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Face muscle system: motor control and expressiveness

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Introduction

Cicero, in his work *de oratore* (2013), described human face expressions as "*Imago animi vultus*", the image of the soul, indicating mimicry of the human face as the most important tool for non-verbal communication. Face muscles contribute significantly to human behavior in a wide range of functions such as, for example, feeding, speech production and communication of affective states (Cattaneo & Pavesi, 2014). For these reasons, these muscles are peculiar and different from any other muscles of the body (Müri, 2016).

Anatomy and physiology of the face motor system

Face muscles are firstly divided into cutaneous and masticatory muscles, the former receive the motor innervation from the facial nerve (VII cranial nerve) and the latter from the trigeminal nerve (V cranial nerve). Masticatory muscles are mainly involved in mastication and positioning of the mandible with respect of the body, while being minimally involved in expressiveness. Therefore, they will not be dealt with here. Cutaneous muscles are in turn divided into muscles of the cranial vault, composed by the temporo-parietal and the occipito-frontal muscles, and face muscles which comprise the muscles around the nose, the auricular pavilion, the mouth and the eyelids (Muri, 2016) (Figure 1). In particular, face muscles consist of a flat web of muscular fascicles, embedded in a variable matrix of connective tissue and packed into a two-dimensional matrix under the facial skin (Cattaneo & Pavesi, 2014). In this respect they differ from the upper limb muscles where a single muscle is characterized by its own bony insertions, the joint upon which it acts, and its individual fascia of connective tissue (D'Andrea & Barbaix, 2006). This feature is more relevant in the superficial layer of the muscles around the mouth, which are largely overlapping and can be defined, from a morphological point of view, as single muscle unit (D'Andrea & Barbaix, 2006).
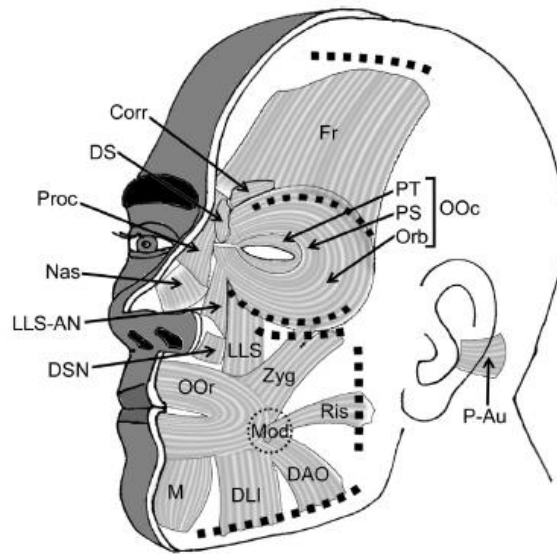
Figure 1. Schematic representation of the face muscular system.

Corr = corrugator; Fr = crontalis; Proc = procerus; Nas = nasalis; DS = depressor supercilii; OOc = orbicularis oculi; PT = pretarsal; PS = preseptal; Orb = orbital; LLS-AN = levator labii superioris alaquae nasi; LLS = levator labii superioris; DSN = depressor speti nasi; Zyg = zygomaticus major and minor; Ris = risorius; M = mentalis; DLI = depressor labi inferioris; DAO = depressor anguli oris; P-Au = posterior auricularis. Bold dashed lines represent the main points of thethering of the facial skin to the skull. Mod = modiolus.

Cattaneo & Pavesi, *Neuroscience & Biobehavioral Reviews*, 2014

In regard of somatosensory afferents and sensorimotor integration of face muscles, they are peculiar and different from limb muscles. For the latter, sensory fibers reach the posterior horns of the spinal cord and then project towards the thalamus and then to the somatosensory cortical areas, contrary to the face muscles where the sensory information from the face is collected by the trigeminal nerve and reaches three different nuclei, located in the brainstem called the mesencephalic, the spinal and the principal nucleus, (Hall & Hall, 2020). In particular, these nuclei play different roles: the mesencephalic nucleus mediates proprioceptive information from the masticatory and extraocular muscles, the principal nucleus receives epicritic information of the face and oral mucosae, while the spinal nucleus collects the protopathic information. Differently from the other body muscles where the proprioceptive information is collected by the muscle spindles and the tendon receptors, that are engaged to monitor instantaneously static and dynamic information about muscle activity, the facial muscles seems to lack these or similar receptor structures. In fact, previous studies demonstrated that proprioceptive system may depend on cutaneous Ruffini-like receptors which are able to detect information of

dynamic and static movements of face muscles (Nordin & Thomander, 1989; Connor & Abbs, 1998) but this issue is still debated.

For the facial muscles, somatomotor information is driven by the proper facial nerve (Figure 2) which originates in the pons from the facial nucleus and emerges from the ventro-lateral part of the ponto-medullary junction (Hall & Hall, 2020). It enters in the facial canal at the level of the parotid gland where it divides into 3 different branches to innervate facial muscles: the intra-cranial, the intra-temporal and the extra-cranial, which dived the facial nerve into several branches that reach the scalp, the auricular pavilion, the face, the *platysma*, the *styloioid* muscle and the posterior abdomen of the *digastric* muscle (Hall & Hall, 2020).
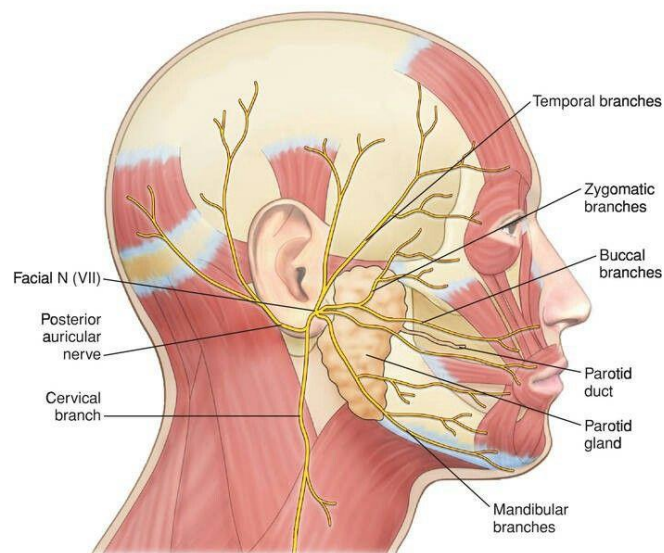

Figure 2. Proper facial nerve (VII)

Superficial branches of facial nerve, Jipa L., web site, <https://ro.pinterest.com/pin/779545016720090746/> (11/01/2022)

The motor nucleus of the facial nerve (Figure 3) is located in the caudal portion of the ventrolateral pontine tegmentum and it contains only motoneurons directed to the mimetic muscles. It has been observed that in humans the facial nucleus is the largest of motor nuclei in the brainstem (Cattaneo & Pavesi, 2014). The facial nucleus is organized in a musculotopic pattern, in which neurons innervating the same facial muscles are arranged together in longitudinal columns, which are oriented craniocaudally (Morecraft et al., 2001). Both in humans and primates the facial nucleus is subdivided in four distinct subnuclei: the dorsal, the medial, the intermediate and the lateral subnuclei. The dorsal

subgroup innervates the muscles around the eyes, while the medial subgroup controls muscles in the area of the ears (Holstege, 2002). The intermediate and the lateral subnuclei of the facial nucleus send fibers innervating the perioral region (Jenny & Saper, 1987). In particular, the lateral subgroup, which is the hugest neuronal subgroup of the facial nucleus is divided into a dorsal and ventral part, which innervates the muscles of the upper and lower part of the mouth respectively (Holstege, 2002).
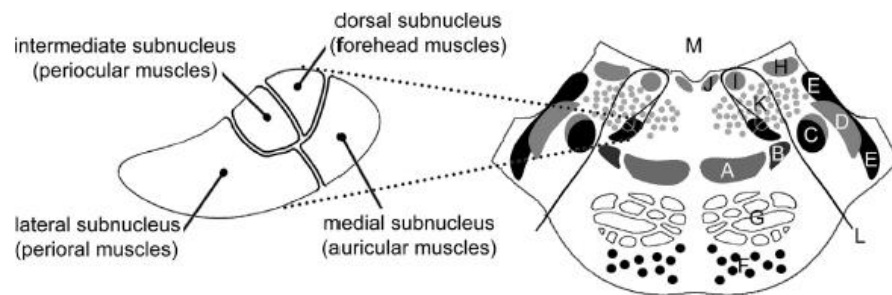

Figure 3. Anatomical representation of the motor nucleus of the facial nerve (VII cranial nerve).

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 an important motor modulation by the face representation of the primary motor cortex (M1). Previous studies showed that humans have thicker and richer local circuitry in face motor cortices than other species and the contraction of their face muscles has the highest rate of dependence on the M1 compared to the other muscle motor representations in M1 (Cattaneo & Pavesi, 2014; Müri, 2016). It is important to remember that face representation of M1 sends bilateral projections with a contralateral predominance (Paradiso et al., 2005; Pilurzi et al., 2013), differently from limb motor control where M1 only projects contralaterally (Rothwell et al., 2021) as demonstrated by studies conducted in non-humans primates (Morecraft et al., 2001) and in humans through transcranial magnetic stimulation (TMS) (Benecke et al., 1988; Meyer et al., 1994; Urban et al., 1997; Liščić & Zidar, 1998; Pilurzi et al., 2013).

Another important difference between limb and face motor control is the transcallosal interaction between the M1s of the two hemispheres. In particular, hand M1 of a

hemisphere is able to inhibit the contralateral M1 to promote asymmetrical movements of the upper limbs (Ferbert et al., 1992). However, a previous TMS-study failed to detect this interaction between face M1s, which lead to hypothesize that it does not occur or at least it is less powerful (Ginatempo et al., 2019a).

Several studies conducted in non-humans primates demonstrated that facial nucleus receives projections also from other areas of the cerebral cortex different from M1 (Morecraft et al., 1996, 2001). In particular, it was shown that the dorsal (dPM) and the ventral (vPM) premotor areas, localized anterior to the face representation of M1, project contralaterally to the lateral subnucleus of the facial nucleus innervating muscles of the lower mouth (Morecraft et al., 2001). Moreover, the caudal portion of the anterior cingulate motor cortex (M4) projects to the lateral subnucleus (Morecraft et al., 2001). M4 projects exclusively to the dorsolateral part of the lateral subnucleus, which innervates the upper portion of the mouth. It was observed that bilateral projections to the medial subnucleus of the facial nucleus, innervating muscular region around the eyes, originate from the supplementary motor area (SMA), located in the midline and anterior to M1, and from the rostral portion of the anterior cingulate cortex (M3) (Morecraft et al., 1996, 2001). Interestingly, cingulate areas are crucial stations for limbic inputs into the facial motor system, as it is demonstrated by both limbic and prefrontal inputs to M3 (Morecraft & van Hoesen, 1993; 2007). Furthermore, a previous study described projections from the amygdala to M3 and from M3 to the facial nucleus of primates (Shima et al., 1991; Shima & Tanji, 1998; Morecraft et al., 2007). This area is believed to be involved in the control of the emotional expressions such as fear, anger, happiness, and sadness of upper facial muscles (Morecraft et al., 2007). This hypothesis was confirmed by previous animals studies that showed the presence of mirror neurons specifically activated during the observation of face movements (Ferrari et al., 2003). These neurons are located in the lateral portion of the vPM and in the opercularis area, and present strong connections with the dPM, the insula, the somatosensory (I and II), infraparietal, ventral prefrontal cortices, the striatum, the amygdala and the thalamus. These connections are important for the correct understanding of face expressions of others (Ferrari et al., 2017). This face mirror circuitry is partially different from the hand which implies projections from the superior temporal sulcus, carrying visual information, directed to parietal areas and which turns finally to the hand representative area in the vPM (Bonini et al., 2010; Gerbella et al.,

2011). The differences between these circuits have been justified by the different developmental of hand and face muscle functions. In fact, during infancy, it seems that the ability to move the hand develops thanks to the visual feedback of the own hand movement, integrating visuomotor information within the parietal-premotor circuits (Del Giudice et al., 2009; Casile et al., 2011; Ferrari et al., 2013). In contrast, infants cannot see their own face, for this reason, their mother/caregiver provides continuous feedback to their face expressions and emotional state, often imitating them and thus reinforcing the emotional content of their face expression (Murray et al., 2016). Face mirror neurons could therefore be formed within a context of communication and emotional exchange.

On the other hand, a dissociation between both emotional and voluntary facial movements and their cerebral pathways was evidenced by patients studies. In particular, the voluntary palsy of face muscles was observed after several type of lesions such as the M1, the descending the pyramidal tract and to the brainstem (Hopf et al., 1992; Töpper et al., 1995; Trepel et al., 1996; Urban et al., 1998, 2001; Bouras et al., 2007; Kappos & Mehling, 2010). Differently, the less frequent emotional palsy of face muscles was described after lesions of the contralateral anterolateral and posterior thalamus, of the anterior striatocapsular region and of the medial frontal lobes (Bogousslavsky et al., 1987; Trosch et al., 1990; Hopf et al., 1992, 2000; Ross & Mathiesen, 1998; Michel et al., 2008). Moreover, emotional palsy can occur also after brainstem lesions in the medulla (Cerrato et al., 2003) and in the ipsilateral pons (Hopf et al., 1992), suggesting that the voluntary and the emotional systems are independent up to the facial nucleus.

Cerebellar influence on the face motor system

It is well known that the cerebellum plays an important role in the coordination of movements especially to adjust planned motor responses to the context, through the integration of peripheral and central information (Hall & Hall, 2020). Cerebellum has a complex structure, composed by a cortical region, in which Purkinje cells receive inputs from the rest of the central nervous system and communicate with the deep nuclei located in the most ventral portion of the cerebellar structure, which represent the exit gates of the information from the cerebellum (Hall & Hall, 2020) (Figure 4).

It has been demonstrated that the plastic changes occurring both in the cortex and in the deep nuclei of the cerebellum, are the basis for several functions such as memory and

learning. As largely demonstrated in the literature, cerebellum plays a major role not only in motor, but also in cognitive and emotional processes (Sacchetti et al., 2004, 2005, 2009; Timmann et al., 2010; Grimaldi et al., 2014). In addition, cerebellum has deep connections with the facial motor system (Stoodley & Schmahmann, 2010). In particular, the cerebellar cortex receives trigeminal projections transporting sensory information of the face to anterior and posterior cerebellar cortices; while, through the deep nuclei activation, it modulates facial motor responses (Stoodley & Schmahmann, 2010).
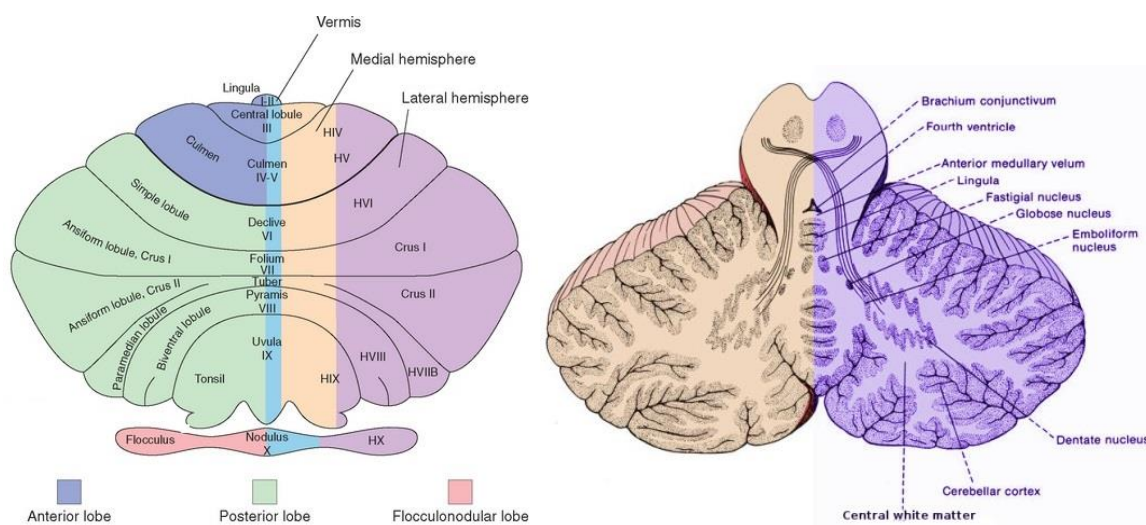

Figure 4. Cerebellar anatomical structure

Left panel represents external cerebellar structure with its lobular stratification. Right panel describes internal cerebellar structure showing differentiation between the cerebellar cortex and the deep nuclei.

