (cathode over the homonymous foramen and anode two cm lateral) connected to a DS7A Stimulator (Digitimer, Welwyn Garden City, Herts, UK). All stimuli were square waves (0.2 ms duration), and stimulus intensity was set at three times the R2 threshold (lowest intensity that evoked at least five R2 responses in 10 consecutive trials). ES was delivered at the SON at variable time intervals (between 20 and 40 s) to minimize habituation of the blink reflex.

To assess SMI in TMS experiment for DAO M1 representation, ES was applied to the mandibular branch of the right facial nerve for LAI protocol, and on the mandibular branch of the trigeminal nerve for SAI protocol (Ginatempo et al., 2024), through a pair of cup electrodes (cathode distal) connected to a constant-current stimulator (model DS7; Digitimer Ltd) (Figure 19A). Single square-wave pulses (0.2 ms duration) were delivered at a frequency of 0.25 Hz, and stimulus intensity was set at a value nearly three times the perceptive threshold (PT) of the subject, which was able to evoke a small twitch in the target muscle for LAI protocol. For AD recordings, both SAI and LAI protocols were obtained following the stimulation of the mandibular branch of the trigeminal nerve at a value nearly three times the PT of the subject (Ginatempo et al., 2024) (Figure 19B).

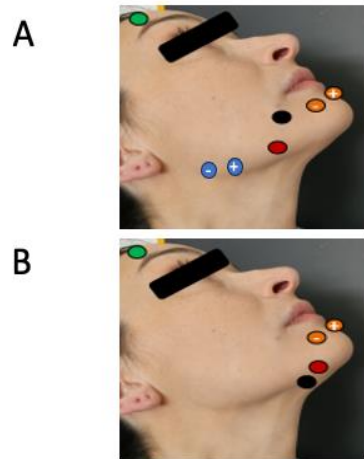


Figure 19. Position of the electrodes for electromyography (EMG) recordings from the depressor anguli oris (DAO), anterior digastric muscle (AD) for the electrical stimulation of the trigeminal, facial nerves. Panel A shows the position of EMG recording electrodes over the DAO: the active electrode (black) is placed at the midpoint between the angle of the mouth and the lower border of the mandible, the reference electrode (red) over the mandible border, 1 cm below the active electrode. Electrodes for stimulation of trigeminal and facial nerves are depicted as orange and blue circles, respectively. Panel B shows the position of EMG recording electrodes over the AD: the active electrode (black) was over the anterior part of the muscle belly and the reference electrode (red) about 2 cm anterior to it, adjacent to the inferior border of the mandible. Electrodes for stimulation of the trigeminal nerve are depicted as orange circles. For both DAO and AD EMG recordings, the ground electrode was placed over the right forehead (green).

Experimental design

The experimental design was composed by four experiments performed in a single session in controls and in two sessions, one before and one after one month from surgery, in patients. Out of the eighteen participants, five patients and five controls were excluded from experiments 2 and 3 due high thresholds of M1 to TMS. Furthermore, two patients couldn't complete all sessions due to heightened post-surgery pain sensitivity.

Experiment 1: Study of brainstem circuits excitability in physiological conditions and in the presence of trigeminal nerve afferents alteration

The excitability of the brainstem was probed using the blink reflex in all eighteen controls and eighteen patients at baseline and in 16 (mean age 26.94 ± 1.30 ; range 22-40 years old; 4 males and 12 females) out of 18 patients after surgery. During recording of the blink reflex, subjects sat calmly in a chair with the eyes open. The early ipsilateral R1 response and the late ipsilateral (iR2) and contralateral (cR2) R2 responses induced by SON stimulation (Kimura, 1983) were assessed. EMG recordings from OO muscles, started 2 s before each stimulus, to allow recognition of excessive background muscle activity and thus rejecting online the trial which showed a clear muscle pre-activation. R2 threshold, R1, iR2 and cR2 areas were calculated in controls and in patients before and after surgery.

Experiment 2: Study of excitability and SMI in DAO M1 representation area in physiological conditions and in the presence of trigeminal nerve afferents alteration

SICI, SAI and LAI protocols were tested in DAO M1 of 13 controls (mean age 23.46 ± 0.92 ; range 21-32 years old; 4 males and 9 females) and 13 patients at baseline (mean age 27.92 ± 1.75 ; range 19-40 years old; 4 males and 9 females) and 11 patients after surgery (mean age 27.36 ± 1.36 ; range 19-40 years old; 3 males and 8 females). For SICI protocol, paired pulse TMS was performed. Stimuli consisted of a subthreshold conditioning stimulus (CS) preceding by a suprathreshold test stimulus (TS) with an interstimulus interval (ISI) of 3 ms. The CS intensity was set at 80% RMT and the TS intensity at 120% RMT.

For SMI assessment, single pulse TMS of the left DAO M1 was preceded by ES on the mental branch of the right trigeminal nerve with 20 ms ISI in the SAI protocol; and by ES on the right facial nerve by 100 (LAI100) and 180 (LAI180) ms ISIs in the LAI protocol. The five blocks and all states (TS alone, SICI, SAI, LAI100, LAI180) were randomized in each subject using a semi-randomized protocol. Ten trials for each state were recorded from the right DAO muscle. SICI, SAI, and LAI were expressed as the ratio between the conditioned MEP amplitude, elicited by the CS, and the unconditioned MEP, evoked by the TS alone.

