DOI: 10.1113/JP278877

Title Page

Title

Role of cutaneous and proprioceptive inputs in sensorimotor integration and plasticity occurring in the facial primary motor cortex.

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Running title: Cutaneous and proprioceptive inputs in facial sensorimotor integration

This is an Accepted Article that has been peer-reviewed and approved for publication in the The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; doi: 10.1113/JP278877.

Key words: cutaneous afferents, proprioceptive afferents, sensorimotor integration, facial muscles

Word count (excluding key points, references and figure legends, including abstract): 5581

Table of Contents Category: Neuroscience – behavioural/systems/cognitive

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Key Points summary

• Previous studies investigating the effects of somatosensory afferent inputs on cortical excitability and neural plasticity often used TMS of hand motor cortex (M1) as a model. In this model it is difficult to separate out the relative contribution of cutaneous and muscle afferent input to each effect.

• In the face, cutaneous and muscle afferents are segregated in the trigeminal and facial nerves respectively. We studied their relative contribution to corticobulbar excitability and neural plasticity in the depressor anguli oris M1.

• Stimulation of trigeminal afferents induced short-latency (SAI) but not long-latency (LAI) afferent inhibition of face M1. In contrast, facial nerve stimulation evoked LAI but not SAI. Plasticity induction was observed only after a paired associative stimulation protocol using the facial nerve.

• Physiological differences in effects of cutaneous and muscle afferent inputs on face M1 excitability suggest they play separate functional roles in behavior.

Abstract:

The lack of conventional muscle spindles in face muscles raises the question of how sensory input from the face is used to control muscle activation. In 16 healthy volunteers, we probed sensorimotor interactions in face motor cortex (fM1) using short-afferent inhibition (SAI), long-afferent inhibition (LAI) and LTP-like plasticity following paired associative stimulation (PAS) in the depressor anguli oris muscle (DAO). Stimulation of low threshold afferents in the trigeminal nerve produced a clear SAI ($p<0.05$) when the interval between trigeminal stimulation and TMS of fM1 was 15-30 ms. However there was no evidence for LAI at longer intervals of 100-200 ms, nor was there any effect of PAS. In contrast, facial nerve stimulation produced significant LAI ($p<0.05$) as well as significant facilitation 10-30 minutes after PAS ($p<0.05$). Given that the facial nerve is a pure motor nerve, we presume that the afferent fibres responsible were those activated by the evoked muscle twitch. The F-wave in DAO was unaffected during both LAI and SAI consistent with their presumed cortical origin. We hypothesise that in fM1, SAI is evoked by activity in low threshold, presumably cutaneous afferents, whereas LAI and PAS require activity in (higher threshold) afferents activated by the muscle twitch evoked by electrical stimulation of the facial nerve. Cutaneous inputs may exert a paucisynaptic inhibitory effect on fM1, while proprioceptive information is likely to target inhibitory and excitatory polysynaptic circuits involved in LAI and PAS. Such information may be relevant to the physiopathology of several disorders involving the cranio-facial system.

Abbreviations.

1, area 1 of SI; 2, area 2 of SI; 3a, area 3a of SI; 3b, area 3b of SI; a, accessory nerve; ANOVA, analysis of variance; BS, brainstem; CMAP, compound muscle action potential; DAO, depressor anguli oris muscle; ES, electrical stimulation; f, facial nerve; FDI, first dorsal interosseus muscle; ISI, interstimulus time interval; LAI, long-afferent inhibition; LTD, long-term depression; LTP, long-term potentiation; M1, primary motor cortex; MEP, motor evoked potential; MSO, maximal stimulator output; PAS, paired associative stimulation; PMN, paramedian nuclei; PPC, posterior parietal cortex; PT, perceptual threshold; RMT, resting motor threshold; SI, primary sensory cortex; SII, secondary sensory cortex; SAI, short-afferent inhibition; SKIN, facial skin; t, trigeminal nerve; TH, thalamus; TMS, transcranial magnetic stimulation; TS, test stimulus; VII, facial motor nucleus; Vcn, fifth cranial nerve; VIIcn, seventh cranial nerve; VPM, ventroposteromedial nuclei.

Introduction

The lack of conventional muscle spindles in face muscles raises the question of how sensory input from the face is used to control muscle activation (Cattaneo & Pavesi, 2014). In this paper we examine sensorimotor integration in the face area of primary motor cortex (fM1) by using standard techniques of transcranial magnetic stimulation (TMS) to measure afferent-evoked short- and long-interval inhibition of motor cortex output (SAI and LAI, respectively), and by using paired associated stimulation (PAS) to investigate sensorimotor plasticity.

SAI, LAI and PAS have been studied extensively in the hand. Here, a single electrical stimulus to a cutaneous (e.g. digital nerve) or mixed nerve (median nerve) can suppress the amplitude of motor evoked potentials (MEPs) in hand muscles by TMS. SAI refers to the period of inhibition that is observed when the peripheral stimulus is given about 20-25 ms before the TMS pulse; LAI refers to a later phase of inhibition that occurs when the interval is >100 ms (Chen et al., 1999; Classen et al., 2000; Tokimura et al., 2000; Kobayashi et al., 2003; Bikmullina et al., 2009; Devanne et al., 2009). Both SAI and LAI are thought to be of

cortical origin since there is no clear suppression of the F-wave, a conventional indicator of the excitability of spinal motoneurons (Classen et al., 2000; Tokimura et al., 2000). PAS involves repetitive pairing of peripheral and cortical stimulation at interstimulus intervals (ISIs) around 20-25ms. This leads to long-lasting increases in MEP amplitude that are thought to be due to early processes of synaptic long-term potentiation (Stefan et al., 2000; Wolters et al., 2003; Kujirai et al., 2006; Quartarone et al., 2006). Again, because no changes are observed in spinal motoneuronal excitability, the changes are assumed to occur in the cerebral cortex (Stefan et al., 2000).

In a previous study on the face we found that electrical stimulation of the facial nerve evokes LAI and PAS in the depressor anguli oris (DAO) muscle (Pilurzi et al. 2013). We argued that although the facial nerve is a pure motor nerve, the responsible sensory input is likely to involve mechanosensitive receptors in skin (and perhaps non-spindle receptors in muscle) that are activated by the muscle twitch. Unexpectedly, facial nerve stimulation did not evoke SAI. One possible explanation is that the muscle twitch evoked by facial nerve stimulation produces a temporally dispersed volley rather than a synchronous input as evoked by electrical stimulation (Pilurzi et al., 2013).

The first aim of the present paper was to test this hypothesis by examining whether SAI can be evoked by electrical stimulation of the (sensory) mentalis branch of the trigeminal nerve. We found that although electrical stimulation of sensory fibres produced SAI, there was no LAI or PAS. One possibility is that LAI and PAS in facial muscles require temporally dispersed afferent input, which occurs during a muscle twitch but is not present after electrical stimulation. However this seems unlikely since electrical nerve stimulation produces clear LAI and PAS in the hand. As we argue below, it may be that sensorimotor integration operates differently in the face area of cortex compared with the hand. In the face, SAI is produced by stimulation of (low threshold) cutaneous fibres, whereas LAI and PAS require activation of mechanoreceptors sensitive to muscle twitches. These mechanosensitive receptors could be cutaneous receptors with a higher electrical threshold than those required for SAI, and/or they could be the “Ruffini-like” intramuscular receptors that have recently been described in human facial muscles.