Yu & Wang, *SatPearls*, 2021

Cerebellar dentate nucleus sends projections that pass along the superior peduncle and makes synapse with the contralateral red nucleus in the midbrain. From the red nucleus the information reaches the ventro-lateral nucleus of the thalamus which, in turn, projects to the premotor cortex and the M1, influencing voluntary movements (Timmann et al., 2010; Grimaldi et al., 2014). Moreover, interpositum cerebellar deep nucleus is connected with the red nucleus which, in turn, is directly linked with the facial nucleus (Freeman & Steinmetz, 2011; Takehara-Nishiuchi, 2018). This circuit underpins the eye blink conditioning, in which an aversive stimulus (unconditioned stimulus), capable of triggering the blink reflex, paired with a neutral stimulus (conditioned stimulus), is able to induce changes in the cerebellar circuits (Thompson and Steinmetz, 2009). The repetition of these combination of stimuli leads to a conditioned learning that results in a conditioned

response following only the administration of the conditioned stimulus. The conditioned response, identifies the formation of a new learning, which depends on plastic changes occurring both in the cerebellar cortex and in the interpositum nucleus (Thompson & Steinmetz, 2009; Freeman & Steinmetz, 2011).

Learning processes influence face motor system

The learning process consists in the acquisition or modification in the information regarding knowledge, behaviors or abilities. This process is due to different biological processes which lead to changes in the brain microstructure (Magee & Grienberger, 2020). This phenomenon is based on synaptic plasticity processes, such as long term potentiation (LTP) and long term depression (LTD) (Magee & Grienberger, 2020) (Figure 5). In particular, LTP and LTD describe changes in synaptic transmission, an increment in LTP and a reduction in LTD, which can be simulated in experimental protocols in order to study the synaptic complexity of a target area (Sacchetti et al., 2004; Rosenkranz et al., 2007). Previous TMS-studies demonstrated that also face M1 is susceptible to plastic changes (Pilurzi et al., 2013; 2020). These studies used a paired associative stimulation (PAS) protocol in which the association of a peripheral electrical stimulation to the facial nerve with a TMS-pulse delivered into the face M1 was able to induce plastic changes in the face M1, observed as increment in amplitude of the motor evoked potential (MEP). The plastic change occurring in the hand M1 is often associated to increased ability in fineness of movements (Zatorre et al., 2007; Penhune et al., 2012), but the behavioral counterpart of these changes in the face M1 has not been investigated.
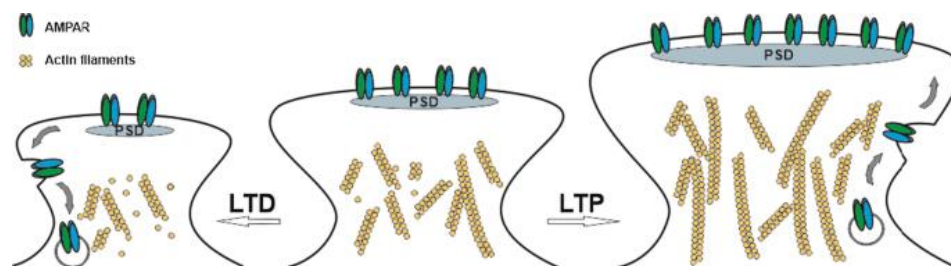

Figure 5. Graphic representation of long term potentiation (LTP) and long term depression (LTD) phenomena

More alfa-Amino-(3-Idrossi-5-Metil-4-isoxazol) propionate receptors (AMPARs) are recruited to the postsynaptic membrane through exocytosis and anchored on the postsynaptic density (PSD), which is also increased in the persistent phase of LTP. Conversely, LTD induces AMPAR endocytosis and consequently PSD depotentiation.

Ji et al., *Molecular Brain*, 2021

An important model of learning acknowledged in the literature is the musician. In fact, playing a musical instrument from an early age plays a crucial role in the motor coordination and fineness. These functional improvements depend on structural modifications in several brain areas such as the cerebellum, dPM, SMA, basal ganglia, corpus callosum and M1 (Schlaug et al., 1995; Zatorre et al., 2007). Previous TMS-studies demonstrated differences between musicians and non-musicians in the motor network. In particular, they show that musicians have steeper MEP recruitment curves, greater sensitivity to plasticity-inducing protocols (Rosenkranz et al., 2007) and less interhemispheric inhibition (Ridding et al., 2000). In particular, changes in M1 representation are related to the type of instrument played, indicating that they are produced by the specific experience of playing the instrument rather than genetic or other causes (Bangert & Schlaug, 2006). For instance, pianists' and violinists' brains can be distinguished from each other even on the gross macroscopic level by examining the shape and size of the part of M1 that contains the representations of the hand (Bangert & Schlaug, 2006).

Face recognition

Recognizing the emotions of an individual from his/her facial expressions is a fundamental processing to understand others facial communication and to better perform social behavior (Blair et al., 2003, 2004). Face expressions are believed to represent innate and automatic behavioral patterns in response to a value of a stimulus. Accordingly, Ekman (1973) identified six innate basic emotions, which appear to be categorized similarly from facial expressions in different cultures (Ekman, 1994). These expressions comprise happiness, sadness, fear, disgust, anger and surprise. There are evidence suggesting that emotional information of faces is extracted by the geometric properties of the stimulus, discriminating emotional content of faces by morphological categories (De Gelder et al., 1997; Calder et al., 2001). Hence, faces with different expressions are morphed with different geometric structures, leading to different categorical perception of facial expressions. Among the information that can be extracted from a face, not only the emotion is identifiable, indeed other information such as gender, identity and age are all dissociable information that are analyzed at different stages of the processing (Adolphs,

2002). One possibility is that different types of information are processed by cerebral subsystems that are already discriminated at the level of categorical perception (Adolphs, 2002). Several neurobiological evidence allowed to determine two principal neuronal pathways describing face expressions processing: a subcortical pathway, that bypass striate cortex, including a rapid, automatic and coarse processing, elicited by highly salient visual stimuli; and a cortical processing involving occipital and temporal visual cortices. This second pathway is believed to be activated when fine-grained perceptual representations need to be constructed (Adolphs, 2002; Blair, 2003; Vuilleumiere & Pourtois, 2007; Smith & Smith, 2019) (Figure 6). The pathways mediating subcortical perception of emotion involve projections from the retina to the superior colliculus, which leads visual information to the pulvinar of the thalamus projecting in turn to extrastriate visual cortices. In support of this subcortical processing, neuronal responses to visual stimuli can be detected in extrastriate cortex despite a complete lack of input from striate cortex (Rodman et al., 1989). Information acquired with this subcortical pathway is useful to process crude features of the stimulus, such as its spatial location, its direction of motion, and some spectral information (Cowey & Stoerig, 1991). The amygdala and, more in general, the limbic circuit seem to influence this subliminal face processing, giving an emotional modulation to this process (Adolphs, 2002; Vuilleumiere & Pourtois, 2007).
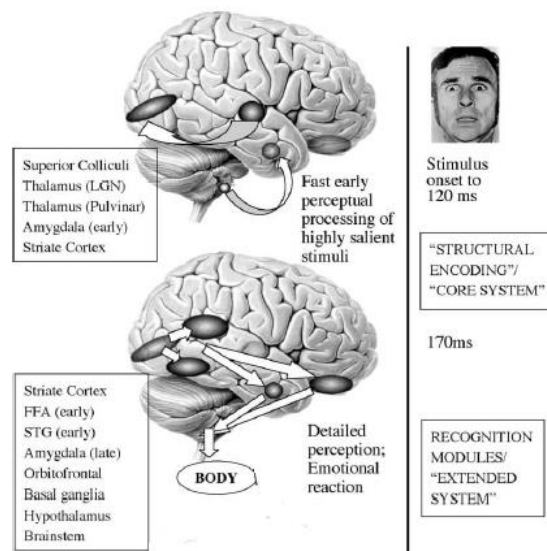

Figure 6. Processing of emotional face expressions

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

Early perceptual processing relevant to categorize faces as distinct from other visual objects is dependent on the activity of the occipito-temporal cortex, importantly including the fusiform gyrus and superior temporal gyrus, which are crucial areas to detect spatial and configural information of faces (Adolphs, 2002; Vuilleumiere & Pourtois, 2007). Studies performed with elettroencephalography (EEG) (in particular with the use of event-related potentials, ERP) and magnetoencephalography (MEG) showed that 45 to 90 ms are needed for the occipital cortex to discriminate some coarse information such as gender (Van Rullen & Thorpe, 2001), while discrimination of expressions was observed in the occipital region between 80-110 ms (Pizzagalli et al., 1999; Halgren, 2000; Herrmann et al., 2005; Moradi et al., 2017) and in temporal region between 130-200 ms (Streit et al., 1999; Andreatta et al., 2012; Hinojosa et al., 2015). However, several studies did not find expression effect in EEG or MEG studies in both occipital and temporal regions (Liao et al., 2017; Gonçalves et al., 2018), suggesting that emotional discrimination does not occur at this early stage of processing. However, even the reported emotional effects were observed in some work, it did not appear to be selective for any specific expression, but rather discriminative from neutral non-specific configural and attentional effects associated with encoding of structural facial cues (Vuilleumiere & Pourtois, 2007). Recent findings indicated later stages of processing as fundamental for emotion discrimination, in a time window subsequent to 200 ms (Adolphs et al., 2002; Vuilleumiere & Pourtois, 2007). Specifically, different effects of emotional versus neutral faces were consistently found in ERP studies in associative and supramodal brain areas and in centro-parietal regions after 300 ms (Carretié & Iglesias, 1995; Krolak-Salmon et al., 2001 Campanella et al., 2013;). These responses may reflect complex cognitive and/or arousal processes triggered by emotional stimuli, beyond more basic perceptual/sensory processing.

Moreover, as demonstrated by lesion studies, expression recognition does not depend only on sensitive cortical areas but also by inferior parietal and premotor cortical areas (Adolphs et al., 1996, 2000). In this light, a previous study suggested a new theory called "somatosensory simulation" (Goldman & Spirada, 2005). The somatosensory simulation theory suggests that to better recognize face expressions, the observer has to assess the expression of another person, estimating the motor representations that gave rise to that observed expression. Then, this representation could in turn trigger conceptual knowledge of the expression observed (Goldman & Spirada, 2005). Hence, the trigger is not given by the representation of someone else expression, but rather, a representation of ourselves

(Adolphs, 2002; Goldman & Spirada, 2005). Supporting this theory, a previous study showed that the premotor cortex was activated during observation of face expressions (Carr et al., 2003) using functional magnetic resonance imaging (fMRI) technique. In addition, a previous TMS-study, using inhibitory plasticity protocol showed an involvement of the premotor cortex in the recognition of face expressions (Balconi & Bortolotti, 2013).

Previous TMS-studies demonstrated that M1 excitability is influenced by the view of emotional images (Oliveri et al., 2003; Baumgartner et al., 2007; Hajcak et al., 2007; Schutter et al., 2008; Hortensius et al., 2016; Ginatempo et al., 2020), and, in more details, the observation of faces expressing negative emotions increases the excitability of hand M1 (Schutter et al., 2008; Hortensius et al., 2016; Ginatempo et al., 2020). Recently, Ginatempo and colleagues (2020) investigated, face M1 excitability following the passive viewing of face expressions and compared results with the hand M1 excitability. They found that the latter was influenced by the view of faces expressing negative emotions, while the former was modulated by the view of happy faces. These results suggested that face M1 has an important role in the processing of affiliative face expressions, in particular for the communication of happy emotions.

It is well known that aging is a process which physiologically impairs the ability to recognize (Sullivan & Ruffman, 2004; Ruffman et al., 2008) and perform (Pedder et al., 2016) face expressions. In particular, aging process seems to reduce the ability to perceive and recognize negative face expressions (Calder et al., 2003; Sullivan & Ruffman, 2004; Ruffman et al., 2008), suggesting physiological differences in the processing of emotions.

As explained above, several studies indicate recognition of face expressions as a more complex processing, which also involves motor cortices. In this light, face M1 seems to be involved in the processing of faces expressing emotions, but there is a lack of studies describing how aging influences M1 in the processing of face expressions.

Aims of the work

As previously described, face and hand motor systems have several functional diversities which are supported by anatomical and physiological differences. While the hand motor system has been widely studied, only few studies have investigated the face motor system in humans due the inherent technical difficulties to assess it non-invasively. For instance, studies investigating the effect of learning within the face M1 are lacking and it is still debated which cortical or subcortical areas play a role in the face expression recognition process and whether this process may be influenced by aging.

To answer these questions, a first study was performed aimed at had the aim to better understand the physiological strategies adopted by the face motor system to adapt to the long term learning and practice of a complex and fine motor task, such as paying a musical instrument.

In a second study, the involvement of the cerebellum was investigated in another complex task, i.e. the recognition of faces expressing emotions, with particular regard to cerebellar circuits underpinning the learning and retention processes. Indeed, the cerebellum has a role in both motor and emotional processing, but no studies investigated how face expressions may affect cerebellar learning processes.

Finally, we focused on the influence exerted by the viewing of faces expressing emotions on M1 excitability in aging. Aging affects the capability to recognize faces, but most of the studies focused on the perceptive aspects of this cognitive decline, while no studies investigated whether face motor responses to face expressions are impaired by aging.

Study 1

What are the strategies of face M1 to adapt to a long-term learning and practice of a complex motor skill?

Rationale

It is generally believed that the acquisition and practice of complex motor skills, such as playing a musical instrument, can induce both functional and structural plastic changes in cerebral cortex (Schlaug et al., 1995; Gaser & Schlaug, 2003; Rosenkranz et al., 2007; Zatorre et al., 2007; Bermudez et al., 2009; Houdayer et al., 2016). Indeed, anatomical changes in motor-related pathways have been seen in the white matter micro-organization of highly trained pianists where the amount of musical practice during childhood is associated with greater integrity of corticospinal tracts (Bengtsson et al., 2005; Zatorre et al., 2007). Changes have also been described in many other parts of the motor network including motor and premotor cortices (Gaser & Schlaug, 2003; Bermudez et al., 2009) the cerebellum (Hutchinson et al., 2003) and the anterior corpus callosum (Schlaug et al., 1995). The latter is presumably a reflection of the importance of bilateral hand movements (Schnitzler et al., 1996), when playing most musical instruments (Ridding et al., 2000).

Neurophysiological studies, using TMS, have also demonstrated differences between musicians and non-musicians. However, most of these have involved hand muscles and keyboard or string players. They show that these musicians have steeper MEP recruitment curves, greater sensitivity to plasticity-inducing protocols (Rosenkranz et al., 2007) and less interhemispheric inhibition (Ridding et al., 2000). In particular, changes in hand M1 representation are related to the type of instrument played, indicating that they are produced by the specific experience of playing the instrument rather than a genetic or other cause (Bangert & Schlaug, 2006). For instance, pianists' and violinists' brains can be distinguished from each other even on the gross macroscopic level by examining the shape and size of the part of M1 that contains the representations of the hand (Bangert & Schlaug, 2006).

In woodwind musicians (WM), little is known about the organisation of hand M1 and there is a lack of knowledge about face M1 in whom control of the embouchure is highly

important. Therefore, the aim of the present study was to compare the excitability and plasticity of both face M1 and hand M1 in WM, string musicians (SM) and non-musicians (NM). We hypothesize that the neurophysiological adaptations in WM and SM are site-specific, and relate to the particular physiological properties of the hand and facial motor systems. For example, interhemispheric inhibition, is normally absent between lower face M1s (Ginatempo et al., 2019a) presumably because bilateral facial muscles are rarely contracted asymmetrically (Cattaneo & Pavesi, 2014). We predict that this may differ in WM and contribute to fine control of the embouchure.

Experimental Procedures

Participants

Experiments were conducted in thirty-three healthy subjects (11 females and 22 males; mean age 33.51±12.03 years) dived into three groups: SM, WM and NM. All subjects gave their informed written consent to participate in the study, which was approved by the local ethical committee and conducted in accordance with the declaration of Helsinki. None of the subjects had history or current signs/symptoms of neurological diseases. The SM group included string instruments players who use all fingers to play their instruments, without any interposition of support such as bow or plectrum, i.e. pianists and classical guitarists. All musicians had commenced their musical training at an early age (8-12 years old), and were professional-level performers. The experiments were all performed in the morning, and none of the musicians had played their instrument beforehand. Subjects sat in a comfortable chair and were asked to stay relaxed but alert during the experiments.

Electromyography

Electromyography (EMG) was recorded from the right depressor anguli oris (DAO) and first dorsal interosseous (FDI) muscles, 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; Ginatempo et al., 2019a, b).

For EMG recordings from the FDI, the active electrode was placed over the muscle belly, the reference electrode at the second finger metacarpo-phalangeal 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 (x1000), 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 70 mm figure-of-eight shaped coil connected to a Magstim 200 stimulator stimulators through a Bistim module (Magstim Co., Whitland, and Dyfed, UK). The optimal stimulation site, for the contralateral DAO or 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 (Pilurzi et al., 2013; 2020; Ginatempo et al., 2019a, b) while for FDI the coil pointed backwards and laterally at 45 deg away from the midline (Rossini et al., 2015). 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) (Rossini et al., 2015). Active motor threshold (AMT) was established as the minimum stimulus intensity able to evoke MEPs >0.2 mV peak-to-peak amplitude in at least five out of ten consecutive trials during isometric contraction of the tested muscle at 10% of maximum voluntary isometric contraction (MVIC) (Rossini et al., 2015). The intensity of the test stimulus (TS) for TMS was 120% of RMT.

