UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE

Coordinatore del Corso: Prof. Andrea Fausto Piana

CURRICULUM IN NEUROSCIENZE

Responsabile di Curriculum: Dott.ssa Rossana Migheli

XXX CICLO

Peripheral neuromodulation for drug-resistant epilepsy: the effect of short-term transcutaneous trigeminal nerve stimulation on EEG activity

Coordinatore:

Prof. Andrea Fausto Piana

Tutors:

Prof. GianPietro Sechi

Prof.ssa Franca Deriu

Tesi di dottorato di:

Dott.ssa Chiara Fois

Anno Accademico 2016 – 2017

CONTENTS

SECOND SECTION: clinical study

SUMMARY

Aim: Trigeminal nerve stimulation (TNS) has been proven to exert beneficial effects on symptoms of drug-resistant epilepsy (DRE). However, whether and how TNS is able to modulate the electroencephalogram (EEG) background activity in DRE patients is still unknown. We aimed to investigate the effect of acute TNS on EEG activity by conducting qualitative (morphologic) and quantitative (power spectra) analyses on two groups of DRE patients, undergoing real or sham TNS.

Methods: Twenty-two DRE patients were randomly divided into a "sham-TNS" or "real-TNS" group. Real-TNS was delivered bilaterally to the infraorbital nerve with trains of a symmetric biphasic square wave pulse (1 to 20 mA, 120 Hz), in a cyclic modality for 20 minutes. The sham-TNS protocol mimicked the real-TNS stimulation but at a zero intensity. EEG recordings were collected for each patient 10 minutes *pre*, 20 minutes *during* and 10 minutes *post* TNS delivery. EEG signal was subsequently visually analysed for interictal epileptiform discharge (IEDs) and processed by spectral analysis (Fast Fourier Transform). A between and within subject repeated-measures ANOVA was used for statistical analyses.

Results: A significant increase of EEG absolute alpha power was observed in the during real-TNS compared to the sham-TNS ($F_{2,18}$=1.748; p=0.006). Conversely, no significant effects were noticed either for quantitative analysis of other frequency bands or for IEDs detection.

Conclusion: Short-term TNS is able to induce an acute effect on EEG background composition of DRE patients. In line with recent evidence, alpha rhythm enhancement might be interpreted as an index of functional inhibition, able to influence cortical activity and thus reduce seizure propensity.

FIRST SECTION

Introduction

1. DRUG-RESISTANT EPILEPSY

1.1 Definition and epidemiology

The *International League Against Epilepsy* (ILAE) *Task Force* defined Epilepsy as "an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological and social consequences of this condition" (Fisher et al., 2005, 2014).

Epilepsy is one of the most common disorders of the brain, affecting worldwide 50 million people of all ages. According to the World Health Organization (WHO) survey, it accounts for 1% of the global burden of disease, higher than Alzheimer's disease, multiple sclerosis and Parkinson's disease combined and similar to breast cancer in women and lung cancer in men, with an estimated prevalence of active forms (i.e. continuing seizures or with the need for treatment) between 4 and 10 per 1000 people and 2.4 million of new epilepsy diagnoses each year (Murray and Lopez, 1994; Murray et al., 2012).

Ideal goals for epilepsy treatment would be achieving complete seizure control while avoiding drug side effects, in order to improve quality of life and reduce disability and

morbidity. Up to 70% of epileptic patients can be successfully and completely treated with first line therapy, consisting in daily medication with one or more anti-epileptic drugs (AEDs) with a global cost of US$ 5 per year (Kwan and Brodie, 2000; World Health Organization atlas, 2005). After 2-year therapy, more than a half of patients can continue to be seizure free following drug discontinuation (Specchio and Beghi, 2004; Strozzi et al., 2015).

However, approximately one-third of epilepsy patients (i.e. 1 million people in the United States) despite adequate pharmacotherapy, continues to suffer from uncontrolled seizures (Kobau et al., 2008; Kwan and Brodie, 2000). This medically refractory condition represents the greatest burden of the disease and accounts for the 80% of the cost of epilepsy (Begley et al., 2000). It is generally termed "pharmaco-resistant", "medically intractable" or "drug-resistant" epilepsy (DRE) and has been recently defined by the ILAE task force as "*the failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom*" (Kwan et al., 2010).

Besides the risks of frequent life-threatening seizures, which increase mortality rate to 5–10 times of the general population, DRE appears associated with developmental delay, severe lifetime disability and morbidity in all ages (Lhatoo and Sander, 2001;

Sperling et al., 2016). Uncontrolled seizures may cause progressive cognitive impairment, eventually resulting in family dependence or institutionalization, with important social and financial burdens. Furthermore, psychological morbidities (such as depression, behavioural disturbances, anxiety and memory loss) may contribute to the risk of physical injuries (Bell et al., 2008; Hermann et al., 2006; Sheth et al., 2004), sudden unexpected death (SUDEP) (Langan et al., 2005; Walczak et al., 2001) and diminished health-related quality of life (Jacoby and Baker, 2008).

In order to early identify and prevent the causes of pharmaco-resistance, some studies focused on predictive factors of poor drug-responsiveness outcome, including the patient's history, the timing of AED response and the epilepsy/seizure features.

In particular, *aetiology of epilepsy* seems to be an important risk factor for DRE development. While childhood and adolescence idiopathic epilepsies (without structural brain damage and with hereditary predisposition) are generally less likely to become drug resistant (13%), symptomatic epilepsy (associated with structural brain lesions or epileptic encephalopathies) tends to be more AED-resistant (Sillanpaa et al., 1999). In general, epileptic encephalopathies such as Ohtahara syndrome, early myoclonic encephalopathy, West syndrome, Dravet syndrome and Lennox-Gastaut syndrome carry more than 50% of chance to become medical refractory (Mohamed and Minassian, 2006; Camfield and Camfield, 2003; Berg et al., 2006); whereas

symptomatic focal epilepsies, such as those secondary to structural brain lesions or malformations (hippocampal sclerosis, cortical dysplasia, brain injuries, haemorrhage, tumors, etc.) are at higher risk (65%) than cryptogenic forms (55%) (Briggs and French, 2003; Semah et al., 1998).

Other important predictors of DRE seem a high frequency of seizures prior to treatment (Elwes et al., 1984; Sillanpaa et al., 1993, 2009; Kwan and Brodie, 2000; Berg et al., 2001; Hitiris et al., 2007), age at onset (Ko TS and Holmes, 1999) and family history of epilepsy (Hitiris et al., 2007), a personal history of infantile spasms, neonatal or febrile seizures or status epilepticus and presence of neurological impairments (Berg et al., 1996, 2001; Oskoui et al., 2005).

While the epileptogenic zone (in particular the temporal lobe and sensorimotor cortices localization) seems to play a role in refractoriness mechanisms, inconsistent are data regarding seizure types and electroencephalogram findings, which did not show a clear-cut prognostic value (Mohanraj and Brodie, 2013).

Importantly, inadequate response to the first AED therapy has been shown to be the most powerful indicator of refractory epilepsy (Mohanraj and Brodie, 2013; Kwan and Brodie, 2000). Generally, seizure-free rates decrease from 61.8% for the first AED to 41.7% after the second AED and to 16.6% after 2–5 subsequent inefficient trials. After 6 AEDs, absolute refractoriness (0% seizure free) is very likely (Schiller and

Najjar, 2008). Overall, patients who do not respond to the first two AEDs trials have only a small percentage chance to become seizure-free with any additional AED.

Therefore, having ruled out pseudo-pharmacoresistance imitators (such as drug intolerance or inadequacy, psychogenic seizures or inadequate compliance) and DRE has been proven, an alternative therapeutic approach is required as soon as possible. At this point, management of DRE may direct towards two different strategies: epilepsy surgery and alternative treatments including neurostimulation, ketogenic diet, and lifestyle changes.

The *American Academy of Neurology* (AAN) Epilepsy Quality Measurement and the ILAE's subcommission on pediatric epilepsy surgery recommend that all patients who continue to be compromised by seizures after the failure of 2 appropriate AED trials, deserve a consultation at a specialized, full-service Epilepsy Center (Fountain et al., 2015; Cross et al., 2006).

For patients suitable for curative epilepsy surgery, in particular for those with distinct resectable lesions, epilepsy surgery has been shown to be superior to the continued use of AEDs (Kwan and Brodie, 2006). However, whilst epilepsy surgery may not be reserved to all DRE patients, since some of them may be considered not good surgical candidates (such as cryptogenic epilepsies with negative brain imaging, multifocal epilepsies or genetic syndromes) (Fois et al., 2015), this procedure cannot guarantee

100% outcome in terms of seizure freedom even in the most suitable candidates (de Tisi et al., 2011).

In this regard come into play alternative treatment approaches principally direct to reduce seizure severity and improve quality of life, such as neurostimulation methods.

A number of peripheral (extracranial) or central (intracranial) neuromodulation devices have been developed over the past few decades. Some of them have obtained US Food and Drug Administration (FDA) and/or European Conformity (CE) approval, such as vagal nerve stimulation (VNS), responsive nerve stimulation (RNS), deep brain stimulation (DBS) and external trigeminal nerve stimulation (TNS) and have been used since then, showing to be efficacy additional treatments for seizure control (Krishna et al., 2016; DeGiorgio and Krahl, 2013b). However, some of these neurostimulation methods imply invasive implantation, thus carrying the risk of surgical procedure and leading to a series of adverse effects which can limit or discontinue their use (Ben-Menachem et al., 2015).

Ketogenic diet is essentially used in children with refractory epilepsy and although some studies showed a high efficacy rate (Neal et al., 2008), it is challenging to administer and not devoid to side effects.

Lifestyle changes consists in avoiding seizure triggers and precipitants, such a sleep deprivation, psychophysical stress, alcohol intake, drugs of abuse and any potential photic stimulation.

1.2 Mechanisms of refractoriness

The importance to understand mechanisms underlying DRE is essentially to prevent and/or to treat it at an early stage of appearance.

There are a number of theories about mechanisms of drug refractoriness, but so far none of them has been proven to be certain and unique. What appears instead clearer is that mechanisms of DRE are rather multifactorial, involving genetic inheritance, environmental factors and disease or drug-related features.

Among a number of drug refractory theories, two represent the most reliable theories so far: (1) the transporter hypothesis and (2) the target hypothesis (Tang et al., 2017; Rogawski et al., 2013).

The *transporter hypothesis* proposes that increased expression or function of drug efflux transporters, such as P-glycoprotein (P-gp) and multidrug resistance–associated proteins (MRPs), reduces the local concentration of AEDs in epileptic brain regions to a sub-therapeutic level (Loscher and Potschka, 2002).

The best known efflux transporters are members of the ABC (ATP-binding cassette) family, specifically P-gp (ABCB1 or MDR1), the MRPs (MRP1, ABCC1; MRP2, ABCC2) and breast cancer resistance protein (BCRP, ABCG2) (Sisodiya et al., 2006). These ABC transmembrane proteins cause drug-resistance while actively extruding substrates (i.e. therapeutic drugs) out from the cell and limiting their entry into the target organs.

Multidrug resistance due to efflux transporters has been first studied in tumor cells, where ABC protein overexpression has been shown to induce chemotherapeutic drug resistance and determine a poor prognosis (Mao and Unadkat, 2015).

In this regard, P-gp is the most studied ABC protein. It is normally expressed in capillary endothelial cells in the brain with the function to actively extrude xenobiotics from intracellular space and so to reduce their cerebral accumulation.

In surgically treated DRE patients, blood-brain barrier and/or glial and neuronal cells P-gp up-regulation have been found in brain resected tissue (Sisodiya et al., 2002; Aronica et al., 2003). It is postulated that aberrant P-gp brain overexpression may cause AED resistance in a way similar to what seen for cancer patients, with active extrusion of some AEDs form the target brain tissue (Loscher and Potschka, 2002).

In animal models an up-regulation of P-gp has been found following seizures and associated to a reduce AED brain concentration level (Tishler et al., 1995; Sisodiya et

al., 2002). However, human studies did not reach the same level of evidence as for animals and whether human P-gp is actually able to bind and extrude AEDs to a significant extent and whether this has clinically relevant effects on AED concentration and efficacy in epilepsy patients remains unclear (Tang et al., 2017; Rogawski et al., 2013).

The *target hypothesis* postulates that alterations in the properties, function or structure of AED targets, such as ion channels and neurotransmitter receptors, result in decreased sensitivity to a drug and leads to treatment refractoriness (Remy et al., 2003, 2006).

According to this theory, two mechanisms of refractoriness can be advanced: the genetic (or intrinsic) form, where the cause of drug-interaction dysfunction has to be found in the inheritance predisposition to drug resistance and the acquired form, which is due to structural target changes secondary to enduring epileptic activity within the affected brain tissue.

For instance, polymorphisms of the SCN2A gene, which encodes the α2 subunit of the neuronal Nav1.1 sodium channels, were found to be associated with resistance to antiepileptic drugs in particular to Carbamazepine and Phenytoin (Tate et al., 2005).

Contrariwise, an altered expression and/or a reduced drug-sensitivity of subtypes of the γ-aminobutyric acid type A (GABAa) receptor and of the Carbamazepine binding

voltage-gated sodium channels have been reported in the pilocarpine model of epilepsy (Jandova et al., 2006; Remy et al., 2003) and found in resected brain tissue of drug-resistant temporal lobe epilepsy patients (Loup et al., 2000). These seizure-induced structural or sensitivity molecular changes might be similar to what seen during prolonged status epilepticus, where resistance to benzodiazepines results to a cellular internalization of synaptic GABAa receptors (Wasterlain and Chen, 2008; Fritsch et al., 2010; Joshi and Kapur, 2012).

Besides them, other two DRE theories deserve to be mentioned: the neural network hypothesis which states that seizure-induced remodelling of the neural network, together with the formation of new excitatory circuits, suppress endogenous anti-seizure mechanisms (Fang et al., 2011) and the intrinsic severity hypothesis which postulates that pharmaco-resistance is an inherent features of some type of epilepsies, particularly related to the severity grade of the disease and in particular to the high frequency of pre-treatment seizures (Rogawski 2008, 2013).

At present, all the theories above appear still controversial and difficult to validate being lacking in strong evidence and reproducibility for different specific drugs, patients and types of epilepsy.

Therefore, an important aim to improve epilepsy prognosis, in terms of morbidity and related mortality, would be the development of new therapeutic strategies targeting

intrinsic, neurobiological or acquired mechanisms underlying DRE and different to currently AED approach. Hopefully, such treatment would be able to interfere with aberrant neuronal network and restore the unbalance between excitatory and inhibitory mechanisms involved in seizure generation and propagation (Rocha, 2013).

Several non-pharmacological strategies are also currently under consideration as adjunctive treatment for DRE, such as neuromodulation approaches which have gained much attention over the past few decades, showing promising results in terms of seizure reduction efficacy and safety (Dalkilic, 2017; Krishna et al., 2016; DeGiorgio and Krahl, 2013b).

2. NEUROSTIMULATION IN EPILEPSY

2.1 Peripheral neurostimuation devices

Neurostimulation has emerged as a potential treatment option for patient with DRE who are not suitable for curative epilepsy surgery or continue to suffer from seizures despite previous inefficient surgical treatment.

The aims of these therapeutic methods are different from those of surgery, having as the main target the reduction of seizure frequency or generalization and so the improvement

of quality of life. They are also called palliative procedure since they have been conceived without curative intention.

The mechanism of action of neurostimulation devices, although apparently different from AEDs, has not been entirely clarified since now but they have been supposed to modulate cortical excitability through the stimulation of subcortical structures (Fanselow, 2012).

At present the neuromodulation modalities approved by FDA and/or CE for DRE treatment are anterior nucleus deep brain stimulation (AN-DBS), responsive neurostimulation (RNS), vagal nerve stimulation (VNS) and trigeminal nerve stimulation (TNS). Whereas the first two modalities provide central (intracranial) stimulation of brain structures and implantation requires very precise localization of seizure foci or site of stimulation, VNS and TNS represent the two different peripheral (extracranial) approaches for DRE treatment, able to influence the brain activity without carrying the invasiveness of intracranial surgery.