Methods

Ethical Approval

Experiments were conducted in sixteen healthy volunteers (10 females and 6 males; mean age 28.69 (4.84 SD: standard deviation) years), all right handed according to the Oldfield inventory scale. All subjects gave their informed written consent to participate in the study, which 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, except for registration in a database. None of the subjects had a history of neurological diseases. Subjects sat in a comfortable chair and were asked to stay relaxed but alert during the experiments.

EMG

EMG was recorded, in different experimental sessions, from the right DAO, from the right first dorsal interosseous (FDI), from the right masseter and from the right trapezius muscles, using 9 mm diameter Ag-AgCl surface electrodes. For the DAO EMG recordings, the active electrode was placed at the midpoint between the angle of the mouth and the lower border of the mandible, with the reference electrode over the mandible border, 1 cm below the active electrode and the ground electrode over the right forehead. For the FDI EMG recordings, 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. For the masseter muscle EMG recording, active electrode was positioned in the lower third of the masseter with reference electrode placed in the middle part of the zygomatic arch. For the trapezius muscle recording electrode was placed in the upper trapezius over the muscle belly and the reference electrode over the acromion-clavicular joint. Unrectified EMG signals were recorded (D360 amplifier, Digitimer Ltd, Welwyn Garden City, UK), amplified (x1000), filtered (bandpass 3-3000 Hz for MEP and 50-5000 Hz for F-waves recordings), and sampled (5 kHz per channel; window frame length: 500 ms for MEPs; window 250 ms for F-waves) using a 1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and Signal 5 software on a computer and stored for off-line analysis.

TMS

TMS of the left hemisphere was performed using a figure-of-eight shaped coil with external loop diameter of 7 cm connected to a Magstim 200 stimulator (Magstim Co., Whitland, Dyfed, UK). The optimal stimulation site, for the contralateral DAO or FDI, was carefully searched and then marked with a soft tip pen over the scalp, to maintain the same coil position throughout the experiments. The handle of the coil pointed posteriorly and laterally, at approximately 30-45 deg to the interhemispheric line (Kujirai et al., 2006; Pilurzi et al., 2013). The resting motor threshold (RMT) was taken as the lowest TMS intensity, expressed as percentage of the maximum stimulator output (MSO), that elicited, in the relaxed muscle, MEPs of 0.05 mV in at least 5 out of 10 consecutive trials. The intensity of the test stimulus (TS) for TMS of face M1 was 120% of RMT. In experiment 3, TS was set at 110% of RMT, adjusted to evoke in the FDI MEPs of nearly 1mV.

Electrical stimulation

Electrical stimulation (square-wave pulses of 0.2 ms duration) was applied through a pair of cup electrodes (cathode distal), connected to a constant current stimulator (model DS7; Digitimer, Welwyn-Garden City, Herts, UK), to the mentalis branch of the right trigeminal nerve, to the marginal branch of the right facial nerve and to the right accessory nerve as a conditioning stimulus (ES) in different sessions (Figure 1). Due to the high interindividual anatomical variability of the mandibular branch of the facial nerve, electrodes position was adjusted in each subject to obtain supramaximal DAO excitation using the lowest stimulus intensity. In order to avoid activation of the ipsilateral masseter muscle by facial nerve stimulation, due to a conducted volume, masseter EMG was recorded (Figure 2).

The intensity of the electrical stimulus was set at an intensity of three times the perceptual threshold (PT) of the subject for the trigeminal nerve; while for both facial and accessory nerve stimulations, ES was set at a value able to evoke a small stable compound muscle action potential (CMAP) in the right DAO and the right trapezius muscle respectively.

Facial F-waves, were evoked through ES of the right marginal branch of the facial nerve at supramaximal intensity (TS).

Experimental design

Main experiments

Experiment 1. Effects of trigeminal versus facial nerve stimulation on DAO MEP in the SAI and LAI protocols.

In all sixteen subjects, the effects of trigeminal and facial nerve stimulation on DAO MEPs were compared in the SAI and LAI paradigms. Single pulse TMS of the left face M1 was preceded by ES of the right trigeminal or facial nerves at various ISIs. The experiment was divided up into four blocks: trigeminal-SAI (tSAI), facial-SAI (fSAI), trigeminal-LAI (tLAI) and facial-LAI (fLAI). In tSAI and fSAI blocks, TS alone and 10, 15, 20, 25, 30 ms ISIs were tested. Each tLAI and fLAI block consisted of TS, 100, 150, 180 and 200 ms ISIs. The four blocks and all states (TS alone and ISIs) were randomized in each subject using a semi-randomized protocol. Ten unconditioned MEPs and 10 conditioned responses for each ISI were recorded from the right DAO at rest.

Experiment 2. After-effects of trigeminal versus facial nerve stimulation on DAO MEP in the PAS protocol.

Fifteen out of the 16 subjects enrolled in experiment 1 participated in experiment 2. Eight subjects (5 females and 3 males; mean age 29.25(4.74) years) underwent facial-PAS (fPAS), seven subjects (4 females and 3 males; mean age 28.22(4.87) years) underwent trigeminal PAS (tPAS). The PAS intervention was administered by pairing ES of the right facial or trigeminal nerves (fPAS and tPAS group, respectively) with TMS of the left face M1 using a ES-TMS ISI of 20 ms. Two hundred pairs of stimuli were given at 0.25 Hz. Subjects were instructed to keep facial muscles relaxed and stay alert. Twenty MEPs were collected from the resting DAO before and immediately (T0), 10 (T10), 20 (T20) and 30 (T30) minutes after PAS delivery.

Control experiments

Control experiments took place at least two weeks apart from the main experiments. SAI and LAI were tested using the same experimental and data collection procedure as experiment 1.

Experiment 3. Effects of trigeminal versus facial nerve electrical stimulation on facial F-Wave

To test the origin of the tSAI and fLAI, the effects of trigeminal and facial nerve stimulation on facial F-waves were investigated in 8 of the subjects who had participated in experiment 1 (5 females and 3 males; mean age 31.86(3.80) years). F-waves were obtained from the right DAO following TS of the marginal branch of the facial nerve for each subject. The same ES used in experiment 1 were given to the mentalis (ISIs of 10-15-20-25-30 ms ISIs) and marginal (ISIs of 100-150-180-200 ms) nerves before the TS. Twenty unconditioned and twenty conditioned recordings were collected for each ISI, in randomized order. Then, the persistence of the facial F waves, expressed as the number of F-waves clearly detectable (amplitude >20 μV) divided by number of recordings, was compared between the two conditions.

Experiment 4. Effects of accessory nerve stimulation on DAO MEP in SAI and LAI protocols

To compare the effects of homotopic and heterotopic cranial nerve stimulations (close and far from the target muscle, respectively), in 11 out of 16 subjects (8 females and 3 males; mean age 29.54(4.55) years), the effects of heterotopic accessory nerve stimulation on DAO MEPs were tested using SAI (aSAI) and LAI (aLAI) paradigms, where the stimulation of the accessory nerve was paired with TMS of face M1, and results compared with SAI and LAI induced by stimulation of homotopic cranial nerves. The accessory nerve was chosen because it is purely motor, it can be easily stimulated by surface electrodes and the effects of its stimulation can be checked by recording a clear CMAP from the ipsilateral trapezius muscle.

Experiment 5. Effects of trigeminal and facial nerve stimulation on FDI MEP in SAI and LAI protocols

Topographic muscle specificity of trigeminal and facial effects was tested in a distant muscle. FDI was chosen because of its accessibility and well standardized use in SAI and LAI protocols. All sixteen subjects underwent trigeminal and facial nerve stimulation (same stimulation procedure described in experiment 1) paired with TMS of hand M1. Results obtained in the FDI were then compared with significant effects obtained in the DAO muscle.