Electrical stimulation

Electrical stimulation (ES) was applied to the mandibular branch of the right facial nerve and to the right median nerve at the wrist, through a pair of cup electrodes (cathode distal) connected to a constant-current stimulator (model DS7; Digitimer Ltd). Single square-

wave pulses (0.2 ms duration) were delivered, and stimulus intensity was set at an intensity able to evoke a small twitch in the target muscle.

Experimental design

This cross-sectional study consisted of three main experiments, which were performed in two experimental TMS sessions (hand M1 and face M1), that were attended by all subjects. The two sessions were separated by at least one week.

Excitability of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

Rest and active short-interval intracortical inhibition (SICI) (Figure 7) were studied in both hand M1 and face M1 TMS. Rest and active SICI were elicited using a paired-pulse TMS protocol with a subthreshold conditioning stimulus (CS) preceding a suprathreshold TS by an interstimulus interval (ISI) of 3 ms. The CS intensity was set at 80% of RMT or AMT and the TS intensity at 120% of RMT or AMT for rest SICI and active SICI respectively. Active SICI was performed during isometric contraction of the tested muscle at 10% of MVIC. The two sessions, the two blocks and all states (TS alone and ISIs) were randomized in each subject. Fifteen unconditioned MEPs and fifteen conditioned responses for each ISI were recorded. Rest SICI and active SICI were expressed as the ratio of MEP amplitude evoked by the conditioned to the unconditioned MEP.
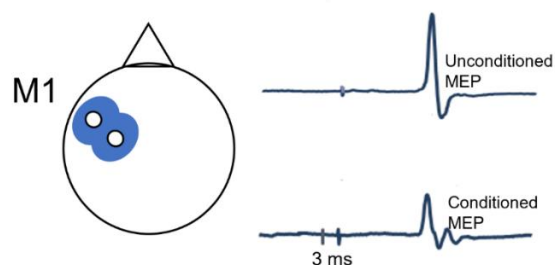

Figure 7. Short-interval intracortical inhibition (SICI)

Schematic representation of SICI. Left image shows the localization of the coil on the scalp. Right image describes electromyographical representation of the protocol: on the top, the unconditioned motor-evoked potential (MEP) obtained with single pulse TMS, on the bottom, the conditioned MEP obtained with paired-pulses TMS with 3 ms of interstimulus interval.

Interhemispheric inhibition of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

Interhemispheric inhibition (IHI) (Figure 8) was probed in both hand M1 and face M1 TMS sessions. IHI was tested using 7-cm double coils and delivering a CS (120% of RMT) to the M1 of one side before the administration of TS to the contralateral M1. The ISIs between the CS and the TS were 4 ms and 10 ms, since the former allows investigation of the contribution of brainstem excitability to DAO responses to the IHI protocol (Ginatempo et al., 2019a) whereas an ISI of 10 ms is effective in producing IHI in hand muscles (Ferbert et al., 1992). The experiment was divided up into two blocks: IHI of DAO M1 and IHI of FDI M1. The two blocks and all states (TS alone and ISIs) were randomized in each subject. Fifteen unconditioned MEPs and fifteen conditioned responses for each ISI were recorded. IHI was expressed as the ratio of MEP amplitude evoked by the conditioned to the unconditioned MEP.
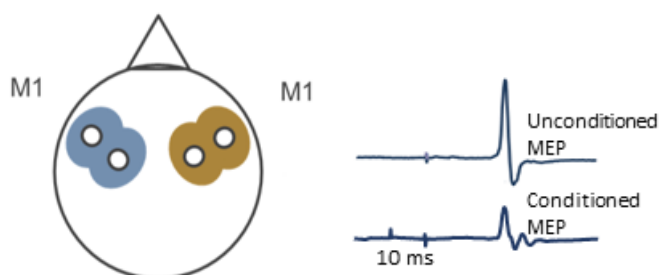

Figure 8. Interhemispheric inhibition (IHI)

Schematic representation of IHI. Left image shows the localization of the coils on the scalp. Right image describes electromyographical representation of the protocol: on the top, the unconditioned motor-evoked potential (MEP) obtained with single pulse TMS, on the bottom, the conditioned MEP obtained with paired-pulses TMS with 10 ms of interstimulus interval.

Plasticity of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

The effect of PAS (Figure 9) intervention was investigated in both hand M1 and face M1 TMS sessions. The PAS intervention was administered by pairing ES of the right facial nerve with TMS of the left face M1 and ES of the right median nerve with TMS of the left hand M1 (face-PAS and hand-PAS respectively) using ES-TMS intervals of 20 and 25 ms, respectively. Two hundred pairs of stimuli were given at 0.25 Hz. Subjects were instructed

to keep muscles relaxed and stay alert. Fifteen MEPs were collected from the resting DAO and FDI before and immediately (T0), 10 (T10), 20 (T20) and 30 (T30) minutes after PAS delivery. Effects of PAS administration were measured by comparing the mean peak-to-peak amplitude of the MEPs collected at each time interval tested after the PAS intervention with that of test MEP (baseline).
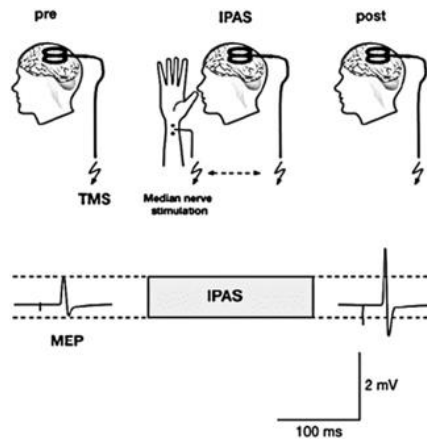

Figure 9. Paired associative stimulation (PAS)

Graphical representation of PAS. On the top are portrayed the stimulation localizations, while on the bottom is shown electromyographical representation of the protocol, analyzing the motor-evoked potential (MEP) before and after plasticity induction.

Stefan et al., *Brain*, 2000

Statistical Analysis

Statistical analysis was performed with SPSS 20 software (SPSS Inc, Chicago, IL, USA). Student's paired t-test, repeated measures analysis of variance (ANOVA) and planned post hoc t-test with Bonferroni correction for multiple comparison were used. Compound symmetry was evaluated with the Mauchly's test and the Greenhouse-Geisser correction was used when required. Significance was set for p value <0.05. Unless otherwise stated, values are expressed as means ± standard deviation (SD). Raw amplitude and amplitude ratio of conditioned to unconditioned MEPs, were used as variables. Differences in demographic features, RMT, AMT intensities were assessed using one-way ANOVA with GROUP (WM, SM and NM) as within-subjects factor.

A preliminary one-way ANOVA on raw TS MEP amplitude at baseline was performed with group (SM, WM and NM) as between-subjects factor separately for each muscle

(DAO and FDI) and each muscle condition (rest and active). In case no significant effect of group on MEP amplitude was detected, a two-way mixed-ANOVA was performed using amplitude ratio as a variable, separately for rest and active SICI, with muscle (FDI and DAO) as within-subjects factor and group (WM, SM and NM) as between-subjects factor.

A preliminary one-way ANOVA on raw TS MEP amplitude at baseline was performed with group (SM, WM and NM) as between-subjects factor separately for each muscle (DAO and FDI). In case no significant effect of group on MEP amplitude was detected, a two way mixed-ANOVA, was performed separately for each ISI (4 and 10 ms) using amplitude ratio as variable, muscle (FDI and DAO) as within-subjects factor and group (WM, SM and NM) as between-subjects factor.

A preliminary one-way ANOVA on raw TS MEP amplitude at baseline was performed with group (WM, SM and NM) as between-subjects factor separately for each muscle. In order to evaluate if a LTP effect occurred after PAS protocol, two-way mixed-ANOVA was performed separately for each muscle using time (pre, T0, T10, T20 and T30) as within-subjects factor and group (WM, SM and NM) as between-subjects factor. In case no significant effect of group on MEP amplitude was detected, a three-way mixed-ANOVA was performed using amplitude ratio as variable with time (T0, T10, T20 and T30) and muscle (FDI and DAO) as within-subjects factor and group (WM, SM and NM) as between-subjects factor.

A correlation analysis was performed between type of instrument (1: WM, 2: SM), practice time, start playing and neurophysiological parameters using Spearman's correlation coefficient.

Results

There were no significant differences in demographics (Table 1) and baseline TMS parameters (Table 2).

Table 1. Demographic characteristics of the study participants

Variables	Hand-musician	Wind-wood musician	Non-musician
Age (y)	29.36±3.03	37.82±4.07	32.45±3.20
Gender (M:F)	6:5	8:3	8:3
Age when started playing (y)	9.45±0.71	11.00±0.62	
Practice intensity (hours/day)	2.14±0.45	2.86±0.30	
TMS parameters			
RMT of FDI M1 (%MSO)	42.00±1.52	38.73±1.64	41.91±1.90
RMT of DAO M1 (%MSO)	56.73±2.04	58.45±2.84	58.55±2.12
AMT of FDI M1 (%MSO)	31.27±1.42	30.27±1.03	32.09±2.01
AMT of DAO M1 (%MSO)	48.45±1.11	51.09±2.14	49.82±2.05

M, male; F, female; y, years; TMS, transcranial magnetic stimulation; RMT, rest motor threshold; AMT, active motor thre
MSO, maximum stimulator output.

Ginatempo et al., *Neuroscience,* 2021

Table 2.   Neurophysiological characteristics of the study participants

Representation area in the Primary Motor Cortex	Muscle condition	TMS parameters	Woodwind musicians	String Musicians	Non-musicians	*Groups diferences (p value)*
Depressor Anguli Oris Muscle	Rest	RMT(%MSO)	58.45±9.41	56.73±6.77	58.55±7.03	*0.829*
		80% RMT (%MSO)	46.91±7.57	45.45±5.47	46.82±5.65	*0.833*
		120% RMT (%MSO)	69.90±11.33	68.09±8.02	70.29±8.39	*0.842*
		Unconditioned MEP Amplitude (mV)	0.15±0.07	0.23±0.18	0.22±0.19	*0.479*
	Active	AMT (%MSO)	51.09±7.69	48.45±3.67	49.82±6.79	*0.622*
		80% AMT(%MSO)	41.18±6.60	38.64±2.94	39.91±5.45	*0.528*
		120% AMT(%MSO)	61.45±9.22	58.27±4.41	59.73±8.15	*0.618*
		Unconditioned MEP Amplitude (mV)	0.57±0.45	0.56±0.39	0.58±0.33	*0.994*
First Dorsalis Interosseous Muscle	Rest	RMT (%MSO)	38.73±5.42	42.00±5.04	41.91±6.33	*0.313*
		80% RMT(%MSO)	31.00±4.45	33.55±4.16	33.73±5.04	*0.307*
		120% RMT(%MSO)	46.45±6.41	50.64±6.10	50.09±7.63	*0.301*
		Unconditioned MEP Amplitude (mV)	0.92±0.66	0.99±0.73	1.26±0.47	*0.518*
	Active	AMT(%MSO)	30.27±3.41	31.27±4.71	32.09±2.01	*0.708*
		80% AMT(%MSO)	28.91±2.84	29.91±4.37	30.64±6.67	*0.699*
		120% AMT(%MSO)	36.09±3.39	37.36±5.63	38.36±8.07	*0.677*
		Unconditioned MEP Amplitude (mV)	0.97±0.64	1.15±0.83	1.32±0.89	*0.609*

TMS, transcranial magnetic stimulation; %MSO, percentage of the maximum stimulator output, RMT, rest motor threshold; AMT, active motor threshold; MEP, motor evoked potential, mV, millivolts, Values are reported as mean ± standard deviation. Significance level set at p < 0.05.

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Excitability of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

There were no significant group differences between groups in the amplitude of the baseline (test) MEPs (main effect of group: DAO rest: $F_{2,32}= 0.756$ p=0.479; FDI rest: $F_{2,32}= 0.674$, p=0.518; DAO active: $F_{2,32} = 0.006$ p=0.994; FDI active: $F_{2,32}= 0.505$, p=0.609). These results allowed us to analyse SICI using the amplitude ratio between conditioned/unconditioned MEPs.

In the resting condition, SICI was stronger in the FDI than DAO but there were no differences between the three groups (Figure 10A). This was reflected in the ANOVA, which revealed a significant effect of muscle ($F_{2,29}$=14.268, p= 0.001), but no effect of group ($F_{2,29}$=0.372, p= 0.693) or interaction between factors ($F_{2,29}$=0.060, p= 0.942). Post-hoc analysis demonstrated a stronger rest SICI in FDI than in DAO muscle (p=0.001).

Similarly, in the active condition, SICI was stronger in the DAO than FDI (Figure 10B). Mixed ANOVA showed a significant effect of muscle ($F_{2,29}$= 14.072, p= 0.001) but a non-significant effect of group ($F_{2,29}$= 0.235, p= 0.792) and a non-significant interaction between the factors ($F_{2,29}$=0.150, p= 0.861). Post-Hoc analysis demonstrated weaker active SICI in FDI than DAO (p=0.001).
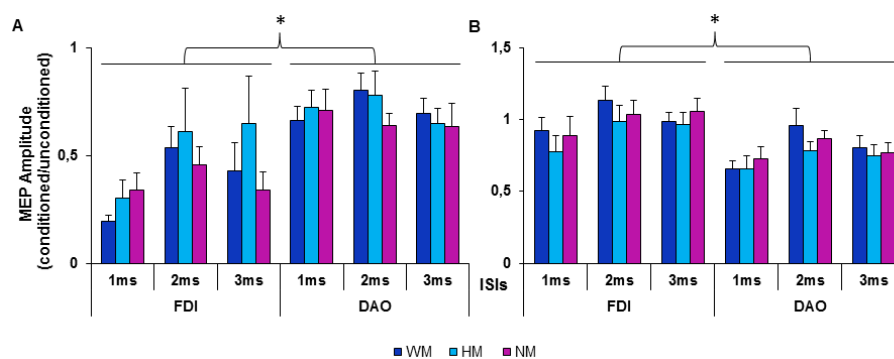

Figure 10. Short-interval intracortical inhibition (SICI) in the resting and active first dorsalis interosseus (FDI) and depressor anguli oris (DAO) muscles of woodwind musicians (WM), string musicians (SM) and non-musicians (NM).

The graphs show data of amplitude of the motor-evoked potential (MEP) (expressed as a ratio of the unconditioned MEP), in both FDI and DAO muscles during the rest (A) and active (B) SICI protocol in WM, SM and NM. A stronger rest SICI was observed in FDI than DAO muscle while a stronger active SICI was observed in the DAO than FDI muscle, with no differences among groups. The graphs represent mean ± SEM. *=p < 0.05.

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Interhemispheric inhibition of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

There were no significant group differences between groups in the amplitude of the baseline (test) MEPs (DAO: $F_{2,32}= 0.314$ p=0.733; FDI: $F_{2,32}= 0.261$, p=0.772). These results allowed us to analyse SICI using the amplitude ratio between conditioned/unconditioned MEPs.

A clear IHI at 10 ms ISI was detected in the FDI muscle of all subjects, with no differences among the three groups. In the DAO, a clear IHI was detected at 10 ms ISI only in the WM but not in the SM and NM. Recordings from representative subjects are shown in Figure 11. In particular, at 4 ms ISI, mixed ANOVA on the amplitude ratio demonstrated a significant effect of muscle ($F_{2,32}= 20.028$, p<0.001) but non-significant effect of group ($F_{2,32}= 1.489$, p= 0.242) and interaction between factors ($F_{2,32}=2.510$, p=0.099). At 10 ms ISI, mixed ANOVA on the amplitude ratio showed a non-significant effect of group ($F_{2,32}= 2.223$, p=0.126) but a significant effect of muscle ($F_{2,32}= 27.155$, p<0.001) and interaction between these factors ($F_{2,32}= 5.110$, p=0.013). Post-hoc analysis of the interaction showed that there were no clear differences among groups in the FDI, while there was a significant difference between NM and WM (p=0.031) in the DAO muscle (Figure 12).
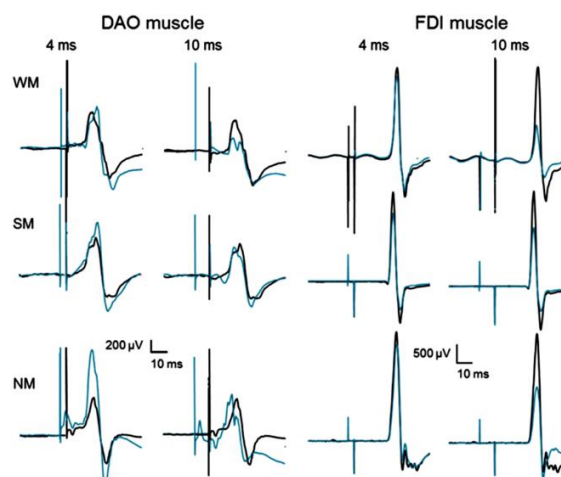

Figure 11. Interhemispheric inhibition (IHI) in the motor cortical representation of the depressor anguli oris (DAO) and first dorsalis interosseus (FDI) muscles of string musicians (SM), woodwind musicians (WM) and non-musicians (NM).

