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Sites and Mechanisms of Trigeminal Nerve Stimulation: a Human and Animal Study

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Summary

Trigeminal nerve stimulation (TNS) has proven efficacious in the treatment of several neurological disorders, but sites and mechanisms of action are still unknown. TNS effects were investigated on: intracortical circuits and sensorimotor integration at cortical level (Study 1), brainstem excitability and plasticity (Study 2), in healthy subjects; hippocampal neurogenesis (Study 3), in rats.

TNS consisted of 20min bilateral stimulation of the infraorbital nerve. Study 1: Short- and long-interval intracortical inhibition, intracortical facilitation, short- and long-afferent inhibition were assessed using transcranial magnetic stimulation in 17 volunteers before and after TNS.

Study 2: The R1 and R2 areas of the blink reflex (BR) were measured before and after 0, 15, 30, 45min from TNS delivery.

Study 3: Hippocampal neurogenesis was evaluated in 18 male Sprague-Dawley rats after 24h from TNS, through immunohistochemical labeling of newly formed brain cells.

Results. Study 1: cortical excitability and sensorimotor integration were unaltered by TNS.

Study 2: The R2 area of the BR was significantly reduced after TNS at all time points tested. By contrast, R1 area was unaffected.

Study 3: The number of newly formed cells in the dentate gyrus was significantly increased after TNS.

These data suggest that TNS mainly acts on brainstem polysynaptic circuits with a minor role in modifying the activity of higher-level structures. Acute TNS induces a long-lasting inhibition of the R2 component of the BR, which resembles a long-term depression-like effects. In the rat TNS promotes new cell proliferation in the hippocampus, which supports the notion of an involvement of hippocampal plasticity in the TNS effects described in several neurological conditions.

Introduction

The use of electrical and/or magnetic stimulation techniques in order to manipulate the activity of the central nervous system (CNS) has a long history.

Different brain stimulation techniques, both invasive and noninvasive, are currently used in neurology and psychiatry. Some common examples of therapeutic application of neurostimulation include:

- deep brain stimulation (DBS) which has now replaced the old methods of ablation used to treat movement disorders and today applied to treatments of Parkinson's disease, dystonia, epilepsy and psychiatric disorders such as some forms of depression, obsessive-compulsive disorder and Tourette's syndrome (Tekriwal and Baltuch, 2015);
- transcranial direct current stimulation (tDCS) used for neuropsychiatric disorders such as depression (Shiozawa et al 2014), motor function and cognitive disorders (Andrews et al., 2011; Elsner et al, 2013);
- sacral nerve stimulation (SNS) for the treatment of imbalances in the pelvic region and incontinence (Bemelmans et al., 1999; Brazzelli et al., 2006);
- repetitive transcranial magnetic stimulation (rTMS) for the treatment of various psychiatric cognitive disorders (Poleszczyk, 2015)
- glossopharyngeal nerve stimulation (GNS) for the treatment of epilepsy (Tubbs et al., 2002).
- vagus nerve stimulation (VNS), for the treatment of some forms of epilepsy (Connor et al., 2012), obesity (Bodenlos et al., 2014) and depression (Beekwilder and Beems, 2010);
- trigeminal nerve stimulation (TNS) as an alternative option to VNS the treatment of drug-resistant epilepsy (DeGiorgio et al., 2003), depression (Shiozawa, 2014) and migraine (Riederer et al., 2015) .

These techniques probably act via different mechanisms; some of these work by directly stimulating the brain, others indirectly through stimulation of peripheral nerves. In particular, the first group of techniques (DBS, tDCS, rTMS) are supposed to act with a top-down mechanism, that modulates brain activity directly through subcortical excitability changes in the activity of primary cortical

network. On the other hand, stimulation of peripheral nerves (VNS, TNS, GNS) may affect brain activity through a bottom-up mechanisms that is, by stimulating cranial nerves nuclei in the brainstem, which, in turn, make extensive connections to higher CNS structures (Shiozawa et al., 2014)

From a medical viewpoint neurostimulation techniques may provide several advantages with respect to conventional drug treatment:

- specificity: stimulation can be targeted to particular areas avoiding the insurgence of systemic side-effects, typical of traditional drug therapies;
- safety: neurostimulation techniques are generally well-tolerated and almost devoid of dangerous side effects:
- flexibility: the treatment can be interrupted at any time.

With regard to the effects of cranial nerve stimulation, the first observation that VNS directly affected central function in cats is from Bailey and Bremer, 1938. This seminal work was confirmed by Dell and Olson in 1951 and primate studies provided evidence of VNS effects on basal limbic structures, thalamus, and cingulate cortex (MacLean, 1990).

Based on these findings it was hypothesized that VNS would have anticonvulsant properties (Zabara, 1985a, Zabara, 1985b), with an impact on both direct termination of an ongoing seizure as well as seizure prevention (Zabara, 1992). Following this basic work, VNS was further developed as an adjunct treatment for seizure disorders, leading to approval by the Food and Drug Administration (FDA) for the treatment of pharmaco-resistant epilepsy in 1997.

VNS-induced mood elevation was serendipitously observed in epilepsy patients and prompted researchers to also examine possible effects of VNS on emotional health (Elger et al., 2000; Goodnick et al., 2001; Gaynes et al., 2011). Several clinical trials were conducted to evaluate the efficacy of VNS in depressed patients resistant to standard antidepressant treatments. The prospective investigation of VNS effects in depressed patients resulted in the FDA approval of VNS as an adjunct therapy for the treatment of drug-resistant major depression in 2005.

The clinical use of VNS has been also shown to suffer from several limitations (Ben-Menachem et al., 2015). Some of them are related to the surgical implantation of the stimulating device (hoarseness, cough, vocal cord paralysis, infections), but the most important limitation to the clinical use of VNS is the presence of a visceral component in the vagus nerve and in particular its role in cardiac function control (Schuurman and Beukers, 2009). Therefore in order to avoid a possible depressive effect on cardiac performance, VNS cannot be applied bilaterally nor at high stimulation frequencies, with an overall decrease in its efficacy.

To overcome the limitations of VNS, during the last decade an increasing number of experimental and clinical studies have focused their attention on TNS, which has been consistently proved to exert beneficial effects in the symptomatic treatment of several neuropsychiatric disorders (De Giorgio et al. 2003, 2009, 2011, 2013; Schoenen et al., 2013; Shiozawa et al., 2014; Cook et al., 2015). In particular, Fanselow et al. (2000) first demonstrated in the rat, that electrical stimulation of the infraorbital branch of the trigeminal nerve (ION) reduces both frequency and duration of pentylenetetrazole-induced seizures. In the same study it was also shown, using field potential recording at the thalamic and cortical level, that TNS administration is able to stop the synchronized burst firing at its initial moment, with a general desynchronizing effect.

Based on these data, DeGiorgio and Coll. (DeGiorgio et al., 2003, 2006, 2009, 2011) proposed for the first time the use of TNS in patients with drug-resistant epilepsy, as adjuvant or alternative to VNS. More recently TNS has been also proposed in the treatment of other neurological and psychiatric disorders such as depression, attention deficit hyperactivity disorder, post-traumatic stress disorder, Lennox Gastaut syndrome, traumatic brain injury, migraine, and tinnitus (Soleymani et al., 2011), for which clinical trials are underway.

1.1 Rationale and aim of the project.

Despite the increasing success and use of TNS for the symptomatological treatment of different neurological and psychiatric affections, the neurobiological mechanisms and levels of action of this treatments are yet to be understood.

It has been proposed that the trigeminal nerve (being the largest cranial nerve), can represent a privileged way to forward modulatory signals to the brain (Cook et al., 2014), with the added value that the absence of a visceral component guarantees against the cardiac side-effects observed with VNS.

So far, the majority of the evidences seem to support the idea of a bottom-up effect of TNS on CNS functions. Therefore, TNS effects on higher brain structures should be secondary to the excitation of the ascending reticular formation (RF), probably induced by *locus coeruleus* (LC) and raphe nuclei (RN) activation, on which trigeminal afferents project through the nucleus of the solitary tract (NST) (Magdaleno-Madrigal et al., 2002; Fanselow, 2012). In particular affiliation to the midbrain reticular formation that would cause desynchronization of cortical firing through generalized activation of the ascending reticular system (Fanselow et al., 2000).

However, another possible explanation is a top-down effect of TNS, based on the fact that through the trigeminal nerve tactile sensations reach the primary somatosensory cortex, via the ventro-posterior medial thalamic nuclei. Indeed a recent imaging study has shown that TNS activates the inferior frontal gyrus, the anterior cingulate and parietotemporal cortices; on the other hand it has also been observed inhibition in the left parahippocampal gyrus, sensorimotor, parietal top right, temporo-occipital and visual cortices (Schrader et al., 2012; Silverman et al., 2011).

These data show that a more thorough evaluation of the neurophysiological mechanisms of TNS at different brain levels is definitely needed. Therefore, the aim of the present study was to examine the effect of acute administration of TNS on the excitability of the motor cortex and brainstem of healthy subjects, in order to clarify the possible origin of the therapeutic effects observed in clinical trials. Further, following the reported positive effect of both

VNS and TNS on patient's mood and the known link between recovery from depression and increased hippocampal neurogenesis, a more in-deep study was performed to assess a possible positive effect of TNS on neural stem cells proliferation.

To this end, the intracortical excitatory and inhibitory circuits, as well as the processes of sensorimotor integration that occurs at the cortical level, were explored using a transcranial magnetic stimulation (TMS) approach (Kujirai et al., 1993; Ziemann et al., 1996; Classen et al., 2000; Tokimura et al., 2000).

Facilitation and inhibition of brainstem interneurons were also tested using the blink reflex (BR) and its recovery cycle (BRRC), a reflection trigeminal-facial with integration center at the level of the brainstem (Kimura, 1989; Berardelli et al., 1999; Cruccu et al., 2000; Cruccu et al., 2005). The effect of TNS administration on hippocampal neurogenesis was studied in the rat using an immunohistochemical technique in order to measure the number of newly formed cells in the dentate gyrus of the hippocampus.

**Study 1: Trigeminal nerve stimulation modulates
brainstem more than cortical excitability in
healthy humans.**

2.1 Introduction

VNS is the only neurostimulation method acknowledged for the treatment of drug-resistant epilepsy (DRE) and of major depression (Howland 2014).

However, a large body of evidence supports TNS as a potentially valid alternative to VNS in the treatment of DRE (DeGiorgio et al., 2003, 2006, 2009, 2013; Pop et al., 2011). In addition to DRE, migraine (Schoenen et al., 2013) and depression (Cook et al., 2013) have evidenced benefit from treatment with TNS.

Despite its proved clinical effectiveness, the sites of action in the CNS and the neurobiological mechanisms by which TNS exerts its therapeutic effects have been poorly investigated so far. Accumulating evidence suggest that, like VNS, TNS ultimately influences the pattern of neuronal activity, with the additional advantage that the V nerve may represent a privileged pathway for conveying neuromodulatory signals to the CNS (Cook et al., 2014). Evidence from experimentally induced epileptic animals show that TNS induces cortical and thalamic desynchronization (Fanselow et al., 2000; De Giorgio et al., 2011). This observation is in line with EEG desynchronization observed in epileptic patients following acute (Todesco S., personal communication) as well as chronic TNS (Moseley and De Giorgio 2014). Hence, it has been proposed that the antiepileptic effect of TNS may be due to cortical desynchronization arising from changes in cortical excitability (Fanselow, 2012). However, as yet, a direct effect of TNS on cortical excitability has not been investigated in epileptic patients. A recent study, using TMS, indicates that acute continuous TNS administration does not affect cortical excitability in healthy subjects (Axelson et al., 2014). These data warrant a further in-depth evaluation of the neurophysiological mechanisms of TNS at different brain levels. In fact, the trigeminal afferent system has multiple targets within the CNS, including brainstem and thalamic nuclei, and from these up to subcortical and cortical structures (Fanselow, 2012). Both the brainstem and the cerebral cortex are accessible to noninvasive neurophysiological investigations in physiological and pathological conditions. Brainstem function is commonly studied by recording the BR and its recovery cycle (BRRC), which are considered reliable tests of brainstem interneuron

excitability (Kimura et al., 1969; Kimura, 1983; Berardelli et al., 1999; Cruccu and Deuschl, 2000). Investigations of cortical circuits are extensively performed using paired-pulse TMS (Valls-Solé et al., 1992; Kujirai et al., 1993; Wassermann et al., 1996; Ziemann et al., 1998). TMS protocols allow the exploration of inhibitory and facilitatory intracortical interneurons underlying the short- and long-interval intracortical inhibition (SICI and LICI, respectively), intracortical facilitation (ICF) and short-interval intracortical facilitation (SICF) phenomena (Valls-Solé et al., 1992; Kujirai et al., 1993; Wassermann et al., 1996; Ziemann et al., 1996; Chen et al., 1998). TMS protocols are also available to investigate sensorimotor integration processes occurring at cortical level, such as short- and long- afferent inhibition (SAI and LAI, respectively) (Tokimura et al., 2000; Classen et al., 2000).

The present study proposes to investigate the after effects of acute TNS administration on brainstem and intracortical excitability as well as on cortical sensorimotor integration, by assessing, before and after TNS, the: (i) BR and BRRC; (ii) SICI, LICI, ICF and SICF; (iii) SAI and LAI.