Experiment 3: Study of excitability and SMI in AD M1 representation area in physiological conditions and in the presence of trigeminal nerve afferents alteration

In 13 controls (mean age 23.46 ± 0.92 ; range 21-32 years old; 4 males and 9 females) and 13 patients at baseline (mean age 27.92 ± 1.75 ; range 19-40 years old; 4 males and 9 females) and 11 patients after surgery (mean age 28.91 ± 1.33 ; range 22-40 years old; 4 males and 7 females), SICI, SAI and LAI protocols were tested in AD M1 representation. For SICI protocol, paired pulse TMS was performed. Stimuli consisted of a subthreshold CS preceding by a suprathreshold TS with an ISI of 3 ms. The CS intensity was set at 80% RMT and the TS intensity at 120% RMT. For SMI assessment, single pulse TMS of the AD M1 was preceded by ES on the mental branch of the right trigeminal nerve with 20 ms ISI in the SAI protocol, and 100 (LAI100) and 180 (LAI180) ms ISIs in the LAI protocol. The five blocks and all states (TS alone, SICI, SAI, LAI100, LAI180) were randomized in each subject using a semi-randomized protocol. Ten trials for each state were recorded from the AD muscle. SICI, SAI, and LAI were expressed as the ratio between the conditioned MEP amplitude, elicited by the CS, and the unconditioned MEP, evoked by the TS alone.

Experiment 4: Effect of the trigeminal nerve alteration on the recognition of facial expressions

In 18 controls (mean age 24.56 ± 1.02 ; range 21-38 years old; 5 males and 13 females), 18 patients at baseline (mean age 27.38 ± 1.39 ; range 19-40 years old; 4 males and 14 females) and 16 patients after surgery (mean age 26.94 ± 1.30 ; range 22-40 years old; 4 males and 12 females), a reaction time task was run by the software “Stim2” (Compumedics, Charlotte, CN, USA), which presented the different FES and recorded the subjects’ responses. Each trial started with a dark background. Then, for each trial, FES appeared for 300 ms on a black screen and were preceded and followed by a black background. Subjects were instructed to keep the fixation on the screen, visualize each picture, and discriminate, as quickly and as accurately as possible, whether the target face expressed happiness or sadness, through the pressing of one of two possible response buttons located in a response box, with their right-hand fingers. The 150 FES showing neutral, happy, and sad facial expressions were shown for a total of 300 trials. Inter-trial interval varied randomly between 4800 and 5200 ms. The number of correct responses (%) for each FES and the average time taken for each response were used as variables for the statistical analysis.

Statistical analysis

Statistical analysis was performed with SPSS 26 software (SPSS Inc, Chicago, IL, USA). Mixed and repeated measures (RM) analysis of variance (ANOVA) and planned post-hoc t-tests with Bonferroni correction for multiple comparisons were used. Compound symmetry was evaluated with the Mauchly’s test, and the Greenhouse-Geisser correction was used when required. A one-way ANOVA was used to evaluate the Age, Sex, RMT and PT difference between groups, while a t-Test was used to evaluate the effect of surgery between patient group.

Experiment 1: At baseline a possible difference between the two groups for R1 latency and area was separately performed using one-way ANOVA with group (controls and patients) as between subject-factor. In patients, to evaluate the effect of surgery on R1 latency and amplitude a paired T-test was used. After surgery a possible difference between the two groups for R1 latency and area was separately performed using one-way ANOVA with group (controls and patients) as between subject-factor.

At baseline, R2 latency and area were separately analysed using two-way RM-ANOVA with side (iR2 and cR2) as within-subject factor and group (controls and patients) as between

subjects' factors. To evaluate the effect of group two-way RM-ANOVA with side (iR2 and cR2) and time (before versus one-month post-surgery) as within-subject factors. After surgery, R2 latency and area were separately performed using two-way RM-ANOVA with side (iR2 and cR2) as within-subject factor and group (controls and patients) as between subjects' factors.

Experiment 2: At baseline one-way ANOVA with group (controls and patients) as between subject-factor was performed using as variable ratio of SICI, SAI, LAI100, LAI180 separately. Paired t-Test was performed to compare the effect of surgery within patient group.

After surgery one-way ANOVA with group (controls and patients) as between subject-factor was performed using as variable ratio of SICI, SAI, LAI100, LAI180 separately.

Experiment 3: At baseline one-way ANOVA with group (controls and patients) as between subject-factor was performed using as variable ratio of SICI, SAI, LAI100, LAI180 separately. Paired t-Test was performed to compare the effect of surgery within patient group.

After surgery one-way ANOVA with group (controls and patients) as between subject-factor was performed using as variable ratio of SICI, SAI, LAI100, LAI180 separately.

Experiment 4: The analysis of the reaction time and accuracy of the facial expressions recognition skills were separately performed through two-way RM ANOVA with emotion (happy or sad) as within subjects' factor and group (controls and patients) as between subjects' factors. To evaluate the effect of the surgery a two-way RM ANOVA with emotion (neutral, happy, and sad) and time (before versus one-month post-surgery) as within subjects' factor was performed. To evaluate any difference between patients after surgery and controls a two-way RM ANOVA with emotion (happy or sad) and group (control versus one-month post-surgery patients) as between subjects' factor was performed.

Correlation analysis: Finally, Pearson's (for parametric variables) and Spearman's (for non-parametric variables) bivariate correlation analyses were performed to explore the link between neurophysiological parameters (SICI, SAI and LAI ratios of AD and DAO M1s) and recognition task parameters (accuracies and reaction times).

Results

Controls and patients were not significantly different as for age ($p=0.111$) and sex ($p=0.99$). The neurophysiological parameters are reported in Table 2 for both controls and patients and, for the latter, before and one month after surgery.