The relative lack of systemic side effects, pharmacokinetic interactions, compliance problems and teratogenicity, together with the potential of on-demand use for immediate intervention during an ongoing seizure, would make peripheral neurostimulation devices use, advantageous compared to the addition of yet another AED (Schulze-Bonhage, 2017).

2.1.1 Vagal nerve stimulation (VNS)

Among peripheral neurostimulation devices, VNS is the one who received, in addition to CE marking, FDA approval in 1997 as adjunctive treatment for partial-onset seizures in DRE patients >12 years (The Vagus Nerve Stimulation Study Group, 1995; Handforth et al., 1998) and it has been proved, since then, in more than 40,000 cases of DRE throughout the world (Amar et al., 2000).

The stimulation device (Cyberonics Inc, Houston, TX, USA) is similar to a cardiac pacemaker and it is implanted, under local or general anaesthesia, under the left clavicle. Two helical electrodes are wrapped around the left vagus nerve which is accessed in the midcervical portion of the neck between the carotid artery and the jugular vein and a delicate surrounding network of small vessels (DeGiorgio and Krahl, 2013b; Ben-Menachem, 2002; Ekmekçi and Kaptan, 2017). The right nerve is generally not used for risks of bradycardia or arrhythmias (Randall and Ardell, 1985). The stimulation has given in open loop cyclic modality with a starting level of stimulation of 0.25 mA (gradually increased up to 1.25–2.00 mA over several weeks); 20–30 Hz; pulse width 250–500 μs; 30 s ON, and 5min OFF. The patient may activate on demand stimulation in all models by swiping a magnet over the device (Heck et al., 2002).

Recently, a non-invasive self-administrable external device which provides transcutaneous VNS (tVNS) by an intra-auricular electrode, received European Union (CE mark) approval for the epilepsy treatment; however a recent prospective multicenter trial, comparing 25 Hz versus 1 Hz stimulation, failed to show superiority of tVNS in the treatment group (high stimulation) compared to the sham group (low stimulation) (Bauer et al., 2016). Further, Cyberonics developed a closed-loop VNS device which incorporates a closed-loop system that detects heart rate increase that may be associated with seizure events and delivers stimulation automatically.

2.1.1.1 Mechanisms of action

The vagus (X cranial) nerve is a mixed parasympathetic nerve, containing both afferent and efferent sensory fibers. An estimated 80% of vagus nerve fibers are afferent and convey visceral, somatic and taste sensations. The efferent tracts innervate striated muscle of the larynx and parasympathetic projections for the heart, lungs, and gastrointestinal tract (Rutecki, 1990). Vagus nerve provides an asymmetrical heart innervation which explains why VNS implants are placed on the left side of the neck (atrioventricular node innervation) and not the right side (sinoatrial node innervation).

The nerve is essentially composed of A- large myelinated fibers and C- small unmyelinated fibers, which carry afferent visceral information to the brain, and B- small myelinated fibers for parasympathetic efferent signals (Foley and DuBois, 1937).

Multiple studies have demonstrated that the mechanism of VNS is essentially mediated by central responses to activation of afferent fibers rather than peripheral projections (Zabara, 1992; Osharina et al., 2006).

What has been largely debated is whether only A and B myelinated fibres or also C afferent fibres are needed for the antiepileptic effect of VNS. Evidence against the C fiber theory is the lack of significant effect on pain sensation, heart rate or blood pressure or other autonomic functions after VNS (Banzett et al., 1999; Lotvall et al., 1994) and evidence that selective capsaicin-induced destruction of these small unmyelinated fibers in rats, does not alter VNS antiepileptic effects (Krahl et al., 2001).

The clinical importance of these experimental data consists on the lower amount of current necessary to produce the antiepileptic effects of VNS, since A- and B-fibers have a much lower activation threshold as compared to C-fibers, which permit to avoid side effects from requiring higher current or longer pulses stimulation parameters (Krahl and Clark, 2012).

The afferent fibres project to the nucleus tractus solitarius (NTS), the dorsal motor nucleus, area postrema, medial reticular formation and the nucleus cunneatus. NTS in

turn, connects with higher centres in the brain such as the hypothalamus, dorsal raphe, nucleus ambigus, dorsal motor nucleus of the vagus nerve, amygdala and thalamus, which in turn projects to the insular cortex (Cheng et al., 2004). Moreover, NTS also projects both directly and indirectly to monoamine nuclei in the brainstem, the locus coeruleus (LC) and the raphe nuclei (RN) (Krahl and Clark, 2012).

Because of its extensive connection with multiple sites of potential seizure genesis throughout the brain, NTS is hypothesized as a central mediator of VNS action (Beekwilder and Beems, 2010).

Increasing evidence suggested that noradrenergic and serotonergic neurons may exert antiepileptic effects in a wide variety of seizure models (Krahl and Clark, 2012) and that LC and RN may mediate the major effect of VNS, as bilateral chemical lesions of these centers abolish the antiepileptic effect of VNS therapy (Krahl et al., 1998; Fornai et al., 2011).

Furthermore, recent studies showed that VNS increased the basal firing rates of LC neurons as well as their propensity to fire in bursts of action potentials (Groves et al., 2005). This support previous finding in rat models treated with VNS, which showed an induced c-fos expression in the LC as an index of recent increase in local neuronal activity (Naritoku et al., 1995).

Norepinephrine and serotonin have been further shown to induce GABA interneuron releasing in the brain, and it has been also previously demonstrated that VNS causes an increase of the free GABA levels in the cerebrospinal fluid, therefore potentially increasing seizure threshold (Ben-Menachem, 1995).

Importantly, also the connection between vagus nerve, thalamus and limbic system would be fundamental in the VNS mechanism of action. The midline and intralaminar nuclei of the thalamus have a widespread influence on neocortex and appear to be related to an "arousal-desynchronized" cortical state (Steriade and Glenn, 1982; van der Werf et al., 2002) which has been implicated in VNS seizure-reduction mechanism (Jaseja, 2010). The thalamic nuclei receive several brainstem afferent inputs, including NTS, LC and RN (Krout et al., 2002). Theoretically any manipulation that interrupts any thalamic pathological activity would also interrupt the spread of the seizure activity as it travels across the neocortex (Fanselow, 2012).

Also the limbic system, which is heavily implicated in many epilepsy types, receives afferent input from LC and norepinephrine concentrations are increased in the limbic system by VNS (Roosevelt et al., 2006).

Studies employing neuroimages such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) have tried to further elucidate the role of cortical and/or subcortical regions associated with VNS, showing

regional metabolism changes in some cerebral areas during treatment, such as the ipsilateral anterior thalamus, cingulate gyrus, cerebellum, hypothalamus and insular cortices (Chae et al., 2003; Henry et al., 1999, 2004; Ring et al., 2000).

Henry and colleagues (1999) also found that PET scans performed during VNS treatment, demonstrated increases in thalamic blood flow with positive correlations with the anticonvulsant effects and seizure responsiveness. Altogether, these findings show that VNS is potentially able to interact with cortical functioning and activity through a series of brainstem-cortical interconnections, such as monoamine nuclei, thalamus and limbic system, all of which may affect the brain propensity to generate seizures (Figure 1).

However, due to the lack of understanding of epilepsy mechanisms and the precise brainstem sites of action, how VNS exactly exerts is therapeutic effect remains essentially an open question.

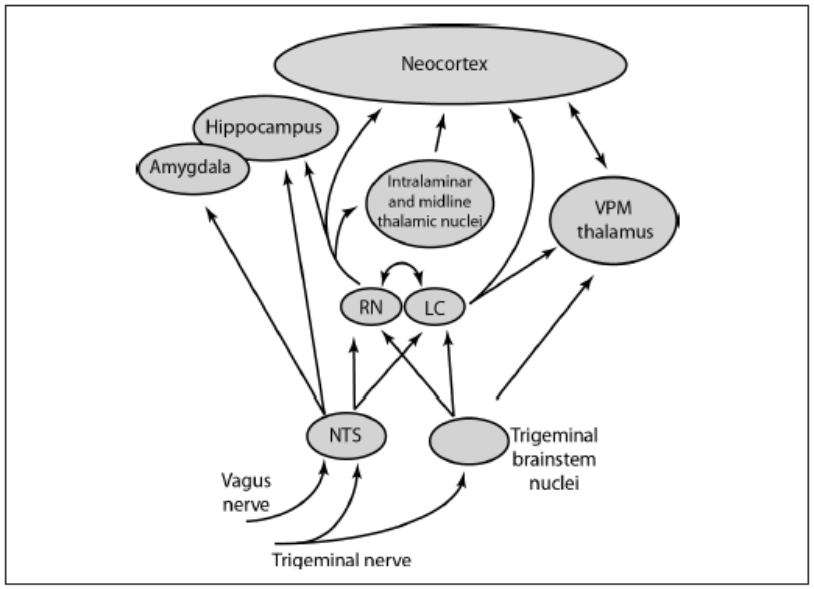


Figure 1: Schematic diagram of ascending projections within the central nervous system that are influenced by input from the trigeminal and vagus nerves. Note that this diagram only depicts a subset of the many divisions of and projections between these highly interconnected brain regions

Figure 1. *From Fanselow, 2012*

2.1.1.2 Clinical indications and efficacy

The incidental findings about the concomitant improvement in mood, cognition, and well-being during VNS therapy in DRE, have raised interest regarding the utility of this treatment in neurological disorders other than epilepsy, such as cognitive, mood and behavioural disturbances, headache and migraine, multiple sclerosis and sleep disorders (Attenello et al., 2016; Beekwilder and Beems, 2010; Yuan and Silberstein, 2016).

Further, according to some authors, VNS might act restoring the imbalance between sympathetic and parasympathetic tone which has been implicated in the

pathophysiology of a number of other disorders, such as heart failure, inflammatory bowel disease and chronic pain syndromes (Ghia et al., 2006; De Ferrari et al., 2011).

However, apart from severe, chronic and recurrent depression in patients >18 years and focal-onset DRE in patients >12 year of age, who already received FDA approval for VNS treatment in 2001 and 1997 respectively (Cyberonics, Inc., 2012), all the other therapeutic aspects are still under investigation and may represent in the future promising indications for VNS therapy.

The first use of VNS in the treatment of DRE in humans was in 1988 by Penry and Dean (1990) which led, during the subsequent years, to a number of clinical trials and publications assessing efficacy, safety, and tolerance of VNS therapy.

The evidence for the efficacy and subsequent the approval of VNS for epilepsy treatment was based on two randomized, double-blind, active-controlled trials named EO3 and EO5, respectively.

EO3 was conducted in 1995 by the *VNS study group* (The Vagus Nerve Stimulation Study Group, 1995) which compared efficacy of VNS in 114 patients divided in two active groups and receiving high (30 s ON and 5 min OFF, 30 Hz, 500 μs, up to 3.5 mA) or low (30 s ON and 90 min OFF, 1 Hz, 130 μs, ≤ 3·5 mA) stimulation current and followed up for 12 weeks. The true placebo group was not possible because VNS output currents are felt by the patients. The mean seizure reduction was 24.5% *vs* 6.1% in high-

and low-stimulation groups, respectively (p = 0.01). Thirty-one percent of patients receiving the high stimulation had a reduction of 50% or more in seizure frequency.

EO5 study (Handforth et al., 1998) was conducted on 190 patients with similar stimulation regimens to the EO3 study, followed up for 12 weeks. After 3 months of stimulation there was a 28% decrease in seizures in the high-stimulation group and a 15% decrease in the low-stimulation group (p=0.039). Between-group comparisons for 50% responders were not significant (23.4% *vs* 15.7%). However, the high-stimulation group was more likely to achieve a 75% seizure reduction than the low stimulation group (10.6% *vs* 2.0%).

Subsequently, there have been two major prospective, long-term follow-up studies that assessed the change in seizure frequency and tolerability from the end of the previous clinical trials (EO3 and EO5) for up to 1 year (DeGiorgio et al., 2000) and up to 3 years (Morris and Muller, 1999). In the study by DeGiorgio et al. (2000) patients were followed-up for 1 year and showed a median seizure reduction of 45% and seizure reduction rates of ≥50% or ≥70% in 34% and 20% respectively. In the EO1–EO5 study (Morris and Muller, 1999), a total of 440 patients were followed up for 3 years. Seizure reduction of more than 50% was seen in 23% of patients at 3 months, 36.8% at 1 year, and 43% at both 2 years and 3 years.

Overall, while efficacy of VNS seems to increase over the time of treatment, reaching a plateau after one to two years (50%-60% of patients achieve ≥50% reduction rate in 2 years) (Morris and Mueller, 1999), side-effects tend to decrease progressively during the follow-up (Englot et al., 2011). Complete seizure freedom is achieved instead in only a small (8%) percentage of patients (Englot et al., 2016).

VNS seems to exert the most of its therapeutic effects with prolonged treatment (long-term) rather than as an immediate consequence of acute stimulation. The facts that efficacy may increase in long-term use suggests that VNS may not only work via immediate stimulation but also through a mechanism of brain network remodulation towards a less epilepsy-prone state (Schulze-Bonhage, 2017).

In 2015, *Cochrane Epilepsy Group* (Panebianco et al., 2015) published updated evidence regarding the efficacy and tolerability of VNS treatment in DRE. Authors recruited a total of five trials and 439 participants and showed that the overall risk ratio (95% CI) for ≥50% reduction in seizure frequency across all studies was 1.73 (1.13 to 2.64) and that high frequency VNS was over one and a half times more effective than low frequency VNS.

According to the AAN guideline published in 2013 (Morris et al., 2013), invasive VNS received level C evidence for achieving >50% seizure reduction in DRE patients and

may be considered for seizures in children, for Lennox-Gastaut syndrome associated-seizures and for improving mood in adults with epilepsy (Level C).

2.1.1.3 Adverse effects

The more VNS device has been used over the years, the more we have learned about the adverse events associated with this neurostimulation method.

Adverse events can be divided into acute, essentially related to surgery procedure and long-term events, principally related to the nerve stimulation *per se*.

Postoperative infections have been reported in 3–6% of patients (Handforth et al., 1998; DeGiorgio et al., 2000; Ben-Menachem et al., 1999) which led to device removal in three of 198 (1.5%) patients in EO5 study (DeGiorgio et al., 2000). Reversible left vocal cord paralysis occurred in one patient in the EO3 study and in 2 patients in the EO5 study. Lower facial weakness was reported in 2 patients in the EO3 study and in 1 patient in the EO5 study. Ventricular asystole during testing of the device on implantation has been estimated one in 875 (0.1%) patients (Ben-Menachem, 2002).

In 1999, Morris and Mueller published all the side effects collected from patients enrolled and followed-up in five previous clinical trials. The most common adverse events were hoarseness (28%) and paresthesias in throat-chin region (12%) after 1 year,

hoarseness (19%) and cough (5.9%) after 2 years and shortness of breath (3.2%) after 3 years (Morris and Mueller, 1999; Beekwilder and Beems, 2010; Ben-Menachem, 2002) (Figure 2).

The symptoms were mild or moderate, often reversible and improved with time, rarely requiring reduction of the stimulation intensity or adjustment of other stimulation variables and seldom the removal of the device. No changes in autonomic function were seen, such as blood pressure, heart rate, Holter monitor measures, lung function, or blood chemistry. No idiosyncratic side-effects have been reported after 12 years of experience, and VNS did not seem to interact with antiepileptic drugs. Central nervous system (CNS) side effects, such as tiredness, psychomotor slowing, irritation, and nervousness have not been frequently reported and they did not stand out as major side-effects of VNS. Importantly, VNS treatment did not show excessive death rates compered to controls and SUDEP were lower than in similar groups of DRE patients not receiving VNS treatment (Ben-Menachem, 2002).