2.2 Methods and materials

2.2.1 Subjects

Seventeen healthy volunteers (9 females and 8 males; 30.0 ± 4.4 years old; range 24–40 years) participated in the study. All the subjects, but one, were right handed. Prior to the study subjects gave their informed written consent and the procedure, approved by the local ethical committee (Bioethics Committee of ASL n.1 Sassari, ID 982/2/L) was in accordance with the Helsinki Declaration. None of the participants had a history of neurological and/or psychiatric diseases, was on medication and presented contraindications to undergo TMS and/or surface electrical stimulation procedures.

2.2.2 EMG recordings

EMG signals were recorded (D360 amplifier; Digitimer Ltd, Welwyn Garden City, UK) using 9-mm-diameter Ag–AgCl surface cup electrodes, placed over the target muscle in a belly tendon montage. Trials with excessive EMG artifact were rejected online. Data were recorded and analyzed using Signal 5.02 software (Cambridge Electronic Design, UK).

In experiment 1, the first (R1) and the second (R2) components of the BR were recorded bilaterally from the orbicularis oculi muscle (OO), with the recording electrode placed over the lower lid, the reference electrode two cm far from the lateral cantus and the ground electrode over the forehead. EMG was amplified ($\times 5000$), filtered (bandpass 50–5000 Hz) and sampled (10 kHz per channel in a window frame of 4000 ms) using a CED1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK). The raw blink recordings were DC-corrected, rectified, and averaged for off-line measurements.

In experiment 2 and 3, motor evoked potentials (MEP) were recorded from the first dorsal interosseous muscle (FDI) of the dominant hand. The recording electrode was placed over the FDI, the reference electrode on the first metacarpophalangeal joint and the ground electrode on the volar surface of the forearm. EMG was amplified ($\times 1000$), filtered (bandpass 3–3000 Hz) and sampled (5 kHz per channel in a window frame length of 250 ms) using a CED1401 power analog-to-digital converter.

2.2.3 Electrical stimulations (ES)

To elicit the BR in experiment 1, ES of the left supraorbital nerve (SON) was delivered at the supraorbital notch, via cup electrodes (cathode over the homonymous foramen and anode two cm lateral) connected to a DS7A Stimulator (Digitimer, Welwyn Garden City, Herts, UK). All stimuli were square waves (0.2 ms duration), and stimulus intensity was set at three times the R2 threshold (lowest intensity that evoked at least five R2 responses in 10 consecutive trials).

ES were delivered to the SON at variable time intervals (between 20 and 40 s) to minimize habituation of the BR. To test sensorimotor integration in experiment 3, the median nerve ipsilateral to the recorded FDI was electrically stimulated at the

wrist through bipolar electrodes (cathode proximal) connected to a Digitimer DS7A constant current stimulator. ES consisted of single square-wave pulses of 0.2 ms width and 0.25 Hz frequency; intensity was set at nearly 2–3 times the perceptual threshold (PT), just above the motor threshold for evoking a visible twitch of the thenar muscles.

2.2.4 TMS

TMS of the motor cortex innervating the dominant hand was performed using a figure-of-eight coil (external loop diameter of 9 cm), with the coil handle pointing backwards and about 45° laterally.

Magnetic stimuli were generated via two Magstim 200 stimulators connected in a Bistim module (Magstim Co., Whitland, Dyfed, UK). The optimal stimulation site for eliciting MEPs in the contralateral FDI was marked on the scalp with a soft tip pen to ensure that the coil remained in the same place throughout the experiments. In all experiments, TMS frequency was 0.25 Hz.

The resting motor threshold (RMT) was taken as the lowest TMS intensity that elicited, in the relaxed FDI, MEPs of 50 μ V in at least 5 out of 10 consecutive trials (Rothwell et al., 1999). Motor threshold was expressed as a percentage of the maximum stimulator output (MSO). The test stimulus (TS) intensity was the intensity sufficient to evoke a motor response in relaxed FDI of 1 mV peak-to-peak amplitude (1 mV MEP).

2.2.5 TNS

TNS was delivered bilaterally to the infraorbital nerve (ION) through 26-mm-diameter disposable, hypoallergenic, silver-gel self-adhesive stimulating electrodes (Globus, Domino s.r.l., Codognè, TV, IT) placed over the ION foramina and connected to a Winner® stimulator (Fisioline biomedical instrumentation, Verduno, CN, IT). According to DeGiorgio's original protocol (DeGiorgio et al., 2003), the stimulus consisted of an asymmetric biphasic squarewave pulse with an electrical mean equal to zero, duration of 0.25 ms, frequency of 120 Hz, delivered in a cyclic modality where 30 s ON and 30 s OFF

were alternated. The total period of TNS was equal to 20 min, according to Schoenen et al. (2013). Stimulation intensities ranged from 1 to 20 mA and corresponded, for each ION, to the maximal pain sub-threshold intensity endurable comfortably by the subject.

2.2.6 Experimental design

BR (Experiment 1) and TMS (Experiment 2 and 3) protocols were performed in all subjects in two distinct experimental sessions. Experiments were carried out in a quiet room by the same operator and at a consistent time of the day. Subjects sat in a comfortable chair with the neck supported and were asked to keep their eyes open and to stay relaxed but alert during data collection.

2.2.7 Experiment 1: TNS effects on brainstem excitability

The early ipsilateral R1 response and the late ipsilateral (iR2) and contralateral (cR2) R2 responses induced by SON stimulation (Kimura, 1983) were assessed before and immediately after TNS. EMG recordings from OO muscles, started 2 s before each stimulus, to allow recognition of excessive background muscle activity and thus rejecting the trial online. R2 threshold, R1 and R2 areas were calculated before and after TNS.

The R2 recovery cycle was investigated using two electrical stimuli of equal intensity delivered to the SON at interstimulus intervals (ISIs) of 250, 500 and 1000 ms (10 trials for each ISI in a randomized order). The R2 inhibition was calculated as a ratio of conditioned/unconditioned R2 area, for each ISI.

2.2.8 Experiment 2: TNS effects on intracortical excitability

RMT, 1 mV MEP, SICI, ICF, SICF and LICI were measured before and immediately after TNS. SICI and ICF were assessed through the classical paired pulse paradigm described by Kujirai et al. (1993). Conditioning stimulus (CS) intensity was 80 % of RMT, while TS intensity was adjusted to elicit 1 mV MEP in the dominant FDI. SICF was tested through the paired-pulse protocol described

by Ziemann et al. (1998). The ISIs tested 1.5 ms for SICF, 3 ms for SICI and 10 ms for ICF were examined in a randomized order. Ten unconditioned MEPs and 10 conditioned MEPs for each ISI were recorded in this experimental block.

LICI was evaluated using the paired-pulse protocol consisting of suprathreshold CS and TS (Valls-Solé et al., 1992; Wassermann et al., 1996). The intensities were both adjusted to elicit 1 mV MEP and separated by 100 ms ISI. Twenty pulses were delivered in a randomized order (10 pulses for conditioned MEP at each ISI and 10 pulses for the test MEP alone).

The MEP peak-to-peak amplitude was measured for each trial and then averaged. Mean amplitude of the conditioned MEP was expressed as a ratio of the averaged test MEP.

2.2.9 Experiment 3: TNS effects on cortical sensorimotor integration

SAI and LAI were induced coupling the ES of the median nerve with TMS of the primary hand motor cortex (Chen et al., 1998; Classen et al., 2000; Tokimura et al., 2000). The 20 ms ISI for SAI and the 200 ms ISI for LAI were examined in a randomized order. Ten unconditioned MEPs and 10 conditioned MEPs for each ISI were recorded and averaged.

The MEP peak-to-peak amplitude was measured for each trial and averaged, before and immediately after TNS administration. Mean amplitude of the conditioned MEP was expressed as a ratio of the averaged test MEP.

2.3 Statistical analysis

Statistical analysis was performed with SPSS 18 software (SPSS Inc, Chicago, IL, USA). In the analysis performed with repeated measures analysis of variance (ANOVA), compound symmetry was evaluated testing the sphericity with the Mauchly's test. The Greenhouse-Geisser correction was used to compensate for non-spherical data. A p value <0.05 was considered significant. Unless otherwise stated, values are expressed as mean \pm standard deviation (SD) of the mean. In experiment 1 and 2, differences between R2 threshold, R1 area and RMT before and after TNS were assessed using Student's paired t test.

In experiment 1, repeated measures ANOVA with TNS (before, after) and SIDE (iR2, cR2) as within-subject factors was performed to evaluate different effects of TNS on the ipsilateral and contralateral OO muscles. In case of any significant effect of SIDE or any significant interaction SIDE*ISI, TNS effects were measured on iR2 and cR2 together. Repeated measures ANOVA and post hoc Student's paired t test were used to test the effect of ISI (250, 500, 1000 ms) and TNS (before, after) on R2 ratio.

In experiment 2 and 3, the effect of TNS (before, after) and ISI (SICI: 3 ms; ICF: 10 ms; SICF: 1.5 ms; LICI: 100 ms; SAI: 20 ms; LAI: 200 ms) on the amplitude of the conditioned MEP (expressed as conditioned amplitude/unconditioned amplitude) was assessed by ANOVA using a model of repeated measures. In case of significant values, Student's paired t test was used for post hoc analysis applying the Bonferroni correction for multiple comparisons when needed.

2.4 Results

No subjects reported any side effects or pain during and/ or after the experimental sessions, apart from sleepiness or relaxation during and immediately after TNS delivery.

2.4.1 Experiment 1: TNS effects on brainstem excitability

Blink reflex

Following unilateral SON stimulation, all subjects showed an early R1 ipsilateral response and a late bilateral R2 response. The BR recorded at baseline and immediately after TNS administration is reported in Fig. 1.

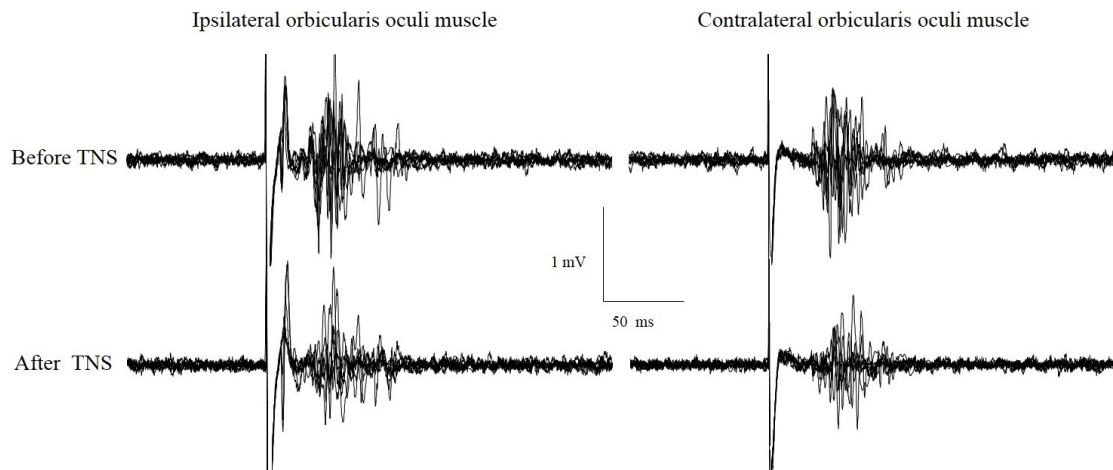


Fig. 1 Blink reflex recorded from a representative subject before and immediately after delivery of trigeminal nerve stimulation. The blink reflex was evoked by the electrical stimulation of the left supraorbital nerve (stimulus parameters: pulse duration 0.2ms, intensity 24 mA, frequency 0.02-0.025 Hz). Trigeminal nerve stimulation was given bilaterally over the infraorbital nerve and consisted of trains (asymmetric biphasic square wave pulses of 0.25 ms, 120 Hz) delivered in a cyclic modality (30 s ON and 30 s OFF) for a total period of 20 minutes, at an intensity of 11 mA.

TNS administration did not affect the R1 component of the BR, by contrast both ipsilateral and contralateral R2 responses appeared reduced (Fig. 1), showing a cumulative significant decrease of their areas to 82 ± 33 and 85 ± 35 % of the pre-TNS values ($p < 0.05$), respectively (Fig. 2).

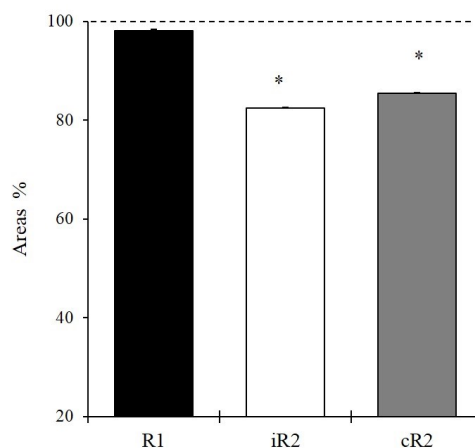


Fig.2 Effects of trigeminal nerve stimulation on the areas of the R1 and R2 components of the blink reflex. Cumulative data showing changes of the R1, ipsilateral R2 (iR2) and contralateral R2 (cR2) areas observed immediately after trigeminal nerve stimulation (TNS). TNS

after effects are represented as mean \pm SEM percentage values of the R1, iR2 and cR2 areas measured before TNS, which were taken as 100% (horizontal dotted line). TNS administration proved to be able to affect only the R2 response, which appeared significantly reduced. *: $p < 0.05$.