Statistical Analysis

Statistical analysis was performed with SPSS 18 software (SPSS Inc, Chicago, IL, USA).

Differences in PT, ES, RMT, TS intensities and test MEP amplitudes were assessed using Student's paired *t* test in experiment 1, 3, 4 and 5 with Student's unpaired *t* test in experiment 2. Values are expressed as a means ± standard deviation (SD).

Data processing

After processing of the EMG signal, each trial was characterized by a single number, i.e. the MEP amplitude. For each subject, each experimental condition contained a series of 10 repeated trials. Given the small number of repetitions we adopted, as a measure of central tendency, the median value. We therefore extracted the median of each pool of MEP amplitudes within each experimental condition. The data from conditioned conditions were then expressed as a ratio of the conditioned MEP over the unconditioned MEP. In this way values between 0 and 1 indicate an inhibitory effect of the conditioning stimulus and values larger than 1 indicate an excitatory effect of the conditioning stimulus. To ensure normality of the distribution, instead of the raw ratio (distributed between 0 and + infinity) we calculated the log of the ratio (distributed between -infinity and +infinity). The log-transformed data indicate inhibition of the conditioning stimulus whenever negative and facilitation whenever positive.

At this point two parallel analyses were performed. One was aimed at finding different distributions of the data according to the factorial designs of each experiment. This was done by feeding the individual data in ANOVAs with different structures according to each experiment. This approach is informative of the different distribution of data between experimental conditions (for example trigeminal stimulation vs facial stimulation) but is not informative of the absolute polarity (inhibition or excitation) of the effects of the conditioning stimulus on the test stimulus. We performed therefore a second, independent analysis consisting of t-tests for single samples applied to the data from each experimental condition against the null hypothesis of mean value = 0 (corresponding to the absence of modulation from the conditioning stimulus on the test stimulus).

Distribution analysis

Experiment 1: Independently for SAI and LAI a two way repeated measure (RM) ANOVA was performed with NERVE (facial or trigeminal) and ISI (SAI: 10, 15, 20, 25 or 30 ms; LAI: 100, 150, 180, or 200 ms) as a within factors.

Experiment 2: A mixed ANOVA was performed with NERVE (facial or trigeminal) as between-subjects factor, and TIME (baseline, 0, 10, 20 or 30 ms) as within-subjects factor.

Experiment 3: A two way RM-ANOVA was performed separately for both SAI and LAI protocols, with NERVE (facial or trigeminal) and ISI (SAI: 10, 15, 20, 25,30 ms; LAI: 100, 150, 180, 200 ms) as within factors.

Experiments 4 and 5: data from these experiments were merged with those from Experiment 1. Being the subjects participants in both the main and control experiments, this made it possible to perform a within-subjects analysis. In Experiment 4, a RM-ANOVA was performed separately for SAI and LAI, with NERVE (accessory, facial or trigeminal) and ISI (SAI: 10, 15, 20, 25 or 30; LAI: 100, 150, 180, 200ms) as within-subjects factors. In Experiment 5, tSAI and fLAI, were analyzed independently using RM ANOVA with MUSCLE (DAO or FDI) and ISI (SAI:10, 15, 20, 25 or 30; LAI: 100, 150, 180, 200 ms) as within-subjects factors.

Data distributions highlighted by significant ANOVA results were explored systematically by Tukey's Honestly Significant Difference Test.

Analysis of the effect of the conditioning stimulus

In each experiment we compared every set of data within each cell of the experimental design to the null hypothesis of mean=0. The significance threshold was adjusted for the number of comparisons using the Bonferroni-Holme method.

Results

Experiment 1. Effects of trigeminal versus facial nerve stimulation on DAO MEP in the SAI and LAI protocols.

SAI: Data indicated a clear difference between facial and trigeminal conditioning stimuli, with SAI being clearly detectable following trigeminal nerve stimulation but not facial nerve stimulation (Figure 3 and 4). The tSAI effect was specific for the 15 ms, 20 ms and 30 ms ISIs (Figure 3). ANOVA showed a significant main effect of NERVE ($F(1,15)=6.84$; $p=0.019$), ISIs ($F(4,15)=6.44$; $p=0.0002$) and a significant interaction NERVE*ISI ($F(4,15)=2.77$;$p=0.03$). Post-hoc analysis indicated a significant difference between trigeminal and facial stimulation at 15 ($p=0.014$), 20 ($p=0.014$) and 30 ms ($p=0.003$) ISIs. The one-sample t-tests indicated absolute inhibitory effects only for trigeminal nerve stimulation at 15 ($p=0.007$), 20 ($p=0.003$) and 30 ms ($p=0.005$) ISIs.

LAI: The results indicated that overall facial stimulation had a different effect comparing to trigeminal stimulation, at all ISIs. In particular, a clear LAI was detected only after facial nerve stimulation at 100 ms ISI (figure 3 and 4). ANOVA showed a main effect of NERVE ($F(1,15)=8.06$; $p=0.012$) but a non-significant effect of ISI and interaction among the factors (all $p>0.26$). The one-sample t-tests indicated absolute inhibitory effects for facial nerve stimulation at 100 ms ISI ($p=0.003$).

Experiment 2. After effects of trigeminal versus facial nerve stimulation on DAO MEP in the PAS protocol.

A clear PAS effect was detected after up to 30 minutes from facial nerve stimulation but not trigeminal stimulation. Statistical analysis showed a significant effect of NERVE ($F(1,13)=18.43$; $p=0.0009$) but a non-significant effect of ISI or interaction among the factors (all $p>0.52$). Compared to trigeminal nerve stimulation, facial stimulation showed a clear PAS effect at all intervals measured. Polarity analysis indicated absolute facilitatory effects, compared with baseline only for facial stimulation, at T10 ($p=0.002$) and T30 ($p=0.005$) time points after PAS (Figure 5).

Experiment 3. Effects of trigeminal versus facial nerve electrical stimulation on facial F-Wave

F-waves were recorded from the right DAO, following supramaximal stimulation (mean intensity: 24(3.9) mA) of the ipsilateral marginal branch of the facial nerve. Each TS evoked a stable CMAP at 1.45 (0.23) ms. Both facial and trigeminal nerve stimulations were not able to influence the mean F-wave persistence value at the tSAI and fLAI intervals. In particular, the mental nerve was stimulated at the SAI ISIs at a mean intensity of 3.8(0.6) mA and the facial nerve at the LAI ISIs at 4.4(1.4) mA. Mean F-wave latency was 14.7(1) ms. The mean F-waves persistence value (number of F-waves/number of stimuli), measured at baseline was 0.56(0.12) and 0.59(0.1) ($p>0.05$) in the trigeminal and facial conditioning trials, respectively. One-way ANOVA with ISI as within-subjects factor, showed no significant effect of both the trigeminal and facial CS at SAI and LAI ISIs, respectively (Figure 6).

Experiment 4. Effects of accessory nerve stimulation on DAO MEP in SAI and LAI protocols.

Data showed that accessory nerve stimulation induced a clear inhibitory effect at 100 ms ISI, likewise facial nerve stimulation in experiment 1. Although polarity analysis did not show a clear inhibition following accessory nerve stimulation at short intervals, an effect similar to tSAI was observed at 15 ms ISI (Figure 7).