Study of brainstem circuits excitability in the presence of trigeminal nerve afferents alteration compared with physiological conditions

The perceptive threshold of the supraorbital nerve was not different between controls and patients at baseline ($F_{1,34}=2.924$; $p=0.096$), between controls and patients after surgery ($F_{1,32}=0.258$; $p=0.615$) and, within the patient group, from pre- to post surgery ($t_{15}=-1.309$; $p=0.210$). Non-significant differences were detected for R1 latency between groups at baseline ($F_{1,34}=0.292$; $p=0.592$), between controls and patients after surgery ($F_{1,32}=0.109$; $p=0.744$) and, in the patient group, from pre- to post surgery ($t_{15}=0.627$; $p=0.540$). Non-significant differences were detected for R1 area between groups at baseline ($F_{1,34}=0.038$; $p=0.847$), after surgery ($F_{1,32}=1.854$; $p=0.183$) and, in the patient group, from pre- to post surgery ($t_{15}=-1.474$; $p=0.161$). The analysis of the latency of R2 between controls and patients at baseline and after surgery showed a non-significant effect of side ($F_{1,34}=1.237$; $p=0.274$; $F_{1,32}=2.327$; $p=0.137$; respectively), group ($F_{1,34}=0.059$; $p=0.810$; $F_{1,32}=0.120$; $p=0.731$; respectively) and interaction between factors. In the patient group, a significant effect of side ($F_{1,15}=6.240$; $p=0.025$) but not of surgery ($F_{1,15}<0.001$; $p=0.991$) and interaction between factors was observed (Table 2).

Statistical analysis of R2 area at baseline and after surgery showed a significant effect of side ($F_{1,34}=28.738$; $p<0.001$; $F_{1,32}=21.046$; $p<0.001$; respectively) but a non-significant effect of group ($F_{1,34}=0.089$; $p=0.768$, $F_{1,32}=1.009$; $p=0.323$; respectively). In the patient group, a significant effect of side ($F_{1,15}=25.231$; $p<0.001$) but not of surgery ($F_{1,15}=3.231$; $p=0.092$) and interaction between factors was observed.

Study of excitability and SMI in DAO M1 representation area in the presence of trigeminal nerve afferents alteration compared with physiological conditions.

Controls and patients were not significantly different as for age ($p=0.084$).

The analysis of perceptive thresholds of the mental branch of the trigeminal nerve showed a significant effect of group at baseline ($F_{1,24}=4.631$; $p=0.042$), after surgery ($F_{1,22}=7.725$; $p=0.011$), and a significant effect of surgery ($t_{10}=3.553$; $p=0.005$) within the patient group (Table 4).

The analysis of perceptive threshold of facial nerve showed a non-significant effect of group at baseline ($F_{1,24}=0.739$; $p=0.399$), after surgery ($F_{1,22}<0.001$; $p=0.987$), and a non-significant effect of surgery ($t_{10}=-0.606$; $p=0.558$) within the patient group (Table 2).

The analysis of stimulation intensity threshold able to induce a compound muscle action potential (cMAP) from the facial nerve showed a non-significant effect of group at baseline ($F_{1,24}=0.671$; $p=0.421$), after surgery ($F_{1,22}=0.182$; $p=0.674$), and a non-significant effect of surgery ($t_{10}=-0.991$; $p=0.345$) within the patient group (Table 2).

The analysis of RMT of the DAO M1 showed a non-significant effect of group at baseline ($F_{1,24}=0.396$; $p=0.535$), after surgery ($F_{1,22}=1.480$; $p=0.237$), and a non-significant effect of surgery within the patient group ($t_{10}=1.067$; $p=0.311$).

Comparing controls with patients at baseline and after surgery, a non-significant effect of group was detected for unconditioned MEP amplitude (baseline: $F_{1,24}=1.007$; $p=0.326$; after surgery: $F_{1,22}=0.041$; $p=0.842$), SICI (baseline: $F_{1,24}=0.806$; $p=0.378$; after surgery: $F_{1,22}=0.161$; $p=0.693$), SAI (baseline: $F_{1,24}=0.082$; $p=0.778$; after surgery: $F_{1,22}=0.236$; $p=0.632$), LAI100 (baseline: $F_{1,24}=0.782$; $p=0.385$; after surgery: $F_{1,22}=1.236$; $p=0.278$) and LAI180 (baseline: $F_{1,24}=0.315$; $p=0.580$; after surgery: $F_{1,22}=0.017$; $p=0.898$).

The analysis of the effect of surgery, within the patient group, failed to detect any significant effect for unconditioned MEP amplitude ($t_{10}=0.874$; $p=0.403$), SICI ($t_{10}=1.172$; $p=0.268$), SAI ($t_{10}=0.285$; $p=0.782$), LAI100 ($t_{10}=0.250$; $p=0.807$) and LAI180 ($t_{10}=0.445$; $p=0.666$).