Proportion of patients with side-effects at stages in long-term treatment

	Proportion (%) of patients with side-effects		
Side-effect	**3 months[18]**	**12 months[30]**	**5 years[51]**
Cough	21	15	1·5
Voice alteration	62	55	18·7
Dyspnoea	16	13	2·3
Pain	17	15	4·7
Paraesthesia	25	15	1·5
Headache	20	16	
Pharyngitis	9	10	
Depression	3	5	
Infection	4	6	

Figure 2. Long-term VNS side-effects based on Handforth et al., 1998(*18), DeGiorgio et al., 2000(*30), Ben-Menachem et al., 1999(*51). *From Ben-Menachem, 2002*

2.1.2 Trigeminal nerve stimulation (TNS)

Transcutaneous TNS represents a relative new neuromodulation approach. It received a CE mark in September 2012 and it has been approved in the European Union as adjunctive treatment for drug-refractory partial-onset epilepsy and depression in adults and children ≥ 9 years of age (DeGiorgio and Krahl, 2013b). VNS has not received FDA approval yet, so it is available only in the European Union.

A pulse generator device is worn externally by the patients and used to deliver bipolar stimulation through transdermal patch electrodes which have been applied to the forehead (supraorbital nerve foramina) or to the patient's cheeks (the infraorbital nerve foramina) in order to bilaterally stimulate the V1 or V2 branches of the trigeminal

nerve, for times between 8 and 12 h, predominantly at night while asleep (Figure 3). Stimulation has given in open loop cyclic modality and parameters are generally set at 120 Hz, pulse width 250 μs, up to 30 s ON and up to 30 s OFF (DeGiorgio et al., 2003; Nune et al., 2015; Cook et al., 2015).

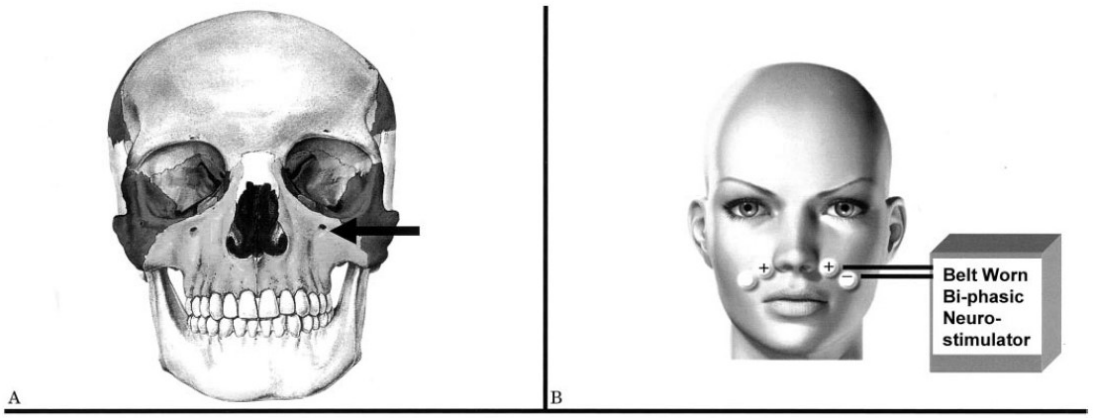


Figure 3. (A) The trigeminal foramen in a frontal view of the skull. The arrow points to the infraorbital foramen. (B) Schematic diagram showing electrode placement. *From DeGiorgio et al.,2003.*

TNS offers some practical advantages compared to VNS. The external transcutaneous stimulation permits a safety neuromodulation effect at low cost and without the challenges of surgical implantation (Nune et al., 2015; DeGiorgio and Krahl, 2013b); also bilateral TNS appeared to be more effective than unilateral and this can permit larger effects at lower intensities (Fanselow et al., 2000; DeGiorgio et al., 2011).

Individual patients may tolerate and respond to various parameter changes so that patients are generally allowed to control the amplitude of the current delivered and are asked to use the maximum comfortable level (DeGiorgio and Krahl, 2013b). It has been shown that seizure-reduction effect increases as the amplitude and frequency of stimulation increase (Fanselow et al., 2000).

There are currently no head-to-head trials comparing the different neuromodulation devices but efficacy appears to be roughly similar; hence, in the a lack of reliable predictors for individual treatment response, the choice of the device is mostly based on individual preferences and clinical features (Nune et al., 2015; Schulze-Bonhage, 2017).

2.1.2.1 Mechanisms of action

Following the promising findings coming from VNS studies, and based on the hypothesis that seizure-reduction effects are not specific to the vagus nerve but can instead be achieved through multimodal stimulation of the reticular activating system (Moruzzi and Magoun, 1949; McLachlan, 1993), scientific interest moved to the development of novel stimulation approaches.

In 2000, Fanselow and colleagues tried to understand whether, like VNS, TNS was able to generate an arousal-like effect in acute seizure models and so to reduce seizure activity in awake rats.

This was a pioneering work in understanding the efficacy and tolerability of TNS in epilepsy treatment, opening a new way for alternative therapeutic options in humans (DeGiorgio et al., 2006).

Fanselow and colleagues (2000) demonstrated that TNS, delivered through a cuff electrode unilaterally or bilateraly implanted in the infraorbital branch of the trigeminal nerve, was able to significantly reduced EEG and behavioural seizure-like activities in pentylenetetrazole (PTZ) treated rats.

This anti-seizure effect became evident for TNS delivered both in closed modality (seizure-triggered stimulus) and in open cyclic modality and appeared effective in reducing the synchronous seizure activity detected on field potential traces which had been recorded from ventral posterior medial thalamus (VPM) and/or primary somatosensory (SI) cortex, through chronically implanted microwires. Bilateral stimulation was more effective than unilateral stimulation, and stimulation was well tolerated.

Once again, the claimed mechanism for explaining seizure-reduction effect was the indirect stimulation of subcortical structures leading to nonspecific generalized arousal via the reticular-activating system.

This was supported by previous work by Moruzzi and Magoun (1949) demonstrating that stimulation of the midbrain reticular formation causes EEG desynchronization and

by the fact that Fanselow and colleagues (2000) interpreted the reduction in frequency and duration of the filed potential seizure-like signals, as an index of desynchronization of thalamic and cortical activity.

The trigeminal nerve has three afferent branches which collect afferent sensory information (e.g. tactile and pain) from the different regions of the face and project them to the CNS via trigeminal ganglion towards trigeminal nuclei and then to the VPM of thalamus and (SI) cortex. Trigeminal nuclei in turns also project to multiple nearby nuclei in the brainstem, including the LC and the NST, which we have already known being fundamental stations interposed from brainstem to CNS with potential neuromodulation and seizure-preventing activity (Krahl and Clark, 2012).

This *buttom-up* mechanism (from peripheral nerve, subcortical structures to the cortex) has been postulated also for VNS, since vagus nerve shared part of the abovementioned widespread projections with trigeminal nerve (Fanselow, 2012).

However, in addition to the vagus nerve, the trigeminal nerve has a more direct and faster pathway (10 ms from the periphery to SI) through which peripheral signals rapidly propagate to the VPM and then to SI.

It is possible that neocortical activity, resulting from these afferent modulating inputs, may cases or prevents spreading of seizures playing a primary role in the therapeutic effects of TNS (Fanselow, 2012).

While it seems to be clear that subcortical structures play a fundamental role in seizure regulation mechanisms by TNS (Mercante et al., 2015, 2017; Pilurzi et al., 2015; Fanselow, 2012), evidence regarding the TNS capacity to alter cortical excitability is scarce and inconclusive (Axelson et al., 2014; Mercante et al., 2015; Fanselow and Noel, 2010).

Recently published works provided evidence regarding TNS-induced brainstem modulation (Mercante et al., 2015, 2017) and brainstem plasticity (Pilurzi et al., 2016) in heathy subjects, while cortical excitability of the primary motor cortex (M1) and sensorimotor integration at cortical level appeared unaffected by acute stimulation, suggesting no direct modulation of TNS on higher structures, at least for M1 (Mercante et al., 2015).

A previous study by Axelson and co-workers (2014) confirmed that TNS does not acutely affect cortical excitability of M1 in healthy subjects studied with transcranial magnetic stimulation (TMS) protocol before, during and after 40 min of 120 Hz bilateral external continuous TNS.

However, a direct and frequency-dependent inhibition of neuronal firing in SI cortex neurons in response to contralateral TNS has been recently shown in rat models (Fanselow and Noel, 2010), providing support to the intrinsic cortical inhibition as a mechanisms for antiepileptic effect of TNS.

Neuroimaging studies also showed a widespread cortical and subcortical influence of TNS at different level without actually clearing this issue. PET studies showed metabolism changes in terms of activation in the inferior e middle frontal gyrus, bilateral parietotemporal cortex and bilateral anterior cingulate gyri and deactivation in the left parahippocampal gyrus, right sensorimotor cortex, right superior parietal area, bilateral temporo-occipital cortex and bilateral visual cortex (Cook et al., 2011; Silverman et al., 2011; Schrader et al., 2012).

2.1.2.2 Clinical indications and efficacy

The first pilot feasibility study that evaluated the safety and efficacy of TNS for epilepsy treatment in humans was published by DeGiorgio and colleagues in 2006.

This study showed promising data, first indicating that transcutaneous stimulation of the supraorbital and infraorbital divisions of the trigeminal nerve was safe and well tolerated for the 6 month treatment periods and also that four (57%) of seven subjects, who completed ≥3 months treatment, experienced a ≥50% reduction in seizure frequency, as observed from clinical diaries.

These findings supported further studies on this field (DeGiorgio et al., 2009) and few years later DeGiorgio and co-workers (2013a) conducted the first double-blind

Randomized Controlled Trial (RCT) providing Class II evidence that TNS may be safe and effective in reducing seizures in people with DRE showing a responder rate (i.e. ≥50% reduction in seizure frequency) of 30.2% for the treatment group *vs* 21.1% for the active control group, for the 18-week treatment period.

Although not significant in between-group results (difference in responder rate in active *vs* control group) the significance in the within-group results (responder rate increased significantly over the 18-week treatment for the active group *vs* controls) was considered sufficient to justify a CE approval.

From 2012 TNS became available in the European Union as adjunctive treatment for drug-refractory partial-onset epilepsy in adults and children ≥ 9 years of age.

The phase II randomized controlled trial was completed with a long-term follow-up study at 1 year, which showed a 50% responder rates at twelve months of 36.8% for the treatment group and 30.6% for all the subjects (Soss et al., 2015).

Currently NeuroSigma has received an investigational device exemption to proceed with a Phase III double-blind pivotal trial in DRE to evaluate the efficacy of adjunctive TNS against sham controls (Cook et al., 2015).

Besides DRE, TNS has been implicated in treatment of different neuropsychiatric disorders (Shiozawa et al., 2014), in particular for migraine (Schoenen et al., 2013) and

depression (Cook et al., 2010, 2013; Shiozawa et al., 2015) but only for the latter TNS has obtained CE approval.

2.2.2.3 Adverse effects

Overall, all the available studies on TNS treatment in DRE patients showed that this is a safe and well-tolerated method for DRE treatment (DeGiorgio et al., 2006, 2009, 2013a; Zare et al., 2014; Soss et al., 2015).

Detailed analysis of adverse events and safety, related to TNS long-term open-label study (DeGiorgio et al., 2009), were summarised within a *Brief Communication* published by Pop and colleagues (2011) in the journal *Epilepsy & Behavior* (Figure 4).

Summary of adverse events.[a]

Subject	Adverse event	Severity	Device related?	Did adverse event resolve in study period?	Comment
1	Sleep disturbance	Mild	Yes	Yes	
2	1. Jaw pain	Moderate	Yes	Yes	Resolved at lower current; switched to supraorbital after 10 months
	2. Eye twitching	Moderate	Yes	Yes	
	3. Tingling left canine tooth	Moderate	Yes	Yes	
	4. Frontal headache	Moderate	Yes	Yes	
3	1. Tooth discomfort	Mild	Yes	Yes	Resolved at lower currents
	2. Twitching of the eye muscle (orbicularis)	Mild	Yes	Yes	
4	1. Tingling left canine tooth	Mild	Yes	Yes	Tingling and sleepiness resolved at lower currents
	2. Mood changes/irritability	Moderate	No	No, preexisting	
	3. Sleepiness	Mild	Yes	Yes	
6	1. Headache	Mild	Yes	Yes	1, 3, 4 resolved with reduction in intensity
	2. Depression	Mild	No	Preexisting	
	3. Sharp bitemporal pain	Moderate	Yes	Yes	
	4. Irritability and anxiety	Mild	No	Yes	
7	1. Behavioral problems	Moderate	No	No	Preexisting behavioral problems, unrelated to device; exited after 1 month
	2. Cluster of seizures	Severe	No	No	
8	Headache	Mild	Yes	Yes	
9	Depression	Moderate	No	Yes	Preexisting, not related to device
10	Frontal headache	Mild	Yes	Yes	Resolved with reduction in current
11	Depression	Moderate	No	No	Mood problems prior to entry into study; started on an antidepressant during study
13	1. Erratic stimulation	Mild	Yes	Yes	1. Device delivered erratic stimulation; device replaced with new stimulator and erratic stimulation ended
	2. Tremor	Mild	No	Yes	2. Tremor caused by valproate
14	Mood changes	Mild	No	Yes	Related to topiramate; topiramate tapered

[a] Subjects 1–4 received infraorbital stimulation initially. Subjects 5–14 received supraorbital stimulation initially. Skin irritation reported in text.

Figure 4. Summary of adverse events reported during long-term open-label TNS study (DeGiorgio et al., 2009). *From Pop et al., 2011*

Mild to moderate skin irritation was the major adverse event, being reported by eight (62%) of the 13 subjects and followed by mild sleepiness or a sleep disturbance (15%), headache (31%), tooth tingling or tooth pain. All of these side effects resolved with decreased stimulation intensity or discontinuation of the device. Pre-existing mood changes or depression was reported in six subjects (46%), unrelated to the device use.

Anxiety (4%), headache (4%) and skin irritation (14%) were also the most common side effects reported in RCT (DeGiorgio et al., 2013a), while sensation of pressure on the head (27.7%), pain (22.2%), skin reaction (16.6%) and tingling (16.6%) were the most common adverse effects reported in the study by Zare et al. (2014).

No significant acute or long-term adverse effects on heart rate or systolic or diastolic blood pressure were warned by all the studies (DeGiorgio et al., 2006, 2009, 2013a; Pop et al., 2011; Soss et al., 2015).

3. EFFECT OF PERIFERIC NEUROSTIMULATION ON BACKGROUND EEG ACTIVITY: STATE OF THE ART

3.1. VNS

The hypothesis that VNS might influence cortical EEG activity took hold in the 20th century from animal studies. In 1938 Bailey and Bremer first described an increased

EEG potentials recorded at the frontobasal cortex of encéphale isolé cat after the stimulation of the central part of the dissected vagus nerve.

Their publication was followed by several other animal studies describing induced EEG background activity changes after VNS, both in terms of EEG desynchronization (Zanchetti et al., 1952; Moruzzi and Magoun, 1949) and synchronisation, depending on the stimulation parameters used and on the histological composition of the nerve fibers receiving the electrode impulses (Chase et al. 1967; Magnes et al., 1961).

Chase and colleagues (1967) found that the EEG background activity was synchronized when cervical VNS was able to activate the faster-conducting, low threshold myelination A- and B-fibers, whereas when stimulus parameters were adjusted to activate C-fibers, the EEG became desynchronized.

Because seizure is characterized by highly degree of EEG synchronous activity, it was hypothesized that desynchronization of the cortical activity would be the mechanism of the anticonvulsant effect exerted by VNS.

Hence, based on these observations C-fibers were initially considered necessary for the anticonvulsive mechanism of VNS.

However, subsequent findings proved that the activation of C-fibers were clearly not required for the anticonvulsant effect of VNS (Krahl et al., 2001) as demonstrated by

the clinical absence of pain sensation and significant autonomic changes after VNS in humans (Banzett et al., 1999; Lotvall et al., 1994).

The induced widespread cortical desynchronization seen after VNS has been linked to a state of *nonspecific arousal,* similar to full vigilance or alertness state induced by indirect activation of reticula system in the brainstem, effect which appeared not exclusive for VNS. Other animal studies previously described similar neuronal desynchronization after stimulation of other peripheral nerves interposed to cortical ascending pathways (Pompeiano and Swett, 1962). Moreover in 1993, McLachlan showed that aspecific sensory or behavioural stimulation, such as placement of rats' tails into hot water, reduced interictal spike frequency in the same manner as VNS, probably thorough an indirect stimulation of reticular activating system.