SAI: ANOVA showed a no significant main effect of NERVE ($F(2, 20)=2.93$, $p=0.077$) but a significant effect of ISI ($F(4, 40)=6.54$; $p=0.0004$) and interaction NERVE*ISI ($F(8, 80)=2.12$, $p=0.044$). Post-hoc analysis showed that at 15 ms ISI the effects induced by accessory nerve stimulation were significantly different from those of trigeminal nerve stimulation ($p=0.04$) but not from those induced by facial nerve stimulation ($p=1.00$). On the contrary, at 20 ms ISI, the effects of accessory nerve stimulation on DAO MEP were significantly different from those induced by facial nerve stimulation ($p=0.02$) but not from those induced by trigeminal nerve stimulation ($p=0.56$). No significant difference between effects of accessory and trigeminal or facial nerve stimulation was found at any other ISIs.

LAI: The effects of accessory nerve stimulation resulted non different from those induced by facial nerve stimulation; a difference was instead detected when compared with the effects of trigeminal nerve stimulation. ANOVA showed a main effect of NERVE ($F(2, 20)=3.47$,

p=0.05) and ISI (F(3, 30)=4.99, p=0.006) but no significant interaction among the factors (p=0.35). Post-hoc analysis using Tukey's HSD to investigate the main effect of NERVE, indicated that the trigeminal stimulation was significantly different form the facial stimulation (p=0.02).

Polarity analysis showed that accessory nerve stimulation was ineffective at SAI ISIs, but induced a clear inhibitory effect at 100 ms ISI (p=0.002) in the LAI protocol.

Experiment 5. Effects of trigeminal and facial nerve stimulation on FDI MEP in SAI and LAI protocols

Results showed that trigeminal and facial nerve stimulations at short- and long intervals, respectively, did not induce any SAI and LAI effects in the FDI muscle (Figure 8).

SAI: ANOVA showed a significant main effect of ISI (F(4,60)=3.78; p=0.008) and an interaction MUSCLE*ISIs (F(4,60)=3.52; p=0.012). Post-hoc analysis detected a different effect exerted by trigeminal stimulation on the FDI MEPs compared to DAO MEPs at 15 ms (p=0.022) and 20 ms (p=0.009) ISIs.

LAI: ANOVA did not show a significant effect of MUSCLE, ISI or interaction among the factors (all p values >0.17).

No absolute inhibitory effect for both tSAI and fLAI on FDI MEPs were found (all p's < 0.05).

Discussion

The main finding of the present study is that SAI could be evoked by stimulation of low-threshold afferents in the trigeminal nerve but was absent after stimulation of distal branches of the facial nerve. In contrast, LAI and PAS required stimulation of facial nerve (see also Pilurzi et al., 2013), but were absent after trigeminal stimulation.

Previously Pilurzi et al. (2013) had argued that stimulation of the facial nerve (a pure motor nerve) generated afferent activity by evoking muscle twitches that were detected by

mechanoreceptors in the overlying skin (Edin & Johansson, 1995) and/or by activation of Ruffini-like receptors that have recently been described in human facial muscles (Cobo et al., 2017a,b). The afferent activity would be conducted by fibres in the trigeminal nerve for the former, and in Ruffini-like afferent fibres in the facial nerve that cross into the trigeminal nerve at the many distal anastomoses that have been described (Cattaneo & Pavesi, 2014; Hwang et al., 2007). This was sufficient to produce LAI and PAS. It was unclear why SAI was absent, but one possibility was that the natural pattern of sensory activity produced by the muscle twitch was insufficient to generate SAI, which might require a more synchronized volley, perhaps in a greater number of fibres.

The present experiments tested this directly by examining the effect of electrical stimulation of the trigeminal nerve. As we had anticipated, this produced SAI, but unexpectedly there was no evidence for LAI or PAS. One possible explanation is that fM1 requires a temporally dispersed afferent input, as generated by a muscle twitch, to evoke LAI and PAS. However, this would be quite unlike the hand where both LAI and PAS can be produced easily by a single electrically elicited afferent volley. Nevertheless it might be useful in future experiments to test this "natural stimulation" hypothesis in more detail. For example, stimulation of pure cutaneous receptors with stimuli such as light brush or skin stretch (Edin et al., 1995; Ito & Ostry, 2010), which are likely to produce a more dispersed afferent volley from slow-adapting receptors, might also produce trigeminal LAI and even PAS.

An alternative explanation is that the afferents responsible for SAI after stimulation of the trigeminal nerve have a low electrical threshold and are readily activated by the peripheral nerve stimulus. In contrast, the mechanosensitive receptors in skin and (possibly) muscle, that are activated by a facial muscle twitch, may have a higher electrical threshold and therefore are not activated by the electrical stimulation. Thus, stimulation of the trigeminal nerve at 3xPT is sufficient to evoke SAI but not PAS. Our hypothesis is that in fM1, SAI may depend on low threshold cutaneous input, whereas LAI and PAS depend on (higher threshold) mechanosensitive receptors activated by muscle contraction.

Interestingly, there is no evidence that activation of muscle afferents is necessary to produce SAI in the hand (Tokimura et al., 2000). All we know is that stimulation of cutaneous fibres, whether in digital nerves or in mixed nerves, can produce SAI, and that the degree of SAI

depends on the amplitude of the nerve afferent volley that is evoked (Turco et al., 2017). Thus, it is possible that SAI in both the face and hand muscles is primarily due to activity in cutaneous afferents.

The situation for LAI and PAS in the hand is less clear. Like SAI, LAI and PAS can be produced by both cutaneous and mixed nerve stimulation (Turco et al., 2017), and at least for LAI, there is no clear evidence in the hand that muscle afferent input plays a dominant role. However, for PAS, digital nerve stimulation leads to smaller effects compared with those obtained with mixed nerve stimulation at an intensity sufficient to generate a muscle twitch (Stefan et al., 2000; Wolters et al., 2003; Kujirai et al., 2006; Quartarone et al., 2006). Thus as in the face, muscle afferents may play a more important role in producing PAS in the hand than pure cutaneous fibres. Indeed, in the hand, PAS evoked with an anterior-posterior TMS pulse cannot be obtained with pure cutaneous stimulation (Kujirai et al., 2006).

Differences in the responsible afferent input would complement other known differences in the mechanism of SAI and LAI (Chen et al., 1999; Sailer et al., 2002, 2003; Paulus et al., 2008; Bailey et al., 2016, Turco et al., 2017). SAI involves GABAa and cholinergic systems (Di Lazzaro et al., 2000, 2007; Paulus et al., 2008), while GABAb pathways may mediate LAI (Sailer et al., 2002, 2003; Paulus et al., 2008). Furthermore, PAS does not alter the expression of SAI but may decrease LAI (Russmann et al., 2009; Meunier et al., 2012).

Cortical origin of tSAI and fLAI in facial muscles

It is well-known that stimulation of trigeminal afferents can produce a silent period in the ongoing EMG activity of voluntarily activated facial muscles. Indeed, a silent period has been described in DAO with an early ipsilateral silence around 15 ms followed by a later bilateral silence around 40 ms after mental nerve stimulation (Pavesi et al., 2000, 2003; Cattaneo et al., 2007; Cattaneo & Pavesi, 2010). Although silent periods are usually evoked with stronger stimulus intensities (about 7xPT), they have also been observed using lower stimulus intensities equivalent to those used in the present experiments.