Table 4. Neurophysiological parameters of participants

Neurophysiology parameters	Controls		Patients				Statistics (controls vs patients baseline)	Statistics (Controls vs patients post-surgery)	Statistics (patients baseline vs post-surgery)
			Before surgery		One-month post-surgery				
Perceptive threshold of supraorbital nerve (mA)	1.49±0.09		1.28±0.08		1.43±0.07		<i>p</i> =0.096	<i>p</i> =0.615	<i>p</i> =0.210
R1 Latency (ms)	10.48±0.78		11.01±0.56		10.79±0.45		<i>p</i> =0.592	<i>p</i> =0.744	<i>p</i> =0.540
R1 Area (ms*mv)	0.003±0.0004		0.003±0.0003		0.004±0.0005		<i>p</i> =0.847	<i>p</i> =0.183	<i>p</i> =0.244
R2 Latency (ms)	(i)	(c)	(i)	(c)	(i)	(c)	<i>p</i> =0.810	<i>p</i> =0.731	<i>p</i> =0.991
	42.0±0.62	42.36±0.91	41.21±1.58	42.40±1.4	41.03±1.48	42.39±1.05			
R2 Area (ms*mv)	(i)	(c)	(i)	(c)	(i)	(c)	<i>p</i> =0.768	<i>p</i> =0.323	<i>p</i> =0.092
	0.014±0.002	0.008±0.001	0.014±0.002	0.010±0.001	0.011±0.001	0.007±0.009			
Perceptive threshold of mental nerve (mA)	1.92±0.17		1.36±0.18		2.79±0.27		<i>p</i> =0.042	<i>p</i> =0.011	<i>p</i> =0.005
Perceptive threshold of facial nerve (mA)	1.46±0.11		1.32±0.10		1.47±0.13		<i>p</i> =0.399	<i>p</i> =0.987	<i>p</i> =0.558
cMAP of facial nerve (mA)	4.23±0.52		3.72±0.32		3.95±0.31		<i>p</i> =0.421	<i>p</i> =0.674	<i>p</i> =0.345
RMT DAO M1 (MSO%)	57.85±1.51		56.31±1.91		55.0±1.80		<i>p</i> =0.535	<i>p</i> =0.237	<i>p</i> =0.311
Unconditioned MEP amplitude of DAO M1 (mV)	0.18±0.01		0.20±0.02		0.19±0.02		<i>p</i> =0.415	<i>p</i> =0.607	<i>p</i> =0.714
RMT AD M1 (MSO%)	55.69±1.80		56.92±2.29		58.18±1.63		<i>p</i> =0.677	<i>p</i> =0.326	<i>p</i> =0.909
Unconditioned MEP amplitude of AD M1 (mV)	0.26±0.04		0.21±0.03		0.18±0.02		<i>p</i> =0.301	<i>p</i> =0.147	<i>p</i> =0.346

cMAP, compound muscle action potential; i, ipsilateral; c, contralateral; RMT, resting motor threshold; MEP, motor evoked potential; AD, anterior digastric muscle; DAO, depressor anguli oris muscle; M1, primary motor area; mA, milliamper; ms, milliseconds; MSO, maximum stimulator output; mV, millivolt. Data are represented as mean ±SEM.

Study of excitability and SMI in AD M1 representation area in the presence of trigeminal nerve afferents alteration compared with physiological conditions.

Controls and patients were not significantly different as for age ($p=0.084$).

The analysis of RMT of AD M1 showed a non-significant effect of group at baseline ($F_{1,24}=0.177$; $p=0.677$), after surgery ($F_{1,22}=1.010$; $p=0.326$) within the patient group ($t_{10}=0.117$; $p=0.909$).

When comparing controls with patients at baseline a non-significant effect of group was detected for unconditioned MEP amplitude ($F_{1,24}=0.446$; $p=0.511$), SICI ($F_{1,24}=1.510$; $p=0.231$), SAI ($F_{1,24}=1.401$; $p=0.248$) LAI100 ($F_{1,24}=0.218$; $p=0.645$) and LAI180 ($F_{1,24}=0.092$; $p=0.764$).

When comparing controls with patients after surgery, a non-significant effect of group was detected for unconditioned MEP amplitude ($F_{1,22}=2.431$; $p=0.133$), LAI100 ($F_{1,22}=2.750$; $p=0.111$) and LAI180 ($F_{1,22}=2.201$; $p=0.152$). On the contrary, a significant effect was observed for SAI ($F_{1,22}=8.944$; $p=0.007$) (Figure 20A) and SICI ($F_{1,22}=10.261$; $p=0.004$) (Figure 20B) which were reduced after surgery.

Within the patient group, the comparison of pre- with post-surgery data failed to detect a significant effect of surgery on unconditioned MEP amplitude ($t_{10}=1.596$; $p=0.142$), LAI100 ($t_{10}=1.486$; $p=0.168$) and LAI180 ($t_{10}=1.501$; $p=0.164$). By contrast, a significant effect of SAI ($t_{10}=3.280$; $p=0.008$) (Figure 20A) and SICI ($t_{10}=2.475$; $p=0.033$) (Figure 20B), was detected showing a reduced SAI and SICI, after surgery.

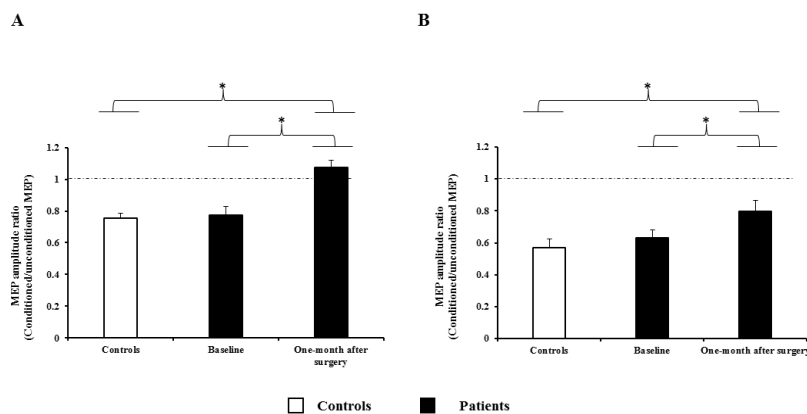


Figure 20. Sensorimotor integration and intracortical inhibition in the representation area of the anterior digastric muscle (AD) in the primary motor cortex of controls and patients. The histograms show the short-afferent inhibition (SAI) (A) and short-interval intracortical inhibition (SICI) (B) recorded from the AD, in controls ($n=13$) and patients at baseline ($n=13$) and post-surgery ($n=11$). The histograms report means $\pm$ standard error of the mean; * $p<0.05$.

When comparing controls with patients at baseline, the analysis of the accuracy in the facial expressions' recognition task showed a significant effect of emotion ($F_{1,34}=5.145$; $p=0.030$) and group ($F_{1,34}=4.628$; $p=0.039$) but no interaction between factors ($F_{1,34}<0.001$; $p=0.987$) (Figure 21A).