Recordings from a representative subject from each group (WM, SM and NM) are reported for each muscle and interstimulus interval (ISI; 4 and 10 ms). The unconditioned motor evoked potential (MEP), elicited by the test stimulus (120% of the resting motor threshold) is represented by the continuous line; the conditioned MEP (conditioning stimulus intensity 120% RMT) is represented by the dashed line. A clear IHI in the DAO muscle was detected only in the WM group at 10 ms ISI while a clear facilitation was observed at 4 ms ISI. In the FDI muscle a clear IHI was observed only at 10 ms in all groups.
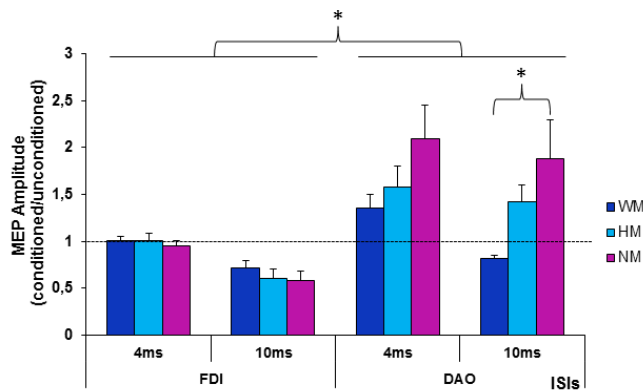
Ginatempo et al., *Neuroscience*, 2021

Figure 12. **Interhemispheric inhibition (IHI) in the motor cortical representation of the depressor anguli oris (DAO) and first dorsalis interosseus (FDI) muscles of woodwind musicians (WM), string musicians (SM) and non-musicians (NM).**

The graph shows data of amplitude of the motor-evoked potential (MEP) expressed as a ratio of the unconditioned MEP, in both FDI and DAO muscles during the IHI protocol at 4 and 10 ms ISI in WM, SM and NM. A significant difference occurs between the two muscle groups for both 4 and 10 ms ISI. At 10 ms ISI a clear difference was detected between WM and the other two groups in the DAO muscle. The graphs represent mean ± SEM. *=p < 0.05.

Plasticity of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

There were no significant differences between groups in the amplitude of the baseline (test) MEPs (DAO: $F_{2,32} = 1.442$ p=0.252; FDI: $F_{2,32}= 1.399$, p=0.263). Mixed ANOVA on the test MEP amplitude showed a clear effect of time (DAO: $F_{2,29}=5.598$; p=0.001; FDI: $F_{2,29}=5.007$; p=0.002) but a non-significant effect of group (DAO: $F_{2,29}=1.563$; p=0.226; FDI: $F_{2,29}=0.177$; p=0.838) and interaction between factors (DAO: $F_{2,29}=1.276$; p=0.274; FDI: $F_{2,29}=1.295$; p=0.265). Post-hoc analysis of the main effect of time showed that in both muscles the amplitude of the post-PAS MEP at T10 (DAO: p=0.032; FDI: p=0.04) and T20 (DAO: p=0.04; FDI: p=0.035) was significantly larger than the amplitude of the pre-PAS MEP.

In order to analyse the significant interaction between the factors we expressed the post-PAS MEP amplitudes as a ratio with the pre-PAS MEP (i.e. baseline). SM showed a stronger PAS-effect than WM and NM in the FDI muscle but not in the DAO. Recordings from a representative subject are shown in Figure 13.

Mixed-ANOVA showed a non-significant effect of muscle ($F_{2,32}= 1.999$, p= 0.168) and time ($F_{2,32}= 2.532$, p= 0.088) but a significant effect of group ($F_{2,32}= 6724$, p= 0.005). Moreover, no significant interactions among the factors were observed except for the muscle*group ($F_{2,32}= 5.655$, p= 0.008). Specifically, significantly larger PAS-effects in the

FDI muscle were observed in SM compared with WM (p=0.006) and NM (p=0.0038). Post-hoc analysis of the muscle*group interaction detected a significant increase of PAS-effect in the FDI muscle in the SM group compared with both WM (p= 0.006) and NM (p=0.009) groups (Figure 14).
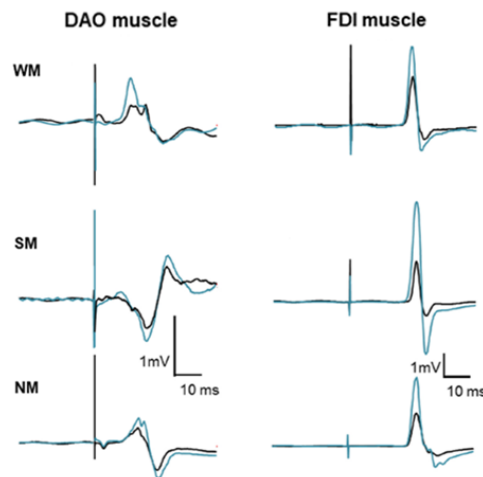

Figure 13. Effect of paired associative stimulation protocol (PAS) on the on the primary motor cortex representation of the depressor anguli oris (DAO) and first dorsalis interosseous (FDI) muscles of string musicians (SM), woodwind musicians (WM) and non-musicians (NM).

Motor-evoked potentials (MEP) recorded from a representative subject belonging to each group (WM, SM, NM) are reported for each muscle at baseline (continuous line) and immediately after (dashed line) the delivery of 20-min PAS protocol using a conditioning stimulus of 120% RMT intensity. A stronger effect of PAS was observed in the SM group than other groups only in the FDI muscle, while no difference among groups was detected in the DAO.

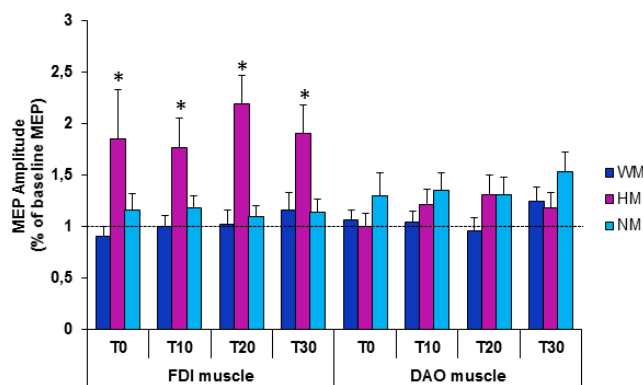Ginatempo et al., *Neuroscience*, 2021

Figure 14. Effect of PAS on the on the primary motor cortex representation of FDI and DAO muscle of woodwind musicians (WM), string musicians (SM) and non-musicians (NM).

The graph shows data for MEP amplitude expressed as a ratio between the peak-to-peak amplitude of the MEPs collected at each time interval tested after the PAS intervention (T0, T10, T20 and T30, after 0, 10, 20 and 30 minutes respectively) with that of test MEP (baseline) for FDI and DAO muscles in WM, SM and NM. The PAS effect was detected in both the FDI and DAO muscle of all groups, with a significantly stronger effect in the SM group. The graphs represent mean ± SEM. *=p < 0.05.

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Correlations with performance parameters in musicians

Correlation analysis between IHI ratio and individual subjects' data revealed that the reduction of IHI ratio significantly correlated with type of instrument (r=-0.819; p<0.001). In particular, the IHI ratio was lower (*i.e.*, the IHI was greater) in WM than SM. Moreover, a positive correlation was observed between the increase of IHI and the amount of musical practice (r=-0.497; p=0.022). An inverse correlation was found between the amount of IHI and start of playing (r=-0.492; p=0.023).

Correlation analysis between PAS ratio and individual features of musicians showed that the increasing of PAS ratio was negatively correlated with type of instrument, being larger in SM (T20: r=0.523; p=0.013; T30: r=0.423; p=0.050) than WM. Moreover, a negative correlation was observed between the intensification of PAS effect and the amount of musical practice (T20: r=-0.482; p=0.023). No significant correlation was found between neurophysiological parameters and start of playing.

Discussion

The results confirm previous work showing that the physiology of M1 differs between skilled musicians and non-musician. The novel result is that the changes differ between woodwind musicians, in whom control of the embouchure is highly important, and string musicians who use only the hands to play their instruments. In particular, woodwind musicians had increased IHI in face M1 but not hand M1, whereas string musicians had heightened plasticity in hand M1 but not face M1.

Short-interval intracortical inhibition of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

There was no significant difference in SICI between string/woodwind musicians and non-musicians in either FDI and DAO, whether measured at rest or during tonic voluntary muscle contraction. This contrasts with a previous study, which reported enhanced SICI at rest and during activation (Nordstrom & Butler, 2002). However, they used CS = 90% AMT, whereas we used 80% RMT at rest and 80% AMT during activation. Such

differences in CS intensity may be important. Rosenkranz et al. (2007) assessed SICI at rest using CS = 70, 80 and 90% AMT, and reported that the important difference between musicians and non-musicians was the steepness of the recruitment curve, with musicians having slightly less SICI at 70% AMT and more SICI at 90% AMT.

Our choice of CS intensities was informed by previous work (Kujirai et al., 1993; Pilurzi et al., 2013) that demonstrated they are effective in both FDI and DAO, which made it possible to compare between muscles at rest and when active. At rest, SICI was stronger in the FDI than DAO, but this is probably due to the small amplitude of the DAO test MEP (0.15 mV): SICI is more effective when evaluated with a larger MEP such as we used in FDI (1 mV) (Sanger et al., 2001; Roshan et al., 2003). Interestingly, although SICI was stronger in the FDI than DAO at rest, the opposite was true during activation. One reason why active SICI is less effective than at rest is that it can overlap with short-interval intracortical facilitation (SICF), which occurs around the same ISIs as SICI (Ortu et al., 2008; Peurala et al., 2008). Since SICF is more easily recruited during activation than at rest, it may be that in the present experiments there is no SICF at rest, when SICI is more powerful in FDI, but during activation, SICF may be more easily recruited in FDI and obscure the clear SICI seen at rest.

Interhemispheric inhibition of face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

In the hand M1, the amount of IHI showed no clear differences in string musicians, woodwind musicians and non-musicians. This result seems to contrast with a previous study, which found less IHI in musicians than non-musicians (Ridding et al., 2000). However, that result (in only 6 individuals) was calculated over a range of ISIs. At the specific interval used here, 10 ms they found no difference in IHI (Ridding et al., 2000).

Differently from hand M1, there was a clear difference in IHI in woodwind musicians compared with string musicians and non-musicians. This finding is supported by the significant correlation between the amount of IHI and the hours of daily practice with wind instruments. In face M1, Ginatempo et al. (2019a) failed to obtain IHI at an ISI of 10 ms and described clear facilitation at an ISI of 4 ms. They suggested that the early facilitation

at 4 ms was due to convergence at the brainstem level between ipsilateral projections, activated by the CS, and contralateral projections from the TS. The lack of IHI at later intervals was attributed either to a true absence of IHI or to the presence of continuing facilitation at the brainstem level that cancels out the effects of later developing IHI at the cortical level. The present results showed that woodwind musicians had a clear IHI at 10 ms ISI, suggesting that they upregulate interhemispheric interactions in face M1, compared to string musicians and non-musicians. We speculate that although facial muscles are usually activated symmetrically, skilled playing of a woodwind musical instrument entails highly fractionated asymmetrical movements of lower face muscles. We suggest that this leads to the enhanced interhemispheric interaction between face M1 that we observed. This hypothesis is supported by the inverse significant correlation found between the amount of IHI and the start of playing. Previous studies proposed that functional maturation of the corpus callosum extends into late childhood and adolescence. In fact, the corpus callosum is one of the latest fiber tracts in the central nervous system to be myelinated and a previous neuroimaging study revealed that increases in callosal size can be seen at least up to the middle of the third decade with a maximum during the first decade of human life (Cowell et al., 1992; Schlaug et al., 1995). This model of corpus callosum maturation may also correspond to a period of cortical plasticity since continuing changes in cortical synaptic density could occur during musical practice (Schlaug et al., 1995).

Plasticity of the face and hand primary motor cortices in string musicians, woodwind musicians and non-musicians

In this study the plasticity of face M1 and hand M1 in both string and woodwind musicians was investigated for the first time. In comparison with woodwind musicians and non-musicians, we found a significantly increased response to the PAS protocol in the hand M1 of string musicians, in agreement with a previous work suggesting that the synapses between interneuronal connections have a higher propensity to undergo changes in synaptic efficacy in hand M1 of string musicians (Rosenkranz et al., 2007). In line with Rosenkranz and colleagues (2007) we found a stronger correlation between the PAS effect and the type of instrument played. By contrast, The PAS effect correlated negatively with the amount of daily practice, which suggests that a ceiling effect for the increase in

plasticity may occur within M1 because of the training, which adds to the PAS-induced MEP increase. In this respect our data contrast with Rosenkranz's study (2007), which found a positive correlation between PAS and daily practice. A number of reasons could contribute to this discrepancy: we calculated daily practice over the last year, whereas Rosenkranz et al. (2007) used an average of the past five years; in addition, the PAS ratio was used for the correlation analysis in our study whereas Rosenkranz et al. (2007) used a more complex measure involving effects of both excitatory (as here) and inhibitory PAS on the slope of the input-output curve plotting MEP amplitude against TMS intensity.

Surprisingly, this propensity was not evident in face M1 of both string and woodwind musicians, which suggests that this adaptive strategy to long-lasting training is specific to hand M1 but not face M1.

Increased plasticity in string and keyboard musicians has been linked to the ability to perform skilled movement sequences with the fingers. The relatively normal levels of plasticity in the hands of woodwind musicians, who also use both hands to play their instruments, may be because higher accuracy is required for playing string instruments. The precise position of the fingers on the strings is important for tuning in string musicians, whereas in woodwind musicians the problem of finger placement is solved by the fixed position of the holes. Woodwind musicians also had normal levels of plasticity in DAO. It may be that although delicate control of the embouchure is necessary in woodwind musicians, they do not need to perform a vast repertoire of movement sequences with their mouth compared with the vast range of finger sequences in string musicians that gives rise to heightened plasticity of the FDI.

A limitation of the study was that the group of WM was quite heterogeneous and included a variety of both woodwind and brass players. However, each instrument has its own requirements: a flute requires different embouchure dynamics than a reed instrument; a single reed instrument differs from a double reed instrument; the size of the bore and its length are also important factors (Carse, 2002). To characterize the physiological properties of M1 according to the different type of wind instrument played, future studies enrolling an adequate number of WM belonging to each category are warranted.

In conclusion, our results suggest that hand and face motor networks of skilled musicians undergo different physiological changes within the primary motor cortex following the

experience of learning and playing a musical instrument and that these changes are topographically specific to the muscular district involved by this complex motor task. An increase in synaptic plasticity in hand M1 and a potentiation of the IHI phenomenon in face M1, appear as the mechanisms mainly involved in string and woodwind players, respectively. Perhaps the former is associated with optimisation of a skill (i.e. finger movement) that is present in everyone but which is greatly refined in string musicians to allow them to produce such a large range of sequential finger movements. The latter may be associated with learning and performing a movement (i.e. control of the embouchure) that is less innate and requires a change in the organisation of motor output.

Study 2

How does the cerebellum influence the processing of face emotional expressions?

Rationale

In humans, perception and reaction to facial expressions are fundamental processes for social communication. In addition, our ability to understand the emotional facial expression of others is key to successfully interpret what they are communicating to us (Blair, 2003, 2004). Several brain networks, including cortical and sub-cortical regions, seem involved in the interpretation of facial expressions and it has been recently observed that also the cerebellum plays an important role in their processing (Fusar-Poli et al. 2009; D'Agata et al., 2011; Ferrucci et al., 2012; Adamaszek et al., 2014, 2015; Ferrari et al., 2018). Apart from the well-known role of the cerebellum in the motor functions, its involvement in emotional processing is much less known. In the later 90s, for the first time, Schmahmann and Sherman (1998) described a consistent pattern of affective deficits in patients with focal cerebellar lesions, and in this kind of patient it has been recently shown also an altered capability of recognizing facial emotions (Adamaszek et al., 2015). In particular, the cerebellum seems to influence the processing of negative facial expressions (Ferrucci et al. 2012; Adamaszek et al., 2014, 2015) as demonstrated by plastic changes induced by neuromodulation in the cerebellar cortex which were able to modulate recognition of negative facial expressions but not of positive ones (Ferrucci et al., 2012).