The effect in resting muscles is unknown, but if a period of inhibition still persisted, it could well contribute to the SAI and LAI described here. However, we found no change in the

persistence of F-waves in DAO during SAI and LAI suggesting that any cutaneous silent period does not affect the excitability of facial motoneurones. Thus inhibition of monosynaptic corticobulbar input during SAI and LAI must be of cortical origin. But facial MEPs could also receive di-or tri-synaptic corticobulbar inputs that also contribute to the amplitude of the evoked MEP. If so there remains the possibility that some part of SAI and LAI have a subcortical contribution.

SAI and LAI produced by stimulation of the accessory nerve

Given that the accessory nerve is usually viewed as a purely motor nerve without sensory fibres, why was it possible to evoke the same amount of SAI as trigeminal stimulation and the same amount of LAI as evoked by facial nerve stimulation? One possibility is that as with facial nerve stimulation, the effect is due to activation of afferents excited by the evoked muscle contraction (in this case, the trapezius muscle). This could account for LAI in the same way as facial nerve stimulation, but is probably not the explanation for SAI since this began only 20 ms after stimulation of the accessory nerve, which is shorter than expected from the combined time taken for efferent conduction to the muscle, excitation-contraction coupling and afferent conduction back to the cortex. One possibility is that the accessory nerve carries some sensory fibres. In particular, visible ganglia or clustered cells have been detected in the accessory nucleus, mainly at C1 spinal level (Boehm & Kondrashov, 2016).

In hand muscles, SAI and LAI are usually stronger when elicited by stimulation of nerves containing afferents from the target muscle or from nearby skin (Classen et al., 2000; Tamburin et al., 2001; Helmich et al., 2005). However, this was not the case in DAO, where the accessory nerve stimulation evoked clear SAI and LAI. The likely explanation is that we used a higher intensity to stimulate the accessory nerve than the facial or trigeminal nerves. In the hand, spatial selectivity is much reduced at higher stimulus intensities (Tamburin et al., 2001; Helmich et al., 2005).

Cranio-facial topographic specificity of tSAI and fLAI

While heterotopic stimulation of the accessory nerve did not reveal a clear topographic effect in the SAI and LAI paradigms, the absence of any effect on the FDI exerted by the activation of trigeminal and facial nerves suggests that some degree of "cranio-facial" selectivity exists. Although a trigeminal-induced MEP inhibition in the relaxed FDI has been previously described (Siebner et al., 1999), it required longer ISIs (30-60 ms versus 10-30 ms) and higher stimulation intensities (10xPT versus 3xPT) than those used in our experiments.

Possible circuits involved in sensorimotor integration and paired associative stimulation protocols

It can be hypothesized that low threshold cutaneous trigeminal inputs activate oligosynaptic circuits which might primarily involve inhibitory connections between areas 3b and 1 of the contralateral primary somatosensory cortex (SI), (Allison et al., 1991; Forss et al., 1994) and layers 5/6 of M1 (Kaneko et al., 1994a, 1994b; Porter, 1996; Classen et al., 2000; Tokimura et al., 2000; Aronoff et al., 2010; Mao et al., 2011; Cash et al., 2015) and that by these connections they mediate the SAI (Porter, 1996; Cash et al., 2015; Kojima et al., 2015; Tsang et al., 2014, 2015; Bailey et al., 2016). Proprioceptive facial inputs activate inhibitory circuits involving areas 3a and 2 of contralateral SI (Friedman & Jones, 1981; Allison et al., 1991) and bilaterally the secondary somatosensory cortex (SII) and the posterior parietal cortex (PPC) (Allison et al., 1991; Forss et al., 1994; Karhu & Tesche, 1999; Chen et al. 1999; Boakye et al., 2000; Sailer et al. 2002), at LAI intervals (Chen et al. 1999; Classen et al., 2000). In line with the idea that LAI and PAS share their underpinning circuits (Russmann et al., 2009; Meunier et al., 2012), it seems reasonable to suppose that the same LAI-inducing proprioceptive input, at short intervals might engage excitatory interneurons in SI and M1 (layers 2/3) mediating PAS-induced LTP-like plasticity (Kaneko et al., 1994b; Cash et al., 2015), but also SII and PPC. The crucial role of SII for sensory processing and sensorimotor integration in face M1 has been confirmed recently by a fMRI study where the Bell's palsy condition induced significant changes in connectivity in SII (Klingner et al., 2014). Fig. 9 attempts to illustrate the possible pathways underlying sensorimotor integration processes and PAS-induced LTP-like plasticity in face M1.

Conclusions

The present findings provide evidence that low threshold cutaneous and muscle twitch-sensitive afferents may play different roles in sensorimotor integration and plasticity of face M1. Cutaneous inputs seem to have a paucisynaptic inhibitory access to face M1. Proprioceptive information is likely to target a more complex higher order network to generate LAI and PAS via excitatory and inhibitory polysynaptic circuits.

Author contributions

The experiments were performed at the laboratories of neurophysiology of the Department of Biomedical Sciences, University of Sassari, Sassari (Italy).

Conception and design of the experiments: G.P., J.C.R and F.D.; acquisition, analysis and interpretation of data: G.P., F.G., B.M., L.C., G.P., J.C.R and F.D. drafting the article or revising it critically for important intellectual content: G.P., L.C., G.P., J.C.R and F.D. All authors approved the final version for publication, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing interests.

The authors do not have any competing interest in and did not receive any funding for this research.

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Figure legends

Figure 1. Position of the electrodes for EMG recordings from the depressor anguli oris (DAO) and trapezius muscles and of electrodes for the electrical stimulation of the facial, trigeminal and accessory nerves.

Electrical stimulation electrodes are shown as white circles while EMG recording electrodes as black circles.

EMG was recorded from the DAO following stimulation of trigeminal and facial nerves. 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 (+). (A) For the electrical stimulation of the trigeminal nerve, the cathode wass positioned on the chin border (+) and the anode electrode on the right mental foramen (-). (B) For the electrical stimulation of the facial nerve, electrodes were placed over the marginal branch of the right facial nerve with cathode distal (+) and anode proximal (-), nearly 2 cm far from the mandibular angle. The correct position was carefully searched for each subject moving 1 cm up and down over the mandible border in order to have a stable compound muscle action potential in the DAO muscle with the lowest intensity, but not conduction volume effect in the masseter muscle.

(C) For the accessory nerve stimulation, EMG fromf the upper trapezius was recorded. The electrical stimulation electrodes were placed in the cervical triangle, 1-2 cm posteriorly to the lateral border of the sternocleidomastoid muscle and anteriorly to the trapezius muscle with cathode distal (+) and anode proximal (-).

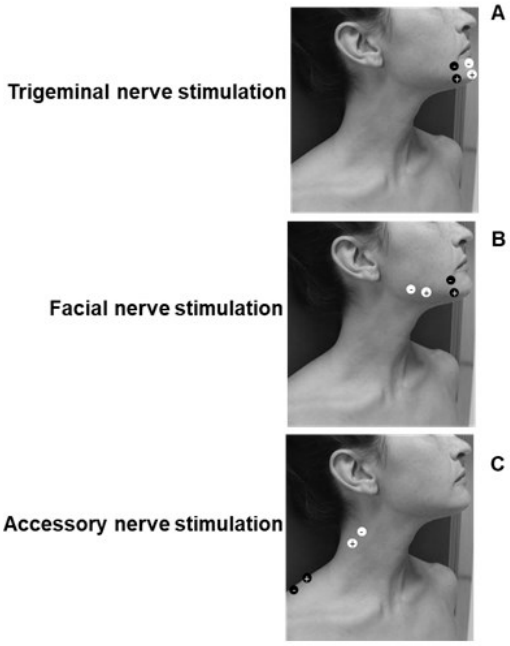


Figure 2. Effects of trigeminal and facial nerve stimulation on depressor anguli oris muscle (DAO) and in the masseter muscle (MM).