The comparison between patients and control after surgery, for the accuracy in the facial expressions' recognition task showed a non-significant effect of emotion ($F_{1,32}=2.363$; $p=0.134$), group ($F_{1,32}=0.413$; $p=0.526$) and interaction between factors ($F_{1,32}=1.265$; $p=0.269$).

The analysis of the accuracy within group, comparing before and after surgery in the patient group, revealed a significant effect of surgery ($F_{1,15}=7.589$; $p=0.015$) showing that the recognition of facial expressions improved after the surgery but a non-significant effect of emotion ($F_{1,15}=0.468$; $p=0.504$) and interaction between factors ($F_{1,15}=0.393$; $p=0.540$).

When comparing controls with patients at baseline the analysis of the reaction time showed a significant effect of emotion ($F_{1,34}=52.704$; $p<0.001$) but a non-significant effect of group ($F_{1,34}=0.005$; $p=0.944$) and no interaction between factors ($F_{1,34}=0.608$; $p=0.441$) (Figure 21B).

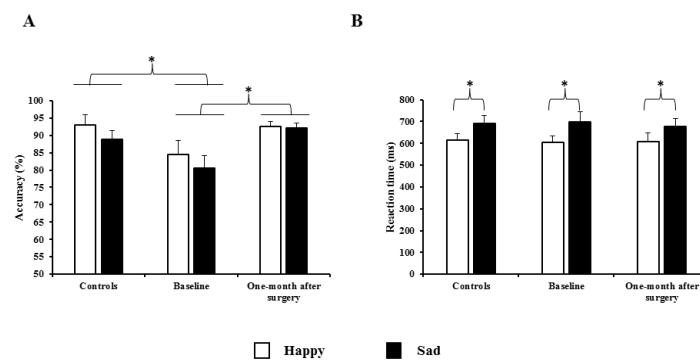


Figure 21. Recognition of facial expressions in controls and patients. The histograms show the percentage of correct responses (A) and the reaction time (B), of controls ($n=18$) and patients ($n=18$) at baseline and post-surgery ($n=16$), during the passive viewing of faces expressing happy or sad emotions in the facial expressions recognition task. The histograms report means $\pm$ standard error of the mean; * $p<0.05$.

When comparing the controls with patients at post-surgery, the analysis of the reaction time showed a significant effect of emotion ($F_{1,32}=11.002$; $p=0.002$) but a non-significant effect of group ($F_{1,32}=0.153$; $p=0.859$) and no interaction between factors ($F_{1,32}=0.249$; $p=0.781$).

The pre- post-surgery analysis of the reaction time within the patients' group revealed a significant effect of emotion ($F_{1,15}=34.556$; $p<0.001$) but a non-significant effect of surgery ($F_{1,15}=2.697$; $p=0.121$) and no interaction between factors ($F_{1,15}=0.060$; $p=0.810$).

Correlation analysis

Spearman's analysis found that before surgery the SAI in the AD M1 was positively correlated with the perceptive threshold of the mental branch of the trigeminal nerve ($r=0.619$; $p=0.024$) (Figure 22A) and negatively correlated with the accuracy in the recognition of happy facial expressions ($r=-0.610$; $p=0.027$) (Figure 22B).

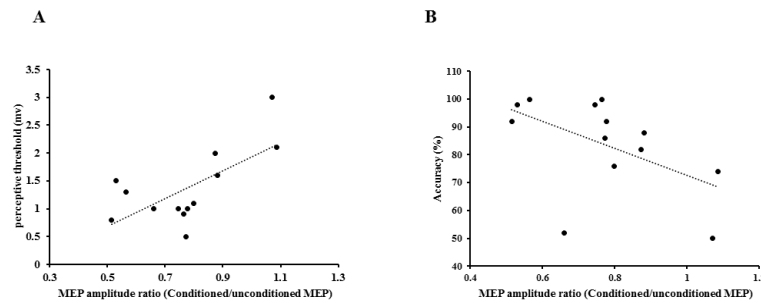


Figure 22. Correlation analysis. The graphs refer to the correlation between the amplitude of the motor evoked potentials recorded in the short-afferent inhibition (SAI) protocol from the of anterior digastric muscle (expressed as ratio between the conditioned and unconditioned motor evoked potentials, MEP) and the perceptive threshold of the mental branch of the trigeminal nerve (A) and the percentage of correct responses during the facial expressions recognition task, with specific regard to happiness (B), within the patient group. Analyses are conducted with Spearman's method, dotted lines express trends of the correlation.

Discussion

Data showed a specific impairment of the short-latency SMI process and of intracortical inhibition in the masticatory representation area in M1 of patients who underwent surgical maxillo-mandibular repositioning, causing an alteration of sensory information carried by the trigeminal nerve, as suggested by specific peripheral alteration at the level of the mental nerve, but not supraorbital and facial nerves. In line with this observation, SMI and excitability of face M1 as well as brainstem excitability were not influenced by the surgical procedure. Interestingly, before surgery, our cohort of patients with chronic DD showed normal cortical, brainstem and peripheral nerve excitability, which were indeed non different from those exhibited by age- and sex-matched healthy controls.

Interestingly, this study also revealed that, despite the apparent adaptation of neurophysiological parameters to the chronic DD, patients displayed a pre-surgery deficit in the recognition of face emotional expressions. Indeed, when compared with healthy controls,

patients exhibited a delayed reaction time in the facial expressions recognition task, regardless the emotion observed. In addition, they specifically showed less accuracy in the recognition of happy facial expressions, which however improved at the post-surgery assessment.

Peripheral and subcortical excitability

Both the condition of DD as well as the mandible repositioning after surgery did not affect both the perceptive threshold of the supraorbital branch of the trigeminal nerve and any of the components of the blink reflex, which exhibited R1 and R2 latencies and areas non significantly different from healthy controls. In addition, in both groups at baseline and in patients after surgery, a lateralization effect for R2 area has been confirmed, aligning with previous studies (Esteban, 1999; Mercante et al., 2015).