It is generally believed that cerebellum is a key structure in the processing of delay eyeblink classical conditioning (EBCC) (Robleto et al., 2004; Thompson & Steinmetz 2009; Freeman & Steinmetz, 2011; Hu et al., 2015 Adamaszek et al., 2017). Delay EBCC is a model of associative memory in which the association of an unconditioned stimulus (US) (such as an electrical stimulation of the trigeminal nerve) with a conditioned stimulus (CS) (auditory, visual or somatosensory) leads to an eyeblink conditioned response (CR) (Thompson & Steinmetz, 2009; Freeman & Steinmetz, 2011; Takehara-Nishiuchi, 2018). Animal studies suggested that plastic modifications of the cerebellar cortex and deep nuclei are fundamental for conditioning processes, being the interpositus nucleus considered the key structure for the CR formation (Thompson & Steinmetz, 2009; Freeman & Steinmetz,

2011; Takehara-Nishiuchi, 2018). Delay EBCC circuit requires that cerebellar outputs from the interpositus nucleus reach the brainstem to eventually activate the facial motor nucleus eliciting the eyeblink response. Several studies used the delay EBCC as a cerebellar learning model to observe how a neutral stimulus (*i.e.*, the CS) is able to determine a CR following association with an aversive stimulus (*i.e.*, the US).

Since the role of the cerebellum in emotional functions, such as the processing of face expressions, has been poorly investigated in physiological conditions we proposed to determine whether the presentation of faces expressing diverse emotions, could modulate the CR elicitation compared to the delay EBCC induced by an auditory CS (classical protocol). Moreover, in the attempt to assess whether the passive viewing of face emotional expressions influences brainstem structures common to the EBCC, we investigated its effects on the blink reflex (BR) and its recovery cycle (BRRC). Finally, the possible involvement of the connection between the cerebellum and the primary motor cortex (M1) in the processing of face emotional expressions has been explored probing the cerebellar brain inhibition (CBI) (Ugawa et al., 1995; Celnik, 2015).

Experimental Procedures

Participants

Forty healthy subjects (22 females and 18 males; mean age 25.28 ± 0.67 years), all right handed, according to the Oldfield inventory scale (Oldfield, 1971), participated in the study. An informed written consent was previously obtained from all subjects and the experimental procedure was approved by the local ethical committee and conducted in accordance with the Helsinki Declaration. None of the participants had history and/or current signs/symptoms of neurological and/or psychiatric diseases. 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.

Electromyography

EMG signals were recorded bilaterally from the orbicularis oculi muscles for the EBCC and BR recordings, using 9 mm diameter Ag-AgCl surface cup electrodes. The recording electrode was placed over the lower lid, the reference electrode 2 cm far from the lateral cantus and the ground electrode located over the forehead. For the CBI protocols, EMG recordings were performed from the FDI muscle of the right hand. The active electrode was placed over the muscle belly, the reference electrode at the second finger metacarpo-phalangeal 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 (EBCC and BR recordings: x3000; CBI: x1000), filtered (bandpass delay EBCC and 2: 5-5000; CBI: 3-3000 Hz), sampled (5 kHz per channel) using a 1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and Signal 5.02 software on a computer and stored for off-line analysis.

Electrical stimulation

For both EBCC and BR protocols, electrical stimulation was delivered to the left supraorbital nerve (SON), 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). Stimuli were square waves (0.2 ms duration), and stimulus intensity was equal to three times the threshold for the R2 component of the BR, *i.e,* the lowest intensity that evoked at least five R2 responses in 10 consecutive trials.

Face emotional expressions stimuli

Face emotional expressions stimuli (FES) consisted of photographs of ten actors taken from the Karolinska directed emotional faces set (Lundqvist et al., 1998). Each actor (10 in total, 5 women) displayed a neutral, sad or happy facial expression for a total of 30 visual stimuli. All stimuli were projected on a 17" LCD monitor, with a 1280x1024 resolution and a 70Hz refresh rate, using PsychToolbox software (Brainard, 1997), running in MATLAB environment (Version 2015b, MathWorks, Inc., Natick, MA, United States).

Transcranial magnetic stimulation

TMS was performed using a figure-of-eight coil with external loop diameter of 7 cm connected to a Magstim 200 stimulator (Magstim Co., Whitland, Dyfed, UK). The optimal stimulation site was carefully searched and then marked with a soft tip pen over the scalp, to maintain the same coil position throughout the experiments; the coil pointed backwards and laterally at 45 deg away from the midline. The handle of the coil pointed posteriorly and laterally, at approximately 30-45 degrees to the interhemispheric line (Rossini et al., 2015). The 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.

Study design

The design of the study comprised a main experiment (the delay EBCC) and two control experiments (BRRC and CBI) which took place one week apart from the main experiment.

Effect of face emotional expressions stimuli on the delay eyeblink classical conditioning

In order to reduce the number of stimuli delivered to each subject, participants were randomly divided into four groups. One group underwent the standard EBCC protocol where the CS used was a loud tone (70–80 dB; 2000 Hz; 400 ms) delivered via binaural headphones. The other three groups underwent recording of the EBCC, but the CS used was a visual stimulus showing one of the following type of FES: neutral, happy or sad. A graphical representation of the EBCC protocol is shown in Figure 15.

The US, was an electrical stimulation, delivered 400 ms after the onset of the CS. The EBCC protocol consisted of an acquisition (or learning) phase and of an extinction phase. In the acquisition phase, pairs of CS and US were delivered in 6 acquisition blocks (each consisting of 9 CS-US pairs, 1 US-only, and 1 CS-only trial). In the extinction phase, a seventh block consisting of 11 CS-only trials was carried out. The inter-trial interval between pairs of CS-US, was randomised between 10 and 30 seconds to reduce habituation. EMG bursts were considered "alpha blinks," i.e., non-conditioned responses

inconsistently produced by the CS, if their amplitude exceeded 50 μV and their latency was <200 ms after the CS, whereas they were regarded as CR if latency was 200–400 ms after the CS (ending before the US). For the CS-only trials, EMG bursts occurring 200–600 ms after the CS were considered CRs. Number of CRs for each block was used as parameter.
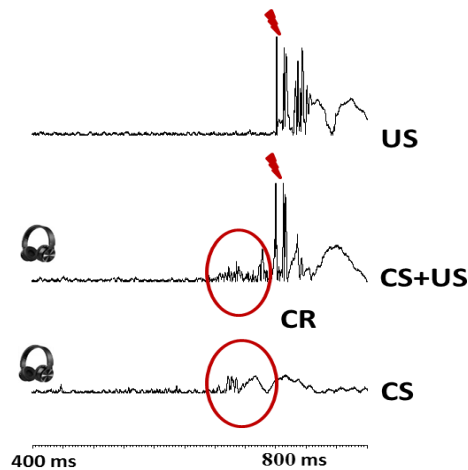

Figure 15. Eye-blink classical conditioning (EBCC)

Electromyographical representation of the EBCC protocol. On the top the unconditioned stimulus (US) is able to elicit the muscular response but not the conditioned response (circled in red) which is instead elicited associating the US with the conditioned stimulus (CS) (middle graph) or delivering the only CS after the conditioning process (bottom graph).

Effect of face emotional expressions stimuli on the blink reflex and blink reflex recovery cycle

Thirty out of the 40 subjects who participated in the first experiment (15 males and 15 females; mean age 25.0 ± 0.75 years) were randomly divided into three groups: neutral, happy and sad group.

The BR was elicited by the stimulation of the left supraorbital nerve. Single or double pulses were given randomly at ISIs of 250, 500 and 1000 ms and at an intertrial interval of 30 ± 10 s to minimize habituation. Ten trials for each ISI were collected. Electrical stimulus was delivered 400 ms after the neutral, happy or sad FES to the respective group.

The area of the early ipsilateral (R1) and late bilateral (ipsilateral, iR2, and contralateral, cR2) BR components, were measured. The recovery cycle (Figure 16) was defined by the R2 area ratio (R2 area of the conditioned response divided by the R2 area of the unconditioned response) for each ISI.
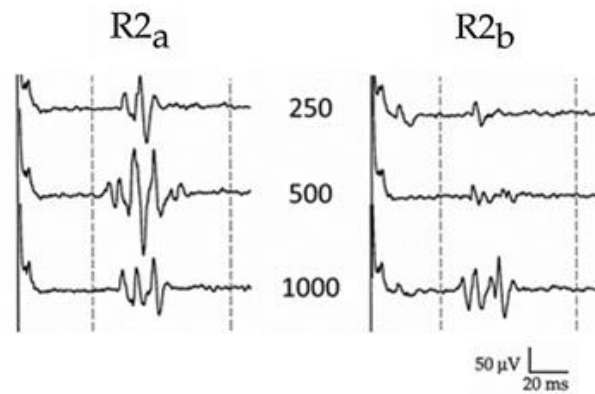
Figure 16. Blink reflex recovery cycle (BRRC)

Electromyographical representation of the BRRC. Muscular response (R2) recorded in the ipsilateral (a) and contralateral (b) orbicularis oculi muscles with 250 ms, 500 ms and 1000 ms of interstimulus intervals.

Effect of face emotional expressions stimuli on the cerebellar brain inhibition

Fifteen out of the subjects who participated in the first experiment (9 females and 6 males; mean age 26.67 ± 0.90 years) underwent CBI recording before and after FES (sad, happy and neutral faces) administration.

CBI was elicited using a standard TMS protocol where a CS is delivered to the cerebellar cortex 5 ms before a TS delivered to the target M1 (Ugawa et al., 1995) (Figure 17).

The intensity of the TS was 120% of RMT. Cerebellar CS was delivered over the right cerebellar cortex, 3 cm lateral and two cm below the inion with a 90 mm diameter double cone coil connected to a Magstim 200 stimulator (Magstim Co., Whitland, Dyfed, UK). The intensity of the CS was set in order to avoid brainstem stimulation. To do so, brainstem activation was first explored at 60% of MSO. In case of no brainstem activation, 70% of MSO was employed as CS intensity. CBI was expressed as the average MEP amplitude evoked by the cerebellar-conditioned stimulation of M1 relative to the average MEP amplitude evoked by the TS of M1. CBI was tested in the hand area of the left M1, after 400 ms from the random delivery of FES (neutral, sad and happy faces) (Ginatempo et al., 2019b). Thirteen unconditioned (TS over M1) and 13 conditioned MEPs (paired cerebellar CS and TS over M1) for each FES type were recorded in randomized order. Raw amplitude and amplitude ratio of conditioned to unconditioned MEPs, were used as variables.
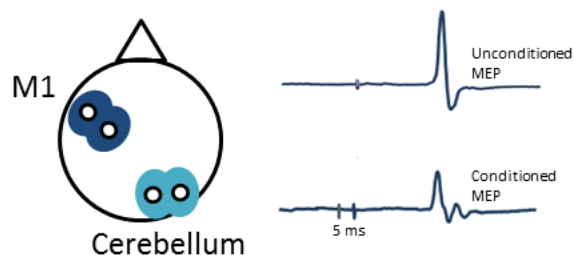
Figure 17. Cerebellar brain inhibition (CBI)

Schematic representation of CBI. Left image shows the localization of the coil on the scalp. Right image describes electromyographical representation of the protocol: on the top, the unconditioned motor-evoked potential (MEP) obtained with single pulse TMS, on the bottom, the conditioned MEP obtained with paired-pulses TMS with 5 ms of interstimulus interval.

Statistical Analysis

Statistical analysis was performed with SPSS 20 software (SPSS Inc, Chicago, IL, USA). Mixed and repeated measures ANOVA and planned post hoc t-tests with Bonferroni correction for multiple comparison were used. Compound symmetry was evaluated with the Mauchly's test and the Greenhouse-Geisser correction was used when required. Significance was set for p values < 0.05. Value are expressed as mean $\pm$ standard error of the mean (SEM). To ensure that there were no differences between experiments and groups in terms of age, one-way between-group ANOVAs were used, while a Pearson's Chi Square test was performed to evaluate differences in gender composition.

Delay EBCC: Two separate mixed factor ANOVA on the number of CRs for each block were performed with BLOCK (form 1st to 6th block) as within subjects factor and GROUP (EBCC, sad EBCC, happy EBCC and neutral EBCC) as a between subjects factor. A separate one-way ANOVA was performed using number of CR in the retention block with GROUP (EBCC, neutral EBCC, happy EBCC and sad EBCC) as a between subjects factor.

BR and BRRC: Three separated ANOVA were performed to evaluate the areas of R1 and R2 components of the BR. A one-way fixed model ANOVA was performed to calculate R1 differences between groups (neutral, happy and sad). A two-way mixed model ANOVA was used to assess R2 differences in the BR, with SIDE (ipsilateral and contralateral) as within subjects factor and GROUP (neutral, happy and sad) as between subjects factor. A three-way mixed model ANOVA was employed to investigate R2 differences in the BRRC using ratio as variable, with SIDE (ipsilateral and contralateral)

and ISI (250 ms, 500 ms and 1000 ms) as within subjects factors and GROUP (neutral, happy and sad) as between subjects factor.

CBI: A preliminary one-way random model ANOVA on MEP amplitude was performed with FES (happy, sad and neutral) as within factor. In case of no significant effect of FES on MEP, a one-way repeated measure random model ANOVA on the ratio conditioned/unconditioned MEP was performed using FES (happy, sad and neutral) as within subjects factor.

Results

Statistical analysis showed no difference between the experiments as for age ($F_{2,83}$=1.169; p=0.316) and gender (X^2_2=1.543; p=0.462) of the participants. Similarly, within each experiment, age (delay EBCC: $F_{3,36}$=1.420; p=0.253, BR and BRRC: $F_{2,27}$=2.099; p=0.142) and gender (delay EBCC X^2_3=1.212; p=0.750, BR and BRRC: X^2_2=0.0; p=1.00) were not different between groups.

Effect of face emotional expressions stimuli on the eyeblink classical conditioning

Statistical analysis showed no differences between the four groups as for somatosensory threshold ($F_{3,36}$=0.364; p=0.780) and intensity of SON stimulation ($F_{3,36}$=0.808; p=0.498).

Results obtained during the acquisition phase, showed a clear learning effect in all groups apart the sad EBCC group, which exhibited instead a significant reduction in learning.

In particular, mixed factor ANOVA showed a significant effect of TIME ($F_{5,32}$=6.170; p<0.001) and GROUP ($F_{3,36}$=4.757; p=0.007) but a non-significant interaction between factors ($F_{5,15}$=1.497; p=0.136). Post-Hoc analysis showed that, for all groups, the first block had significantly lower percentage of CR than the sixth block (p=0.005). Moreover, a significant difference between EBCC and sad EBCC groups (p=0.009) was detected (Figure 18A).

During the extinction phase both happy and sad EBCC groups presented a smaller number of CRs than the EBCC group. In particular, one-way ANOVA on the number of CR showed a significant effect of GROUP ($F_{3,36}$=6.501; p=0.001). Post-hoc analysis

highlighted that both happy (p=0.006) and sad (p=0.002) EBCC groups had lower CRs than the EBCC group (Figure 18B).
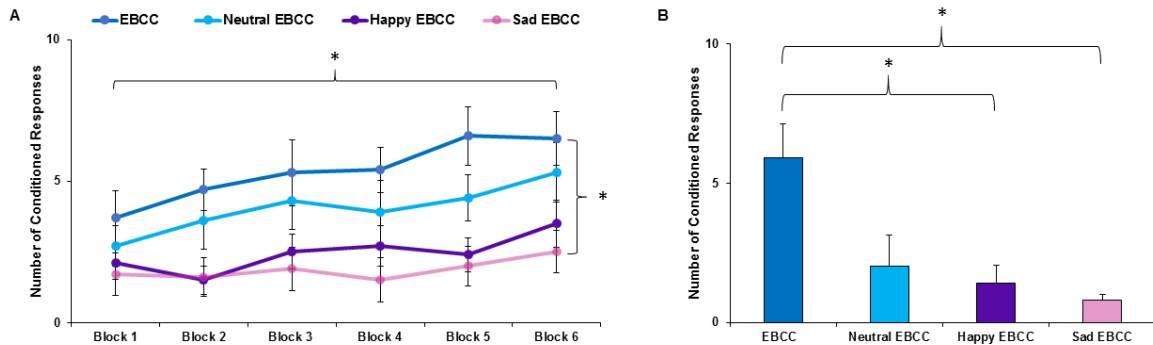

Figure 18. Effect of emotional visual stimuli on delay eye-blink conditioning (EBCC) in both learning and retention phases.