Therefore, supported by a large amount of animal evidence, for the subsequent years the EEG-desynchronization effect was considered the major mechanism of anti-epileptic action of vagal nerve stimulation in patients with DRE (Jaseja, 2010).

Being clear since now that long-term VNS is able to clinically reduce seizure frequency (Panebianco et al., 2015) and that such effect might be mediate from an aspecific cortical desynchronization induced through the stimulation of different ascending subcortical projection systems (Jaseja, 2010; Fanselow, 2012), understanding whether

and how VNS exactly modifies human background EEG activity have been the aim of different studies over the last decades.

3.1.1 Epileptiform activity changes

The anti-seizure effects of short-term, and less often long-term, VNS protocols have been largely documented from animal experiments comprise stimulation administered just before, during or after the onset of induced seizures, which showed that VNS is able to suppress many different seizure types as demonstrated by ictal EEG findings (Aalbers et al., 2011).

Human studies objectively showing interruption or reduction of ictal EEG seizure activity by VNS are instead very scarce (Ravan et al., 2017a, 2017b; Hallbook et al., 2005) or even only anecdotal (Hammond et al., 1992; Salinsky and Burchiel, 1993), probably because of the difficulties in evoking or predicting seizures and so in conceiving and developing a reliable study design on this regard. Hence, the efficacy of VNS, in terms of seizure reduction during clinical trials, has been generally based on clinical diary reporting.

Most of the human studies have been instead focused on the short- and long-term effect of VNS on interictal epileptiform discharge (IEDs) change detection in DRE patients; however showing inconsistent or conflicting results.

In 1992 Hammond and colleagues failed to show any acute changes in IEDs burst duration or morphology during VNS in 9 patients with DRE.

This finding was later contradicted by Koo (2001) which observed a statistically significant reduction in IEDs and an increased periods of spike-free intervals, in subsequent EEGs collected from 21 VNS-implanted DRE patients which had been followed up to 3, 6 and 12 months. This EEG interictal changes have been found higher in patients who demonstrate active and frequent epileptiform activity, irrespective of the patients' seizure control.

One year later, also Kuba and colleagues (2002) found that short-term VNS was able to reduce IEDs significantly and that the value of IEDs reduction was higher in DRE patients who showed to clinically respond to VNS. The same effect was then confirmed from the same group also in a long-term analysis, in patients chronically stimulated with VNS (Kuba et al., 2010).

In 2004, Rizzo and colleagues again failed to detect significant changes in IEDs reduction during long-term VNS treatment, although they described a trend towards a

decrease in the number of IEDs and a significant shortening in the duration of the discharges (Rizzo et al., 2004).

Also Santiago-Rodrıguez and colleagues (2006) showed inconclusive results from their work, acute VNS modified number and duration of IEDs in 80% of patients undergoing the 30 s/5 min cycle stimulation but it did not show any effect at 7 s/18 s cycle; however in 20% of patients, both cycles increase the epileptiform activity.

However, the subsequent works from Hallbook et al. (2005) and Wang et al. (2009) appeared concordant while showing that long-term VNS is able to induce a statistically significant progressive decrease in the number of IEDs in treated DRE patients.

In conclusion, although no definitive and conclusive results can be drown from the findings above, this does not mean that acute or long-term VNS have any therapeutic effect on DRE patients. Indeed, it is already well known that clinical outcome does not necessary correlate with EEG interictal findings and also, since VNS efficacy has been shown to increase over the time, probably with a mechanism of network remodulation (Schulze-Bonhage, 2017; Fraschini et al., 2014), long-term EEG VNS-effect may not be detected even after several months of follow-up.

3.1.2 Quantitative EEG analysis

Besides VNS-induced IEDs pattern modulation, another interesting field of investigation is whether and how VNS may affect the architecture of background EEG dynamics, in terms of quantitative spectral analysis and correlations between signals. This issue has been investigated by different authors over the years.

Starting from the hypothesis that VNS is able to induce a cortical desynchronization on EEG activity of animal models (Aalbers et al., 2011), Salinsky and Burchiel (1993) tried to define the acute effects of VNS on human EEG background rhythm composition. They concluded that VNS, at the parameters in current clinical use, does not alter the awake EEG background rhythms, either in term of EEG total power and median frequency, or in power of any conventional frequency bands, also in those patients with apparent clinical response. Therefore their results did not support the hypothesis that VNS produces acute desynchronization in human awake EEG.

Also Hammond and colleagues (1992) showed that acute VNS (at stimulus frequencies of 1, 5, 10, and 50 Hz) had no effect on the EEG background rhythm composition of awake, sleep or anesthetized patients.

However, in 2004 Rizzo and colleagues evaluating the impact of chronic VNS on sleep/wake background EEG activity showed an increase in the EEG total power and a statistically significant increase in delta and theta rhythm in NREM sleep, and of alpha

in wakefulness and REM sleep. They suggested that only long-term VNS produces, instead of an EEG desynchronization effect, an enhancement in normal background rhythms which characterised each sleep/wake state, maybe enhancing brain's ability to generate a better structured electrical activity. Contrariwise, no direct effect of acute VNS was presented in sleep/wake EEG, in line with findings of previous authors.

One year later, Marrosu and colleagues (2005) investigated in their work both the EEG power spectra and the inter- and intra-hemispheric synchronization (coherences of EEG signals) changes of the EEG frequency bands in 19 long-term VNS-treated DRE patients, compared to a control group of 10, only AED treated, DRE patients. They found that VNS decreased synchronization of theta frequencies and increased power spectrum and synchronization of gamma bands (20–50 Hz) in the VNS treated group, compared to controls. Authors proposed these findings as a possible explanation of VNS anticonvulsant mechanism, considering the gamma band enhancement as a kind of "protective effect" against seizures.

The same group, in a subsequent paper (Fraschini et al., 2013), investigated the correlation between variations of global EEG synchronization and the clinical outcome (VNS responders *vs* non-responders) in 10 long-term VNS-treated DRE patients. The investigation was conducted with a different methodological approach, the phase lag index (PLI), which allowed the study of the global rate of synchronicity among the EEG

signals before and after VNS implantation and lead to different interpretation of the EEG analysis. They found that VNS induced a desynchronization in the gamma frequency band which was statistically significant in VNS responders compared to the non-responder group and they interpreted the global desynchronization in gamma band as a potential part of the antiepileptic effect exerted by VNS, other than a possible new tool in assessing the efficacy of VNS treatment.

The PLI index was later used also by Bodin and colleagues (2015) who studied the level of synchronicity of inter-ictal EEG rhythms in 19 patients undergoing chronic VNS therapy, between pre- and post-VNS treatment, ON and OFF stimulation phases and between responder (R) and non-responder (NR) patients. The main results were that patients with a good response to VNS have a lower level of synchronization in the interictal state than non-responders (particularly in delta and alpha bands). They also found that stimulation (ON) periods were associated with a decrease in synchrony in comparison with the non-stimulation (OFF) periods. Once again, the hypothesis of VNS-induced EEG desynchronization was confirmed from a human study and interpreted as a mechanism of seizure prevention.

Overall, to summarize findings above we can conclude that the inconsistency reported in the study results has probably to be attributed to the large variability and differences in study design, including individual and group characteristics, VNS stimulus

parameters, different methodological approach for the analyses and the result interpretation.

Therefore, after almost two decades of VNS utilization, its mechanisms of action, and so how it is able to clinically determine a significant seizure reduction in DRE patients (i.e. interfering with interictal/ictal discharges or modulating background rhythm composition and/or synchronisation), appears still unclear.

1.2 TNS

Evidence regarding EEG background changes following TNS protocol in human and animal models is very scares, also compared to the large amount of studies relative to VNS, as seen above. This might be due to the relative lower widespread availability of the novel method (European Union only), which has determined its lower use, and the more recent launch and approval obtained from CE.

3.2.1 Epileptiform activity changes

The first evidence regarding objective EEG epileptiform activity changes after TNS comes from the study of Fanselow et al. (2000) where they showed that the number and

the frequency of PTZ-induced seizure activities were reduced during the stimulation of the infraorbital nerve after cyclic and automatic seizure-detection delivery.

To demonstrate that, authors implanted several microwires in VPM of the thalamus and SI cortex in rat brains, in order to recorded field potential changes as index of seizure activity. The two indicators of seizure activity were highly synchronous, large-amplitude above-threshold activity in the thalamic and cortical field potential traces and the corresponding presence of behavioural changes (i.e. clonic jerking of the body and forelimb).

They found that stimulation of the infraorbital nerve was able to stop synchronous seizure-like activity and to reduced frequency and duration of seizures in a current-dependent manner by up to 78%.

Recently some animal studies focused on TNS-induced behavioural changes in rats after PTZ or Pilocarpine injections, showed promising and consistent results in terms of seizure reduction and TNS effectiveness compared to sham controls (Wang et al., 2016; Mercante et al., 2017). However, epileptiform EEG changes following TNS have been not evaluated in either of those mentioned studies.

Mercante and co-workers (2017) performed a behavioural pattern analysis, by means of the Racine's scale visual scores, in 10 male rats treated with real or sham TNS, during pentylenetetrazole (PTZ)-induced seizures. They found that in comparison with sham

groups, TNS significantly decreased the duration of PTZ-induced seizures ($p<0.05$) and promoted a faster recovery ($p<0.001$) by reducing the most severe seizure types.

Regarding humans, there is only one published study which anecdotally reported a refractory status epilepticus (RSE) ceasing and EEG improvement after the adjunctive treatment with TNS in a DRE patient (Moseley and DeGiorgio, 2014).

3.2.2 Quantitative EEG analysis

So far, only one clinical case has been published on quantitative EEG analysis after TNS treatment in a 60-year old female patient with drug-resistant depression (Shiozawa, 2016).

Ten daily TNS sessions (with an interval in the weekend), of 30 min each was performed. The electrodes were placed over the two supraorbital branches of the trigeminal nerve and the stimulus parameters were set a 120 Hz, 250 μs, with current intensity adjusted for a nonpainful paresthesia as reported by the patient. Five minutes of continuous EEG was recorded at baseline and after the 10-day TNS protocol. A fast Fourier transform was used to calculate absolute power (AP = μV2) in each of the six frequency bands (delta: 0.5–4 Hz, theta: 4–8 Hz, alpha 1: 8–10 Hz, alpha 2: 10–2 Hz, beta: 12-30 Hz, gamma: 30–60 Hz). After 10-day TNS protocol a cortical widespread

increase in delta band power and a parieto-occipital increase for alpha 1 and 2 power were noted during the eye close condition.

To the best of our knowledge, data on qualitative and quantitative EEG analysis following TNS protocol in DRE patients, have never been published yet.

SECOND SECTION

Clinical study

1. BACKGROUND AND AIMS

Drug-resistant epilepsy (DRE) accounts for 30% of the worldwide epileptic patients for whom, despite adequate pharmacological therapies, seizures are not completely controlled (Kwan et al., 2010). Excluding a little percentage of DRE patients eventually suitable for curative epilepsy surgery, most of them continue to have lifelong disabling seizures with a significant burden on quality of life (Fois et al., 2015; de Tisi et al., 2011).

Following the increasing need for novel therapeutic options for DRE and based on historical observations that electrical stimulation of peripheral nerves and subcortical structures could modify cortical excitability (Pompeiano and Swett, 1962; Moruzzi and Magoun, 1949; McLachlan, 1993), a number of neuromodulation devices have been developed over the past few decades.

Vagal nerve stimulation (VNS) represents the most widely used neurostimulation method, being the only peripheral approach currently approved by the US Food and Drug Administration (FDA) as an adjunctive treatment for partial-onset DRE and showing a 1-year responder rate (≥50% reduction in seizure frequency) by up to 36.8% of the treated patients (DeGiorgio et al., 2000; Morris and Mueller, 1999).

However, in the last decade a large body of evidence has supported the use of an alternative peripheral neuromodulation method, named trigeminal nerve stimulation (TNS).TNS has recently received European Conformity (CE) marking as a promising additional treatment for intractable epilepsy (1-year responder rate of 36.8%) (Soss et al., 2015).

It has been reported that TNS has some advantages in comparison with the well acknowledged VNS. While VNS implantation represents an invasive procedure, carrying the risk of surgical intervention and different adverse effects (Ben-Menachem 2002, 2015), TNS is an external peripheral method which can be safely used and easily self-administered by the patient (Pop et al., 2011). Moreover, the absence of autonomic fibers in the trigeminal nerve allows a bilateral stimulation with larger effects at lower intensities compared to unilateral stimulation as routinely used for VNS (DeGiorgio et al., 2003; Fanselow et al., 2000).

Despite their clinical utilization, yet mechanisms underlying the anticonvulsant effect of cranial nerve stimulations are not completely understood.

A number of animal and human epilepsy models suggested that VNS may modulate cerebral activity by inducing an electroencephalographic (EEG) desynchronization and so a cortical inhibition, lowering the excitability of the epileptogenic tissue (Zanchetti et al., 1952; Aalbers et al., 2011; Jaseja, 2010; Fraschini et al., 2013; Bodin et al., 2015).

As well as VNS, also TNS has been shown to influence cortical activity in a single, pioneering experimental epilepsy model, by means of a thalamo-cortical, stepwise, EEG desynchronization effect (Fanselow et al., 2000).

This is in line with the *bottom-up* hypothesis which attributed the cortical neuronal modulation to an indirect stimulation of subcortical stations interposed to higher ascending pathways (Shiozawa et al., 2014; Fanselow, 2012).

Trigeminal and vagus cranial nerves share widespread projections to different subcortical structures with cortical neuromodulatory effects; therefore they may be also supposed to share some of the anticonvulsant mechanisms (Fanselow et al., 2000; Fanselow, 2012; Mercante et al., 2015, 2017; Pilurzi et al., 2016).

However, whether and how TNS is able to modulate human cortical excitability and background EEG dynamics, in DRE patients is still unknown.

Recently neurophysiology works provided evidence regarding TNS-induced brainstem modulation (Mercante et al., 2015, 2017) and brainstem plasticity (Pilurzi et al., 2016) in heathy subjects, while primary motor cortex (M1) excitability and sensorimotor integration at cortical level appeared unaffected by acute stimulation, suggesting no effect of TNS on higher structures, at least on M1 (Mercante et al., 2015; Axelson et al., 2014).

Contrariwise, a frequency-dependent inhibition of neuronal firing in primary somatosensory cortex (SI) neurons, in response to contralateral TNS, has been recently shown in rat models (Fanselow and Noel, 2010), in accordance with the previous work which showed EEG spike suppression in SI and thalamus after TNS delivery (Fanselow et al., 2000).

Therefore, while it seems clear that subcortical structures play a fundamental role in seizure regulation mechanisms by TNS (Mercante et al., 2015, 2017; Pilurzi et al., 2016; Fanselow, 2012), evidence regarding the TNS capacity to alter cortical excitability and/or EEG dynamics is scarce and inconclusive (Axelson et al., 2014; Mercante et al., 2015; Fanselow and Noel, 2010; Fanselow et al., 2000) and cannot be easily translated to DRE patients as explanation of TNS clinical benefits.

Indeed, TNS cortical modulation effects, by means of background EEG activity changes in DRE patients, has neither be proved nor investigated.

With this study, we aimed to evaluate the effect of acute TNS protocol on cortical EEG background activity in DRE patients, by conducting a qualitative (morphologic) and quantitative (power spectra) EEG analyses in an active vs a sham-TNS group, to better clarify the underlying neuromodulation mechanism of this method.

2. MATERIALS AND METHODS

2.1 Subjects

Twenty-two subjects affected by DRE (11 females and 11 males; 48.77 ± 13.16 years old; range 27-68 years), who attended the Center for Diagnosis and Treatment of Epilepsy at the Unit of Neurology, University of Sassari (Italy), were recruited for the study. They were selected based on the following *inclusion criteria*: age 18–70 years; diagnosis of focal DRE (Kwan et al., 2010) with ≥2 partial or secondary generalized tonic-clonic seizures (GTC) per month for the last 2 consecutive months and concurrent use of ≥1 antiepileptic drug (AED).