This result allowed to rule out a possible impact of both DD and surgery on brainstem circuits mediating the blink reflex, which afferent branch is the supraorbital branch of the trigeminal nerve, the R1 circuit is thought to be restricted to the pons, with an ipsilateral pathway connecting the main trigeminal sensory nucleus and the facial motor nucleus (Aramideh and Ongerboer De Visser, 2002; Cruccu et al., 2005) and the R2 response arising out of a complex bilateral polysynaptic circuit, which includes the spinal trigeminal nucleus, neurons in the lateral tegmental field and the lateral reticular formation (Aramideh and Ongerboer De Visser, 2002; Bourque and Kolta, 2001; Cruccu et al., 2005).

Differently, both the pathological condition as well as the surgery, affected the function of the somatosensory fibres travelling in the mental branch of the trigeminal nerve, as demonstrated by the increase in perceptive thresholds of this nerve in patients, both before and, even more, after surgery. It cannot be excluded that in the latter condition the further increase of the nerve threshold could be due to the post-surgery oedema. However, a single role for the post-surgery oedema in the worsening of the mental nerve perceptive threshold after the mandible repositioning can be excluded, since the facial nerve, which travels closely to the mental nerve, did not appear to be not affected either before or after surgery.

Intracortical excitability and SMI interactions

Data showed a modulation of SMI and intracortical inhibition in the M1 representation area of the AD but not of the DAO after the surgery, suggesting that the alteration of peripheral afferent information, both muscular and cutaneous, modifies central SMI with topographic specificity. In fact, the alteration of the inhibitory circuits underlying SICI and SAI found in

AD M1 representation can be largely attributed to the considerable impact of the surgical procedure on the masticatory muscles, due to either an elongation or a reduction in their length during the repositioning of the mandible (Grunheid et al., 2009). In addition, the specific alteration of SAI could be the result of the impact of surgery on the mental nerve branch excitability as demonstrated by the alteration of its perceptive threshold, both before and after surgery. Noteworthy, the trigeminal nerve plays a crucial role in innervating both the motor and sensory components of the AD muscle.

Studies have demonstrated that SAI is modulated by cholinergic activity in the S1 (Tamburin et al., 2001; Tokimura et al., 2000; Turco et al., 2018), providing robust evidence that implicates acetylcholine (Ach) in SAI generation, as administration of a muscarinic antagonist intravenously reduces it (Oliviero et al., 2005). Furthermore, these studies highlighted the ability of GABA-a receptor agonists to diminish SAI (Rossini et al., 2015; Turco et al., 2018), providing also evidence of an involvement of GABA in SAI generation. The present work revealed that SMI in IDD was impaired as indicated by the reduction of SAI, implying potential dysfunction in GABA-A and/or Ach interneuron activity within masticatory M1. It has been demonstrated that SAI evoked in the cranial district is due to the stimulation of pure cutaneous fibres (trigeminal nerve) (Pilurzi et al., 2013, 2020) and it is known that it can become greater with an increase of nerve stimulation intensity (Turco et al., 2018). However, a reduction of SAI was observed when stimulating pain fibres (Burns et al., 2016), which suggest that the facilitation of nociceptive sensory fibres due to surgery could be responsible for the reduction in SAI observed in the present work. On the other hand, various factors, such as the intensity of nerve stimulation, nerve composition, and the specific muscle and receptors activated, can influence SAI (Turco et al., 2018). Specifically, in the AD it has been recently demonstrated that SAI depends on the activity of low threshold, presumably cutaneous, afferents (Ginatempo et al., 2024), in line with the observation that muscle spindles are rare or absent in AD (Saverino et al., 2014). The observed reduction in SAI that we observed in the post-surgery assessment suggests that alterations in cutaneous fibres may underlie changes in SAI, confirming the study from Ginatempo and Colleagues (2024) showing that this phenomenon is physiologically mediated mainly by cutaneous afferent information.

Not only SAI but also SICI was impaired in patients one-month post-surgery, suggesting that the abnormal processing of sensory inputs and SMI exhibited by the patients after surgery had also an impact on the activity of intracortical inhibitory interneurons. A previous study on

humans examined the impact of somatosensory inputs on cortical motor output using TMS and recording the descending corticospinal volleys (Tokimura et al., 2000). This study showed that the earliest volleys had latencies of 3.4-3.8 ms and were therefore called I1 waves. Later volleys were numbered according to their order of appearance. Furthermore, Tokimura and colleagues (2000) revealed a decrease in both I2 and I3 waves, aligning with SAI intervals, while the I1 wave remained unaffected (Reis et al., 2008). Considering this evidence, it appears plausible that a diminished corticofugal output could result with a reduction in MEP amplitude. In addition, it has been demonstrated that MEPs were inhibited by a TMS pulse delivered 25–50 ms after homotopic stimulation of a digital nerve (Classen et al., 2000; Tamburin et al., 2005). However, if the cutaneous stimulus was applied roughly 35 ms before a conditioning TMS pulse this led to a decrease in SICI (Ridding and Rothwell, 1999). This suggests that afferent input from the digital stimulus directly influences circuits involved in SICI, potentially disrupting later I-waves, and weakening the efficacy of cortical conditioning stimulus (Reis et al., 2008). Consequently, we propose that the peripheral trauma exerted by the surgery, causing the abnormality of sensory inputs could have resulted in a reduction in SICI through the modulation of late I-waves. Whereby, subjects undergoing maxillofacial surgery provide a perfect model to explain how sensory alterations in the cranio-facial region can lead to dysfunctions in motor inhibitory control with topographic specificity.