The raw values of R2 threshold R1, iR2 and cR2 areas are reported in Table 1. Two-way repeated measures ANOVA with TNS (before, after) and SIDE (iR2, cR2) showed a significant effect of TNS ($p = 0.018$) on the R2 area but no effect of SIDE or interaction TNS*SIDE. Student's paired t test revealed a significant reduction in iR2 ($p = 0.02$) and cR2 ($p = 0.015$) areas after TNS.

Table 1 Mean \pm SD values (n=17) of the blink reflex parameters, before and after Trigeminal Nerve Stimulation

BR Parameters	Before TNS	After TNS	<i>p value*</i>
R2 threshold (mA)	4.26 \pm 0.39	4.32 \pm 0.40	0.37
R1 area (mV.s)	0.006 \pm 0.004	0.006 \pm 0.004	0.95
iR2 area (mV.s)	0.018 \pm 0.015	0.012 \pm 0.009	0.02
cR2 area (mV.s)	0.015 \pm 0.012	0.011 \pm 0.007	0.015

TNS = Trigeminal Nerve Stimulation; BR = Blink Reflex; R2= R2 component of the BR; iR2 = ipsilateral R2 area; cR2 = contralateral R2 area; *Student t test.

Blink reflex recovery cycle

All subjects showed a normal BRRC, with an almost complete recovery of the response R2 response at 1000 ms ISI, which appeared not affected by TNS delivery at any ISI used (Fig. 3). A three-way repeated measures ANOVA with TNS (before, after), SIDE (iR2, cR2) and ISI (250, 500, 1000 ms) as within-subject factors, showed no interaction SIDE*ISI and TNS*SIDE*ISI, demonstrating that TNS effects on iR2 and cR2 ratios were not significantly different, according to SIDE. The averaged R2 ratios were then compared by means of two-way repeated measures ANOVA with ISIs and TNS as within-subject factors.

Statistical analysis showed a significant effect of ISI ($p < 0.001$) but no significant interaction TNS*ISI. Posthoc analysis revealed inhibition of conditioned R2 at 250 and 500 ms ($p < 0.001$).

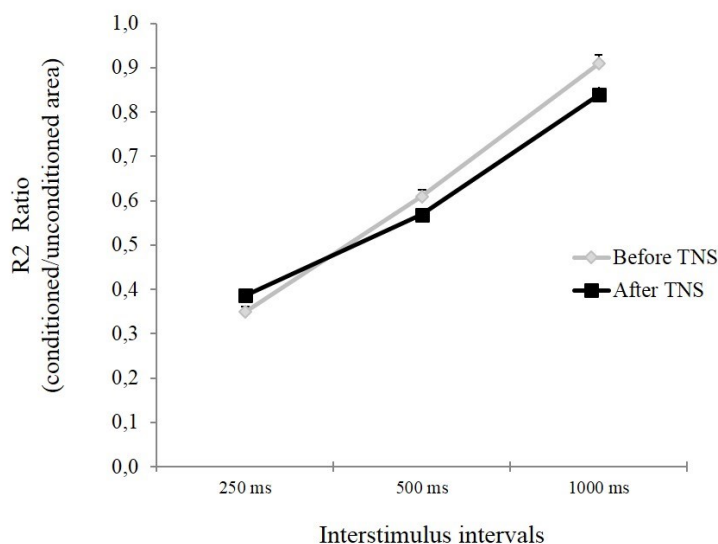


Fig. 3 Effects of trigeminal nerve stimulation (TNS) on the R2 recovery cycle.

The graphs report mean \pm SEM values of the conditioned R2 area of the blink reflex, expressed as ratio of the unconditioned response. The left supraorbital nerve was stimulated at interstimulus intervals of 250, 500 and 1000 ms and the ratio between R2 conditioned area/unconditioned area was calculated at each ISI. With respect to baseline, no significant changes of the R2 recovery cycle were observed after TNS administration at any ISI tested.

2.4.2 Experiment 2: TNS effects on intracortical excitability

Student's paired t test showed that RMT and 1 mV MEP intensity values were not significantly different before and after TNS (Table 2).

Table 2 Mean \pm SD (n = 17) of motor and perceptual thresholds, intensities of magnetic and electrical stimulations before and after Trigeminal Nerve Stimulation.

Stimuli	Parameters	Before TNS	After TNS	<i>p value*</i>
TMS (%MSO)	RMT	40.12 \pm 5.66	40.06 \pm 5.63	0.79
	1mV MEP	47.59 \pm 8.22	47.65 \pm 8.40	0.35
ES (mA)	PT	1.84 \pm 0.30	1.76 \pm 0.30	0.24
	MNS	5.55 \pm 0.91	5.39 \pm 0.88	0.40

*TNS: Trigeminal Nerve Stimulation; TMS: transcranial magnetic stimulation, ES: electrical stimulation; RMT: resting motor threshold; 1 mV MEP: TMS intensity that evokes motor potential of 1 mV; %MSO: percentage of maximal stimulator output; PT: perceptual threshold; MNS: intensity of median nerve stimulation; *Student t test.*

Two-way repeated measures ANOVA comparing TNS effects (before vs. after) and ISI in the paired-pulse protocols testing SICF, SICI, ICF and LICI (at ISIs of 1.5, 3, 10 and 100 ms, respectively) revealed a significant effect of ISI ($p < 0.001$) but no significant effect of TNS or interaction between TNS and ISI. Because of this lack of interaction, averaged MEP amplitudes measured before and after TNS were compared by means of a one-way ANOVA with ISI as main factor. Results showed a significant effect of ISI ($p < 0.001$) and planned post hoc Student t test showed a significant ($p < 0.001$) inhibition at 3 and 100 ms (SICI and LICI, respectively) and a significant facilitation ($p < 0.001$) at 1.5 and 10 and ms (SICF and ICF, respectively) (Fig. 4).

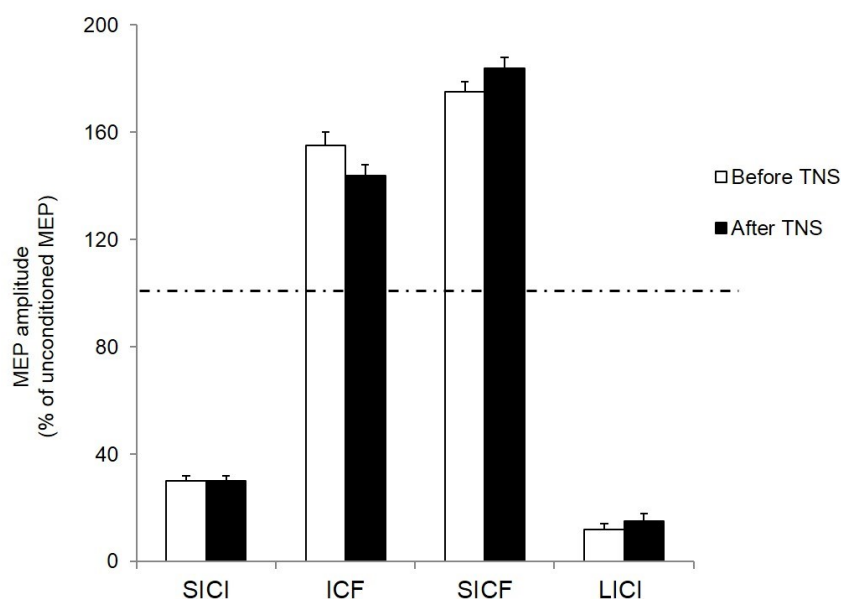


Fig. 4 Effect of trigeminal nerve stimulation on intracortical excitability. Histograms report short interval intracortical inhibition (SICI), intracortical facilitation (ICF), short interval intracortical facilitation (SICF) and long interval intracortical facilitation (LICI) measured from the dominant FDI muscle, before (white columns) and after (black columns) 20 minutes of trigeminal nerve stimulation (TNS). Paired TMS induced a significant inhibition of motor evoked potentials (MEPs) at 3 ms (SICI) and 100 ms (LICI) intervals and a significant facilitation at 10 ms (ICF) and 1,5 ms (SICF) interstimulus intervals. Ordinates indicate MEP amplitude expressed as a mean \pm SEM percentage value of the unconditioned MEP, induced by a single pulse TMS, taken as 100% (dotted horizontal line). All parameters of intracortical excitability appeared substantially unchanged after TNS administration

2.4.3 Experiment 3: TNS effects on cortical sensorimotor integration

TNS administration did not significantly affect PT and ES intensity values (Table 2) as well as the short-latency (SAI) and long-latency (LAI) inhibitory effects of a conditioning ES of the median nerve on MEPs induced in the FDI by TMS of the contralateral hand motor area (Fig. 5).

A two-way repeated measures ANOVA with TNS (before, after) and ISI (20 ms for SAI, 200 ms for LAI) as within-subject factors showed a significant effect of ISI ($p < 0.001$) but no significant interaction between TNS and ISI. Post hoc analysis revealed significant inhibitory effects on the conditioned MEP amplitude both at 20 and 200 ms ISI ($p < 0.001$).

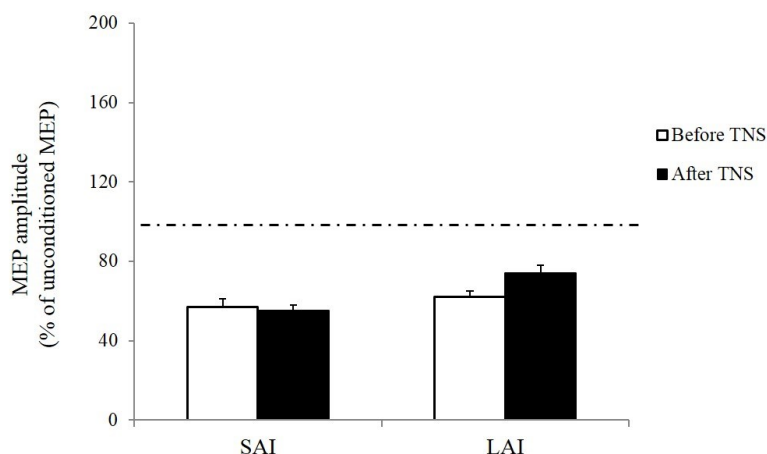


Fig. 5 Effect of trigeminal nerve stimulation on sensory-motor integration occurring at cortical level. Histograms show values of short afferent inhibition (SAI) and long afferent inhibition (LAI) measured from the dominant FDI muscle before (white column) and after (black columns) trigeminal nerve stimulation (TNS). MEP amplitudes were significantly reduced when TMS of the cortical motor area innervating the dominant hand was preceded by electrical stimulation of the ipsilateral median nerve by 20 ms (SAI) and 200 ms (LAI) intervals. However, SAI and LAI did not change significantly after TNS. Ordinates indicate MEP amplitude (mean \pm SEM) expressed as a percentage of unconditioned MEP induced by the TMS alone, taken as 100% (dotted horizontal line).

2.5 Discussion

The present study examined brainstem and cortical excitability following acute TNS. Results showed that TNS significantly depresses brainstem circuits mediating the R2 but not the R1 component of the BR. By contrast, the activity of both facilitatory and inhibitory intracortical interneurons as well as processes of sensorimotor integration, occurring at cortical level, appeared unaltered after TNS.

2.5.1 Effects of TNS on brainstem excitability

To the best of our knowledge, the current study is the first to examine the effects of acute TNS on brainstem excitability. Our data showed that acute, cyclic, 20-

min TNS administration significantly alters the magnitude of the R2 response of the BR, leaving the R1 component unaffected.

These different effects exerted by TNS on the BR components should be interpreted in light of the different circuits they involve and of the functional properties of the nervous structures which modulate them. The R1 circuit is thought to be restricted to the pons, where an ipsilateral pathway connects the sensitive afference, the main trigeminal sensitive nucleus and the facial motor nucleus (Aramideh and Ongerboer de Visser, 2002; Cruccu et al., 2005). The consistency of the R1 response after TNS, in comparison with the baseline, indicates that no changes in the excitability status of the R1 oligosynaptic circuit occurred. Indeed, the R1 response is regarded as a stable component of the BR, insensitive to supratentorial influences, including supratentorial lesions, disorders of consciousness and cognitive factors (Cruccu and Deuschl, 2000).