The graphs illustrate the number of Conditioned Responses (CRs) elicited by EBCC learning (A) and extinction (B) following loud acoustic stimuli (EBCC), neutral (neutral EBCC), happy (happy EBCC) and sad (sad EBCC) face expressions. In all groups, the number of CRs was significantly higher in blocks VI than in block I. A clear difference between sad EBCC and EBCC was also observed in the learning phase. In the retention phase EBCC showed higher number of CRs than sad and happy EBCC groups. The graph represents means + SEM. *p < 0.05.

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Effect of face emotional expressions stimuli on the blink reflex and blink reflex recovery cycle

One-way ANOVA for SON threshold was not different between the EBCC and the BR and BRRC experiments ($F_{1,27}=1.812$; p=0.190) nor between the three groups of the BR and BRRC experiment ($F_{2,27}=0.769$; p=0.573). Statistical analysis on the intensity of SON stimulation failed to detect significant difference between EBCC and the BR and BRRC experiments ($F_{1,27}=0.941$; p=0.341) and between the groups of the BR and BRRC experiment ($F_{2,27}=0.453$; p=0.640).

No significant effect of FES was detected in both the BR and BRRC (Figure 19). In particular, one-way ANOVA on R1 area showed a non-significant effect of GROUP ($F_{2,27}=0.104$; p=0.902). The two-way ANOVA for the R2 component of the BR, revealed a statistically significant effect of SIDE ($F=_{2,27}11.020$; p=0.003) but no main effect of GROUP ($F_{2,27}=0.150$; p=0.861) nor interaction between factors (Figure 19A).

Three-way ANOVA used to assess ratios of the R2 recovery in the BRRC failed to detect any significant difference for SIDE ($F_{2,27}=1.291$; p=0.266) and GROUP ($F_{2,27}=0.052$; p=0.949), with no interaction between factors (Figure 19B). By contrast, a significant

effect for ISI ($F_{2,27}=6.253$; p=0.006) was found and post-hoc analysis highlighted a higher R2 area at 1000 ms ISI than at 250 ms (p=0.005) and at 500 ms (p=0.0234). Moreover, R2 area at 500 ms ISI was also observed significantly higher than at 250 ms ISI (p=0.038).
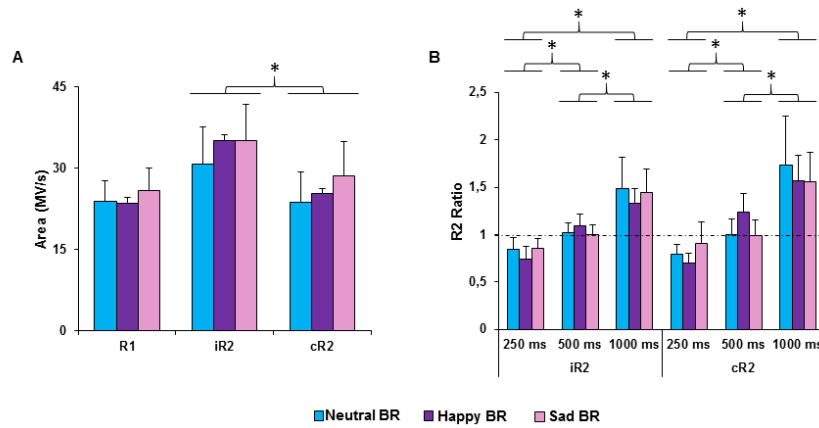

Figure 19. Effect of emotional visual stimuli on blink reflex and its recovery cycle.

The graph describes the differences between areas of R1 and both ipsilateral (iR2) and contralateral (cR2) R2 components of the blink reflex (A), and its recovery cycle (B), following viewing of neutral, happy and sad face emotions. Panel B showed the ratio between conditioned stimulus delivered at 250, 500 and 1000 ms, and unconditioned stimulus. Significant differences were found in the blink reflex where iR2 area was larger than cR2, and in the recovery cycle in which 1000 ms presented higher ratio amplitude compared to 250 and 500 ms and 500 ms more than 250 ms. No differences of emotion were observed. The graph represents means + SEM. *p < 0.05.

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Effect of face emotional expressions stimuli on the cerebellar brain inhibition

The passive viewing of FES did not affect CBI. A preliminary analysis on raw MEP amplitude showed a non-significant effect of FES ($F_{2,13}=1.643$, p=0.231). This result allowed to perform the analysis on the ratio conditioned/unconditioned MEP, which appeared not significantly affected by FES ($F_{2,13}=0.005$; p=0.995) as shown in Figure 20.
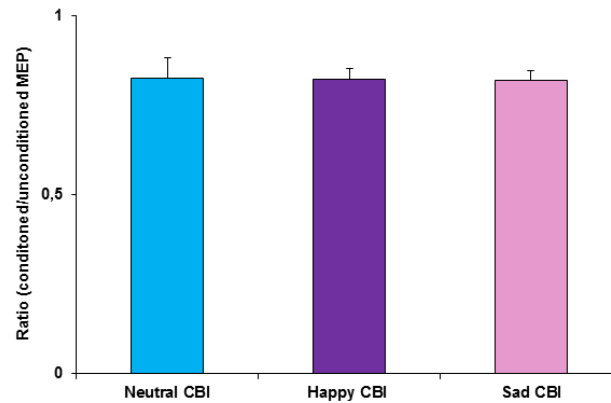
Figure 20. Effect of emotional visual stimuli on cerebellar-motor cortex inhibition.

The graph shows the effect of the passive viewing of neutral, happy and sad faces on the amplitude of motor-evoked potentials (MEP) recorded in the first dorsalis interosseus muscle (expressed as ratio between conditioned and unconditioned MEP amplitudes). No significant effects were observed. The graph represents means + SEM.

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Discussion

This work investigated for the first time the effect of the passive viewing of face expressing emotions on cerebellar learning and on the cerebello-M1 inhibitory pathway.

Overall, the present study provides first evidence that the passive viewing of faces displaying sadness influences significantly the cerebellum-brainstem circuit underlying the delay EBCC. This influence seemed to be not reflected neither in the cerebello-cortical inhibition nor in brainstem circuitry subtending the delay EBCC.

Results showed that in the learning phase of the EBCC, the association of visual stimuli, consisting of sad faces as CS with an electrical stimulus as US, was able to reduce significantly the number of CRs compared to the classical EBCC protocol, where the CS consisted of a standard auditory stimulus. It is well known that the cerebellum plays an essential role in the delay EBCC and that the performance of the unconditioned response, elicited by the aversive electrical US, is not influenced by lesions that affect the CR (Takehara-Nishiuchi, 2018). In particular, experimental evidence indicates the anterior interpositus nucleus as the neural structure necessary for acquisition and retention of the CR (Thompson & Steinmetz, 2009; Freeman & Steinmetz, 2011; Takehara-Nishiuchi, 2018). Of note that the neural pathways that lead the US and CS stimuli to the cerebellum are different. The US pathway includes the trigeminal nucleus, the dorsal accessory division of the inferior olive and the climbing fiber excitatory projection to the anterior

interpositus nucleus and to Purkinje cells in the cerebellar cortex, which in turn send inhibitory projections to the interpositus nucleus. From this deep nucleus an excitatory projection is sent to the red nucleus in the midbrain and, from here, to the facial motor nucleus in the pons to elicit the blink response in the orbicularis oculi muscles (Thompson & Steinmetz, 2009; Freeman & Steinmetz, 2011; Takehara-Nishiuchi, 2018). The CS information is collected by the pontine nuclei which relay it to the interpositus nucleus through the middle cerebellar peduncle and, via excitatory mossy fibers to the Purkinje cells, which in turn send inhibitory projections to the interpositus nucleus (Thompson & Steinmetz, 2009; Freeman & Steinmetz, 2011; Takehara-Nishiuchi, 2018).

It has been reported that, following the combined action of the US-CS pathways, plastic changes that are created in the cerebellar cortex with the connection between Purkinje cells and parallel fibers and in the interpositus nucleus with the mossy fibers, are responsible for the learning process that allows the CS alone to elicit the blink response (Weeks et al., 2007; Thompson & Steinmetz, 2009; Takehara-Nishiuchi, 2018). Our results indicate that the view of faces expressing sadness, associated with the US, is able to disrupt the plastic changes in the cerebellar cortex and interpositus nucleus which occur in the learning phase.

A possible role for brainstem circuits in these changes can be ruled out, based on the results of the BR and BRRC experiment, which failed to detect any effect of the FES on the BR. This is a complex reflex response induced by an electrical stimulation on the supraorbital terminations of the trigeminal nerve which elicits mainly two different motor responses in the orbicularis oculi muscles: an early ispilateral R1, which reflects a disynaptic arch between the sensory trigeminal nucleus and the facial motor nucleus, and a late bilateral R2 (Ongerboer De Visser & Kuypers, 1978). The R2 is mediated by an extensive polysynaptic circuitry which crosses both the pontine and bulbar tracts of the spinal trigeminal nucleus, neurons in the lateral tegmental field and the lateral reticular formation before reaching the facial motor nucleus in the pons; (Kimura, 1983; Bourque & Kolta, 2001; Aramideh & Ongerboer De Visser, 2002; Cruccu et al., 2005).

In this framework, our results suggest that faces expressing emotions have no influence on those brainstem neurons mediating the BR, particularly the R2 component, which are a common relay station for the eyeblink CR (Pellegrini & Evinger, 1997). Moreover, the lack of effect of FES on the BRRC, suggests that other structures engaged in this

physiological process, such as basal ganglia and superior colliculus (Pauletti et al., 1993; Basso et al., 1996; Berardelli et al., 1999) are not involved in the consistent reduction of the eyeblink CR induced by the passive view of sad faces.

Based on these results it is reasonable to suggest a main role of the cerebellum in the processing faces expressing sadness.

The specific inhibitory effect exerted by the view of sad faces on the learning phase of the EBCC was not detected in the extinction phase, where both sad and happy face expressions were able to reduce the retention of the CR, compared to the classical protocol using the auditory CS. This finding, apparently opposed to the associative learning results, may be explained by the different neural bases underlying the acquisition and extinction phases of the delay EBCC. Indeed, whereas the cerebellar cortex and the interpositus nucleus are essential for the former, the latter is more dependent on the inhibitory transmission from the interpositus nucleus to the inferior olive (Medina et al., 2002). Besides the inferior olive and the facial motor nucleus (Krupa & Thompson, 2003), several other brain areas, such as the hippocampus (Akase et al., 1989; Christiansen & Schmajuk, 1992) and the medial prefrontal cortex (Weible et al., 2000; Schreurs et al., 2001) are thought to be crucial in this phase, suggesting that acquisition and extinction phases are determined by at least partially different learning processes (Robleto et al., 2004; Hu et al., 2015). Therefore, it is likely that both happy and sad facial expressions modulate the connection between the cerebellum with deep limbic structures (Freeman & Steinmetz, 2011), whereas only faces expressing sad emotions modulate the circuitry underling the connection between the interpositus nucleus and the cerebellar cortex. This hypothesis is in line with several studies suggesting that the cerebellum is particularly involved in the processing of negative emotions (Schutter et al. 2008; Park et al., 2010; Ferrucci et al., 2012; Adamaszek et al., 2014, 2015). In particular, a previous functional magnetic resonance imaging study showed that the passive viewing of faces expressing happiness, activated the middle temporal gyrus, parahippocampal gyrus, hippocampus, claustrum, inferior parietal lobule, cuneus, middle frontal gyrus, inferior frontal gyrus, and anterior cingulate gyrus; while the view of faces expressing negative emotions, such as anger, activated the posterior cingulate, fusiform gyrus, and the cerebellum (Park et al., 2010).

The modulatory action exerted by the view of faces expressing emotions appeared not to be reflected in the inhibitory connection between the cerebellum and hand M1, as assessed by the CBI. CBI allow to assess the inhibitory projections from the cerebellar lateral cortex to the motor and premotor areas, via the dentate nucleus, the superior cerebellar peduncle and the contralateral ventrolateral nucleus of the thalamus (Ugawa et al., 1995; Daskalakis et al., 2004). Several studies demonstrated that the cerebellar cortex is involved in the processing of face expressions (Fusar-Poli et al., 2009; Ferrucci et al., 2012; Uono et al. 2017; Ferrari et al., 2018, 2020) and one study showed that cerebellar cortex activity influences M1 excitability during viewing of face expressions (Uono et al., 2017). However, our results suggest that the latter effect may not depend on the CBI.

Study 3

Faces emotional expressions: from perceptive to motor areas in aged and young subjects

Rationale

In humans, the ability to rapidly recognize and react to facial expressions is crucial for survival and social interactions (Blair, 2003, 2004). Neuroimaging and neuropsychological studies indicate that face perception involves the coordinated participation of multiple cortical regions, including an inferooccipital-inferotemporal connection, where relatively invariant aspects of faces are represented, and an inferooccipital-superotemporal connection, where changeable aspects of faces, resulting from movement of the facial muscles, are processed (Haxby et al., 2000; Winston et al., 2003; Rossion et al., 2003; Steeves et al., 2006; Engell & Haxby, 2007). In spite of the large number of brain areas involved, the recognition of emotional face expressions is a fast procedure, which takes only 300 ms (Smith & Smith, 2019). However, it does not only involve the occipito-temporal areas, as proposed by the sensorimotor simulation theoretical model, which suggests that motor processing and its correlated cortical areas appear directly connected to emotion recognition (Gallese et al. 1996; Adolphs, 2002; Goldman & Sripada, 2005; Argaud et al., 2016, Wood et al. 2016a, b). Although experimental studies supported this model showing a direct influence of premotor cortex activity in the recognition and processing of face expressions (Carr et al., 2003; Balconi & Bortolotti, 2013), the anatomo-functional connection between perceptive and motor cortical areas is still debated. Several human studies have demonstrated that during passive viewing of face expressions, once the visual stimulus reaches limbic areas, such as cingulate cortex, prefrontal cortex and the amygdala (Adolphs, 2002; Dalgleish, 2004; Vuilleumier & Pourtois, 2007), it activates premotor/motor areas and brainstem nuclei involved in arousal responses and muscle activation (Dimberg & Thunberg, 1998; Dimberg et al., 2000) following emotional stimuli (Adolphs, 2002). These observations were confirmed by previous neurophysiological studies using TMS, which showed increased excitability of cortico-spinal (Oliveri et al., 2003; Baumgartner et al., 2007; Hajcak et al. 2007; Schutter

et al., 2008; Coombes et al., 2009; Hortensius et al., 2016) and cortico-bulbar tracts (Ginatempo et al., 2020) in response to emotional stimuli.

While there is general support to the hypothesis of a right hemisphere dominance for all components of the emotional system, including the perception of faces expressing emotions (Adolphs, 2002; Hinojosa et al., 2015; Gainotti et al., 2019), it is not clear whether responses in the M1 are lateralized.

Both perception and motor reaction to face expressions are affected by different factors, being aging one of the most debated. It has been reported that emotional recognition is impaired in aged adults (Sullivan & Ruffman, 2004; Ruffman et al., 2008; Bailey et al., 2009), in line with the general decline of a wide range of complex cognitive functions with age (Ruffman et al., 2008). In particular, compared to young adults, aged subjects present an impaired recognition of negative face expressions (MacPherson et al., 2002; Phillips et al., 2002; Sullivan & Ruffman, 2004; Isaacowitz et al., 2007) as well as longer reaction times (Liao et al., 2017). Although an influence of the age-related decline in cognitive and sensory functions on emotional processing is widely described, whether it reflects on the responses of motor areas to visual stimuli with emotional content remains unclear. In addition, there is a lack of studies investigating the influence of age on M1 response to the observation of faces expressing different emotions.

Based on the above background, we hypothesized that aging would impair motor responses of face M1 to the view of face emotional expressions, along with their declined sensory processing. Therefore, we proposed to investigate emotional recognition processes and the effect of passive viewing of different face expressions on the excitability of the face M1 in aged and young adults.

Experimental Procedures

Participants

Thirty-four healthy subjects all right-handed according to the Oldfield inventory scale, participated in the study. To distinguish young from aged subjects, an age cut-off of 55 years was used and subjects were divided into two groups: young (7 males and 10 females;

mean age 24.41±0.71 years, range 21-32 years) and aged (6 males and 11 females; mean age 63.82±0.99 years, range 57-73 years). An informed written consent was previously obtained from all subjects. The experimental procedure was approved by the local ethical committee (Bioethics Committee of ASL n. 1 – Sassari, ID 2075/CE/2014), and conducted in accordance with the Helsinki Declaration. None of the participants had history and/or current signs/symptoms of neurological and/or psychiatric diseases. 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.