Exclusion criteria included history of diabetes, migraine or trigeminal neuralgia; presence of cardiovascular diseases (such as arrhythmia, heart failure or ischemic heart disease) or other serious medical or psychiatric conditions and ongoing pregnancy. Characteristics of selected patients are summarized in Table 1.

The experimental procedure was approved by the local ethical committee (Bioethics Committee of ASL n.1 Sassari, Prot n. 982/CE) and conducted in accordance with the Helsinki Declaration. An informed written consent was obtained from all the subjects before conducting the study. Patients were divided into two groups "sham-"or "real-TNS": after baseline evaluation, 22 opaque envelopes were numbered consecutively and randomly assigned to an intervention (Real-TNS; n = 11) or to a no-intervention (Sham-

TNS; n = 11) group, with a blocking procedure employing the Research Randomizer 3.0 software. Both subject and statistician were blinded for the type of intervention.

Table1. Characteristics of subjects divided for Sham- and Real-group.

SHAM-GROUP						
Case N	**Sex**	**Age (years)**	**Type of Epilepsy**	**Lesion**	**N of seizures (/month)**	**AEDs**
1	F	52	RFr	Malacic	7	LEV+LTG
2	M	65	LT	HS	2	VPA+PER+PRI+LEV
3	M	67	LFr	Schizencephaly	3	OXC+LEV+PGB
4	M	44	LO	Gliosis	2	PER+LTG+LAC
5	M	61	RT	HSE	15	LAC+LEV+CBZ
6	M	42	LFr	Malacic	15	VPA+LTG+TPM
7	M	53	RT	Crypto	7	CBZ+LAC+VPA+LEV
8	F	54	RP	SAH	8	CBZ+LEV
9	F	46	RT	Crypto	8	LTG
10	F	66	LT	Crypto	18	LEV+LTG
11	M	35	LT	Crypto	35	LEV+LTG
REAL- GROUP						
1	M	31	LFr	Crypto	2	CBZ+LTG
2	M	65	LT	Crypto	2	CBZ
3	F	27	RT	HS	4	LEV+LAC
4	M	68	BFr	Crypto	2	LEV+PB+CBZ
5	M	49	RFr	RE	3	LEV+CBZ
6	F	37	RO	Crypto	2	TPM
7	F	31	RFr	Malacic	10	VPA+ LTC+LAC
8	F	57	LT	Crypto	15	CBZ+ZNS
9	F	51	RT	Crypto	17	CBZ+LEV
10	F	35	BT	NH	3	LEV+LTG+LAC
11	F	37	RT	FCD	40	LEV

F, female; M, male; R, right; L, left; B, bilateral; Fr, frontal; T, temporal; P, parietal; O, occipital; Crypto, cryptogenic; FCD, focal cortical dysplasia; HS, hippocampal sclerosis; HSE, Herpes simplex encephalitis; NH, nodular heterotopia; RE, Rasmussen encephalitis, SAH, subarachnoid hemorrhage; CBZ, carbamazepine; LAC, lacosamide; LEV, levetiracetam; LTG, Lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PER, perampanel; PGB, pregabalin; PRI, primidone; TPM, topiramate; VPA, valproic acid; ZNS, zonisamide.

2.2 TNS protocol

Real-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). The stimulus consisted of trains of a symmetric biphasic square wave pulse (duration 0.25 ms, frequency 120 Hz), delivered in a cyclic modality (30 s ON and 30 s OFF) for 20 min, according to previous works (Pilurzi et al., 2016; Mercante et al., 2015; Ginatempo et al., 2017). 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.

The sham-TNS protocol mimicked the initial bilateral real-TNS stimulation and consisted of a previous calculation of both perceptual and pain threshold, followed by 20s of TNS, the intensity of which was subsequently gradually decreased down to zero, which corresponded to the OFF position of the stimulator.

2.3 EEG recording

EEG signals were recorded with head caps using a 19 channel EEG system (Brain Quick System, Micromed, Mogliano-Veneto, Italy) in physical reference with successive reconstructions of bipolar derivations (FP1-F3, F3-C3, C3-P3, P3-O1, FP1-F7,F7-T3,T3-T5,T5-O1,FP2-F4,F4-C4,C4-P4,P4-O2,FP2-F8,F8-T4,T4-T6,T6-O2,FZ-CZ,CZ-PZ) according to the international 10–20 system. EEG was acquired in eyes-closed resting state, band pass filtered between 0.5 and 70 Hz and digitized with sample frequency set at 256 Hz. EEG was continuously recorded for a 40-minutes period comprising 3 phases: (i) 10 minutes of baseline EEG, recorded before TNS (pre) (ii) 20 minutes during the intervention (real- or sham-TNS) (iii) 10 minutes after intervention (post). Electrocardiogram and electrooculogram (EOG) were simultaneously monitored in all subjects (time constant: 0.3 s, filter 30 Hz). EOG was recorded from the orbicularis oculi muscle by surface electrodes, with the active electrode placed over the mid lower eyelid and the reference 2–3 cm lateral.

2.3.1 Qualitative analysis (IEDs detection)

The EEG recordings were visually analyzed. Artifacts due to eye blinks, horizontal–vertical eye movements, muscle contractions and possible signs of sleepiness were detected by visual inspection and deleted.

Only definite spikes, sharp waves, and spike-wave complexes were considered epileptiform abnormalities and distinguished from the background activity due to their morphology and/or amplitude. Two different paradigms were visually analyzed: the absolute number of interictal epileptiform discharges (IEDs) for each time points and the ratio between the number of IEDs and the minutes of time points in which they appeared. Both calculations were included in the statistical analysis.

2.3.2 Quantitative analysis (power spectra)

Bipolar EEG signals of each recording were processed by spectral analysis: Fast Fourier Transform (FFT) was applied to 4-sec artifact-free basic epochs and mean spectra were computed according to the Welch's method (with Tukey window) for each phase (pre, during and post). Due to the overlapping of the stimulation artifact with EEG signals, the analysis of data collected during TNS (real or sham) was limited to the OFF phase only, with the exclusion of the 3-second periods at the beginning and end of it. Based on mean spectra, mean power was computed for each frequency band: delta (0.5-3 Hz), theta (3.1-7.0 Hz), alpha (7.1-13 Hz), beta (13.1-30 Hz) and gamma (30-48 Hz). Power-weighted mean frequency was computed in a large band (0.5-48 Hz) in order to measure a possible global frequency shift (Figure 1).

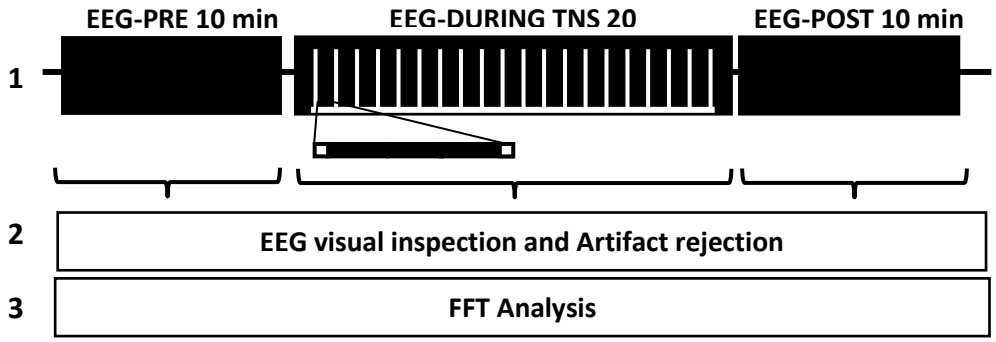


Figure 1. Schematic representation of EEG recording and analysis process in pre, during and post TNS. FFT, Fast Fourier Transform Analysis.

2.4 Statistical analysis

Paired t test was performed to evaluate demographic features between groups for age, sex, number of seizures and number of AEDs. A between and within subject repeated-measures ANOVA was used with TIME (pre, during and post TNS), CONDITION (sham, real) and DERIVATION (spatial position) as factors for each variable (mean frequency and absolute power of each frequency band).

Given the large number of comparisons, values were corrected for multiple comparisons using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995) which is less conservative than Bonferroni correction, and better suitable for assessing global variations. Data analysis was performed using the SPSS 18 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1 Demographics

No significant difference in any of the demographic features or in EEG quantitative and qualitative patterns was observed between sham- and real-TNS groups in either condition at baseline (age p=0.14; sex p=0.19; number of seizure p=0.49; AEDs p=0.11).

3.2 IEDs detection

Qualitative analysis of IEDs in terms of ratio and absolute values showed no significant effect for TIME ($F_{2,20}$=0.077, p=0.46; $F_{2,20}$=3.885,p=0.05 respectively), CONDITION ($F_{2,20}$=0.227,p=0.64; $F_{2,20}$=0.503,p=0.49 respectively) or interaction among the factors (Table 2).

		Absolute Value			**Ratio**		
Condition	Patient	Pre	During	Post	Pre	During	Post
Sham-TNS	1	18	9	4	1.44	0.91	0.38
	2	9	10	12	0.78	1.06	1.05
	3	0	1	1	0.00	0.11	0.08
	4	31	29	28	2.55	2.9	3.13
	5	0	0	2	0.00	0	0.14
	6	23	14	23	2.10	1.44	2.01
	7	40	46	56	4.00	4.66	4.72
	8	23	23	7	2.03	2.33	0.92
	9	8	7	12	0.65	0.75	1.27
	10	89	86	147	5.10	8.6	8.75
	11	18	14	19	15.52	26.25	20.07
Real-TNS	1	19	1	24	1.72	0.19	2.5
	2	45	1	36	3.18	0.17	3.10
	3	42	4	3	58.33	21.43	28.13
	4	4	3	1	0.31	0.59	0.13
	5	2	1	1	0.17	0.17	0.09
	6	6	2	3	0.51	0.39	0.3
	7	7	3	3	0.5	0.35	0.3
	8	6	6	3	11.20	23.68	45
	9	57	33	72	4.07	6.19	7.30
	10	25	5	11	2.08	0.94	1.1
	11	18	11	10	1.55	1.79	1.07

Table 2. Absolute and ratio values of IEDs shown for time (pre, during, post) and condition (Sham- and Real-TNS).

3.3 Spectral analysis

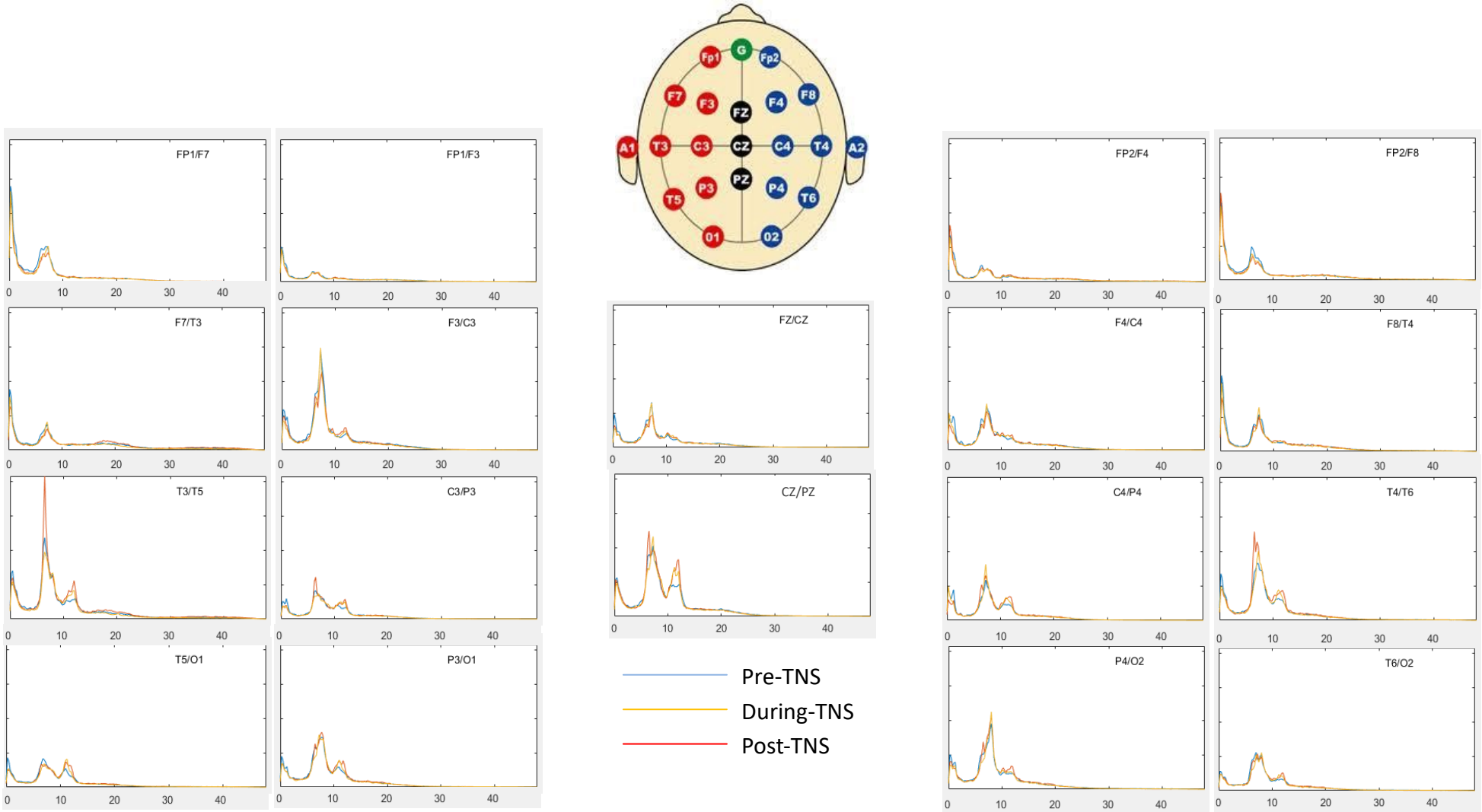
Repeated-measures ANOVA showed a significant effect of DERIVATION, which was observed for all variables. Absolute power of each band was not significantly affected by TIME and CONDITION and no significant TIME*CONDITION interactions were observed for any variable, except for the absolute power of alpha band.

The alpha band showed a significant increase for TIME in the Real condition during TNS, but no significant difference between CONDITIONS. A significant interaction DERIVATION*TIME and DERIVATION*TIME*CONDITION was detected (Table 3; Figure 2).