On the other hand, the R2 response arises out of a complex bilateral polysynaptic circuit, which includes the spinal trigeminal nucleus, neurons in the lateral tegmental field and the lateral reticular formation (Kimura, 1983; Bourque and Kolta, 2001; Aramideh and Ongerboer de Visser, 2002; Cruccu et al., 2005). The R2 response is less stable than the R1 response and appears very sensitive to sensory inputs and to supratentorial influences, consciousness status and cognitive factors (Cruccu and Deuschl, 2000), probably because of the high number of synapses in the reflex circuits (Berardelli et al., 1999). Our data offer no indication on the possible origin of the TNS induced inhibitory effect on the R2 circuit. However, since the R2 components share the same motoneurons with the R1 response, the TNS-induced bilateral and symmetric reduction in the R2 area cannot be explained by possible changes in the excitability of neurons in the facial motor nucleus. Instead, this effect is likely due to an increased inhibitory weight on those structures (spinal trigeminal nucleus, interneurons in the lateral reticular formation and in the lateral tegmental field) underpinning the R2 circuit, which are not in common with the R1 pathway (Aramideh and Ongerboer de Visser, 2002; Cruccu et al., 2005). All these areas can be possible targets for TNS action. Afferent trigeminal fibers project primarily to the trigeminal nuclei in the brainstem, from where facial and oral somatosensory information is

sent to the thalamus and then conveyed to the somatosensory cortex. Trigeminal nuclei are also connected to brain areas which are thought to modulate the lateral reticular formation, such as the NTS and the LC. The NTS is the main recipient of vagal afferents (Nieuwenhuys et al., 2008), which in part also projects to the spinal trigeminal nucleus (Kiernan, 2009). The NTS is, in turn, connected to noradrenergic and serotonergic systems associated with the regulation of mood, anxiety, emotions (Ruffoli et al., 2011) and to glutamatergic and GABAergic systems regulating the susceptibility to seizures (Walker et al., 1999). The LC provides virtually all noradrenergic innervation to the brain by means of its widespread varicosities and plays a crucial role for vigilance (Halliday, 2004). Both LC and NTS are considered as nuclei which disseminate neuromodulatory compound (Fanselow, 2012) in the CNS, since they profoundly affect its excitability at virtually all levels. In addition, the LC and the NTS are believed to play a key role in mediating the clinical benefits observed following TNS in several neurological and neuropsychiatric disorders such as epilepsy, depression, anxiety and migraine (Shiozawa et al., 2014; Fanselow, 2012). In addition to the indirect effects on the lateral reticular formation, TNS can also modulate the activity of interneurons in the lateral tegmental field. This is suggested by the sedative effects described during SON stimulation (Piquet et al., 2011) which aligns with the relaxing effects following ION stimulation in our subjects. It has been shown that the level of arousal influences in a different manner the early and late components of the BR (Shahani, 1968). For instance, during sleep the R1 component of the BR is absent, while the second component is preserved. On the other hand, an apprehension state results in a marked increase in the amplitude of the R2 component of the BR.

Conversely, when the subject is relaxed and assured that the electrical stimulus would not be painful, only the first component is elicitable. The LC, which is recognized as a major wakefulness-promoting nucleus (Samuels and Szabadi, 2008) and is a relays station between trigeminal afferent projecting neurons and facial motoneurons involved in the BR circuit (Dauvergne et al., 2008; Steidl et al., 2004; Bourque and Kolta, 2001), could modulate the occurrence of the blinking, facilitating or inhibiting the BR, depending on the arousal state,

emotions or alertness (Ueno and Uchikawa, 2004). Basing on the above reported literature, it is reasonable to speculate that an overall TNS-induced decrease in the level of arousal could explain the observed reduction in the R2 area.

The excitability of the BR has been experimentally modulated with other methods using repetitive stimulation of the trigeminal nerve, like the high-frequency stimulation (HFS) of the SON (Mao and Evinger, 2001). HFS has been demonstrated to facilitate or attenuate the R2 response of the BR, depending on the time the burst was applied. These effects were attributed to a long-term potentiation or to a long-term depression of the wide dynamic range neurons involved in the BR circuit (Mao and Evinger, 2001; Quartarone et al., 2006; Zeuner et al., 2010; Bologna et al., 2010; Suppa et al., 2011, 2014) These data allow speculating that the TNS-induced reduction in the R2-area might be mediated by plastic changes in the BR, although long-term after effects on the BR were not possible to be investigated in the present experimental set up.

Although the TNS protocol used in this study significantly reduced the R2 area of the BR, it proved unable to affect the activity of brainstem inhibitory interneurons that modulate the R2 recovery cycle. Our subjects showed a normal R2 recovery curve after TNS, suggesting that trigeminal stimulation was not able to influence passive mechanisms or activation of negative feedback circuits which are accounted for suppression of the R2 response (Cruccu and Deuschl, 2000). As pointed out above, the excitability of the R2 component of the BR is modulated by inputs other than the local ones originating in the brainstem, like descending projections coming from suprasegmental levels (Kumru et al., 2009; Valls-Solé et al., 2004; Kimura, 1973). In particular, a key role in the modulation of the excitability of brainstem interneurons involved in the R2 recovery cycle seems to be played by the basal ganglia, via the superior colliculus (Basso et al., 1996). This assumption is supported by the demonstration that in pathological conditions, characterized by a basal ganglia dysfunction, the R2 recovery cycle is altered, while the R1 and R2 areas are unaffected (Pauletti et al., 1993; Berardelli et al., 1999; Nisticò et al., 2014). Therefore, it is conceivable that TNS

effects are mainly exerted on brainstem structures and are unlikely to involve basal ganglia circuits.

2.5.2 Effects of TNS on cortical excitability and sensorimotor integration

Our study did not provide evidence of any effect exerted by TNS on the resting and active motor thresholds as well as on the activity of cortical interneurons mediating SICF, SICI, ICF and LICI phenomena. These findings, obtained following 20-min of cyclic bilateral TNS of the ION, are in agreement with data reported by a recent work, performed in healthy subjects, where a 40-min continuous TNS delivered to the SON, failed to show any alteration of the excitability of SICI, ICF and LICI circuits recorded in the hand motor cortex (Axelson et al., 2014). Our study demonstrated for the first time that sensorimotor integration processes occurring at cortical level (i.e. SAI and LAI) were also unaffected by TNS. It should be taken into account that the hand motor cortex may not be a specific “spatial” target for TNS. However, this area was used in this study as a model to assess cortical excitability for several reasons: first, all single and paired TMS protocols have been widely investigated and hence standardized in the hand motor cortex (Rossini et al., 2015); second, the same protocols have been also studied in cranial muscles but there is not universal agreement yet on the cortical origin as well as on reproducibility of motor evoked responses (Cruccu et al., 1989, 1990, 1997; Kobayashi et al., 2001; Dubach et al., 2004; Sohn et al., 2004; Paradiso et al., 2005; Ortu et al., 2008; Pilurzi et al., 2013); third, recordings of TMS-induced responses from cranial muscles are technically challenging due to the difficulty or impossibility to evoke stable MEPs in resting conditions, to cross-talk from neighboring muscles, high motor threshold of masseter and facial motor area (Ortu et al., 2008; Cattaneo and Pavesi, 2014); and finally, most of studies performed in neurological and psychiatric conditions not involving the motor cortex used TMS over the hand motor area and concluded that this procedure is the most suited one to disclose cortical excitability changes without any spatial specificity (Kobayashi and Pascual-Leone, 2003; Di Lazzaro et al., 2004; Badawy et al., 2014).

TNS parameters used in the present study are proved to be clinically efficacious and safe in epilepsy (DeGiorgio et al., 2006; Pop et al., 2011), in major depressive disorders (Cook et al., 2013) and in migraine (Schoenen et al., 2013), similar to the well-established VNS therapy (Groves and Brown, 2005). The similarity of clinical effects induced by VNS and TNS has given rise to the hypothesis that these neuromodulatory interventions may share not only crucial anatomical structures but also neurobiological mechanisms (Fanselow, 2012; Bari and Pouratian, 2012). The effects of VNS on hand cortical excitability, namely on SICI were explored by only one study (Di Lazzaro et al., 2004). Di Lazzaro and Coll. studied VNS effects on SICI of epileptic patients, and interestingly, they found that only those patients with a pathologically reduced SICI responded to VNS with a SICI increase. Conversely, patients with a normal SICI had a lack of VNS effect. These findings lead these authors to hypothesize that VNS could be of limited value when the baseline levels of intracortical inhibition are high, probably because of a “floor effect” (Di Lazzaro et al., 2004). It is reasonable to assume that also the inefficacy of TNS on the cortical excitability of healthy subjects, observed in the present study and in the study of Axelson et al. (2014), might be explained in terms of baseline levels, which may account for a quasi “floor” or “ceiling” effect, so that the potential influence of TNS cannot be demonstrated physiologically.

In most of clinical studies (DeGiorgio et al., 2006, 2013; Pop et al., 2011; Cook et al., 2013; Schoenen et al., 2013), TNS was delivered for long periods, thus our findings cannot be generalized to the clinical context. In the present study, a short-lasting TNS rather than a chronic TNS was delivered since our aim was to investigate the site of action, cortical and/or subcortical, of the TNS rather than exploring its clinical effects in the short-term period. However, 20-min TNS have been also proved to be able to induce short-term effects in patients with migraine (Piquet et al., 2011; Schoenen et al., 2013).

In conclusion, in normal subjects 20 min of cyclic bilateral TNS of the ION was able to induce after effects on brainstem polysynaptic circuits mediating the R2 component of the BR. By contrast, it proved inefficacious in modifying the activity of higher-level structures involved in the R2 recovery cycle and in modulation of

cortical excitability. Further experiments performed in pathological conditions presenting these last parameters altered, might provide more information on TNS sites and mechanisms of action. Moreover, it may be worth investigating whether TNS administered chronically has a higher potential in producing measurable after effects on its CNS targets.

**Study 2: Transcutaneous trigeminal nerve stimulation
induces a long-term depression-like plasticity of the
human blink reflex.**

3.1 Introduction

Transcutaneous stimulation of cranial nerves has been used to treat several neurological conditions. In particular, TNS is a noninvasive neuromodulation method which has been recently proposed for the treatment of drug-resistant epilepsy (DeGiorgio et al., 2003, 2006, 2009, 2013; Pop et al., 2011; Moseley and DeGiorgio, 2014; Soss et al., 2015; Cook et al., 2015), migraine (Schoenen et al., 2013), and depression (Schrader et al., 2011; Cook et al., 2013, 2014; Shiozawa et al., 2015). TNS has been proved to be safe, not associated with adverse cardiovascular events (Pop et al., 2011), and to have some advantages in comparison with the well acknowledged VNS. In fact, differently from VNS, TNS can be applied bilaterally with larger effects than unilateral stimulation (Fanselow et al., 2000; DeGiorgio et al., 2003) and none of the autonomic disadvantages of a bilateral VNS (Pop et al., 2011; Howland, 2014).

Despite its proved clinical effectiveness, the neurophysiological mechanisms by which TNS modulates brain activity are still under debate. TNS is thought to involve a number of different actions on the nervous system, including changes in the levels of inhibitory or excitatory networks (Faingold, 2008a, b; Shiozawa et al., 2014). Furthermore, recent animal and human studies suggested that cranial nerve stimulation causes neuroplasticity and neurogenesis in the brain (Marrosu et al., 2003; Follesa et al., 2007; Faingold, 2008a; Biggio et al., 2009; Shiozawa et al., 2014; Grimonprez et al., 2015). Stimulation techniques can be used to modulate disease symptoms inducing short- and long-lasting increase (long-term potentiation, LTP) or decrease (long-term depression, LTD) in synaptic transmission (Cooke and Bliss, 2006; Bliss et al., 2013). In the last decade, LTP-like plasticity and LTD-like plasticity have been studied at subcortical level using the BR as a model suitable to document some neurophysiological mechanisms underlying brain stem function (Aramideh and Ongerboer de Visser, 2002; Valls-Solé, 2005). The BR consists of two electromyographic (EMG) responses evoked in the orbicularis oculi (OO) muscles by the percutaneous electrical stimulation of the supraorbital branch of the trigeminal nerve (supraorbital nerve, SON). The early R1 response is mediated by an oligosynaptic pontine circuitry,

whereas the late R2 component results from the activation of a polysynaptic chain of brain stem interneurons extending in the lateral reticular formation at the pontine and medullar level (Aramideh and Ongerboer de Visser, 2002; Cruccu et al., 2005). Low-frequency stimulation (LFS) of the SON has been proved to induce a significant reduction in the BR integral and the sensory-evoked cortical potentials (Schorr and Ellrich, 2002; Ellrich and Schorr, 2004). Furthermore, noxious LFS of the mental nerve also causes a decrease in the masseteric inhibitory reflex (Ellrich and Schorr, 2002). Both these effects lasted for at least 1 h, meeting the criteria for an LTD-like effect (Hess and Donoghue, 1996), which led the authors to conclude that LTD-like plasticity of synaptic transmission in the brain stem can be reliably induced by LFS of trigeminal afferents (Schorr and Ellrich, 2002; Ellrich and Schorr, 2002). In addition to LFS, high-frequency stimulation (HFS) of the SON also has been demonstrated to facilitate or attenuate the R2 response of the BR, depending on the time the burst was applied. In fact, an LTP- or an LTD-like effect was observed when the burst coincided or preceded the reflex blink, respectively (Mao and Evinger, 2001). Another method able to alter brain stem excitability is a cyclic 20-min TNS, which recently has been proved to depress significantly the area of the R2 component of the BR, without modifying its recovery cycle (Mercante et al., 2015). Plastic mechanisms mediating this TNS-induced effect have been hypothesized, but evidence of this phenomenon is still lacking. This hypothesis was here tested by investigating whether such a protocol of TNS exerts long-term plastic changes on brain stem excitability.

3.2 Materials and methods

3.2.1 Subjects

Thirty-one volunteers (18 females and 13 males; 28 ± 4.5 years old; range 20–37 years) participated in the study. Informed written consent was obtained from all subjects and the procedure, approved by the local ethical committee (Bioethics Committee of ASL n.1 Sassari, Prot n. 2078/ce), was in accordance with the Helsinki Declaration.

None of the participants had a history of neurological and/or psychiatric diseases. Experiments were carried out in a quiet room. Subjects sat in a comfortable chair with headrest and were asked to keep their eyes open and to stay relaxed but alert during data collection.