Electromyography

EMG was recorded from the DAO 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. 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 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). Since previous studies demonstrated that TMS on face M1 produces larger, more consistent and stable MEP in the contralateral than ipsilateral lower face muscles (Paradiso et al. 2005; Pilurzi et al. 2013, 2020, Ginatempo et al., 2019a), we chose to analyze only MEP recorded in the contralateral DAO following TMS over the face M1 in right and left hemispheres. The optimal stimulation site for the contralateral DAO, defined as the cortical spot where larger MEP were obtained, was carefully searched and then marked with a soft tip pen over the scalp, to maintain the same coil position throughout the experiment. The handle of the coil pointed posteriorly and laterally, at approximately 30-45 deg to the

interhemispheric line (Pilurzi et al., 2013, 2020; Ginatempo et al., 2019a, 2020). The RMT was defined as the lowest TMS intensity that elicited, in the relaxed muscle, MEP of at least 0.05 mV in at least 5 out of 10 consecutive trials and was expressed in percentage of the MSO (Rossini et al., 2015; Pilurzi et al., 2013, 2020). The paired-pulse TMS protocol was delivered with the same coil, CS and TS were delivered separated by an ISI of 3 ms for SICI and 10 ms for intracortical facilitation (ICF). In both cases, the CS intensity was set at 80% RMT and the TS intensity at 120% RMT. SICI and ICF were expressed as the ratio of conditioned MEP amplitude evoked by the CS to the unconditioned MEP evoked by the TS alone (Figure 21).
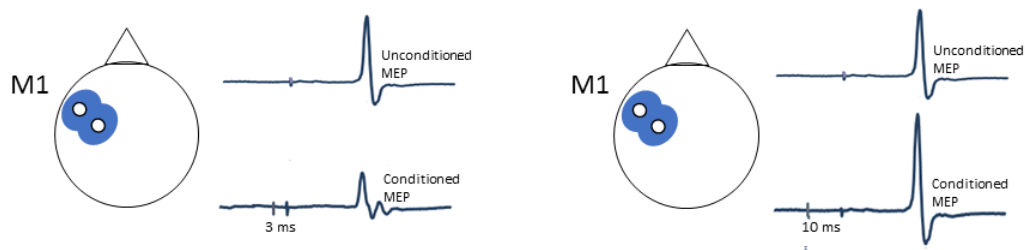

Figure 21. Short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF)

Schematic representations of SICI and ICF. Electromyographic traces show the unconditioned motor-evoked potential (MEP) obtained with single pulse TMS on the top, while, on the bottom, is showed the conditioned MEP obtained with paired-pulses TMS with 3 ms of interstimulus interval (ISI) for SICI and 10 ms of ISI for ICF.

Face emotional expressions stimuli

Visual stimuli reporting FES were used. The visual stimuli consisted of photographs of ten actors taken from the Karolinska directed emotional faces set (Lundqvist et al., 1998). Each actor (10 in total, 5 women) displayed a neutral, sad or happy facial expression for a total of 30 visual stimuli. Stimuli were shown on a 17" LCD monitor, with a 1280x1024 resolution and a 70 Hz refresh rate, by using PsychToolbox software (Brainard, 1997), running in MATLAB environment (Version 2015b, MathWorks, Inc., Natick, MA, United States). The duration of the visual stimulus was set at 300 ms, based on previous studies, which showed 300 ms as an adequate time window to allow the stimulus reaching M1 (Cuthbert et al., 2000; Schutter et al., 2008; Smith & Smith, 2019; Ginatempo et al., 2020). Although current models suggest that the information sufficient to distinguish faces from other objects is encoded within 120 ms and that the construction of a detailed perceptual

representation of a face requires approximately 170 ms, the conceptual knowledge of the emotion signaled by the face requires at least 300 ms of stimulus exposure (Adolphs, 2002).

Event Related Potentials

Event Related Potentials (ERP) were recorded to assess whether the FES were effectively perceived by the subjects. Two markers of face processing were recorded: i) the P100 wave (*i.e.,* the positive wave occurring 100 ms post stimulus), which is a visually-evoked component involving extra-striate visual regions, that is typically linked to low-level stimulus properties and attention (Smith & Smith, 2019); ii) the N170 wave (*i.e.,* a negative wave that appears after 170 ms from the stimulus onset), which is known to be elicited by the passive viewing of facial expressions (Itier & Taylor, 2004) and is attributed to the face recognition process through activation of the superior temporal sulcus and the fusiform gyrus (Itier & Taylor, 2004; Sadeh et al., 2010). Electroencephalogram (EEG) was recorded using a Neuroscan Synamps system (Compumedics, Charlotte, CN, USA) connected to a 32 channels EEG cap with 32 Ag/AgCl electrodes (Compumedics, Charlotte, CN, USA). Recorded data were streamed to a PC and the signal was monitored online through Neuroscan software. Recording electrodes were arranged according to the 10–20 international EEG system and included 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 offline referenced to the mean reference and the ground electrode was placed on Fpz. In the offline analysis, an average reference was used. Skin impedances were kept below 5 kΩ and the sampling frequency during recording was 1000 Hz. Off-line EEG processing was performed with EEGLAB 14.1.1 (Delorme & Makeig, 2004) running in MATLAB environment (Version 2015b, MathWorks, Inc., Natick, MA, United States). The EEG signal was 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) fourth order Butterworth filter. Epochs were visually inspected and those with excessively noisy signal were excluded (less than 5% 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 distribution, and

amplitude criteria (Rogasch et al., 2013, 2014). The analysis was focused on the latency and amplitude of P100 and N170 (Figure 22) in every single subject for each condition, thus EEG signals from electrodes T6, T5, and Oz were averaged separately for each FES. P100 peak was identified as a positive peak with latency between 80 and 100 ms in Oz (Adolphs, 2002) while the N170 wave as a negative peak with latency between 130 and 200 ms in both T5 and T6 (Hinojosa et al., 2015). Latency and amplitude were used as variables.
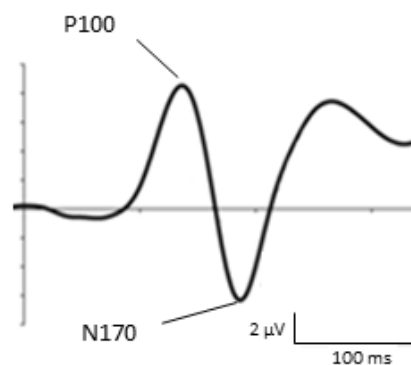

Figure 22. Event-related potentials (ERP)

Graphical representation of ERP components P100 and N170.

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Experimental design

The study comprised three experiments which were performed in random order and separated by at least one week.

Experiment 1: Influence exerted by face emotional expressions stimuli on the P100 and N170 waves

Experiment 1 consisted of an ERP session. EEG activity was continuously recorded over 300 trials: Each trial started with a dark background, and then a FES (neutral, sad and happy faces) appeared for 300 ms on the black background. Inter-trial intervals varied randomly between 4800 and 5200 ms. ERP analysis was focused on the period in which FES were presented. A total of 300 trials were recorded (100 per each FES).

Experiment 2: Influence exerted by face emotional expressions stimuli on the M1 representation area of left and right depressor anguli oris muscles

This experiment was planned to investigate a possible influence of age on the modulatory action exerted by passive viewing of images reporting emotional face expressions on face M1. To reduce the number of the stimuli delivered in each session, the experiment was divided into two randomized sessions, separated by at least one week: i) TMS on the right face M1 and ii) TMS on the left face M1. In both sessions, TMS-induced MEP were recorded in the contralateral DAO muscle. Both SICI and ICF were tested in face M1 after 300 ms from FES (neutral, sad and happy faces) delivery. This time interval was chosen based on previous studies assessing the time necessary from complex visual stimuli to reach motor/premotor areas (Carretié et al., 1997; Cuthbert et al., 2000; Smith & Smith, 2019). The inter-trial interval varied randomly between 4800 and 5200 ms.

Twenty unconditioned and twenty conditioned MEP for each ISI (3 ms for SICI and 10 ms for ICF) and each condition (happy, sad and neutral) for a total of 180 trials per session were collected. The two experimental sessions (TMS on left face M1 and TMS on right face M1) were performed in randomized order. RMT, raw MEP amplitudes and amplitude ratios of conditioned to unconditioned MEP, were used as variables.

Experiment 3. Valence ratings and reaction time to face emotional expressions stimuli

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, the FES appeared for 300 ms on a black background and were preceded and followed by a black background. Subjects were instructed to maintain fixation on the screen, visualize each picture, and judge as quickly and as accurately as possible whether the target face expressed happiness or sadness by pressing with their right-hand fingers one of two possible response buttons located in a response box. Therefore, the 100 FES showing sad, happy and neutral facial expressions used in experiment 1 and 2 were presented for a total of 300 trials. Inter-trial interval varied randomly between 4800 and 5200 ms, while image duration lasted 300 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 20 software (SPSS Inc, Chicago, IL, USA). Mixed and repeated measures 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. Differences in age and gender were assessed using Student's paired t-test and chi-square test, respectively. Significance was set for p value <0.05. Values are expressed as mean ± standard error of the mean.

Experiment 1: A two-way mixed-ANOVA on P100 waves using separately amplitude and latency as variables, with FES (happy, sad and neutral) as within-subjects factor and group (young and aged) as between-subjects factor was performed. A three-way mixed-ANOVA on N170 waves using separately amplitude and latency as variables, with side (T5 and T6) and FES (happy, sad and neutral) as within-subjects factor and group (young and aged) as between-subjects factor was performed.

Experiment 2: A two-way mixed-ANOVA on RMT value was performed with side (left and right DAO) as within-subjects factor and group (young and aged) as between-subjects factor.

A preliminary mixed-ANOVA on raw TS MEP amplitude was performed with side (left and right DAO) and FES (happy, sad and neutral) as within-subjects factor and group (young and aged) as between-subjects factor. In case of no significant effect of FES on MEP amplitude was detected, a three-way mixed-ANOVA for SICI and ICF, was performed separately using amplitude ratio as variable with side (left and right DAO) and FES (happy, sad and neutral) as within-subjects factor and group (young and aged) as between-subjects factor.

Experiment 3: A two-way mixed-ANOVA on the percentage of correct responses with FES (happy and sad) as within-subjects and group (young and aged) as between-subjects factor was performed. A two-way mixed-ANOVA on the reaction time with FES (happy

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and sad) as within-subjects and group (young and aged) as between-subjects factor was performed, separately.

Finally, Pearson's correlation analysis was performed to explore the link between perceptive and motor areas using ERP (P100, N170 amplitudes and latencies), TMS (SICI and ICF ratios) and recognition task (accuracies and reaction times) as variables.

Results

The two groups differed significantly for age (T_{33}=32.207; p<0.001) but not for gender composition (Chi=0.1245; p=0.72). Given the imbalance between the number of male and female subjects for both groups, Chi-Square analysis was performed to detect differences in gender frequencies. No differences were detected neither for the aged (p=0.225) nor for the young group (p=0.467), nor for both groups pulled together (p=0.170).

Experiment 1: Influence exerted by face emotional expressions stimuli on the P100 and N170 waves

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

P100 wave – P100 latency was not influenced by group or FES. However, P100 amplitude was smaller in aged than young subjects only after the view of sad expressions A two-way mixed-ANOVA on P100 latency showed a non-significant effect of FES ($F_{1,32}$=0.505; p=0.609), group ($F_{1,32}$=0.226; p=0.638) nor interaction between factors (Figure 23A). Two-way mixed-ANOVA on P100 amplitude showed a non-significant effect of FES ($F_{1,32}$=1.038; p=0.367) and group ($F_{1,32}$=0.417; p=0.523) but a significant interaction between the factors ($F_{1,32}$=3.671; p=0.038). Post-hoc analysis revealed a smaller P100 following sad than neutral FES (p=0.032) only in the aged subjects (Figure 23B).
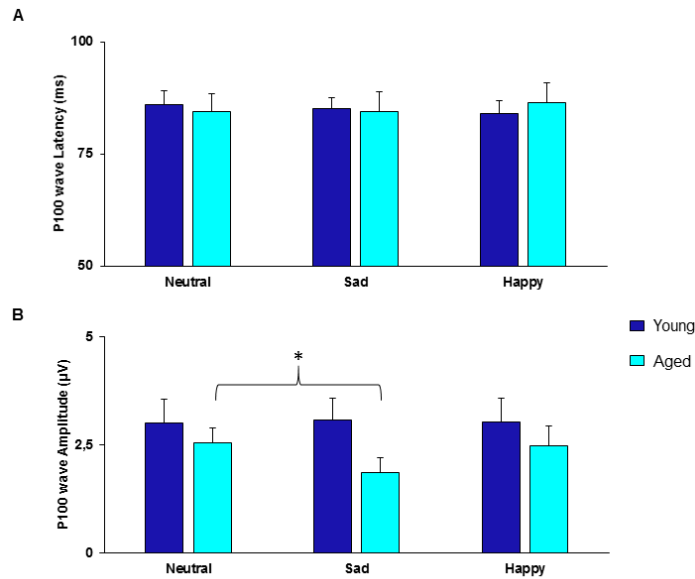
Figure 23. Influence exerted by the passive view of facial emotional expressions on the P100 wave in aged and young subjects.

Histograms report for the young (n = 17) and aged (n = 17) groups' latency (A) and amplitude (B) of the P100 wave during the passive view of faces expressing happiness, sadness or neutral emotions. The graph represents means + SEM. *p<0.05; **p<0.01; ***p<0.001.

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N170 wave - Aged subjects showed longer latency of N170 without any differences by side or FES. On the other hand, a clear lateralization effect on N170 amplitude was detected regardless of the group. In fact, three-way mixed-ANOVA on the latency of the N170 showed a significant effect of group ($F_{1,32}$=5.508; p=0.029) but no significant effect of side ($F_{1,32}$=1.049; p=0.313), FES ($F_{1,32}$=2.031; p=0.148) nor interaction between the factors. Bonferroni test showed a longer N170 latency in the aged than in the young subjects (p=0.029) (Figure 24A). The statistical analysis on the N170 amplitude showed a significant effect of side ($F_{1,32}$=14.694; p=0.001) but no significant effect of FES ($F_{1,32}$=0.585; p=0.563), group ($F_{1,32}$=1.538; p=0.224) nor interaction between the factors (Figure 24B).
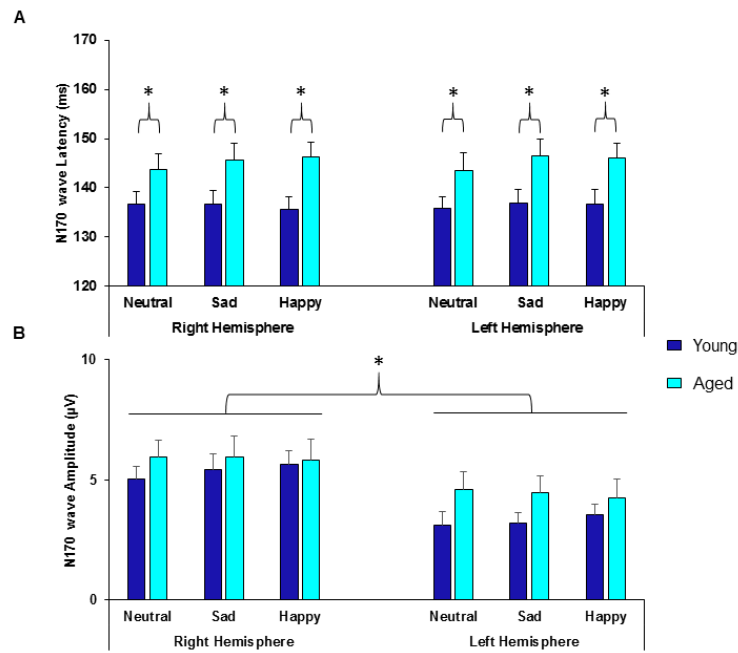
Figure 24. Influence exerted by the passive view of facial emotional expressions on the N170 wave in aged and young subjects.

Histograms report for the young (n = 17) and aged (n = 17) groups' latency (A) and amplitude (B) of the N170 wave during the passive view of faces expressing happiness, sadness or neutral emotions. The graph represents means + SEM. *p<0.05; **p<0.01; ***p<0.001.

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Experiment 2: Influence exerted by face emotional expressions stimuli on the primary motor cortex representation area of left and right depressor anguli oris muscles

Neurophysiological features of face M1, as assessed by TMS, are described by age and hemisphere side in Table 3. Statistical analysis on RMT values showed no significant effect of side ($F_{1,32}$=2.968, p=0.095), group ($F_{1,32}$=0.668, p=0.420) nor interaction between factors. A preliminary analysis on the absolute MEP amplitude showed no significant effect of group ($F_{1,32}$ =0.281, p=0.600), side ($F_{1,32}$=0.529, p=0.472) and FES ($F_{1,32}$=0.680, p=0.539) nor interaction among the factors. This result allowed performing the analysis on the ratio conditioned/unconditioned MEP amplitude for both SICI and ICF.

Table 3. Neurophysiological parameters of the right and left face M1 in young and aged subjects.