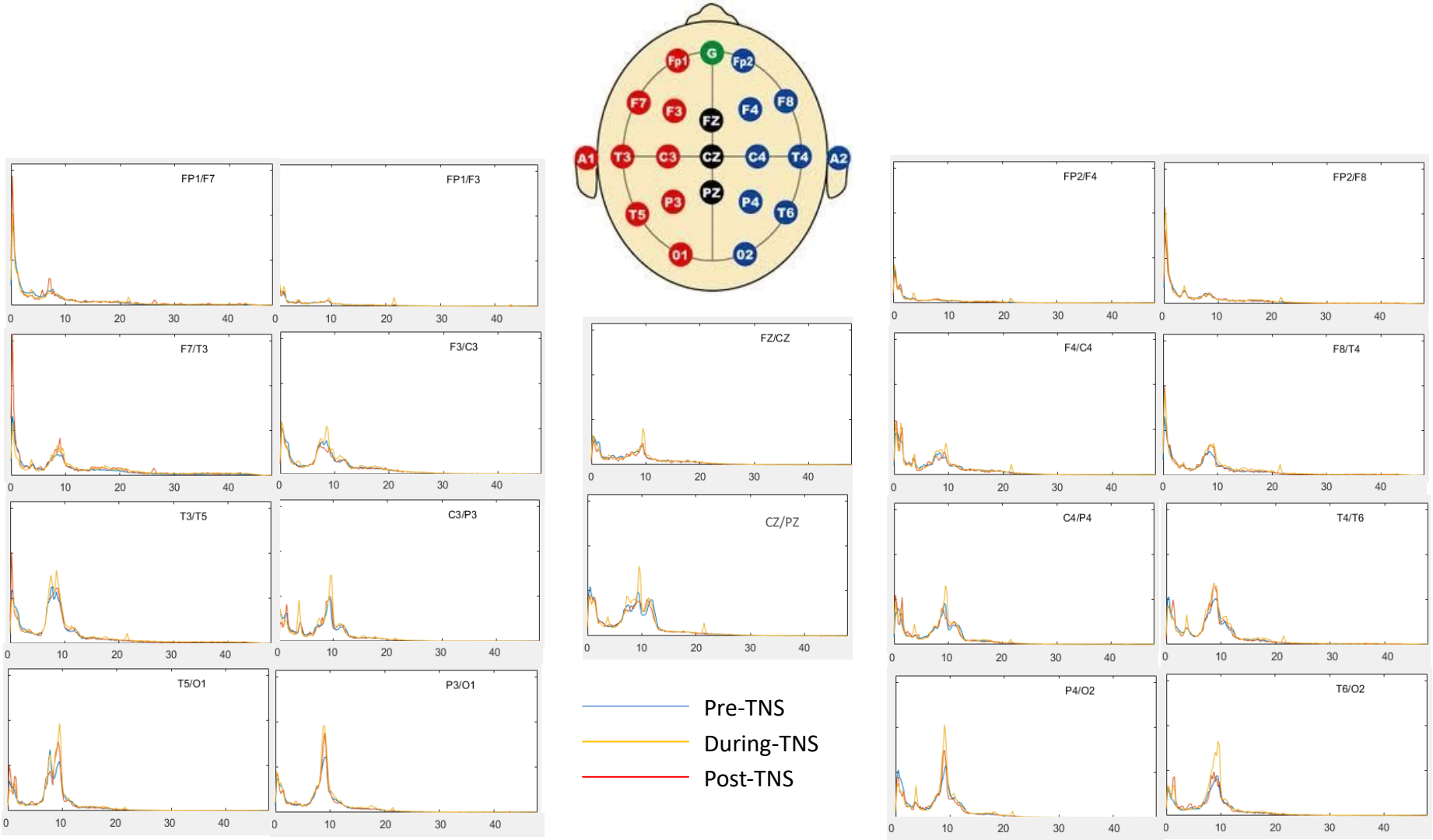
Table 3. Repeated-measures ANOVA analysis between and within subject of mean frequency and absolute power for each band (delta, theta, alpha, beta and gamma) measured before (PRE), DURING and after (POST) acute TNS. The table shows the results of ANOVA for main effect of Derivation, Time, Condition and interaction.

Variable	Band	Derivation	Time (pre, during, post)	Condition (real-TNS, sham-TNS)	Time x Condition	Derivation x Condition	Derivation x Time	Derivation x Time x Condition
Absolute Power	Delta (0.5-3.0 Hz)	$F_{2,18}$=4.440 p<0.001	$F_{2,18}$=0.837 p=0.44	$F_{2,18}$=3.197 p=0.09	$F_{2,18}$=0.362 p=0.70	$F_{2,18}$=0.756 p=0.74	$F_{2,18}$=0.772 p=0.84	$F_{2,18}$=1.181 p=0.22
	Theta (3.1-7.0 Hz)	$F_{2,18}$=4.785 p<0.001	$F_{2,18}$=1.269 p=0.29	$F_{2,18}$=0.250 p=0.62	$F_{2,18}$=0.187 p=0.83	$F_{2,18}$=0.819 p=0.671	$F_{2,18}$=1.052 p=0.39	$F_{2,18}$=1.339 p=0.09
	Alpha (7.1-13.0 Hz)	$F_{2,18}$=11.03 3 p<0.001	$F_{2,18}$=3.445 p=0.04	$F_{2,18}$=0.397 p=0.54	$F_{2,18}$=1.676 p=0.20	$F_{2,18}$=0.966 p=0.496	$F_{2,18}$=2.208 p=0.001	$F_{2,18}$=1.748 p=0.006
	Beta (13.1-30.0 Hz)	$F_{2,18}$=6.302 p<0.001	$F_{2,18}$=1.308 p=0.282	$F_{2,18}$=0.028 p=0.868	$F_{2,18}$=2.416 p=0.10	$F_{2,18}$=0.516 p=0.94	$F_{2,18}$=1.099 p=0.32	$F_{2,18}$=1.181 p=0.22
	Gamma (30.1-48.0 Hz)	$F_{2,18}$=9.128 p<0.001	$F_{2,18}$=2.092 p=0.14	$F_{2,18}$=0.742 p=0.40	$F_{2,18}$=1.117 p=0.21	$F_{2,18}$=0.739 p=0.76	$F_{2,18}$=1.036 p=0.41	$F_{2,18}$=1.091 p=0.33
Mean frequency (0.5-48.0 Hz)		$F_{2,18}$=5.669 p<0.001	$F_{2,18}$=1.873 p=0.17	$F_{2,18}$=1.238 p=0.28	$F_{2,18}$=1.199 p=0.31	$F_{2,18}$=1.475 p=0.10	$F_{2,18}$=0.817 p=0.76	$F_{2,18}$=0.725 p=0.88

Figure 2. Graphic representation of mean power spectra for different conditions (**A.** Sham- and **B.** Real-Group) and time (pre, during, post TNS).

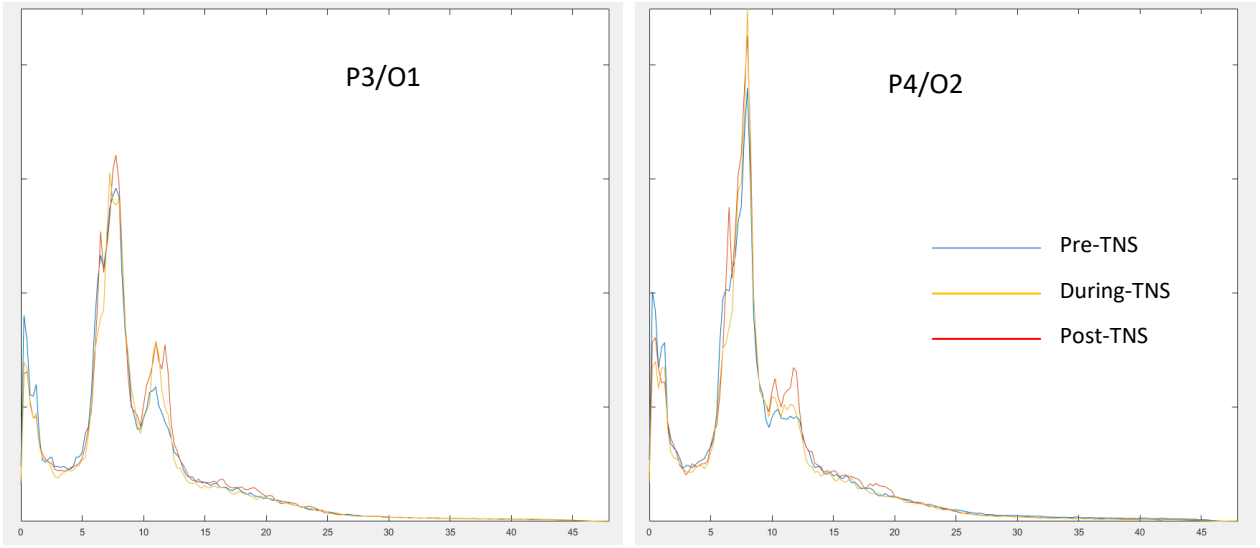


A. Sham Group

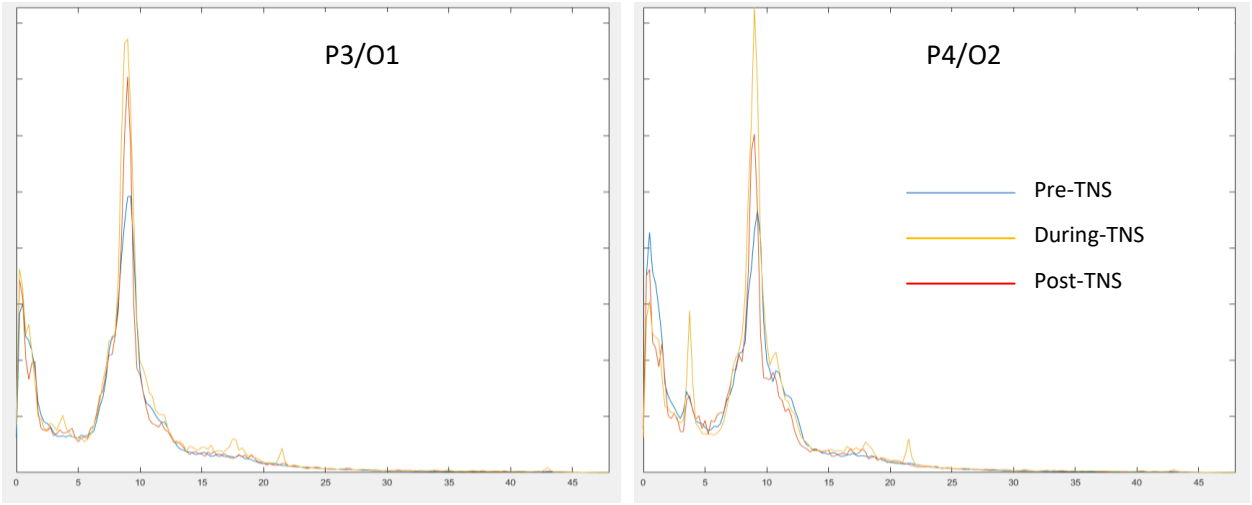

FP1/F7
FP1/F3
F7/T3
F3/C3
T3/T5
C3/P3
T5/O1
P3/O1
G
Fp1
Fp2
F7
F3
FZ
F4
F8
A1
T3
C3
CZ
C4
T4
A2
PZ
P3
P4
T5
T6
O1
O2
FZ/CZ
CZ/PZ
Pre-TNS
During-TNS
Post-TNS
FP2/F4
FP2/F8
F4/C4
F8/T4
C4/P4
T4/T6
P4/O2
T6/O2

B. Real Group

In particular, an increase of absolute power of alpha band was detected in parieto-occipital and frontal areas (Figure 3).



A. Sham Group



B. Real Group

Figure 3. Graphic representation of mean absolute power spectra grouped in different conditions (**A.** Sham- and **B.** Real-Group) and time (pre, during, post-TNS). Details for single derivations P3/O1;P4/O2.

DISCUSSION AND CONCLUSION

Besides IEDs detection, a number of intrinsic background EEG abnormalities in quantitative and power spectra analyses have been reported in epileptic patients compared to healthy controls. Overall a global increase of the spectral power in the slow frequency range (i.e. theta and delta) and a decrease in the alpha band have been variously reported by many authors, possibly unrelated to AED consumption or syndromic variants (Pyrzowski et al., 2015; Diaz et al., 1998; Gelety et al., 1985). Neuromodulation methods are supposed to influence the propensity to generate seizures by interfering with the background EEG activity in epileptic patients, raising the threshold of epileptogenic tissue and thus restoring the balance between excitatory and inhibitory mechanisms (Krishna et al., 2016; Dalkilic, 2017; Rocha, 2013).

The trigeminal nerve projects to a widespread number of subcortical and brainstem regions with documented cortical neuromodulatory effects (Fanselow, 2012; Mercante et al., 2017); however, the neurobiological mechanisms by which TNS modulates brain function in humans and how this actually affects background EEG rhythms is unknown.

Overall, data from this study showed that a short-term TNS protocol is able to induce an acute effect on the EEG background composition of focal DRE patients and its effect is mainly expressed as a modulation of the distribution of the signal power over frequencies, as demonstrated by the spectral power analysis.

Indeed, a consistent and significant increase of the absolute power of alpha band during the real TNS was observed in patients' EEGs compared to the sham group. Alpha enhancement appeared during the 20 minutes of TNS-ON and partially maintained during the post-TNS period (10 minutes after stimulation).The alpha power enhancement appeared particularly localized in the parietal-occipital and frontal areas, unrelated to epileptogenic focus (Figure 2 and 3). Conversely, no significant effects were noticed either for quantitative analysis of other frequency bands (delta, theta, beta and gamma) or for qualitative analyses (IEDs detection).

This positive alpha modulatory effect has been previously described following chronic stimulation of the vagus nerve in epileptic patients (Rizzo et al., 2004). In their work the authors demonstrated that VNS induced a better structured composition of the background EEG in these patients, while enhancing the power of the EEG rhythms which characterize each sleep-wake state (i.e. delta and sigma in non-REM sleep, alpha in both REM sleep and wakefulness). These authors suggested that VNS is able to induce an improvement of cortical electrogenesis, thus enhancing the brain's ability to generate a normal electrical activity, possibly through metabolic changes on the thalamo-cortical EEG generating system.

Interestingly, neuroimaging human studies variously reported that increased EEG alpha power correlates with a decreased metabolic rate in multiple regions of the occipital,

superior temporal, inferior frontal and cingulate cortex, but with an increased activity in the thalamus and insula (Goldman et al., 2002; Bazanova and Vernon, 2014).

Extensive literature regarding the functional aspect of the alpha rhythm proposed that regional alpha enhancement (in terms of increased EEG amplitude) would represent a cortical inhibitory process useful to control cortical activation and excitability in a top-down manner (Klimesch et al., 2007; Bazanova and Vernon, 2014). It has been suggested that greater levels of regional alpha amplitude are associated with inhibition of cortical non-essential or conflicting processing or activities and, in turn, with the facilitation of performance on different, more fruitful tasks (Cooper et al., 2003).

The functional inhibition of the alpha-amplitude oscillations would play an important role in a variety of cognitive processes (VanRullen and Koch, 2003) as well as in regulating motor activity (Klimesch et al., 2007). The increase in alpha power across sensorimotor regions showed a strong inhibitory influence on the generation of neuronal firing and action potentials in monkey models (Haegens et al., 2011).

Since a high level of excitatory neurotransmission could be a neurobiological factor that may underlie augmented susceptibility to develop pharmacoresistance (Rocha, 2013), a possible increase of inhibitory cortical activity can underpin the clinical effect of TNS in epileptic patients.

Accumulating evidence suggest that, like VNS, TNS ultimately influences the pattern of neuronal activity while connecting to brain areas which are thought to modulate the lateral reticular formation, such as the nucleus tractus solitarii (NTS) and the locus coeruleus (LC) (Mercante et al., 2017). These are, in turn, connected to noradrenergic and serotoninergic systems associated with the regulation of mood, anxiety (Ruffoli et al., 2011) and to glutamatergic and GABAergic systems regulating the susceptibility to seizures (Walker et al., 1999). NTS and LC are also considered as nuclei which disseminate neuromodulatory compound in the central nervous system (CNS), since they profoundly affect its excitability at virtually all levels and are believed to play a key role in mediating the clinical benefits observed from TNS procedure (Mercante et al 2017; Krahl and Clark, 2012; Krahl et al., 1998; Fornai et al., 2011).

Importantly, sensory input which relayed via the trigeminal nerve to the ventral posterior medial (VPM) thalamus, could also trigger intra-thalamic inhibition within a given nucleus or activate the inhibitory cells that comprise the reticular nucleus of the thalamus (Fanselow, 2012). It is thus possible that afferent trigeminal input arising from TNS could also evoke sufficient inhibition to interrupt any pathological oscillatory activity within the thalamus that could contribute to seizure activity (Fanselow, 2012).

In this view, the alpha-activity oscillation in the awake brain, modulated by thalamo-cortical firing and brainstem afferent inputs (Başar, 2012; Cooper et al., 2003), may act

as a seizure-preventing mechanism, inhibiting cortical abnormal excitability and ictal spreading. Indeed, the alpha-power enhancement seems to favour a talamo-cortical stabilization (Palva and Palva, 2007) similar to the "transient functional deafferentation" achieved during phases of stable synchronized Non-REM sleep (i.e. 'closed thalamic gate' theory) in which seizures are known to generally not occur (Gibbs et al., 2016).

Moreover, some recent studies from intracranial EEG recording in epileptic patients demonstrated that the enhancement of alpha activity is able to modulate and temporally break ongoing gamma activity with a mechanism of regional pulsed inhibition (Jensen and Mazaheri, 2010; Osipova et al., 2008) and so attenuating the highly rhythmic components which have been found to often proceed epileptiform discharges (Medvedev, 2002; Fisher et al., 1992).