3.2.2 EMG recordings

Reflex responses were recorded from both OO, using 8-mm diameter Ag–AgCl surface cup electrodes, with the recording electrode placed over the lower lid, the reference electrode 2 cm away from the lateral cantus with the ground electrode over the forehead. EMG signals were amplified (x5000; D360 amplifier; Digitimer Ltd, Welwyn Garden City, UK), filtered (bandpass 50–5000 Hz), and sampled (10 kHz per channel in a window frame of 2500 ms) using a CED1401 power analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK). The raw blink recordings were DC-corrected and rectified for off-line measurements.

EMG recordings from OO muscles started 2 s before each stimulus, to allow online rejection of trials with excessive activity. Data were stored on a personal computer and analyzed using Signal 5.02 software (Cambridge Electronic Design, UK).

3.2.3 Electrical stimulations

Electrical stimulation of the left SON was delivered at the supraorbital notch, using silver chloride disc surface electrodes (cathode over the homonymous foramen and anode two cm lateral) connected to a constant current stimulator (DS7A Stimulator, Digitimer, Welwyn Garden City, Herts, UK). Square-wave pulses (0.2-ms duration) were delivered to the SON at variable time intervals (between 20 and 40 s) to minimize habituation of the BR, and stimulus intensity was set at three times the R2 threshold (lowest intensity that evoked at least five R2 responses in 10 consecutive trials).

3.2.4 TNS

In light of the similar effects induced by SON and infraorbital nerve (ION) stimulation (DeGiorgio et al., 2006; Pop et al., 2011) and of the effectiveness of ION stimulation on the R2 component of the BR (Mercante et al., 2015), in the present study TNS was delivered to the ION through disposable, hypoallergenic, silver gel self-adhesive stimulating electrodes (30 mm diameter, SEI-EMG s.r.l. Cittadella, IT) with the cathode placed over the ION foramina and the anode 2 cm lateral, and connected to a Winner® stimulator (Fisioline biomedical instrumentation, Verduno, CN, IT).

The stimulation of the ION instead of the SON allowed the delivery of both TNS and BR stimuli (to the SON) without changing the electrode position during the experiment.

A TNS protocol, which proved to be able to inhibit the area of the R2 component of the BR (Mercante et al., 2015), was used. It consisted of asymmetric biphasic square-wave pulses (0.25-ms duration, 120 Hz) with an electrical mean equal to zero delivered in a cyclic modality (30-s ON and 30-s OFF) for 20 min, which was demonstrated to inhibit the R2 area (Mercante et al., 2015). In each subject, perceptual and pain thresholds were bilaterally calculated. Stimulation intensity corresponded, for each ION, to the maximal intensity endurable comfortably by the subject, always below the pain threshold (range 1–20 mA).

3.2.5 Experimental design

The study was composed of three experimental sessions. For those subjects who participated in more than one experiment, sessions took place at least 2 weeks apart.

3.2.6 Experiment 1: Aftereffects of bilateral real-TNS on BR

Twenty volunteers (12 females and 8 males; 29.0 ± 4.2 years old; range 21–37 years) participated in this experiment, which was aimed at evaluating the effect of 20-min bilateral TNS on the BR over a 45-min time period. The early ipsilateral

R1 and the late ipsilateral (iR2) and contralateral (cR2) R2 responses of the BR evoked by SON stimulation (Aramideh and Ongerboer de Visser, 2002) were assessed before, immediately after (T0), and at 15 (T15), 30 (T30), and 45 (T45) min following TNS. Ten BRs were collected at each time interval, and R1 and R2 areas were calculated. Three out of the 20 subjects who participated in this experiment (two females and 1 male; 31.3 ± 2.5 years old; range 28–34 years) underwent the same stimulation protocol with data collection at baseline, T0, T15, T30, 60 (T60), and 90 (T90) min after TNS.

3.2.7 Experiment 2: Effects of sham-TNS versus real-TNS on BR

The aim of experiment 2 was to verify whether the prolonged inhibition of the R2 component of the BR, observed following TNS administration (Mercante et al., 2015), could be due to a reflex habituation. Eleven participants (six females and five males; 26.1 ± 4.2 years old; range 20–33 years) underwent an ineffective 20-min bilateral TNS (sham-TNS). The sham-TNS protocol mimicked the initial bilateral real-TNS stimulus perception and consisted of a previous calculation of both perceptual and pain threshold, followed by 20 s of TNS, the intensity of which was subsequently gradually decreased down to zero, corresponding to the OFF position of the stimulator. The BR was assessed using the same experimental procedure and measurements as experiment 1. The R2 area after the shamTNS was calculated as a ratio of the R2 baseline area at each time point and compared with R2 ratios obtained after the real-TNS.

3.2.8 Experiment 3: Aftereffects of unilateral real-TNS on BR

In 12 subjects who participated in experiment 1 (seven females and five males; 30.4 ± 4.0 years old; range 21–37 years), the BR induced by the stimulation of the left and of the right SON was randomly recorded before and after unilateral TNS. Twenty BRs (10 BR per SON) were recorded in the same experimental session at baseline and at 0–45 min after TNS. Perceptual and pain thresholds were calculated for the left ION, and TNS was then applied at a sub-painful intensity for 20 min. The R2 areas obtained following left and right SON

stimulation were recorded before and after unilateral TNS to test effects on the R2 circuits ipsilateral and contralateral to the TNS stimulated ION. Effects of unilateral and bilateral TNS on the R2 ratios calculated following left SON stimulation were compared at each time point (T0–T45).

3.3 Statistics

Statistical analysis was performed with SPSS 18 software (SPSS Inc, Chicago, IL, USA). In the analysis performed with repeated measures analysis of variance (ANOVA), compound symmetry was evaluated testing the sphericity with the Mauchly's test. The Greenhouse–Geisser correction was used to compensate for non-spherical data. A p value <0.05 was considered significant. Unless otherwise stated, values are expressed as mean \pm standard deviation (SD) of the mean.

In experiment 1, to analyze the effects of TNS within R1 and R2 components of BR, repeated measures ANOVA (RM-ANOVA) was performed separately on raw data for R1 and R2. For R1 analysis, one-way RM-ANOVA was performed with TIME (before, T0, T15, T30, and T45) as within-subject factor. Two-way RM-ANOVA with TIME (before, T0, T15, T30, and T45) and SIDE (iR2, cR2) as within-subject factors was performed to evaluate different effects of TNS on the ipsilateral and contralateral OO muscles. In case of no significant interaction SIDE*TIME, TNS effects were measured on iR2 and cR2 together. In case of significant values, Student's paired t test was used for post hoc analysis applying the Bonferroni correction for multiple comparisons. In the three subjects, who underwent 90 min post-TNS recordings, a separate oneway RM-ANOVA with TIME (baseline, T0, T15, T30, T60, T90) on averaged iR2 and cR2 areas and on R1 area was performed.

In experiment 2, the effects of TNS on R2 ratio was tested performing RM-ANOVA with TIME (T0-T45) as within-subject factor and TREATMENT (real- vs. shamTNS) as between-subject factor. Planned post hoc Student's independent sample t test with Bonferroni correction was used in case of significant values.

In experiment 3, three-way RM-ANOVA with TIME (before, T0, T15, T30, and T45), SIDE (iR2, cR2), and NERVE (left and right SON) as within-subject factors

was performed to evaluate different effects of unilateral TNS on the ipsilateral and contralateral SON stimulation and iR2 and cR2 responses. In case of no significant interaction TIME*SIDE*NERVE, TNS effects on iR2 and cR2 after left and right SON were measured together. To test the different effect of unilateral and bilateral TNS on R2 ratio, RM-ANOVA with TIME (T0-T45) and TNS (bilateral vs. unilateral) as within-subject factors was performed. In case of significant values, Student's paired t test was used for post hoc analysis applying the Bonferroni correction for multiple comparisons.

5.4 Results

The electrical stimulation of the left SON evoked ipsilateral R1 and bilateral R2 responses in all 31 subjects tested (Fig. 1).

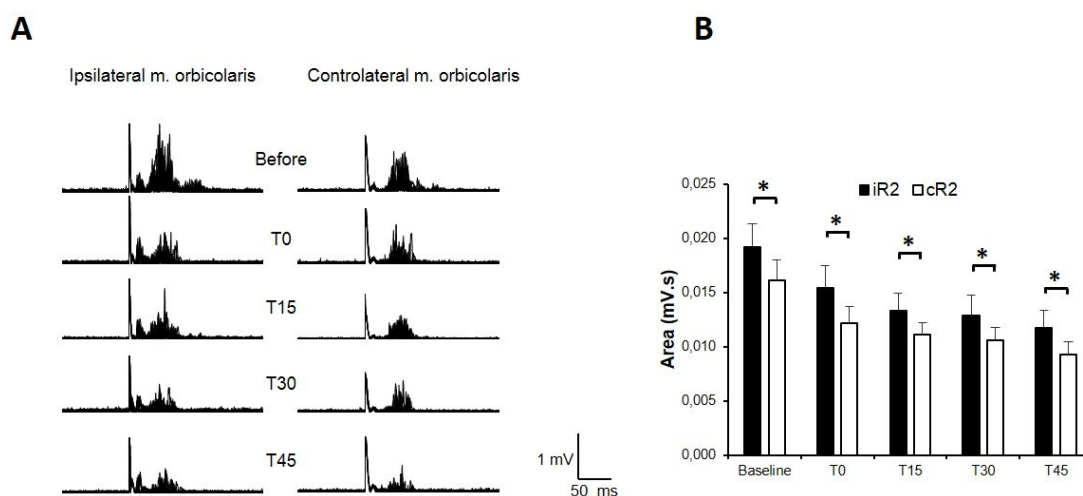


Fig. 1 Effects of transcutaneous trigeminal nerve stimulation (TNS) on the Blink Reflex recorded from the orbicularis oculi (OO) following electrical stimulation of the left supraorbital nerve (SON). A. EMG Recordings from a representative subject. In each trace of 10 single trials were superimposed. B. The histograms report mean R2 data collected from 20 subjects. Clear ipsilateral R1 and bilateral R2 responses were visible in the OO EMG after SON stimulation, with the ipsilateral R2 (iR2) significantly larger than the contralateral R2 (cR2) at any time-point tested. The figure reports the BR recorded before (baseline) and after real-TNS at zero (T0), 15 (T15), 30 (T30) and 45 (T45) minutes. In comparison to baseline, both iR2 and cR2 areas were significantly inhibited following TNS, while the R1 response was unaffected. Asterisks: $p < 0.05$. Error bars represent mean \pm standard error.

None experienced pain or side effects during and/or after the experimental procedures. Mean age of the participants as well as values of stimulus intensities used were not significantly different between the bilateral real-TNS, unilateral real-TNS, and bilateral sham-TNS groups (Table 1).

Table 1. Mean \pm SD values of stimuli parameters in the bilateral real-TNS, bilateral sham-TNS and unilateral real-TNS groups.

<i>Stimulus intensity</i> (mA)	Bilateral real-TNS (n = 20)	Bilateral sham-TNS (n = 11)	Unilateral real-TNS (n=12)
<i>R2 Threshold</i>	4.49 \pm 1.76	3.54 \pm 1.04	3.28 \pm 1.06
<i>SON Stimulation</i>	13.54 \pm 5.32	10.44 \pm 3.25	10.04 \pm 2.89
<i>TNS</i>	12.00 \pm 1.99	11.25 \pm 1.60	11.25 \pm 1

SON: supraorbital nerve; TNS: trigeminal nerve stimulation

3.4.1 Experiment 1: Aftereffects of bilateral real-TNS on BR

Two-way RM-ANOVA with TIME (before, T0, T15, T30, T45) and SIDE (iR2, cR2) showed a significant effect of TIME ($p < 0.001$) and SIDE ($p = 0.008$) on the R2 area but no interaction TIME*SIDE, being TNS effects on iR2 and cR2 not statistically different. The significant effect of SIDE is due to the well-known difference in amplitude between iR2 and cR2, with the former being larger than the latter (Peddireddy et al., 2006; Kofler et al., 2013). Student's paired t test showed a significant difference in R2 areas with iR2 bigger than cR2 area at each time point tested (baseline: $p = 0.042$; T0: $p = 0.005$; T15: $p = 0.034$; T30: $p = 0.031$; T45: $p = 0.008$) (Fig. 1). As a result of the lack of interaction TIME*SIDE, averaged iR2 and cR2 areas were analyzed together by post hoc planned Student's t test with Bonferroni correction. A significant inhibition of R2 area at T15 ($p = 0.013$), T30 ($p = 0.002$), and T45 ($p = 0.001$) was found after 20 min of TNS administration (Fig. 2).