Face M1	TMS parameters*	Young subjects (n =17)	Aged subjects (n =17)	Young vs Aged (p values)
Left face M1	RMT (%MSO)	56.9±2.2	60.5±2.3	0.24
	MEP amplitude (mV)	0.21±0.03	0.24±0.05	0.94
Right face M1	RMT (%MSO)	56.3±2.7	57.5±2.1	0.71
	MEP amplitude (mV)	0.25±0.04	0.26±0.04	0.75

M1, primary motor cortex; TMS, trancranial magnetic stimulation; *TMS parameters measured from the contralateral Depressor Anguli Oris muscle; RMT, Resting Motor Threshold; MSO, maximum stimulator output; mV, millivolts. The table represents means ±SEM

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SICI – Aged subjects showed less SICI than young subjects. However, weaker SICI was detected following happy expressions in both groups. In particular, mixed-ANOVA on MEP amplitude ratio showed a significant effect of FES ($F_{1,32}$=7.414, p=0.002) and group ($F_{1,32}$=5.629, p=0.024), but no significant effect of side ($F_{1,32}$=0.005, p=0.942) and no interaction between the factors. Post-hoc comparisons revealed that SICI was significantly reduced following the view of happy facial expressions (p= 0.001) compared with neutral faces (Figure 25A).

ICF – No effects of age on ICF were detected. Mixed-ANOVA on MEP amplitude ratios showed a significant effect of FES ($F_{1,32}$=3.894, p=0.031), and a trend towards significance for side ($F_{1,32}$=4.270, p=0.05). By contrast, no significant effect of group ($F_{1,32}$=2.153, p=0.152), nor interaction between the factors (Figure 25B) were detected. Post-hoc comparisons for emotion showed enhanced ICF following the view of happy faces compared to neutral ones (p=0.024).
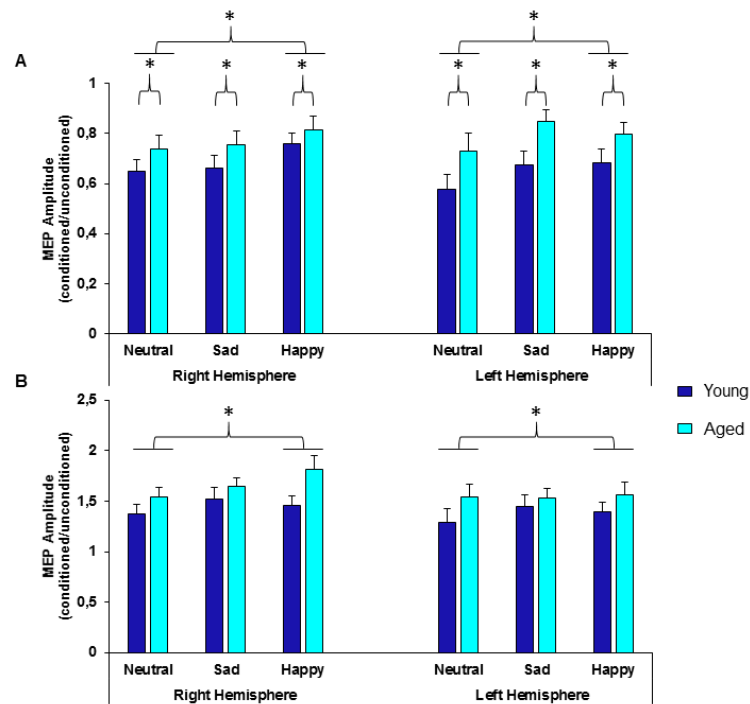
Figure 25. Influence exerted by facial emotional expressions on short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF), in aged and young subjects.

The histograms show SICI (A) and ICF (B) during the passive viewing of faces expressing happiness, sadness or neutral emotions. Motor evoked potentials (MEP) were recorded from the right and left depressor anguli oris muscle of the young (n = 17) and aged (n = 17) groups following paired pulse stimulation of the contralateral face M1. MEP amplitude (means + SEM) is expressed as ratio between the conditioned and unconditioned MEP. *p<0.05; **p<0.01; ***p<0.001.

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Experiment 3. Valence ratings and reaction time to face emotional expressions stimuli

Aged subjects showed less accuracy than young subjects in the recognition of FES (Figure 26A). Moreover, the former took longer time to answer and recognize FES, with specific regard to sad facial expressions (Figure 26B).

Statistical analysis on the percentage of correct responses showed a significant effect of group ($F_{1,32}$= 5.816; p=0.023) but no significant effect of FES ($F_{1,32}$=0.234; p=0.632) nor interaction between the factors. Post-hoc comparisons showed a lower percentage of correct responses in aged than young subjects (p=0.023). Two-way mixed-ANOVA on the reaction time showed a significant effect of FES ($F_{1,32}$=32.639, p<0.001), group ($F_{1,32}$=5.013, p=0.034) and interaction between the factors ($F_{1,32}$=32.639, p<0.001). Post-hoc comparisons showed longer reaction time in the aged than young group (p=0.034). Both groups took more time to recognize sad than happy expressions (p<0.001). However, post-hoc analysis of the interaction between factors revealed that the differences between groups were significant only for sad expressions (p=0.015).
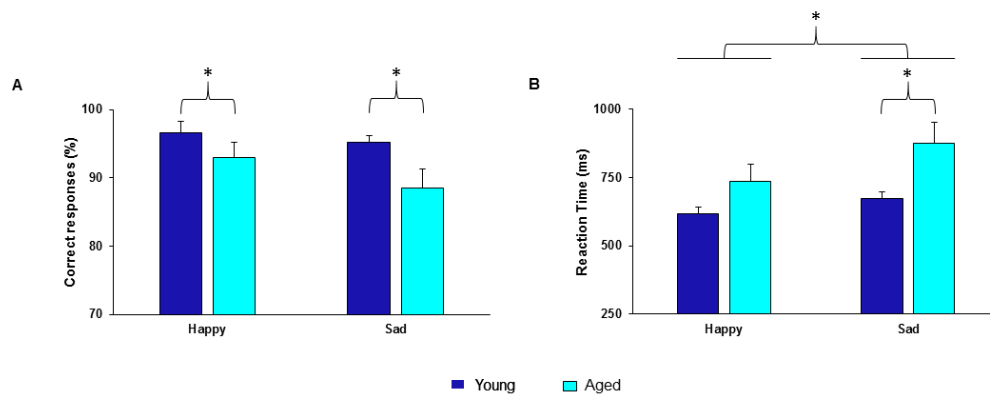
Figure 26. Influence exerted by facial emotional expressions on face recognition task in young and aged subjects.

The histograms report (A) the accuracy of responses in the discrimination between happy and sad faces, expressed as % of correct responses and (B) the reaction times exhibited in the discrimination task, comparing performances of the young (n= 17) and aged (n= 17) groups. The graph represents means + SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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Correlation analysis

Bivariate correlation analyses failed to detect any significant correlation between ERP (P100 amplitudes and latencies, N170 amplitudes and latencies) TMS (SICI and ICF ratios) and recognition task (accuracies and reaction times) variables.

Discussion

This work investigated the role played by age in the perception and recognition of face expressions passively viewed, along with their effect on the excitability of face M1. Results provided first evidence that visual perceptive cortical areas are influenced by sad face expressions in aged but not young subjects, while the excitability of face M1 is modulated by happy expressions, regardless of age.

A clear lateralization effect was observed in perceptive cortical areas. In particular, in agreement with several studies, a larger N170 wave was detected in the right than left hemisphere, regardless of age and emotional expression (Gao et al., 2009; Daniel & Bentin, 2012; Hinojosa et al., 2015). Although the recognition of emotional pictures seems distributed in multiple structures, between the left and right hemispheres, the latter appears as the most critical component of the system, with specific regard to the inferior temporal regions (Adolphs, 200; Rossion et al., 2003; Gainotti et al., 2019), as confirmed by our findings.

The ERP study revealed a reduced P100 amplitude following the view of sad expressions, but only in aged subjects. These findings are in line with previous studies reporting that P100 amplitude may be affected by emotional stimuli (Itier & Taylor, 2002; Herrmann et al., 2005; Moradi et al., 2017). However, it is still unclear if an interaction between this wave and emotional processes of face expressions exists, since several studies excluded it (Liao et al., 2017; Gonçalves et al., 2018). Although the P100 does not measure attention explicitly, in the present study it was employed to gain information on the attention paid by the subjects to visual stimuli, according to the pertinent literature which associates the P100 with attention-related enhancements of the processing of intrinsically salient stimuli (Hillyard & Anllo-Vento, 1998; Luck et al., 2000; Di Russo et al., 2003; Luo et al., 2010; Rossion & Jacques, 2012). The visual stimuli used in the present study were all featured by the same color and luminance composition, which are known to affect the P100 (Herrmann et al., 2005). This excludes that the FES-induced P100 modulation that we observed may be due to changes in low-level visual features of the stimulus. Data indicate that sad faces attract visual attention of aged and young subjects differently. In particular, the emotional content of the face observed was able to reduce the P100 in aged but not in young subjects, suggesting that the former had impaired visual attention for sad faces, which may be responsible for the longer time they needed in the face expression recognition task. It cannot be ruled out a possible role of different face exploration patterns, leading to attention impairment, in aged subjects, as demonstrated by previous eye-tracking studies. In particular, aged people tend to fix their gaze on the mouth region, whereas young people spend more time on the eye areas (Wong et al., 2005; Sullivan et al., 2007; Murphy & Isaacowitz, 2010), which play a central role in decoding negative emotions such as anger, sadness and fear.

Contrary to what was hypothesized, we failed to detect any significant differences between age groups in N170 amplitude following the passive view of face expressions. This finding is in line with previous studies (Krolak-Salmon et al., 2001; Herrmann et al., 2002; Eimer et al., 2003), but in contrast with a work showing that the N170 wave presents a heterogeneous sensitivity to facial expressions (Andreatta et al., 2012). However, our and these studies cannot be directly compared, since they differ in the required task, types of face expressions to be recognized and recording protocols (Hinojosa et al., 2015). Our cohort of aged subjects presented a delayed N170 wave in comparison with the young

group, suggesting that the perception of face expressions takes longer with age. This finding is in contrast with the only study which assessed N170 latency in young and aged subjects for face expressions and found no group differences (Tsolaki et al., 2017). However, this study evaluated the effect of only angry and fearful faces in a group of women.

The N170 wave is thought to express an internal face representation (*e.g.,* the spatial configuration of faces) built in the temporal gyrus (Hinojosa et al., 2015), and used for subsequent expression categorization (Calvo & Beltrán, 2014; Rossion, 2014). Regardless of any emotion discrimination, aged subjects have more difficulties than young subjects in the perception of spatial configurations of faces (Gao et al. 2009; Daniel & Bentin 2012). Therefore, the delay of N170 observed in the aged group could be accountable for a decline of the configural processing of the intrinsic characteristics of faces, rather than the emotion expressed by the face itself. Notably, when relevant information on the configural features of the face is spatially distorted or missing, also young people present increased difficulty in the configural processing of faces, resulting in a delayed N170 wave (Bentin et al., 1996, 2006; Rossion et al., 2000).

Interestingly, while the visual attention to sad faces was different between aged and young subjects, being impaired in the former, the visual perception of happiness did not differ between groups. On the motor side, happiness was the only face expression able to increase the excitability of face M1. Unexpectedly, this effect was observed regardless of age, as suggested by the reduced SICI and enhanced ICF exhibited by both aged and young subjects following the view of happy faces. This finding is in agreement with a recent study demonstrating that face M1 is selectively modulated by passive viewing of happy faces, while hand M1 is modulated specifically by the view of faces expressing sadness (Ginatempo et al., 2020) or fear (Schutter et al., 2008). It has been proposed that the ventral lateral premotor cortex may serve as the integration centre mediating the connection between perceptive areas and face M1 (Ginatempo et al., 2020). However, the correlation analysis failed to detect any significant link between perceptive and motor areas involved in the recognition and production of face expressions, respectively. This may be due to the inadequacy of the experimental set up used in the present study, not designed for this specific purpose, for which connectivity studies would be more adequate.

The motor control of facial muscles is different from that of other muscles of the body in many respects (Cattaneo & Pavesi, 2014; Müri, 2016). M1 projections to the lower face muscles are bilateral, with a contralateral predominance (Paradiso et al., 2005; Pilurzi et al., 2013, 2020; Ginatempo et al., 2019a). Moreover, face muscles are rarely activated in asymmetrical way. In fact, during voluntary movements, facial muscles' groups are recruited together to produce a facial posture, especially when performing facial expressions (Cattaneo & Pavesi, 2014). Therefore, the lateralization effect detected in the visual perception phase might be cancelled-out when the information reaches the premotor cortices. Consequently, when the emotional information reaches M1, the modulation of the facial expression is bilateral to prepare the movement, resulting in an appropriate facial posture, which must be coordinated bilaterally.

The study of face M1 excitability in the two age groups revealed that young subjects had stronger SICI than aged subjects, without any significant difference among emotions expressed by faces. This novel finding of reduced SICI in face M1 is in line with a previous study, which investigated the effect of age in hand M1 (Peinemann et al., 2001), but in contrast with other studies demonstrating a stronger SICI in aged subjects (Smith et al., 2009; McGinley et al., 2010). However, the different CS intensities used in these (95% of the RMT) and our study (80% RMT) makes data not directly comparable. Several studies demonstrated that SICI is specifically involved in the intracortical inhibitory processes, which seem mediated by gamma-aminobutyric acid type A (GABA-A) receptors (Ziemann et al., 1996; Di Lazzaro et al., 2007). Animal studies suggested a strong relationship between aging and decline of GABA-mediated inhibition (Ling et al., 2005; Yu et al., 2006; Hua et al., 2008; Schmidt et al., 2010). Accordingly, human studies demonstrated that aged adults showed a reduction in the ability to coordinate movements (Greene & Williams, 1996; Serrien et al., 2000; Heuninckx et al., 2004) and slower reaction times (Morgan et al., 1994; Salthouse, 1996; Hunter et al,. 2001), which are necessarily correlated to the modulatory capacity of cortical inhibition (Baldissera & Esposti, 2005; Fujiyama et al., 2009; Heise et al., 2013). In this light, we suggest that SICI reduction detected in aged subjects could be attributed to impaired intracortical inhibitory circuit activity, which possibly results in reduced coordination and velocity of movements. The longer reaction time observed in aged than young subjects supports this view. Moreover, the analysis of the rating of the recognition task revealed less accuracy in aged

than in young subjects, in agreement with previous studies reporting reduced accuracy (Gunning-Dixon et al., 2003; Keightley et al., 2007; Ruffman et al., 2008; Liao et al., 2017), longer reaction times (Gunning-Dixon et al., 2003; Hilimire et al., 2014; Liao et al., 2017) in aged than young subjects and no differences between them for happy face expressions compared with neutral ones (Liao et al., 2017). Importantly, although SICI was basically impaired in aged subjects, it proved still significantly responsive to the view of faces expressing happiness. These data, along with the observed modulation of ICF, suggest that responsiveness of face M1 to visual stimuli of happiness is preserved.

Results indicate a differential encoding of facial information in the aged group compared to the young group, however, the exact mechanism is still unclear. Data suggest that aged subjects show impaired visual attention for sad face expressions, possibly due to attention-related changes of the processing of salient stimuli and to increased difficulty in processing configural characteristics of faces. By contrast, responses of face M1 to the view of face emotional expressions are enhanced by happy but not sad expressions, with no differences between age groups.

Conclusions

These studies investigated the physiological characteristics of the face motor system with particular regard to learning and recognition of emotional face expressions.

The first study (Ginatempo et al., 2021) demonstrated that face motor system can be modified by the process of learning and training to play a musical instrument. Wind professional musicians, through constant work since infancy, developed the ability to differently modulate face movements compared to non-musicians and string musicians, changing the functional aspects of face muscles. These findings suggests that learning processes, carried out with face muscles, allow performing asymmetrical movements that are not reproducible by the other people. Future studies can focus on patients with cortical impairment to the face motor area, in which taking up a wind musical instrument may represent a rehabilitation strategy.

The second study (Loi et al., 2021a) demonstrated that the cerebellar circuits underpinning conditioning learning processes are influenced by the view of sad face expressions. This finding may suggest that the emotional content of the faces is able to influence our mnemonic and learning processes performed by the cerebellum. It could be possible that cerebellum, through learning processes, provide an alarm system against potentially aversive stimuli, such as faces expressing negative emotions. Future studies are necessary to investigate whether learning processes in the cerebellum, may influence also the face motor system in response to visual stimuli representing face expressions.

Finally, the third study (Loi et al., 2021b) highlighted that, although aging impairs the perception and the recognition of faces expressing emotions, the face M1 excitability in response to these expressions is preserved. This may suggest that aging does not affect face emotional processing in cortical motor areas modulating face mimicry. Future studies can be directed to assess whether changes in face M1 excitability occur in pathological conditions, such as Parkinson's disease that is often featured by an ipoamimia or amimia.

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