Naturally, it would be interesting to verify whether these changes are restored with a complete recovery in longer follow-up assessments.

The absence of significant effects at the level of the DAO representation area in M1 is noteworthy. The post-surgery alteration of the trigeminal sensory information seems not to exert a substantial influence on the facial motor system, suggesting that a distortion of the peripheral nervous and muscle districts determine a specific alteration of SAI and SICI in the masticatory M1, but not in the contiguous face M1. Importantly, the surgical procedure on the stomatognathic system appears to have preserved the information originating from the facial muscles. As a result, despite changes in the trigeminal sensory pathways following surgery, the motor components, which are crucial for the execution of facial expressions, remained unaffected.

Trigeminal nerve alteration and its influence on facial expressions recognition

Before surgery, patients exhibited a deficit in the recognition of facial expressions when compared with controls. After one month from the surgery, this impairment improved, although it did not appear completely recovered.

The process of facial expressions recognition involves the complex construction of motor representations by individuals when visually perceiving actions, a phenomenon integral to their performance (Halberstadt and Niedenthal, 2001). Notably, studies have pointed out the flexibility of this process, revealing that direct manipulations of facial expressions, such as the physical act of holding a pencil between one's teeth, can exert a discernible influence on an individual's ability to judge the facial expressions of others (Halberstadt and Niedenthal, 2001). Carr and colleagues (2003) expanded this understanding by demonstrating that both passive perception and intentional mimicry of facial expressions activate shared brain structures. These structures encompass the ventral premotor cortex, the insula, the amygdala, the basal ganglia, the left frontal operculum, and the right frontal cortices (Carr et al., 2003). Simultaneously, regions associated with somatosensory functions play a pivotal role in representing the body state defining an emotion. Activation of these areas becomes prominent in instances where there is a substantial emotional reaction to a facial expressions, aiding in the comprehension of emotions through simulation (Niedenthal et al., 2010; Pourtois et al., 2004).

The facial expressions recognition deficiency that we found, were linked to changes in sensory motor integration with specific regards to happiness. These findings strongly imply that shifts in sensory experiences have the potential to bring about changes in the way faces are recognized. Since integration dynamics are closely tied to an individual's perception, it can be suggested that modifications in the sensory pathways directly impact the process of facial expressions recognition. Additionally, it was observed that before the surgery the accuracy in identifying happy faces exhibited a negative correlation with the SAI of the AD and the latter was also positively correlated with the perceptive threshold of the mental branch of the trigeminal nerve. This demonstrates that alterations in the peripheral sensory component induce significant topographic modifications in the sensory motor system which lead to an impaired facial expressions recognition.

Limitations of the study

This study presents some limitations that need to be acknowledged. First and foremost, the sample size is relatively limited. This constraint might have introduced a Type II statistical error, potentially obscuring significant findings that could have emerged in a larger sample series. Therefore, the results from this study should be regarded as preliminary in nature and need to be confirmed in a larger sample. Second, in the present study, the excitability of brainstem circuits was probed using the blink reflex, which was elicited stimulating the supraorbital branch of the trigeminal nerve, which was not involved in the DD. This model was chosen since the blink reflex is a more stable response than the masseteric inhibitory reflex (Crucchi et al., 2005) and, differently from the latter, does not need teeth occlusion, which the patients were not able to perform steadily, due to the post-surgery condition. Although testing a brainstem reflex involving the V3 trigeminal afferents, it has to be noted that the R2 component of the blink reflex, has its reflex arch extended polysynaptically to the lower medulla. Third, improved mastication following surgery may have broader implications for attention, as it has been reported that masticatory function can influence sustained attention (Mirdamadi et al., 2017). Although our study did not directly assess attentional changes, the observed improvements in neurophysiological measures might partially reflect changes in attentional modulation. Future studies could integrate direct measures of attention to further elucidate its role in SMI and cortical plasticity. Fourth, the duration of the follow-up post-surgery is short. To accurately assess the definitive effects of the intervention, both on cortical excitability and face recognition skills, the follow-up period should have been extended to at least six months, since the recovery from the temporary alterations induced by the repositioning manoeuvres of the jaws in the territories innervated by the maxillary and mandibular branches of the trigeminal nerve, spans across the first six months post-surgery. Fifth, the short follow-up duration at 30 days post-surgery limited the ability to assess other important outcomes of orthognathic surgery, such as facial appearance and patient satisfaction. These factors are typically influenced by the resolution of postoperative edema and the restoration of normal dietary habits, which occur over a longer recovery period. The use of a single time point at 30 days prevented a comprehensive evaluation of these outcomes, which may also contribute to changes in cortical excitability and face recognition skills. Given this, it's plausible that the results obtained one-month post-surgery might not be conclusive, and further changes may manifest as the follow-up period progresses.

Conclusions

The repositioning of the jaw, due to surgical treatment of DD disorders, induces a notable decrease in both SICI and SAI in the representation area of the AD, but not DAO, in M1, indicating that changes in sensory information coming from the affected part of the face can result in disrupted control of cortical inhibitory circuits activity, with topographic specificity. This disturbance plays a role in challenges related to facial expressions recognition, underscoring how DD affects the integration of sensory, motor and behavioural functions.