EMG recordings from the DAO and MM muscles of a representative subject are reported for each stimulation condition. The electrical stimuli (duration 0.2 ms, intensity 3xT, frequency 0.25 Hz) were applied over the right facial and trigeminal nerves.

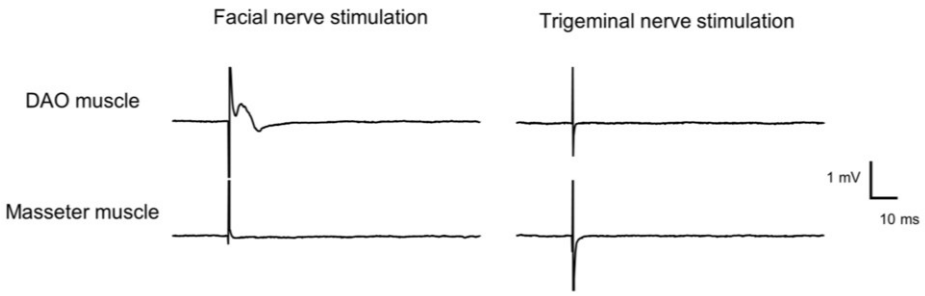


Figure 3. Effects of trigeminal and facial nerve stimulation on motor evoked potentials (MEP) of the depressor anguli oris muscle (DAO) in the short afferent inhibition (SAI) and long afferent inhibition (LAI) paradigms.

A – In the SAI protocol (10-30 ms interstimulus intervals, ISI), the amplitude of DAO MEPs was significantly reduced by trigeminal stimulation (tSAI, black line) at 15, 20 and 30 ms ISIs while it appeared unaffected by facial nerve stimulation (fSAI, grey line).

B- In the LAI protocol (100-200 ms ISI), DAO MEPs showed a significant inhibition at each ISI tested after facial nerve stimulation (fLAI, while trigeminal stimulation was ineffective at any ISI tested.

Ordinates report MEP amplitude expressed as a ratio of the unconditioned MEP. * $p<0.05$. The graphs report the group means (N = 16 subjects) ± standard deviation.

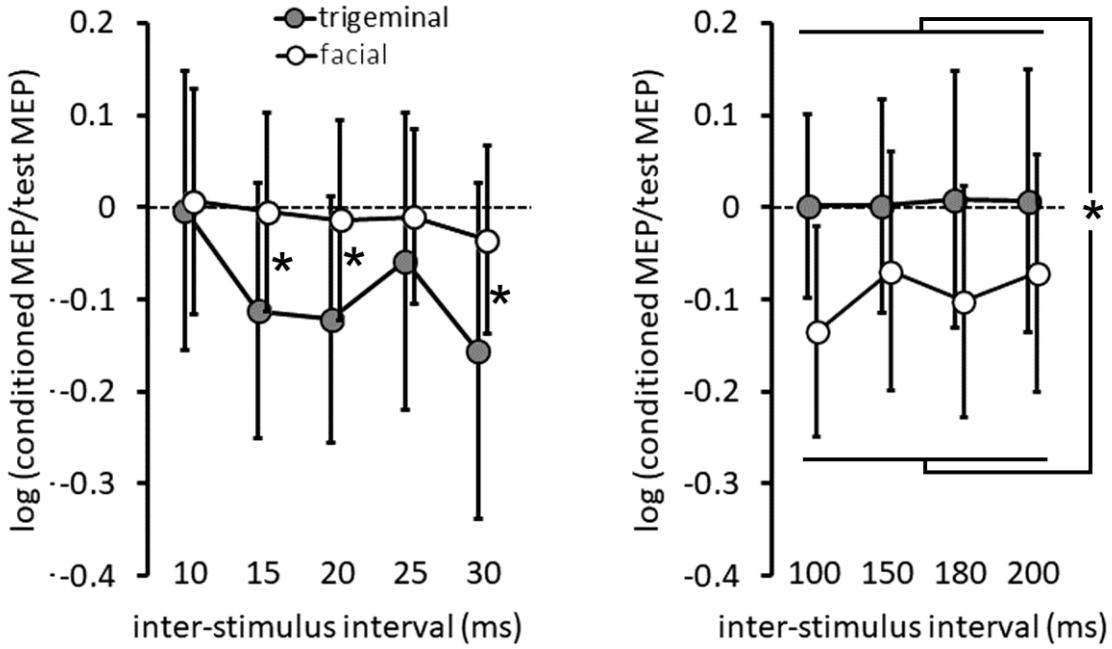


Figure 4. Effects of trigeminal and facial nerve stimulation on motor evoked potentials (MEP) of the depressor anguli oris muscle (DAO) with a paired stimulation in short afferent inhibition (SAI) and long afferent inhibition (LAI) paradigms.

Recordings of MEPs from the DAO of a representative subject are reported for each condition (unconditioned MEP, induced by the test stimulus (TS), and conditioned MEPs at interstimulus intervals (ISIs) of 20 and 100 ms. Conditioning stimulus was applied over the right facial and trigeminal nerves.

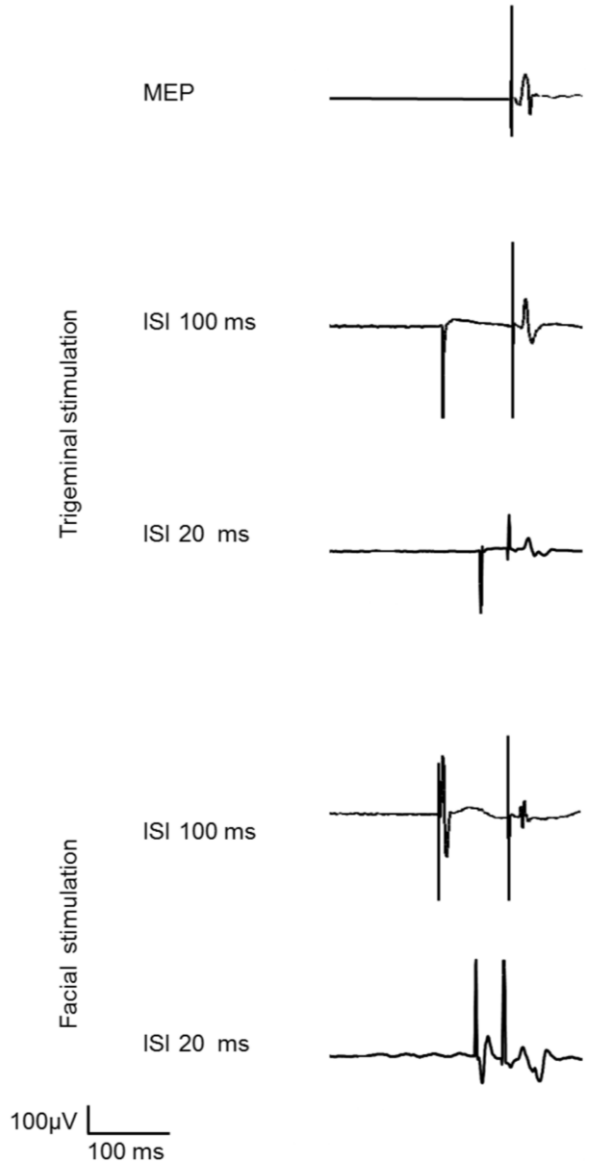


Figure 5. Effects of facial and trigeminal paired associative stimulation (fPAS and tPAS, respectively) on the magnitude of motor evoked potentials (MEP) recorded from the depressor anguli oris muscle (DAO).