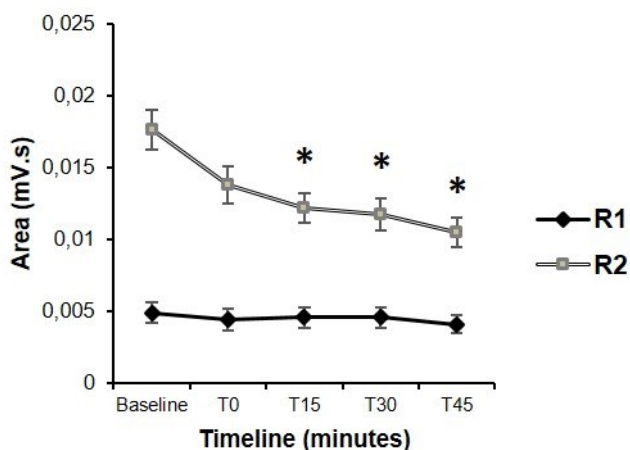


Fig. 2 Time course of mean changes induced by real TNS intervention on the early and late components of the Blink Reflex. The graph reports mean data obtained from twenty subjects who underwent real-TNS administration. Time course of changes in the mean R1 (black line) and R2 (white line) areas from baseline to 0 min, 15 min, 30 min and 45 min after TNS are reported. *iR2* and *cR2* areas were pooled. Asterisks: $p < 0.05$. Error bars represent mean \pm standard error.

By contrast, the one-way RM-ANOVA showed that the R1 component of the BR was unaffected by TNS at any time point tested (Fig. 2). Three out of 20 subjects underwent baseline and 90-min recordings (T0, T15, T30, T60, and T90) after TNS delivery (Fig. 3). One-way RMANOVA was performed separately for R1 and R2 components.

Statistical analysis showed a significant effect of TIME ($p = 0.001$) on R2 areas recorded in six muscles (ipsi- and contralateral OO). One-way RM-ANOVA used to analyze R1 area over time in three muscles (ipsilateral response) was not statistically significant.

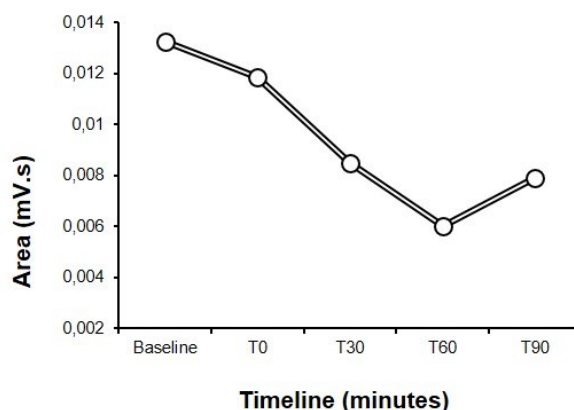


Fig. 3 Time course of TNS-induced inhibition of R2 area, over 90 minutes. The blink reflex was recorded from 3 subjects at baseline and after 0 (T0), 15 (T15), 30 (T30), 60 (T60) and 90 (T90) minutes after TNS administration. The graph shows a significant inhibition of R2 area after TNS lasting up to 90 minutes, with a tendency to recovery after 90 minutes.

3.4.2 Experiment 2: Effects of sham-TNS versus real-TNS on BR

RM-ANOVA with TIME (T0, T15, T30, and T45) as within-subject factor and TREATMENT (real- vs. shamTNS) as between-subject factor showed a significant interaction TIME*TREATMENT ($p = 0.005$). Independent sample Student's t test showed a significant difference in R2 ratios at T30 ($p = 0.012$) and T45 ($p = 0.002$), while, although showing a trend, at T0 ($p = 0.05$) and T15 ($p = 0.069$), it was not statistically significant (Fig. 4).

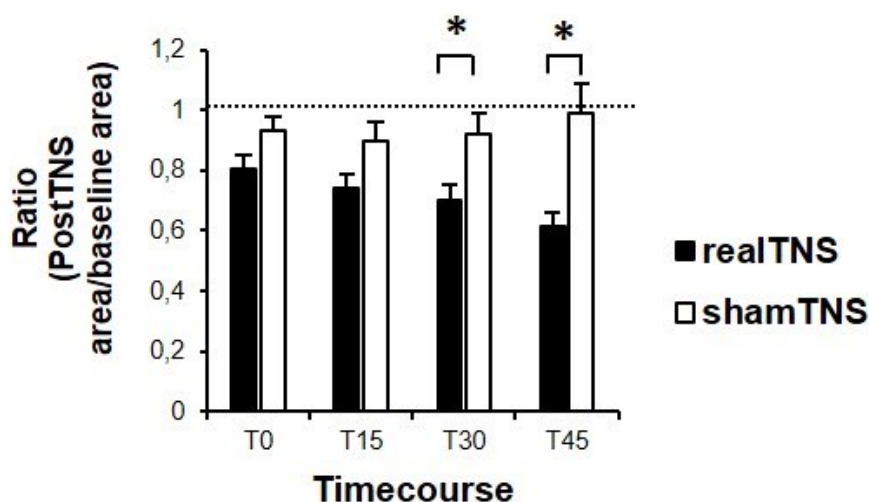


Fig. 4 Effects of real-TNS and of sham-TNS on the magnitude of R2 response of the blink reflex. The histogram shows time course of effects of real-TNS ($n=20$; black columns) and of sham-TNS ($n=11$; white columns). Ratios of R2 areas (post TNS R2 area/baseline R2 area) collected after 0, 15, 30 and 45 minutes are reported for both TNS protocols. Compared to baseline, a significant inhibitory effect was observed only after real TNS, whereas no consistent effects were detected after sham TNS. Comparing R2 ratios in the two experimental conditions, a significant difference was detected at 30 and 45 min after TNS. Asterisks: $p < 0.05$. Error bars represent mean \pm standard error.

3.4.3 Experiment 3: Aftereffects of unilateral real-TNS on BR

Three-way RM-ANOVA with TIME (before, T0, T15, T30, and T45), SIDE (iR2, cR2), and NERVE (left and right SON) was performed for unilateral TNS applied to the left ION. Statistical analysis showed a significant effect of TIME ($p < 0.001$) and SIDE ($p = 0.001$) but no interaction TIME*SIDE, TIME*NERVE, TIME*SIDE*NERVE, with a similar inhibitory effect exerted on iR2 and cR2 areas evoked by left and right SON stimulation. When comparing ratios of R2 areas after unilateral versus bilateral TNS, two-way RM-ANOVA with TIME(T0-T45) and TNS (bilateral, unilateral) as within-subject factors showed a significant effect of TIME ($p < 0.001$) and a significant interaction TIME*TNS ($p = 0.009$). Planned post hoc paired Student's t test showed a difference between R2 ratios, being the amount of inhibition significantly smaller after unilateral than bilateral TNS only at T45 ($p = 0.034$), with a trend at T30 ($p = 0.051$).

3.5 Discussion

This study demonstrates a LTD-like effect of acute TNS on the neural circuitry mediating the R2 components of the BR in healthy individuals.

Effects of TNS on BR excitability

Data showed that 20-min TNS administration significantly alters the magnitude of the R2, but not the R1, response of the BR for 45 min and in a small sample, for at least 90 min. Bilateral TNS was more effective than unilateral TNS, confirming previous data obtained in awake rats with pentylentetrazole-induced seizure activity (Fanselow et al., 2000) as well as in patients with refractory epilepsy (DeGiorgio et al., 2003). The reduction in the R2 area observed after TNS administration cannot be ascribed to habituation of the BR following continuous SON stimulations, since it remained stable in the control group undergoing sham-TNS delivery. The consistency of the R1 response for 45–90 min after TNS, in comparison with baseline, indicates that no technical-based recording losses of the signal occurred during the experiments. Based on what has been stated above and considering that no effect was produced on the BR by the sham-TNS, it is likely that the inhibitory effect observed on the R2 component of the BR after TNS is a true TNS induced aftereffect. It cannot be completely excluded that a TNS-induced reduction in the alertness (Piquet et al., 2011; Mercante et al., 2015) could be responsible, at least in part, for the prolonged depression of the BR observed following TNS. In our experimental setup, a confounding unspecific effect of sleepiness was minimized asking the subjects to stay relaxed but alert for the whole duration of both real- and sham-TNS experiments, which were then possibly affected by a similar level of sleepiness or boredom. By contrast, specific changes in the arousal state induced by the real-TNS seems a plausible mechanism according to the evidence that TNS acts on brain stem nuclei deeply involved in the control of the arousal/sleeping state, such as the ascending reticular system and the LC (Halliday, 2004; Samuels and Szabadi, 2008). However, our experimental setup was not suitable to measure any TNS effect on the arousal state since the electrical stimulation of the SON, like that of other peripheral nerves, has been reported to induce an increase

rather than a decrease in the arousal level (Kwon et al., 2000; Piquet et al., 2011). It is conceivable that the prolonged bilateral inhibition of the R2 observed following TNS delivery is due to an LTD-like phenomenon (Hess and Donoghue, 1996) given the short-time period of the stimulus administration, its effectiveness compared to the sham protocol, the long-lasting inhibitory effect (>1 h) and its reversibility, revealed by a trend to recovery 90 min after TNS administration. Several forms of trigeminal nerve stimulation have been already proven to be able to modulate the plasticity of the BR circuit in humans, both in healthy subjects (Mao and Evinger, 2001; Schorr and Ellrich, 2002; Aymanns et al., 2009) and in neurological conditions, such as blepharospasm (Quartarone et al., 2006), Huntington's disease (Crupi et al., 2008), and Tourette Syndrome (Suppa et al., 2011, 2014). In these conditions, abnormal plasticity of brain stem neurons has been shown to play a role in physiopathological mechanisms. The first evidence of this phenomenon was provided by Mao and Evinger (2001) who demonstrated that high-frequency bursts applied to the SON (trains of nine stimuli, 400 Hz, every 10 s), before or concurrent to the BR, induced a LTD or a LTP of the BR, respectively, for at least 30 min. Therefore, the authors concluded that supraorbital HFS induced LTP- and LTD-like associative plasticity in the wide dynamic range neurons belonging to the BR circuit (Mao and Evinger, 2001). Besides HFS, LFS of the SON (1200 continuous stimuli for 20 min, 1 Hz) was also demonstrated to produce a significant reduction in R2 integrals, lasting for at least 1 h, namely an LTD-like plasticity (Schorr and Ellrich, 2002).

The modulatory effect induced by TNS on the BR is comparable to those of LFS and paired HFS of trigeminal nerve afferents as for the LTD-like feature and duration (more than 80 min after the conditioning protocol of Schorr and Ellrich 2002), up to 90 min in some of our subjects as well. Differently from HFS, LFS and the TNS protocol used in our experiments do not imply any associative time dependent mechanism to evoke plastic changes, with the LFS based on repetitive continuous low-frequency stimulation and the TNS on cyclic high-frequency bursts. Interestingly, the modulatory effect of the HFS was proved to be restricted to the trigeminal complex ipsilateral to the stimulated afferents (Mao

and Evinger, 2001), while TNS seems to modulate the excitability of R2 circuit in a generalized fashion, since a bilateral effect was observed after unilateral TNS. In this regard, it should be pointed out that both LFS and HFS were applied to the SON, while TNS was here delivered to the ION. However, in TNS clinical studies, the stimulation of the SON or of the ION was applied alternatively during the day in several cases, according to the patient's preference, being the effects observed following the stimulation of these two nerves not significantly different (DeGiorgio et al., 2003, 2006; Pop et al., 2011).

3.5.1 Sites of action and possible mechanisms

Theoretically, LTD of the BR may be due to a TNS-induced decrease in activity of any neuron involved in its reflex arc. Nervous pathways common to the R1 and R2 components of the BR are not likely to be responsive to TNS action, since the R1 response was not altered by TNS. Previous studies showed that cortical excitability (Axelson et al., 2014; Mercante et al., 2015) and sensorimotor integration at cortical level (Mercante et al., 2015) are not affected by acute TNS administration, suggesting no direct modulation of TNS on higher structures. Moreover, it has been recently demonstrated that inhibitory interneurons mediating the BR recovery cycle are not influenced by TNS (Mercante et al., 2015). This finding further restricts the number of the possible anatomic-functional sites prone to TNS-induced modulation. An interesting insight is provided by a recent study demonstrating that slow-frequency repetitive TMS (rTMS) applied to the motor cortex is able to induce a long-lasting inhibition of brain stem interneuronal circuits responsible for the BR recovery cycle, but not those mediating the R2 response (De Vito et al., 2009). This is probably related to a depression of cortical excitability. Basing on these reports (De Vito et al., 2009; Axelson et al., 2014; Mercante et al., 2015), it seems reasonable to hypothesize that TNS has a specific target in those brain stem neurons mediating the R2 component of the BR, but not in its recovery cycle. The R2 response of the BR is mediated by a bilateral polysynaptic circuit that includes the spinal trigeminal nucleus, neurons in the lateral tegmental field and in the lateral reticular formation (Bourque and Kolta, 2001; Aramideh and Ongerboer