These results are consistent with our findings which consider the alpha rhythm enhancement, during TNS stimulation, as a sign of cortical "functional inhibition", generated by modulation of subcortical structures (Başar, 2012; Bazanova and Vernon, 2014; Hughes and Crunelli, 2005).

In keeping with the hypothesis of Diaz and co-workers (1998), a possible explanation of our results is that in patients with focal DRE, some unpredictable changes in the intrinsic dynamic properties of neural activity has led to a global reorganization across frequency power possibly with a lowering in alpha band, which may reflect a

dysfunction in thalamic–cortical circuits and eventually affect the cortical susceptibility to initiate seizures (Pyrzowski et al., 2015; Hughes and Crunelli, 2005; Klimesch et al., 2007).

In this substrate TNS could exert its effect by globally enhancing alpha power and so restoring the normal balance between excitatory and inhibitory mechanisms.

This TNS effect has been documented only for the acute stimulus protocol in a relative small patient group, further TNS studies focused on the EEG chronic effect on epileptic subjects may be worthily performed in the perspective of shading new insights in the mechanisms underpinning the TNS antiepileptic effects.

REFERENCES

Aalbers M, Vles J, Klinkenberg S et al. Animal models for vagus nerve stimulation in epilepsy. *Experimental Neurology* 2011;230:167–175

Amar AP, Levy ML, Apuzzoc MLJ. Vagus nerve stimulation for intractable epilepsy, in Neurosurgical Operative Atlas, S. Rengechary, Ed. Chicago, IL: American Association of Neurological Surgeons, 2000;9:179–188

Aronica E, Gorter JA, Jansen GH, et al. Expression and cellular distribution of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors. *Neuroscience* 2003;118:417-29

Attenello F, Amar AP, Liu C et al. Slavin KV (ed): Stimulation of the Peripheral Nervous System. The Neuromodulation Frontier Prog Neurol Surg. Basel, Karger, 2016, vol 29, pp 20–28

Axelson HW, Isberg M, Flink R et al. Trigeminal Nerve Stimulation Does Not Acutely Affect Cortical Excitability in Healthy Subjects. *Brain Stimulation* 2014;7:613e617

Bailey P, Bremer A. Sensory cortical representation of the vagus nerve: with a note on the effects of low blood pressure on the cortical electrogram. *J Neurophysiol* 1938;1:405–412.

Banzett RB, Guz A, Paydarfar D, et al. Cardiorespiratory variables and sensation during stimulation of the left vagus in patients with epilepsy. *Epilepsy Res* 1999;35:1–11

Başar E. A review of alpha activity in integrative brain function: Fundamental physiology, sensory coding, cognition and pathology. *Int J Psychophysiol* 2012;86:1-24

Bauer S, Baier H, Baumgartner C et al. Transcutaneous vagus nerve stimulation (tVNS) for treatment of drug resistant epilepsy: a randomized, double-blind clinical trial (cMPsE02). Brain Stimul 2016, doi:http://dx.doi.org/10.1016/j.brs.2015.11.003

Bazanova OM, Vernon D. Interpreting EEG alpha activity. *Neurosci Biobehav Rev* 2014;44:94-110

Beekwilder JP, Beems T. Overview of the Clinical Applications of Vagus Nerve Stimulation. *J Clin Neurophysiol* 2010;27: 130–138

Begley CE, Famulari M, Annegers JF, et al. The cost of epilepsy in the United States: an estimate from population-based clinical and survey data. *Epilepsia* 2000;41:342–351

Bell GS, Gaitatzis A, Bell CL, et al. Drowning in people with epilepsy: How great is the risk? *Neurology* 2008;71:578

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc Ser B* 1995;57:289–300

Ben-Menachem E, Hamberger A, Hedner T et al. Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. *Epilepsy Res* 1995;20:221–227

Ben-Menachem E, Hellström K, Waldton C et al. Evaluation of refractory epilepsy treated with vagus nerve stimulation for up to 5 years. *Neurology* 1999;52: 1265–67

Ben-Menachem E. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurology* 2002;1:477–82

Ben-Menachem E, Revesz D, Simon BJ, Silberstein S. Surgically implanted and non-invasive vagus nerve stimulation: a review of efficacy, safety and tolerability. *Eur J Neurol* 2015;22:1260-8

Berg AT, Levy SR, Novotny EJ et al. Predictors of intractable epilepsy in childhood: a case–control study. *Epilepsia* 1996;37:24–30

Berg AT, Shinnar S, Levy SR et al. Early development of intractable epilepsy in children: a prospective study. *Neurology* 2001;56:1445–1452

Berg AT, Vickrey BG, Testa FM, et al. How long does it take epilepsy to become intractable? A prospective investigation. *Ann Neurol* 2006;60:73

Bodin C, Aubert S, Daquin G. Responders to vagus nerve stimulation (VNS) in refractory epilepsy have reduced interictal cortical synchronicity on scalp EEG. *Epilepsy Res* 2015;113:98-103

Briggs DE, French JA. What makes epilepsy drug refractory? *Expert Rev Neurother* 2003;3:127–31

Camfield P, Camfield C. Nova Scotia pediatric epilepsy study. In: Jallon, P.; Berg, A.; Dulac, O., et al., editors. Prognosis of epilepsies. Montrouge France: John Libbey, Eurotext; 2003. p. 113

Chae JH, Nahas Z, Lomarev M et al. A review of functional neuroimaging studies of vagus nerve stimulation (VNS*). J Psychiatr Res* 2003;37:443-55

Chase MH, Nakamura Y, Clemente CD et al. Afferent vagal stimulation: neurographic correlates of induced EEG synchronization and desynchronization. *Brain Res* 1967;5:236 –249

Cheng G, Zhou X, Qu J et al. Central vagal sensory and motor connections: human embryonic and fetal development*. Auton Neurosci* 2004; 114: 83–96

Cook IA, DeGiorgio CM, Miller PM et al. Noninvasive neuromodulation with trigeminal nerve stimulation: a novel treatment for major depressive disorder. Poster presented at the NCDEU Annual Meeting. Boca Raton (FL), June 14–17, 2010

Cook IA, Schrader LM, Geist CL et al. Regional PET Activation with Trigeminal Nerve Stimulation in Major Depression. Boca Raton FL: NCDEU Annual Meeting; 2011

Cook IA, Schrader LM, Degiorgio CM et al. Trigeminal nerve stimulation in major depressive disorder: acute outcomes in an open pilot study. *Epilepsy Behav* 2013;28:221–226

Cook IA, Kealey CP, DeGiorgio CM. The potential use of trigeminal nerve stimulation in the treatment of epilepsy. *Ther Deliv* 2015;6:273–275

Cooper NR, Croft RJ, Dominey SJ et al. Paradox lost? Exploring the role of alpha oscillations during externally vs. internally directed attention and the implications for idling and inhibition hypotheses. *Int. J. Psychophysiol* 2003;47:65–74

Cross JH, Jaykar P, Nordli D, et al. Proposed criteria for referral and evaluation of children for epilepsy surgery: Recommendations of the Subcomission for Pediatric Epilepsy Surgery. *Epilepsia* 2006;47:953

Cyberonics, Inc. (2012) VNS therapy products manuals and safety alerts: part I—introduction—indications, warnings, and precautions, pp 7–13. http://dynamic.cyberonics.com/manuals/. Accessed 1 Oct 2012

Dalkilic EB. Neurostimulation Devices Used in Treatment of Epilepsy. C*urr Treat Options Neurol* 2017;19:7

De Ferrari GM, Crijns HJ, Borggrefe M et al. CardioFit Multicenter Trial Investigators. Chronic vagus nerve stimulation: a new and promising therapeutic approach for chronic heart failure. *Eur Heart J* 2011;32: 847–55

de Tisi J, Bell GS, Peacock JL, et al. The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: a cohort study. *Lancet* 2011;378:1388–95

DeGiorgio CM, Schachter SC, Handforth A, et al. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. *Epilepsia* 2000; 41: 1195–2000

DeGiorgio MC, Shewmon A, Whitehurst T. Trigeminal nerve stimulation for epilepsy. *Neurology* 2003;61;421-422

DeGiorgio CM, Shewmon A, Murray D et al. Pilot Study of Trigeminal Nerve Stimulation (TNS) for Epilepsy: A Proof-of-Concept Trial. *Epilepsia* 2006;47:1213–1215

DeGiorgio CM, Murray D, Markovic D et al. Trigeminal nerve stimulation for epilepsy: long-term feasibility and efficacy. *Neurology* 2009;72:936–938

DeGiorgio CM, Fanselow EE, Schrader LM et al. Trigeminal nerve stimulation: seminal animal and human studies for epilepsy and depression. *Neurosurg Clin N Am* 2011;22:449–456

DeGiorgio CM, Soss J, Cook IA et al. Randomized controlled trial of trigeminal nerve stimulation for drug-resistant epilepsy. *Neurology* 2013a;80:786–791

DeGiorgio CM and Krahl SE. Neurostimulation for Drug-Resistant Epilepsy. Continuum (Minneap Minn) 2013b;19:743–755

Diaz GF, Virues T, San Martin M et al. Generalized background qEEG abnormalities in localized symptomatic epilepsy. *Electroencephalogr Clin Neurophysiol* 1998;106:501–507

Ekmekçi H, Kaptan H. Vagus Nerve Stimulation. *Open Access Maced J Med Sci* 2017;5:391-394

Elwes RD, Johnson AL, Shorvon SD et al. The prognosis for seizure control in newly diagnosed epilepsy. *N Engl J Med* 1984;311:944–947

Englot DJ, Han SJ, Lawton MT et al. Predictors of seizure freedom in the surgical treatment of supratentorial cavernous malformations. *J Neurosurg*. 2011;115:1169-1174

Englot DJ, Rolston JD,Wright CW et al. Rates and Predictors of Seizure Freedom With Vagus Nerve Stimulation for Intractable Epilepsy. *Neurosurgery* 2016;79:345–353

Fang M, Xi ZQ, Wu Y et al. A new hypothesis of drug refractory epilepsy: neural network hypothesis. *Med Hypotheses* 2011;76:871–6

Fanselow EE, Noel D. Reduction of neocortical neuronal firing by therapeutic trigeminal nerve stimulation. San Antonio, US; 2010. p. Abst. 2.220

Fanselow EE, Reid AP, Nicolelis MA. Reduction of pentylenetetrazolinduced seizure activity in awake rats by seizure-triggered trigeminal nerve stimulation. *J Neurosci* 2000;20:8160–8168

Fanselow EE. Central mechanisms of cranial nerve stimulation for epilepsy. *Surg Neurol Int* 2012;3:S247-54

Fisher RS, Webber WR, Lesser RP et al. High-frequency EEG activity at the start of seizures. *J Clin Neurophysiol* 1992;9:441–448

Fisher RS, van Emde Boas W, Blume W, et al. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 2005;46:470–472

Fisher RS, Acevedo C, Arzimanoglou A et al. A practical clinical definition of epilepsy. *Epilepsia* 2014;55:475–482

Fois C, Kovac S, Khalil A et al. Predictors for being offered epilepsy surgery: 5-year experience of a tertiary referral centre. *J Neurol Neurosurg Psychiatry* 2015;0:1–3

Foley JO, DuBois F. Quantitative studies of the vagus nerve in the cat: I, the ratio of sensory to motor fibers. *J Comp Neurol* 1937;67: 49–97

Fornai F, Ruffoli R, Giorgi SF et al. The role of locus coeruleus in the antiepileptic activity induced by vagus nerve stimulation. *European Journal of Neuroscience* 2011;33:2169–2178

Fountain NB, Van Ness PC, Bennett A, et al. Quality improvement in neurology: epilepsy update quality measurement set. *Neurology* 2015;84:1483–1487

Fraschini M, Puligheddu M, Demuru M et al. VNS induced desynchronization in gamma bands correlates with positive clinical outcome in temporal lobe pharmacoresistant epilepsy. *Neurosci Lett* 2013;536:14–18

Fraschini M, Demuru M, Puligheddu M et al. The re-organization of functional brain networks in pharmaco-resistant epileptic patients who respond to VNS. *Neuroscience Letters* 2014;580:153–157

Fritsch B, Stott JJ, Donofrio JJ et al. Treatment of early and late kainic acid-induced status epilepticus with the noncompetitive AMPA receptor antagonist GYKI 52466. *Epilepsia* 2010;51:108–117

Gelety TJ, Burgess RJ, Drake ME et al. Computerized spectral analysis of the interictal EEG in epilepsy. *Clinical Electroencephalography* 1985;16: 94–97

Ghia JE, Blennerhassett P, Kumar-Ondiveeran H et al. The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. *Gastroenterology* 2006;131:1122–30

Gibbs SA, Proserpio P, Terzaghi M et al. Sleep-related epileptic behaviors and non-REM-related parasomnias: Insights from stereo-EEG. *Sleep Med Rev* 2016;25:4-20

Ginatempo F, Pirina P, Melis F et al. Short-term trigeminal neuromodulation does not alter sleep latency in healthy subjects: a pilot study. *Neurol Sci* 2017 Sep 30. doi: 10.1007/s10072-017-3134-z.

Goldman RI, Stern JM, Engel J Jr et al. Simultaneous EEG and fMRI of the alpha rhythm. *Neuroreport* 2002;13:2487-92

Groves DA, Bowman EM, Brown VJ. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. *Neurosci Lett* 2005;379:174-9

Haegens S. et al. a-Oscillations in the monkey sensorimotor network influence discrimination performance by rhythmical inhibition of neuronal spiking. Proc. Natl. Acad. Sci. U.S.A. 2011;108,19377–19382

Hallbook T, Lundgren J, Blennow G et al. Long term effects on epileptiform activity with vagus nerve stimulation in children. *Seizure* 2005;14:527—533

Hammond EJ, Uthman BM, Reid SA et al. Electrophysiological studies of cervical vagus nerve stimulation in humans: I. EEG effects. *Epilepsia* 1992;33:1013–20

Handforth A, DeGiorgio CM, Schachter SC et al. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. *Neurology* 1998; 51: 48–55

Heck C, Helmers SL, DeGiorgio CM. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. *Neurology* 2002;59:S31–7

Henry TR, Votaw JR, Pennell PB et al. Acute blood flow changes and efficacy of vagus nerve stimulation in partial epilepsy. Neurology 1999;52:1166–1173.

Henry TR, Bakay RA, Pennell PB, Epstein CM, Votaw JR. Brain blood-flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: II. prolonged effects at high and low levels of stimulation. *Epilepsia* 2004;45:1064-70

Hermann BP, Seidenberg M, Dow C et al. Cognitive prognosis in chronic temporal lobe epilepsy. *Ann Neurol* 2006;60:80–87

Hitiris N, Mohanraj R, Norrie J et al. Predictors of pharmacoresistant epilepsy. *Epilepsy Res* 2007;75:192–196

Hughes SW and Crunelli V. Thalamic Mechanisms of EEG Alpha Rhythms and Their Pathological Implications. *Neuroscientist* 2005;11:357-72

Jacoby A, Baker GA. Quality-of-life trajectories in epilepsy: a review of the literature. *Epilepsy Behav* 2008;12:557–571

Jandova K, Pasler D, Antonio LL et al. Carbamazepine-resistance in the epileptic dentate gyrus of human hippocampal slices. *Brain* 2006;129:3290–3306

Jaseja H. EEG-desynchronization as the major mechanism of anti-epileptic action of vagal nerve stimulation in patients with intractable seizures: Clinical neurophysiological evidence. *Med Hypotheses* 2010;74:855–856

Jensen O, Mazaheri A. Shaping functional architecture by oscillatory alpha activity: gating by inhibition. *Front Hum Neurosci* 2010;186:1-8

Joshi S, Kapur J. (2012) GABAA receptor plasticity during status epilepticus. In Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV (Eds) Jasper's basic mechanisms of the epilepsies [Internet]. 4th ed. National Center for Biotechnology Information (US), Bethesda, MD. Available at: http://www.ncbi.nlm. nih.gov/books/NBK98149/.