The graphs show the time course of effects on DAO MEP amplitudes after 0 (T0), 10 (T10), 20 (T20), 30 (T30) minutes from fPAS (white boxes) and tPAS (grey boxes) interventions. Compared with each other, MEP ratio after fPAS and tPAS were significantly different at all time points, being significantly increased following the fPAS intervention. *p<0.05. The graphs report the group means (N = 15 subjects) ± standard deviation.

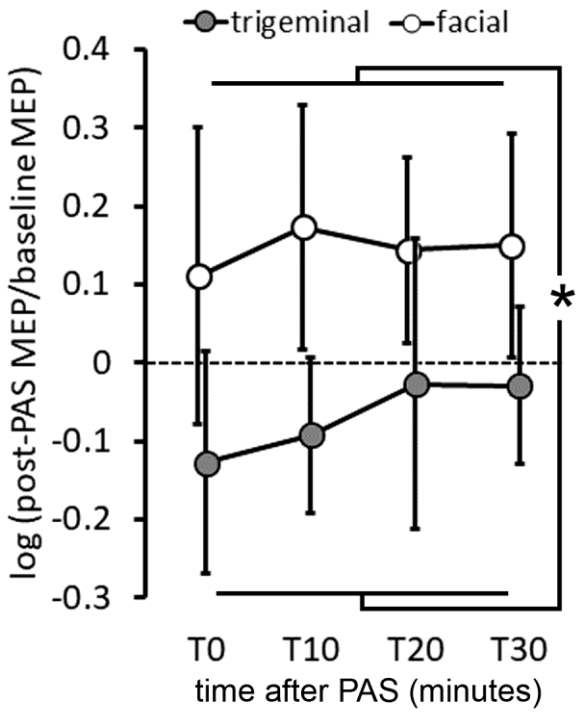


Figure 6. F-waves in the depressor anguli oris muscle (DAO) after stimulation of the trigeminal and facial nerves at SAI and LAI intervals, respectively.

The graphs report the F wave persistence expressed as percentage number of trials eliciting an F-wave following 20 facial nerve stimuli. We report data from unconditioned stimuli (baseline) and stimuli preceded by trigeminal stimulation at SAI intervals (A- left panel) and facial stimulation at LAI intervals (B- right panel). F-waves persistence was not altered by either of the two conditioning stimuli, at any ISI tested. The graphs report the group means (N = 8 subjects) ± standard deviation. The dashed line indicates the mean baseline value.3

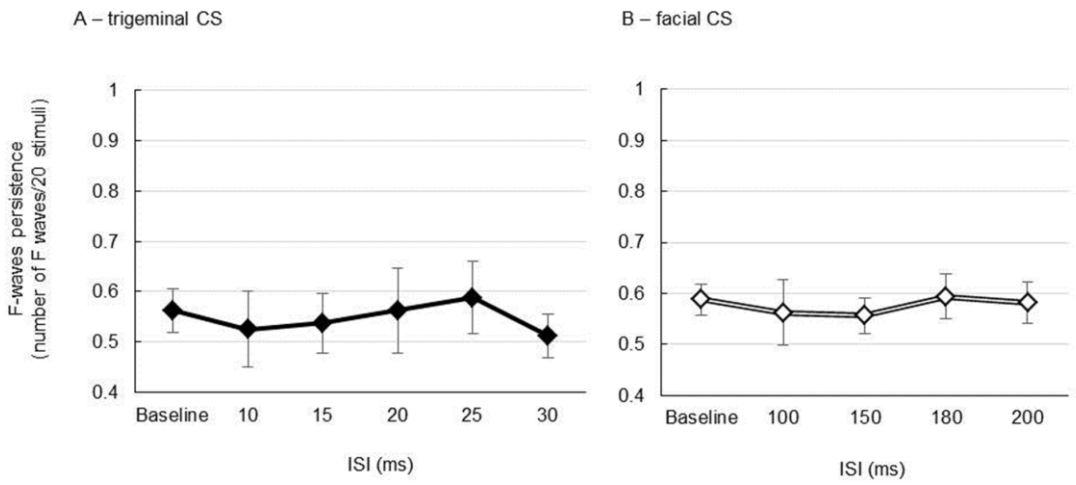


Figure 7. Effects of homotopic and heterotopic nerve stimulation on motor evoked potentials (MEP) of the depressor anguli oris muscle (DAO).

A- In the short afferent inhibition (SAI) protocol, the amplitude of DAO MEPs was significantly reduced at 20 ms interstimulus interval (ISI) by stimulation of both homotopic trigeminal (tSAI, grey boxes) and heterotopic accessory (aSAI, black boxes) nerve stimulation

B- In the long afferent inhibition (LAI) protocol DAO MEPs were significantly inhibited by both homotopic facial (fLAI, white boxes) and heterotopic accessory (aLAI, black boxes) nerve stimulations.

Ordinates report MEP amplitude expressed as a ratio of the unconditioned MEP. *p<0.05. The graphs report the group means (N = 11 subjects) ± standard deviation.

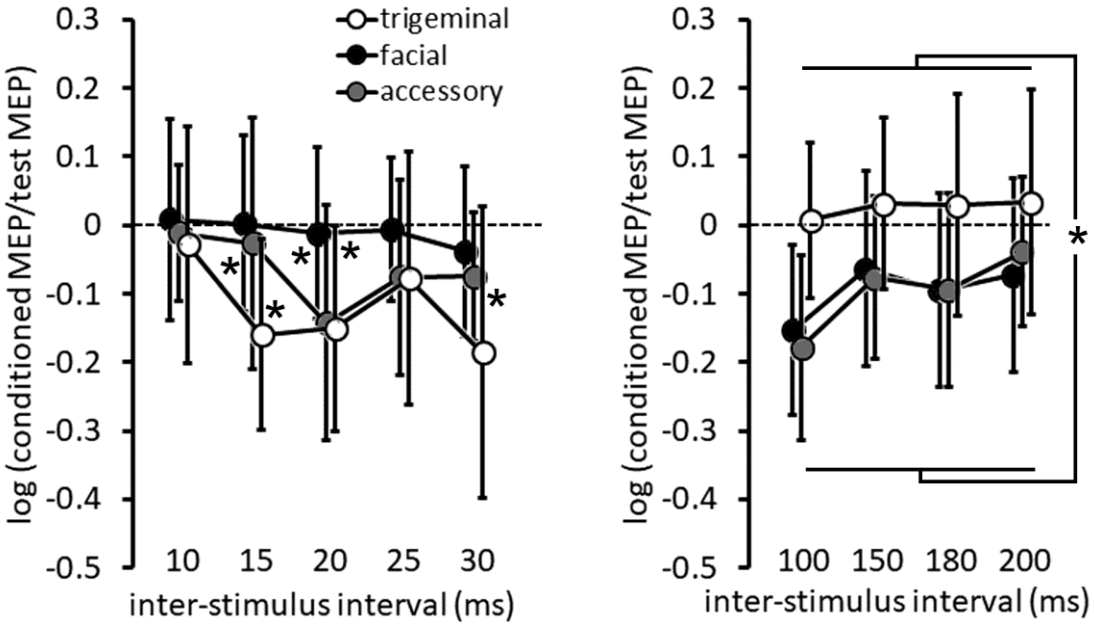


Figure 8. Muscular somatotopy of trigeminal short afferent inhibition (tSAI) and of facial long afferent inhibition (fLAI) in the cortical representation of the depressor anguli oris muscle (DAO) and first dorsal interosseous muscle (FDI)

A- Effects of trigeminal nerve stimulation on motor evoked potentials (MEP) recorded from the DAO (white boxes) and from the FDI (grey boxes) at SAI inter-stimulus intervals (ISI). The DAO exhibited a significant SAI at 15 and 20 ms ISIs, while the FDI was unaffected at any ISI tested.