de Visser, 2002; Cruccu et al., 2005). For this reason, the R2 response appears very responsive to sensory inputs (Cruccu and Deuschl, 2000) and susceptible to plastic changes, probably because of the large number of synapses engaged in the reflex circuits (Ongerboer de Visser and Cruccu, 1993). Various brain stem areas can be possible targets for a TNS modulatory action. Afferent trigeminal fibers project primarily to the trigeminal nuclei in the brain stem, from where facial and oral somatosensory information is sent to the thalamus and then conveyed to the somatosensory cortex. Trigeminal nuclei are also connected to brain areas such as the NTS and the LC which are thought to modulate the reticular formation (Foote et al. 1983; Aston-Jones et al. 1991; Van Bockstaele et al., 1999; Fanselow, 2012) and disseminate neuromodulatory compound in the central nervous system, since they profoundly affect its excitability at virtually all levels (Moruzzi and Magoun, 1949; Foote et al., 1983; Vertes, 1991; Vertes et al., 1999; Ruffoli et al., 2011; Fanselow, 2012). In addition, the LC and the NTS are believed to play a key role in mediating the clinical benefits observed following TNS in several neurological and neuropsychiatric disorders (Faingold, 2008a; Fanselow, 2012; Shiozawa et al., 2014; Trevizol et al., 2015). In our work, we demonstrated that TNS has direct modulatory effects at subcortical level (Mercante et al., 2015). According to recent hypothesis, the propagation of the afferent signal from the trigeminal nerve is thought to travel from peripheral nerves toward the brain stem and then higher central structures, named “bottom-up” mechanisms (Fanselow, 2012; Shiozawa et al., 2014). Peripheral stimulation techniques can be used acutely or chronically to, respectively, abort or prevent pathological symptoms (Fanselow et al., 2000; Faingold, 2008a, b; DeGiorgio et al., 2011). In particular, TNS has been proved effective to terminate symptoms that are impending or actively occurring (Moseley and DeGiorgio, 2014; Shiozawa et al., 2014; Trevizol et al., 2015). This finding is supported by the evidence, reported in animal models of epilepsy (Faingold, 2008a, b), that a direct stimulation within the “disease network,” particularly at high frequencies, can result in disruptive effects on the network, and that this stimulation may also modulate, simultaneously or sequentially, another network which can compete with the “disease network.” The authors hypothesized that the stimulation

procedure might initially involve acute mechanisms, as the LTD-like effect seen in our experiment, that can affect directly or indirectly the “disease network” (Faingold, 2008a, b). Several works suggest that cranial nerve stimulation causes neuroplasticity in the central nervous system (Follesa et al., 2007; Faingold, 2008a; Revesz et al., 2008; Biggio et al., 2009; Grimonprez et al., 2015) with chronic stimulation protocols involving more long-lasting processes than acute protocols. These neurostimulation paradigms contribute to neuronal network activation and neurogenesis (Follesa et al., 2007; Faingold, 2008a, b; Biggio et al., 2009) and have the clinical counterpart in symptoms’ control obtained with chronic TNS used in epilepsy, migraine, and depression.

Study 3: Effects of trigeminal nerve stimulation on rat hippocampal neurogenesis

4.1 Introduction

Although TNS is commonly seen as a treatment for epilepsy, it has been reported by several authors that its administration also positively affects mood, resembling an antidepressant effect (Cook et al., 2014; Shiozawa et al., 2015). Indeed this putative effect of TNS appears to be in common with VNS, which has been approved by FDA in 2005 (Groves and Brown, 2005) for the treatment of pharmacoresistant depression (Howland, 2014).

Among the different brain areas involved in the pathophysiology of depression, the hippocampus has recently received much attention (Malykhin and Coupland, 2015). This brain area is involved in mood and memory functions as well as in epileptogenesis, and show some unique properties among all other brain structures. In particular the process called neurogenesis, i.e. the birth of new neurons from neuronal stem cells, appears to be especially prominent in the hippocampus and has been observed in the adult brains of both rodents and humans (Eriksson et al., 1998 and Gould et al., 1999).

Several evidences suggest that neurogenesis plays a role in depression and imply that reduction of neurogenesis in the hippocampus is a causality factor in depression while stimulated neurogenesis is part of the recovery process (Jacobs et al., 2000). In particular, magnetic resonance imaging studies in depressed patients showed smaller hippocampal volumes (Sheline et al., 1996; Shah et al., 1998, Travis et al., 2014), possibly related to the duration of the depressive state (Sheline et al., 1999). Treatment with antidepressants increases neurogenesis in animal models of rats (Malberg et al., 2000) and primates (Perera et al., 2007; Salomon and Cowan, 2013) and intact hippocampal neurogenesis is required for the antidepressant effects (Santarelli et al., 2003). Interestingly, electric brain stimulation by electroconvulsive treatment has also been shown to be very effective in increasing stem cell proliferation in the adult rat brain (Scott et al., 2000; Madsen et al., 2000). These observations provided a rationale for studying the effects on neurogenesis of other neurostimulation techniques, and indeed several studies have already shown that VNS increases neuron proliferation in the rat hippocampus (Resvez et al., 2008; Biggio et al.,

2009). Although the neurobiological mechanisms underlying this effect are still unclear, it has been hypothesized that serotonin (5HT) may play a major role. In fact, while neurogenesis is stimulated by 5HT (probably through 5HT-1A or 5HT-3 receptors (Brezun and Daszuta, 1999; Kondo et al., 2015), 5HT system activity is decreased in depressed patients (Morrisette and Stahl, 2014; Ressler and Nemeroff, 2000) and major antidepressant drugs (such as tricyclics and SSRI) are known to increase brain 5HT levels (Salomon and Cowan, 2013).

VNS increases RN (which is the major brainstem nucleus for 5HT cells) activity in the rat brain (Dorr and Debonnel, 2006) and 5-HIAA (5-hydroxyindoleacetic acid, 5HT metabolite), in the cerebrospinal fluid of patients with refractory epilepsy (Ben-Menachem et al., 1995), indicating a positive modulation of 5HT system activity. Therefore, it seems sensible to speculate that a common 5HT-mediated mechanism could explain the antidepressive effects of VNS as well as its positive effect on neurogenesis. Further, a VNS-induced increase of neurogenesis could also have relevance in understanding the antiepileptic effects of VNS, since newborn neurons in the dentate gyrus of the adult rat brain have been found to reduce excitability in epileptic brain regions (Jakubs et al., 2006).

The similarity of clinical effects induced by VNS and TNS has risen the hypothesis that these neuromodulatory interventions may share not only crucial anatomical structures but also neurobiological mechanisms (Fanselow 2012; Bari and Pouratian 2012). Therefore we hypothesized that, similarly to VNS, TNS could also affect the rat brain hippocampal progenitor proliferation, which might represent part of the TNS antidepressive mechanism.

4.2 Materials and Methods

4.2.1 Animal and surgical procedure

Experiments were carried out on adult male rats in accordance with the current Italian legislation (D.L. 116, 1992), that allows experimentation on laboratory animals only after submission and approval of a research project to the Ministry of Health (Rome, Italy), and in accordance with European Council directives on

the matter (n. 2007/526/CE). All possible efforts were made in order to minimize animal pain and discomfort and to reduce the number of experimental animals used.

Male Sprague Dawley rats (Harlan Nossan, Italy) weighing 275/350 g at the beginning of the experiments were housed in group under controlled environmental conditions (temperature 22 ± 2 °C; humidity 60–65%) and kept on a 12h/12 h light/dark cycle with food and water ad libitum. Rats were randomly divided in three experimental groups (Control, Sham and TNS).

4.2.2 Nerve cuff electrodes.

The infraorbital branch of the trigeminal nerve was stimulated unilaterally via chronically implanted nerve cuff electrodes (Fanselow et al., 2000). These electrodes were constructed in-house and consisted of two platinum bands (7 mm long, 0.5 mm wide and 0.025 mm thick, 0.8 mm separation between bands) that ran circumferentially around the infraorbital nerve (ION). Platinum bands were held in place and electrically insulated by a thin polyethylene coating. Each platinum band was connected to a flexible, three-stranded Teflon-coated silver wire that was used to pass current between the bands.

4.2.3 Electrode implantation.

Cuff electrodes were surgically implanted around the left infraorbital branch of the trigeminal nerve. Briefly, rats (in both Sham and TNS experimental groups) were anesthetized by intraperitoneal injection of equithesin (1 ml/300g of body mass). Tissue was dissected until the ION was exposed, and the cuff electrode was positioned around the nerve such that the nerve lay inside the cuff. The cuff was then tied around the nerve to hold it in place, and the wound was sutured. The Teflon-coated leads from the platinum bands were run subcutaneously to the top of the head where they were attached to connector pins and affixed to the skull.

After recovering from surgery animals were inspected to assess well-being and pain absence.

4.2.4 TNS

Two days after surgery, subjects in the TNS group were connected to a pulse generator and acutely (3 h) administered with TNS. Stimulation parameters conformed to values used in VNS therapy in both humans (Binnie, 2000; Marrosu et al., 2003) and rats (Follesa et al., 2007; Biggio et al., 2009): 30 s ON, 5 min OFF; pulse frequency 30 Hz; pulse width 500 μ s; pulse amplitude, 1.5 mA. Subjects in the Sham group underwent the surgical procedure for electrodes implantation but did not receive stimulation during the experiment. The control group did not undergo either to surgery or stimulation.

4.2.5 Drug treatment

In order to label newly formed cells in the hippocampus, rats of all experimental groups were injected with bromodeoxyuridine (BrDU, 70 mg/kg in saline, i.p.). BrDU was administered to rats in TNS group three times at intervals of 3h using the following schedule: first dose 3 hours before TNS, second dose at the start of TNS and last dose 3 hours after TNS. Rats in Control and Sham groups were administered with BrDU using the same intervals of 3 hours.

The animals were sacrificed 24 h after the last BrDU administration.

4.2.6 Immunostaining

Rats were deeply anesthetized with Equithesin and then perfused transcardially with ice-cold paraformaldehyde (4%) in phosphate-buffered saline (PBS). After perfusion the brains were removed and fixed overnight in 4% paraformaldehyde and thereafter kept in 30% sucrose solution at 4 °C.

Coronal sectioning was performed on a freeze microtome and serial cross-sections (30 μ m) of the dorsal hippocampus (Paxinos and Watson, 2007) were prepared and processed for immunohistochemical analysis as follows.

Sections were first subjected to DNA denaturation with SSC-formamide solution (50%), followed by 2N HCl for 30 min at 37 °C and washed first with borate buffer (0.1 M sodium borate in deionised water, pH 8.5) and then with PBS.

Sections were treated with 0.3% H₂O₂ and 0.3% goat serum in PBS for 5 min at room temperature to block endogenous peroxidase activity. Afterward sections were incubated for 30 min at room temperature with PBS containing 0.3% Triton X-100 and 0.3% normal horse serum (NHS) and then overnight at 4°C with mouse monoclonal antibodies to BrDU 100 µg/ml (Santa Cruz Biotechnology) in PBS containing 2% NHS and 0.3% Triton X-100. Sections were then washed with PBS before incubation for 1 h at room temperature with biotinylated anti-mouse secondary antibodies (Santa Cruz Biotechnology). Antibody complexes were amplified using the Elite Vectastain ABC kit (Vector Laboratories), and visualized by using diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories).

Stained sections were finally mounted with a nonaqueous mounting medium and examined with a Zeiss Axioplan2 microscope (Carl Zeiss, Munich, Germany) equipped with a video camera.

The number of BrDU-positive cells in the granule cell layer and the subgranular zone of the dentate gyrus was determined in 24 coronal sections (30 µm in thickness and separated by 120 µm) from each brain. BrDU positive cells were counted using ImageJ 1.40g software (NIH, Bethesda, MD). Cells in the uppermost focal plane were ignored by focusing through the thickness of the section (optical dissector principle) in order to avoid oversampling errors (Coggeshall and Lekan, 1996).

4.3 Statistical analysis

Data are expressed as BrDU-positive cells and charted as mean±S.E.M.. Data were analysed with a one-way analysis of variance (ANOVA) for repeated measures and a post-hoc test (Bonferroni's test for multiple pairwise comparison). ANOVA analyses revealing significant main effects were followed by unpaired t-tests (two tailed) to enable pairwise group comparison. A probability level (p) of less or equal to 0.05 was considered significant. All statistics were calculated using GraphPad Prism version 6.0f.

4.4 Results

Effect of TNS administration on hippocampal neurogenesis.

Although the electrodes were implanted on the left ION, a left-sided dominance of hippocampal proliferation was, for technical reasons, not possible to determine. Therefore, BrDU-positive cells count refers to the sum of BrDU-positive cells in the dentate gyrus of both left and right hippocampus (Fig 1, 2).