Klimesch W, Sauseng P, Hanslmayr S. EEG alpha oscillations: theinhibition–timing hypothesis. *Brain Res Rev* 2007;53:63–88

Ko TS, Holmes GL. EEG and clinical predictors of medically intractable childhood epilepsy. *Clin Neurophysiol* 1999; 110: 1245–1251

Koo B. EEG Changes With Vagus Nerve Stimulation. *Journal of Clinical Neurophysiology* 2001; 18:434 –441

Kobau R, Zahran H, Thurman DJ et al. Epilepsy surveillance among adults: 19 states. MMWR Surveill Summ 2008;57:1–20

Krahl SE, Clark KB, Smith DC et al. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia* 1998;39:709–714

Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. *Epilepsia* 2001;42, 586–589

Krahl SE, Clark KB. Vagus nerve stimulation for epilepsy: A review of central mechanisms. *Surg Neurol Int* 2012;3:S255-9

Krishna V, Sammartino F, Kon Kam King N et al. Neuromodulation for Epilepsy. *Neurosurg Clin N Am* 2016;27:123-31

Krout KE, Belzer RE, Loewy AD. Brainstem projections to midline and intralaminar thalamic nuclei of the rat. *J Comp Neurol* 2002;448:53-101

Kuba R, Guzaninová M, Brázdil M et al. Effect of Vagal Nerve Stimulation on Interictal Epileptiform Discharges: A Scalp EEG Study. *Epilepsia* 2002;43:1181–1188

Kuba R, Nesvadba D, Brazdil M et al. Effect of chronic vagal nerve stimulation on interictal epileptiform discharges. *Seizure* 2010;19:352–355

Kwan P, Brodie MJ. Early Identification of refractory epilepsy. New England Journal of Medicine 2000;343:314–319

Kwan P, Brodie MJ. Refractory epilepsy: mechanisms and solutions. Expert Rev Neurother 2006;6:397–406

Kwan P, Arzimanoglou A, Berg AT, et al. Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on therapeutic strategies. *Epilepsia* 2010;51:1069–1077

Langan Y, Nashef L, Sander JW. Case-control study of SUDEP. *Neurology* 2005;64:1131

Lhatoo S, Sander J. The epidemiology of epilepsy and learning disablility. *Epilepsia* 2001;42:6

Loscher W, Potschka H. Role of multidrug transporters in pharmacoresistance to antiepileptic drugs. *J Pharmacol Exp Ther* 2002;301:7–14

Lotvall J, Lunde H, Augustinson LE et al. Airway effects of direct left-sided cervical vagal stimulation in patients with complex partial seizures. *Epilepsy Res* 1994;18:149–54

Loup F, Wieser HG, Yonekawa Y et al. Selective alterations in GABAA receptor subtypes in human temporal lobe epilepsy. *J Neurosci* 2000;20:5401–19

Magnes J, Moruzzi G, Pompeino O. Synchronization of the EEG produced by low frequency stimulation of the region of the solitary tract. *Arch Ital Biol* 1961;99:33–67.

Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ ABCG2) in drug transport – an update. *AAPS J* 2015;17:65–82

Marrosu F, Santoni F, Puligheddu M et al. Increase in 20–50 Hz (gamma frequencies) power spectrum and synchronization after chronic vagal nerve stimulation. *Clinical Neurophysiology* 2005;116:2026–2036

McLachlan RS. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. *Epilepsia* 1993;34:918–23

Medvedev AV. Epileptiform spikes desynchronize and diminish fast (gamma) activity of the brain An "anti-binding" mechanism? *Brain Res Bull* 2002;58:115-28

Mercante B, Pilurzi G, Ginatempo F et al. Trigeminal nerve stimulation modulates brainstem more than cortical excitability in healthy humans. *Exp Brain Res* 2015;233:3301-11

Mercante B, Enrico P, Floris G et al. Trigeminal nerve stimulation induces Fos immunoreactivity in selected brain regions, increases hippocampal cell proliferation and reduces seizure severity in rats. *Neuroscience* 2017;361:69-80

Mohamed IS, Minassian BA: What intractability information is there in the type of generalized seizure? *Adv Neurol* 2006;97:141–147

Mohanraj R, Brodie MJ. Early predictors of outcome in newly diagnosed epilepsy. *Seizure* 2013;22:333–44

Morris GL, Mueller WM, and the Vagus Nerve Stimulation Study Group EO1–EO5. Long-term treatment with vagus nerve stimulation in patients with refractory epilepsy. *Neurology* 1999; 53: 1731–35

Morris GL, Gloss D, Buchhalter J et al. Evidence- based guideline update: vagus nerve stimulation for the treatment of epilepsy: Report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology* 2013;81:1453-1459

Moruzzi G, Magoun HW. Brainstem reticular formation and activation of the EEG. *Electroencephalogr Clin Neurophysiol* 1949;1:455– 473

Moseley BD, Degiorgio CM. Refractory status epilepticus treated with trigeminal nerve stimulation. *Epilepsy Res* 2014;108:600–603

Murray CJL, Lopez AD, eds. Global Comparative Assessment in the Health Sector; Disease Burden, Expenditures, and Intervention Packages. Geneva: World Health Organization; 1994

Murray CJL, Vos T, Lozano R et al. Disability adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012;380:2197–2223

Naritoku DK, Terry WJ, Helfert RH. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. *Epilepsy Res* 1995;22:53–62

Neal EG, Chaffe H, Schwartz RH, et al. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. *Lancet Neurol* 2008;7:500-6

Nune G, DeGiorgio C, Heck C. Neuromodulation in the Treatment of Epilepsy. *Curr Treat Options Neurol* 2015;17:43

Osharina V, Bagaev V, Wallois F et al. Autonomic response and Fos expression in the NTS following intermittent vagal stimulation: Importance of pulse frequency. *Auton Neurosci* 2006;126-127:72-80

Osipova D, Hermes D, and Jensen O. Gamma power is phase locked to posterior alpha activity. *PLoSONE* 2008;3, e3990. doi: 10.1371/journal. pone.0003990

Oskoui M, Webster RI, Zhang X et al. Factors predictive of outcome in childhood epilepsy. *J Child Neurol* 2005;20:898–904

Palva S and Palva JM. New vistas for a-frequency band oscillations. TRENDS Neurosci 2007;30:150-158

Panebianco M, Rigby A,Weston et al. Vagus nerve stimulation for partial seizures. Cochrane Database of Systematic Reviews 2015, Issue 4. Art. No.: CD002896

Penry JK, Dean JC. Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. *Epilepsia* 1990;31: S40–S43

Pilurzi G, Mercante B, Ginatempo F et a. Transcutaneous trigeminal nerve stimulation induces a long-term depression-like plasticity of the human blink reflex. *Exp Brain Res.* 2016;234:453-61

Pompeiano O, Swett JE. EEG and behavioral manifestations of sleep induced by cutaneous nerve stimulation in normal cats. *Arch Ital Biol* 1962;100:311-342

Pop J, Murray D, Markovic D et al. Acute and long-term safety of external trigeminal nerve stimulation for drug-resistant epilepsy. *Epilepsy & Behavior* 2011;22:574–576

Pyrzowski J, Siemiński M, Sarnowska A et al. Interval analysis of interictal EEG: pathology of the alpha rhythm in focal epilepsy. *Sci. Rep*. 2015;5:16230

Randall WC, Ardell JL. Differential innervation of the heart. In: Sipes D, Jalife J, eds. Cardiac electrophysiology and arrhythmias. New York: Grune and Stratton, 1985: 137–44

Ravan M, Sabesan S, D'Cruz ON. On Quantitative Biomarkers of VNS Therapy using EEG and ECG Signals, IEEE. *Trans Biomed Eng* 2017a;64:419–428

Ravan M. Investigating the correlation between short-term effectiveness of VNS Therapy in reducing the severity of seizures and long-term responsiveness. Epilepsy Research 2017b http://dx.doi.org/10.1016/j.eplepsyres.2017.04.008

Remy S, Gabriel S, Urban BW et al. A novel mechanism underlying drug resistance in chronic epilepsy. *Ann Neurol* 2003;53:469–79

Remy S, Beck H. Molecular and cellular mechanisms of pharmacoresistance in epilepsy. *Brain* 2006;129:18–35

Ring HA, White S, Costa DC et al. A SPECT study of the effect of vagal nerve stimulation on thalamic activity in patients with epilepsy. *Seizure* 2000;9:380-4

Rizzo P, Beelkea M, De Carli F et al. Modifications of sleep EEG induced by chronic vagus nerve stimulation in patients affected by refractory epilepsy. *Clin Neurophysiol* 2004;115:658–664

Rocha L. Interaction between electrical modulation of the brain and pharmacotherapy to control pharmacoresistant epilepsy. *Pharmacol Ther* 2013;130:211-28

Rogawski MA, Johnson MR. Intrinsic severity as a determinant of antiepileptic drug refractoriness. *Epilepsy Curr* 2008;8:127–130

Rogawski MA. The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. *Epilepsia* 2013;54:33–40

Roosevelt RW, Smith DC, Clough RW et al. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res* 2006;1119:124-32

Ruffoli R, Giorgi FS, Pizzanelli C et al. The chemical neuroanatomy of vagus nerve stimulation. *J Chem Neuroanat 2011*;42(4): 288-296

Rutecki P. Anatomical, physiological, and theoretical basis for the antiepileptic effect of vagus nerve stimulation. *Epilepsia* 1990;31:S1–S6

Salinsky MC and Burchiel KJ. Vagus Nerve Stimulation Has No Effect on Awake EEG Rhythms in Humans. *Epilepsia* 1993;34:29-304

Santiago-Rodríguez E, Alonso-Vanegas M, Cárdenas-Morales L et al. Effects of two different cycles of vagus nerve stimulation on interictal epileptiform discharges. *Seizure* 2006;15:615-20

Schiller Y, Najjar Y: Quantifying the response to antiepileptic drugs: effect of past treatment history. *Neurology* 2008;70:54–65

Schoenen J, Vandersmissen B, Jeangette S et al. Migraine prevention with a supraorbital transcutaneous stimulator: a randomized controlled trial. *Neurology* 2013;80:697–704

Schrader LM, Geist CL, DeGiorgio CM, et al. Regional PET activations with trigeminal nerve stimulation (TNS) and correlation with therapeutic response. *Epilepsy Curr* 2012;12:235-6

Schulze-Bonhage A. Brain stimulation as a neuromodulatory epilepsy therapy. Seizure 2017;44:169–175

Semah F, Picot MC, Adam C et al. Is the underlying cause of epilepsy a major prognostic factor for recurrence? *Neurology* 1998; 51: 1256-62

Sheth GS, Krauss G, Krumholz A et al. Mortality in epilepsy: driving fatalities vs other causes of death in patients with epilepsy. *Neurology* 2004;63:1002

Shiozawa P, da Silva ME, de CarvalhoTC et al. Transcutaneous vagus and trigeminal nerve stimulation for neuropsychiatric disorders: a systematic review. *Arq Neuropsiquiatr* 2014;72:542-547

Shiozawa P, da Silva ME, Netto GT et al. Effect of a 10-day trigeminal nerve stimulation (TNS) protocol for treating major depressive disorder: a phase II, sham-controlled, randomized clinical trial. Epilepsy Behav 2015;44:23-6. doi: 10.1016/j.yebeh.2014.12.024.

Shiozawa P. Electroencephalographic changes following a trigeminal nerve stimulation (TNS) protocol: Assessing a novel depression treatment. *Epilepsy & Behavior* 2016;58:141–142

Sillanpaa M. Remission of seizures and predictors of intractability in long-term follow-up. *Epilepsia* 1993; 34: 930-6

Sillanpaa M, Jalava M, Shinnar S. Epilepsy syndromes in patients with childhood-onset seizures in Finland. *Pediatr Neurol* 1999;21:533–537

Sillanpaa M, Schmidt D. Early seizure frequency and aetiology predict long-term medical outcome in childhood-onset epilepsy. *Brain* 2009;132:989–998

Silverman DH, Geist CL, Cook I et al. Effects on regional cerebral blood flow of depressed patients undergoing trigeminal nerve stimulation. *J Nucl Med* 2011;52:1288

Sisodiya SM, Lin WR, Harding BN et al. Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* 2002;125:22-31

Sisodiya SM, Martinian L, Scheffer GL et al. Vascular colocalization of P-glycoprotein, multidrug- resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathol Appl Neurobiol* 2006;32:51–63

Soss J, Heck C, Murray D et al. A prospective long-term study of external trigeminal nerve stimulation for drug-resistant epilepsy. *Epilepsy & Behavior* 2015;42:44–47

Specchio LM, Beghi E. Should antiepileptic drugs be withdrawn in seizure-free patients? *CNS Drugs* 2004;18:201– 212

Sperling MR, Barshow S, Nei M et al. A reappraisal of mortality after epilepsy surgery. *Neurology* 2016;86:1938–1944

Steriade M, Glenn LL. Neocortical and caudate projections of intralaminar thalamic neurons and their synaptic excitation from midbrain reticular core. *J Neurophysiol* 1982;48:352-71

Strozzi I, Nolan SJ, Sperling MR et al. Early versus late antiepileptic drug withdrawal for people with epilepsy in remission. Cochrane Database of Systematic Reviews 2015, Issue 2. Art. No.: CD001902. DOI:10.1002/14651858.CD001902.pub2

Tang F, Hartz AMS, Bauer B. Drug-Resistant epilepsy: Multiple Hypotheses, Few Answers. *Front Neurol* 2017;8:301. doi: 10.3389/fneur.2017.00301

Tate SK, Depondt C, Sisodiya SM et al. Genetic predictors of the maximum doses patients receive during clinical use of the anti-epileptic drugs carbamazepine and phenytoin. Proc Natl Acad Sci USA 2005; 102: 5507–5512

The Vagus Nerve Stimulation Study Group. A randomized controlled trial of chronic vagus nerve stimulation for the treatment of medically intractable seizures. *Neurology* 1995;45:224–30

Tishler DM, Weinberg KI, Hinton DR et al. MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* 1995;36:1–6

van der Werf YD, Witter MP, Groenewegen HJ. The intralaminar and midline nuclei of the thalamus. Anatomical and functional evidence for participation in processes of arousal and awareness. *Brain Res Brain Res Rev* 2002;39:107-40

VanRullen R, Koch C. Competition and selection during visual processing of natural scenes and objects. *J. Vis.* 2003;3, 75–85

Walczak TS, Leppik IE, D'Amelio M, et al. Incidence and risk factors in sudden unexpected death in epilepsy: a prospective cohort study. *Neurology* 2001;56:519

Walker BR, Easton A, Gale K. Regulation of limbic motor seizuresnby GABA and glutamate transmission in nucleus tractus solitarius. *Epilepsia* 1999;40:1051–1057

Wang H, Chen X, Lin Z et al. Long-term effect of vagus nerve stimulation on interictal epileptiform discharges in refractory epilepsy. *Journal of the Neurological Sciences* 2009;284:96–102

Wang Q, Zhu L, Wang X et al. Chronic Trigeminal Nerve Stimulation Protects Against Seizures, Cognitive Impairments, Hippocampal Apoptosis, and Inflammatory Responses in Epileptic Rats. *J Mol Neurosci* 2016;59:78–89

Wasterlain CG, Chen JW. Mechanistic and pharmacologic aspects of status epilepticus and its treatment with new antiepileptic drugs. *Epilepsia* 2008;49:63–73

World Health Organization. Atlas: Epilepsy Care in the World. Geneva: World Health Organization; 2005:91

Yuan H, Silberstein SD. Vagus Nerve and Vagus Nerve Stimulation, a Comprehensive Review: Part III. *Headache* 2016;56:479-490

Zabara J. Inhibition of experimental seizures in canines by repetitive vagal stimulation. *Epilepsia* 1992;33:1005-12

Zanchetti A, Wang SC, Moruzzi G. The effect of vagal afferent stimulation on the EEG pattern of the cat. *Electroencephalogr Clin Neurophysiol* 1952;4:357

Zare M, Salehi M, Mahvari J et al. Trigeminal nerve stimulation: A new way of treatment of refractory seizures. *Adv Biomed Res* 2014;3:81.