B- Effects of facial nerve stimulation on DAO and FDI MEPs in the LAI protocol. The box plot shows no significant difference between the two muscles.

Ordinates report MEP amplitude expressed as a ratio of the unconditioned MEP. *p<0.05. The graphs report the group means (N = 16 subjects) ± standard deviation.

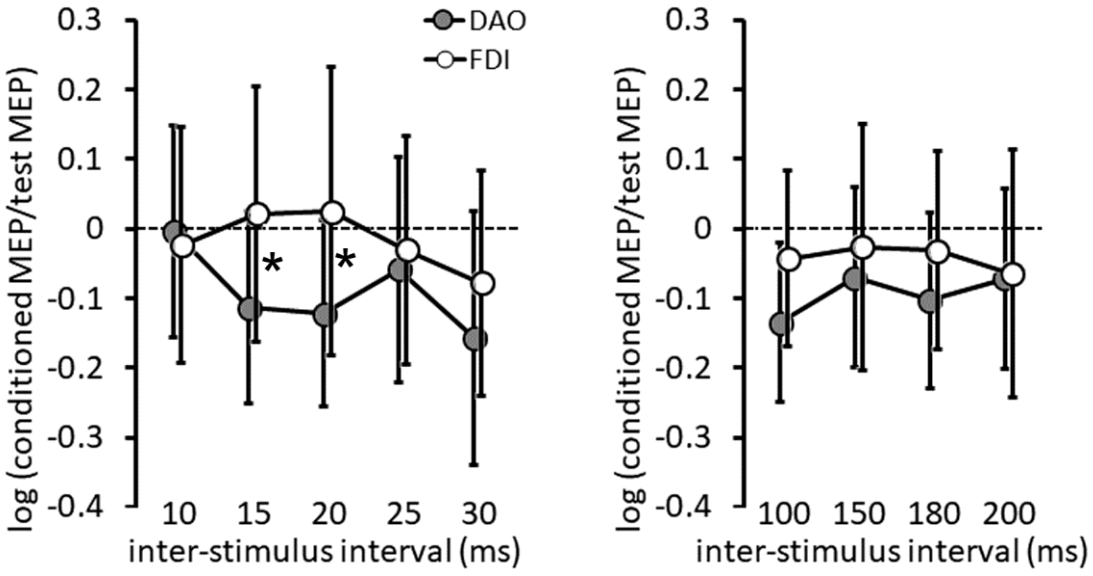
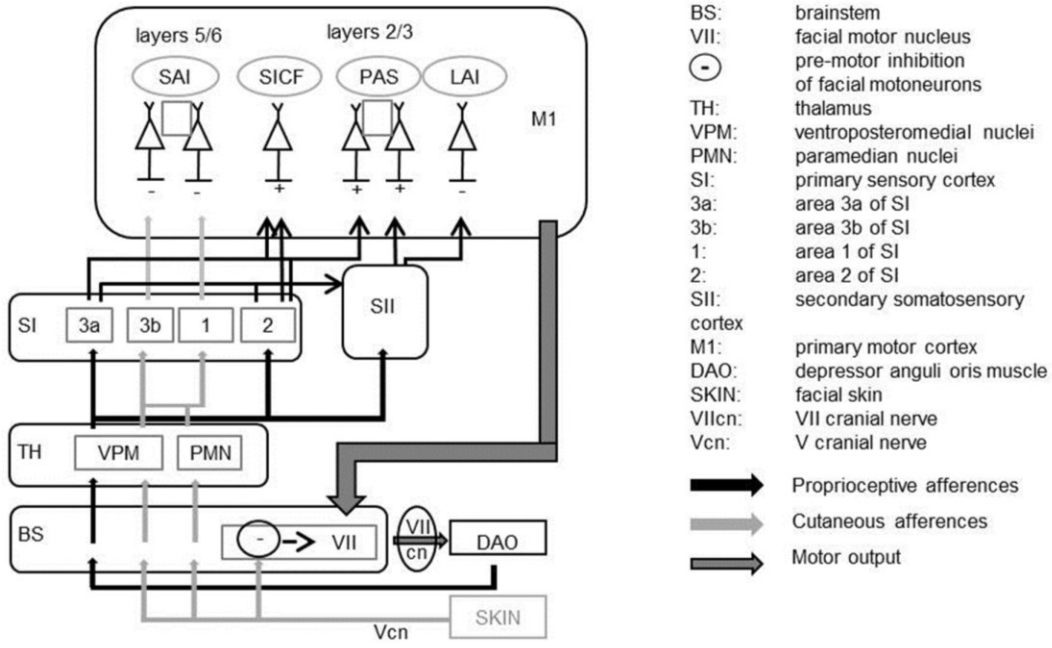


Figure 9. Schematic model of circuits in the facial motor system engaged by SAI, LAI and PAS paradigms. Cutaneous inputs from the facial skin, carried by the Vth cranial nerve (Vcn) join areas 3b and 1 of the primary somatosensory cortex (SI), via the ventral postero-medial nucleus (VPM) of the thalamus (TH). From SI-3b and SI-1, oligosynaptic pathways project to layers 5/6 of the facial primary motor cortex (M1) exerting a short afferent inhibition (SAI) on pyramidal cells innervating the facial motor nucleus (VII) in the brainstem (BS). The same inputs, may produce a SAI phenomenon in the depressor angulis oris muscle (DAO), via sensory-motor integration processes occurring at brainstem (BS) level or mediated by the paramedian nuclei (PMN) of the TH. Single pulse stimulation of the VIIth cranial nerve (VIIcn) excites proprioceptive afferents that project to neurons in the SI areas 3a and 2. These neurons modulate the activity of cortical interneurons in layers 2/3 of M1 producing a short-latency cortical facilitation (SICF) and also send connections to the secondary somatosensory cortex (SII). From SI-3a, SI-2 and SII polysynaptic projections to layers 5/6 of M1 produce a long afferent inhibition (LAI) on the DAO. Paired associative stimulation (PAS) of M1 and of the VIIcn acts via polysynaptic excitatory circuits on both M1 layers 2/3 and SII inducing a long-term potentiation (LTP)-like plasticity in M1.



Dr Giovanna Pilurzi (on the left) obtained her PhD in Neurophysiology at the University of Sassari. She did her residency fellowship in Neurology at the University of Sassari. She works as Neurologist at Fidenza Hospital, where she is mainly involved in the clinical neurophysiology lab. Her research activity was focused on the study of corticobulbar motor control and plasticity in healthy humans and cranial dystonia.

Dr Francesca Ginatempo (on the right) obtained her PhD in Neuroscience at the University of Sassari. She is now a post-Doc at University of Sassari. Since the beginning of her research activity she has investigated the voluntary and emotional control of facial muscles both in physiological conditions as well as in neurological patients.