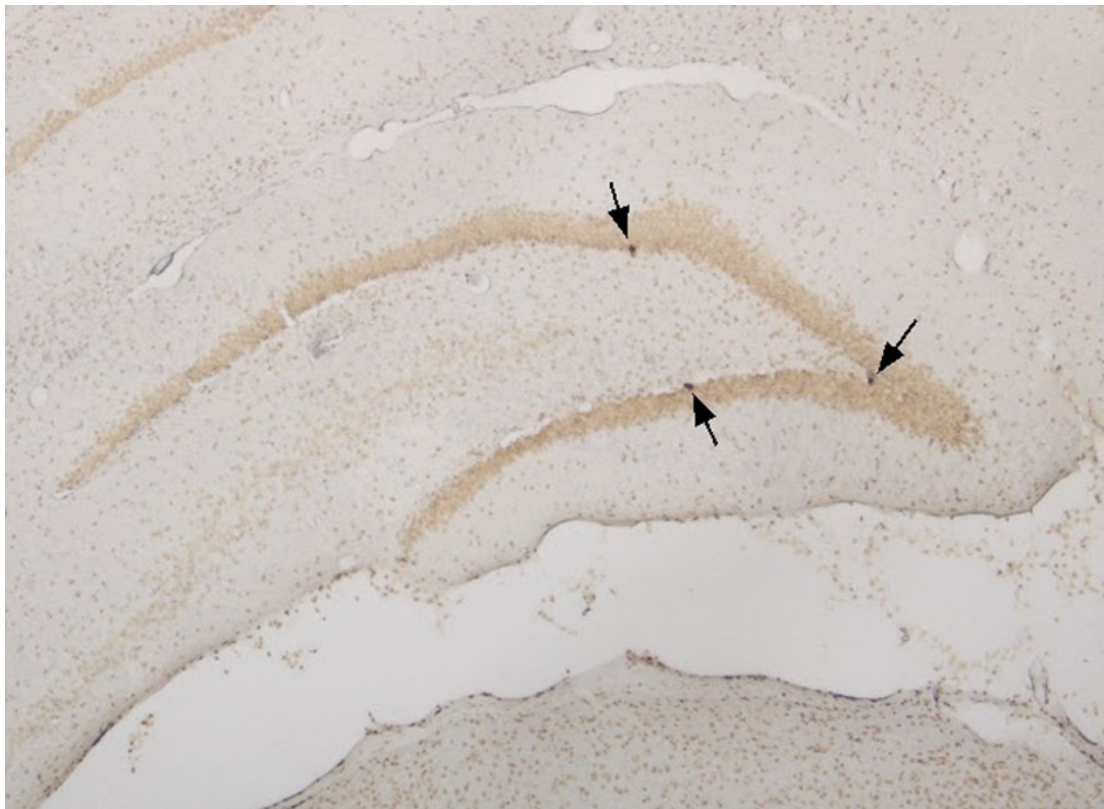


Fig. 1. *BrDU-positive cells in the dentate gyrus of the hippocampus in sham group rats. Newborn cells in the subgranular zone are stained dark blue for BrdU immunoreactivity (arrows).*



Fig. 2. BrDU-positive cells in the dentate gyrus of hippocampus in TNS group rat after acute TNS stimulation. Newborn cells in the subgranular zone are stained dark blue for BrDU immunoreactivity (arrows).

BrDU positive cells counts: Control = 46 ± 2.03 ; Sham = 49.50 ± 3.11 ; TNS = 86.33 ± 8.36 ; n = 6 in all groups.

One-way ANOVA revealed a significant effect of TNS treatment ($F(2, 15) = 18.85$; $p = 0.0001$, $n = 6$). No significant differences were found in the number of positive BrDU cells in Control vs Sham [$+7.07\%$, $t = 0.9405$, $p = 0.369$. Two-tailed, $DF = 10$, $n = 6$]; however BrDU cells in the TNS group was significantly increased with respect to both Control [$+46.71\%$, $t = 4.683$, $p < 0.001$. Two-tailed, $DF = 10$, $n = 6$], and Sham [$+42.66\%$, $t = 4.124$, $p < 0.002$. Two-tailed, $DF = 10$, $n = 6$] groups (Fig. 3).

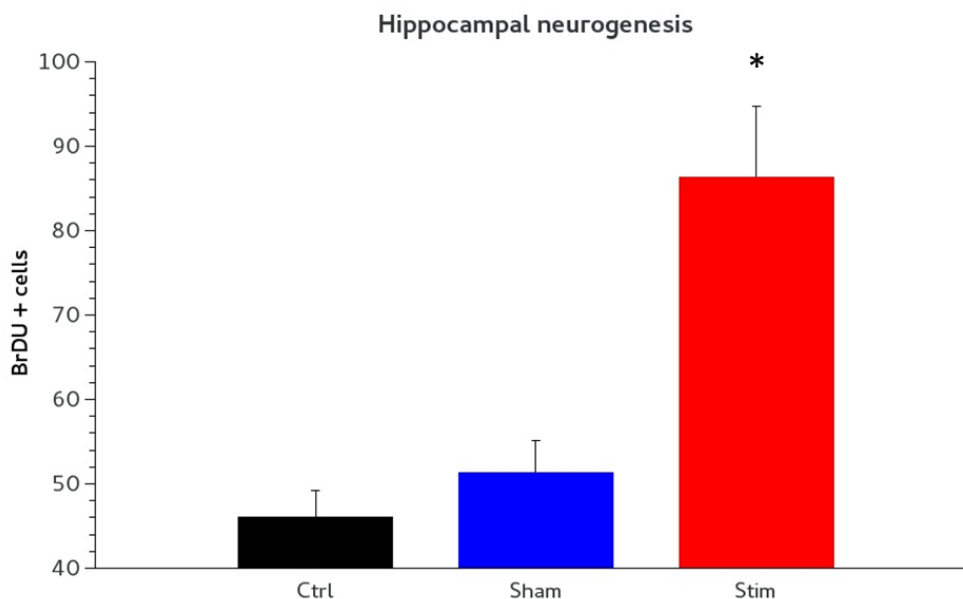


Fig. 3. Quantitation of newborn cells in the dentate gyrus of the rat hippocampus. BrdU+ cells were determined 24 h after administration of TNS. The number of positive cells counted in 24 sections with a thickness of 30 μm and spaced 96 μm apart. Data are charted as mean \pm SEM, $n = 6$ in all groups. * $p < 0.001$ vs control.

4.5 Discussion

Here we report on the effect of acute TNS on hippocampal neurogenesis in the rat. Our data show that 3 hours of TNS administration positively modulates hippocampal progenitor proliferation as indexed by the significant increase in BrDU positive cells found in the dentate gyrus of the TNS group vs both Control and Sham groups (Fig. 3). To the extent of our knowledge, this is the first study to assess this phenomenon.

Although the stimulating electrodes were implanted on the left trigeminal nerve, no side differences in the count of BrDU-positive cells could be seen between the two hippocampi suggesting that, at least in our experimental setup, TNS may cause a bilateral induction of hippocampal neurogenesis. This finding is in agreement with other studies of VNS effects on hippocampal neurogenesis (Revesz et al., 2008), as well as with other evidences of VNS-induced bilateral effects on c-fos expression (Naritoku et al., 1995) and norepinephrine release (Roosevelt et al., 2006).

The results of the present study support the positive effect of this neurostimulation technique on patient's mood (Cook et al., 2014; Shiozawa et al., 2015) as well as the well-known link between recovery from depression and increased hippocampal neurogenesis (Santarelli et al., 2003). However despite the fact that our data clearly show an increase in newly formed cells in the hippocampus, its significance on mood amelioration as well as the underlying neurobiological mechanisms remains obscure.

Since VNS and TNS probably elicit a similar response pattern in the brainstem, it is reasonable to hypothesize that their effect on hippocampal neurogenesis may be mediated via a shared pathway (Castle et al., 2005). In particular, among the various structures in the brainstem, stimulation of both vagus and trigeminal nerves affects the activity of the NTS (Magdaleno-Madrigal et al., 2010; Fanselow, 2012). This latter structure is a key gateway nucleus for many primary afferents and project widely throughout the brain and, of particular relevance for our study, to hippocampus, LC, and RN (Fanselow, 2012; Fornai et al., 2011). Both LC and RN also project to the hippocampus, and it has been shown that LC functional integrity is paramount for the anticonvulsant and (possibly) for the antidepressant effect of VNS (Krahl et al., 1998; Roosevelt et al., 2006).

The LC is the main source of noradrenergic fibres in the CNS and the hippocampus receives a dense projection (both ipsi- and bilateral) from LC by three pathways (Loy et al., 1980). Beta-adrenergic receptors as well as noradrenergic afferents appear to be unevenly distributed in the hippocampal structure, with the highest density on the dentate gyrus relative to the other hippocampal subregions (Booze et al., 1993; Milner et al., 2000). Noradrenaline plays a key role in regulating hippocampal cell functions (Hansen and Manahan-Vaughan, 2015) and changes in noradrenaline concentrations in the CNS have been also shown to affect hippocampal neurogenesis. In particular selective noradrenergic depletion decreases neurogenesis in the dentate gyrus of the rat (Kulkarni et al., 2002) and in agreement with this, increased noradrenaline synaptic levels by selective noradrenaline reuptake inhibitors, enhances neurogenesis (Malberg et al., 2000). Further, hippocampal progenitor survival

was also found to be increased by enhanced noradrenaline levels (Rizk et al., 2006).

VNS administration increases extracellular noradrenaline levels in discrete brain areas. Microdialysis experiments have shown that VNS transiently increases noradrenaline levels in both the cortex and hippocampus, also using stimulation parameters similar to those used in the present study (Roosevelt et al., 2006; Follesa et al., 2007).

Taken together, these findings suggest that a mechanism behind the observed increase in neurogenesis with TNS could be mediated in part by an increase in hippocampal noradrenergic activity.

The RN is the main source of 5HT in the brain and a large amount of evidence show that VNS enhances its activity increasing 5HT levels in the brain (Manta et al., 2009; Dorr and Debonnel, 2006; Fornai et al., 2011). It is well known that 5HT plays a major role in the pathophysiology of depressive states and therefore this phenomenon may be involved in both the antidepressant as well as the seizure inhibition effect of VNS and TNS (Merlet et al., 2004; Borowicz et al., 2007; Mainardi et al., 2008).

Neurogenesis is increased by enhanced hippocampal 5HT concentrations possibly via 5HT-1A or 5HT3 receptor (Brezun and Daszuta, 1999; Alenina and Klempin, 2015; Kondo et al., 2015). Although TNS effects on hippocampal 5HT levels have not been characterized yet, but a TNS-induced increased 5HT levels in the brain is to be expected due to the increased neuronal firing rates in the RN, as shown in VNS-treated rats (Dorr and Debonnel, 2006). In humans, trends for increases in 5-HIAA were seen in the cerebrospinal fluid of VNS-treated epilepsy patients (Ben-Menachem et al., 1995), which suggests a potential increase in serotonergic activity that, therefore, cannot be excluded from a possible role in TNS-induced increases in hippocampal neurogenesis.

An increase in hippocampal neurogenesis may either result from increased dentate gyrus progenitor proliferation or from enhanced progenitor survival, with a decrease in the number of those newly formed cells that normally disappear sometime after the cell division (or from a combination of both mechanisms) (Lehmann et al., 2005). The increase in cell proliferation is not necessarily linked

to an enhancement in the newly formed cell survival, which is a different process mainly regulated by changes in apoptosis rates.

Since cell proliferation and cell survival are controlled by separate intracellular pathways an effect of TNS administration on the survival of newly formed cells could not be excluded, since our experimental setup was intended for proliferation analysis only. However, the relatively short time span between BrDU administrations and sacrifice minimizes the likelihood of detecting changes in survival, suggesting that the effects of TNS on hippocampal progenitors are proliferative in nature.

4.6 Conclusions

Neurostimulation techniques such as VNS and TNS, were originally developed and applied as an alternative therapeutical approach to pharmacoresistant epilepsy, and their potential use in the therapy of depressive states was exploited only later. Hippocampal neurogenesis is a complex phenomenon profoundly involved in the neurobiological basis of depression. Intact neurogenesis is required for successful antidepressant therapy, and it has been hypothesized that TNS positive effects on mood could involve an action on hippocampal neurogenesis.

Our results show that acute TNS administration in the rat enhances stem cell proliferation in the hippocampus, which supports the notion of involvement of hippocampal plasticity in the TNS treatment for depression. Further, this finding might represent an interesting path in the search for mechanisms behind the actions of TNS.

Conclusions

Why it is so important to understand the neurobiological mechanisms by which TNS affects the activity of the CNS? Apart from obvious “basic science” considerations, probably the most compelling reason is the need of robust data to fine tune stimulation parameters in order to be able to tailor therapeutic interventions to patients’ needs. Nowadays the methods for setting stimulus parameters are somewhat empirical, starting with recommended standards and modifying these on a patient-by-patient basis.

Clearly, an understanding of why and how TNS affects brain activity will allow for evidence-based selection of stimulus parameters. Further, it may also be possible that different stimulation patterns could (differently?) affect discrete regions of the brain. Therefore, different stimulus paradigms may reveal to be ideal for specific needs (such as epilepsy or depression or migraine, etc, etc).

The aim of this study was to try to shed some light on how and/or where TNS exert its effects on CNS. Since there are several possible loci for influence, which are not mutually exclusive, we tried to use an “open” approach which included human as well as animal study.

Our data on the effects of TNS on healthy volunteers show that the activity of both facilitatory and inhibitory intracortical interneurons as well as processes of sensorimotor integration, occurring at cortical level, appeared unaltered after acute TNS administration. By contrast, we showed that TNS significantly depresses brainstem circuits mediating the R2 but not the R1 component of the BR. More in detail, we found for the first time that 20-min TNS administration induces a form of LTD-like plasticity of the human BR. Plastic changes at brainstem level detected following TNS may play a role in the clinical effects observed in neurological and neuropsychiatric disorders after TNS administration.

We also found that acute TNS administration in the rat enhances stem cell proliferation in the hippocampus, which supports the notion of involvement of hippocampal plasticity in the TNS treatment for depression. Indeed, this finding may seem to be in contrast with our reported LTD-like effect of TNS in the brainstem of healthy volunteers, however several differences could explain this

discrepancy. In particular the differences in stimulation frequencies and the duration of TNS administration between humans and rats, may play a key role in inducing different effects. Still, the increase in hippocampal neurogenesis might represent an interesting path in the search for the neurobiological basis of the actions of TNS on CNS.

It is clear that, in order to translate the present findings to neurophysiological mechanisms mediating the acknowledged clinical benefits of TNS, further investigations are required mainly to test the effects of chronic TNS on brainstem excitability, in both healthy subjects and neurological patients, in whom the real efficacy of neuromodulation on disease networks remains to be demonstrated.

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