General Discussion

The overarching aim of the 3-year PhD work depicted in this thesis was to investigate the role of the face motor system in emotion recognition and social interaction, with a special emphasis on sensorimotor integration, interhemispheric connectivity, and the predictive engagement of M1 during the perception of facial expressions. Three complementary projects were designed to address how transcallosal connectivity, aging, and sensory input shape both cortical excitability and behavioural performance in face-related tasks. Taken together, these studies build a coherent picture in which the face motor system emerges as a highly specialized core that integrates sensory, motor, and anticipatory processes, thereby enabling humans to interpret and predict the emotions of others in real time. Building on this framework, the first study examined how the interhemispheric transcallosal connectivity between the homologous face motor areas differs from that of the hand system, revealing a functionally distinct and weakly coupled transcallosal pathway underlying face motor control. At the level of peripheral responses, interhemispheric inhibition (IHI) was strong for hand M1 but absent and even facilitatory for face M1 across short interstimulus intervals, replicating the known hand–face dissociation (Ferbert et al., 1992; Ziemann and Hallett, 2001; Matthews et al., 2013; Meyer et al., 1995; Ginatempo et al., 2019). Moreover, TMS–EEG analyses revealed similar interhemispheric signal propagation (ISP) of TEPs for both systems, consistent with the presence of anatomical callosal pathways between the two face M1s whose functional gain is low at rest (Voineskos et al., 2010; Casula et al., 2020, 2021; Jarczok et al., 2016; Määttä et al., 2017). In hand M1, IHI and ISP correlated in the dominant (left) hemisphere, in line with models of left-hemisphere motor dominance (Hayashi et al., 2008; Daligadu et al., 2013; Casula et al., 2020), whereas in face M1 they did not, supporting an anatomical but functionally lightly coupled callosal link that does not impose strong

bilateral inhibition on bulbar output. Non-human primate data reinforce this dissociation, showing sparse but present callosal projections between face representations (Gould et al., 1986; Rouiller et al., 1994). This network organization provides a functional framework to interpret the following results, explaining how local changes in inhibition and afferent processing remain somatotopically confined without producing strong interhemispheric suppression in face muscles.

In line with this interpretation, the second study focused on how aging influences facial expressions recognition and its neural correlates. Older adults adopted different viewing strategies to interpret facial expressions and were specifically disadvantaged for sadness. They preferentially fixated the lower part of the face, showed smaller pupil responses, and exhibited impaired early processing of sad faces, indexed by reduced P100 and delayed N170, while young adults maintained broader fixations over the eye–nose region (Sullivan et al., 2007; Wong et al., 2005; Mantantzis et al., 2020; Murphy and Isaacowitz, 2010; Sullivan et al., 2017). Since the stimulus luminance and contrast were controlled, the P100 reduction points to attentional rather than low-level visual factors (Herrmann et al., 2005; Itier and Taylor, 2002, 2004; Hillyard and Anllo-Vento, 1998; Luck et al., 2000; Rossion and Jacques, 2012). The delayed, right-lateralized N170 aligns with right-hemisphere dominance for faces and age-related challenges in configural processing (Gainotti, 2019; Hinojosa et al., 2015; Rossion, 2014; Bentin et al., 1996, 2006; Calvo and Beltrán, 2014; Rossion et al., 2000; Loi et al., 2021; Tsolaki et al., 2017; Daniel and Bentin, 2012; Gao et al., 2009; Itier et al., 2007). Reduced pupil dilation in older adults is consistent with a lower locus-coeruleus noradrenergic tone and diminished arousal modulation of attention (Bitsios et al., 1996; Eriksson et al., 1965; Daneault et al., 2012; Chen et al., 2022; Sara and Bouret, 2012; Hayat et al., 2020; Joshi et al., 2016; Liu et al., 2017; Murphy et al., 2014; Oliva and Anikin, 2018). Importantly, these perceptual changes co-occurred with increased excitability in face M1 reduced SICI and enhanced ICF particularly when viewing sad faces (Loi et al., 2021). Such inhibitory reductions link perceptual decline to altered intracortical inhibition (Peinemann et al., 2001; McGinley et al., 2010; Smith et al., 2009; Hua et al., 2008; Ling et al., 2005; Schmidt et al., 2010; Yu et al., 2006; Hermans et al., 2018). Behaviourally, older adults were slower and less accurate, especially for sadness, and reduced SICI correlated with poorer recognition, showing that altered early attention and perception can propagate to intracortical circuits and impair decoding (Isaacowitz and Stanley, 2011; Sullivan and Ruffman, 2004; Calder et al., 2003; Gunning-Dixon et al., 2003; Ruffman et al., 2008).

Extending these observations, the third study explored how changing sensory afferents impacts facial expression processing. Patients with dentofacial deformities undergoing maxillo-mandibular repositioning provided a model where orofacial input changes without global motor damage. One-month post-surgery, we observed reduced SAI and SICI in the anterior digastric representation but not in the neighbouring *depressor anguli oris muscle*, indicating topographic specificity where biomechanical repositioning most affected cutaneous input (Grunheid et al., 2009). The SAI reduction is consistent with cholinergic and gabaergic mechanisms underlying sensorimotor integration and with the reliance of cranial SAI on low-threshold cutaneous afferents (Tokimura et al., 2000; Tamburin et al., 2001; Oliviero et al., 2005; Rossini et al., 2015; Turco et al., 2018; Pilurzi et al., 2013, 2020; Saverino et al., 2014; Ginatempo et al., 2024). Nociceptive facilitation can reduce SAI (Burns et al., 2016), and afferent timing modulates SICI via late I-waves (Classen et al., 2000; Tamburin et al., 2005; Ridding and Rothwell, 1999; Reis et al., 2008). Behaviourally, patients were slower and less accurate pre-surgery, especially for happiness, with improvement after surgery. Pre-surgery SAI for the masticatory M1 correlated negatively with accuracy for happy faces and positively with mental nerve thresholds, directly linking peripheral sensory status to cortical sensorimotor integration and recognition (Halberstadt and Niedenthal, 2001; Carr et al., 2003; Niedenthal et al., 2010; Pourtois et al., 2004).

Together, these findings reveal two convergent pathways: via aging and altered sensory input through which inhibitory balance in face M1 becomes disrupted, impairing emotion recognition and highlighting the centrality of sensorimotor integration in social perception.

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