*It always seems impossible until it's done*

*Nelson Mandela - 1918-2013*

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# VAGUS NERVE STIMULATION FOR EPILEPSY AND DEPRESSION: MECHANISM OF ACTION AND STIMULATION PARAMETERS

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# List of abbreviations

| | |
|---|---|
| BDNF | brain derived neurotrophic factor |
| bFGF | basic fibroblast growth factor |
| CA3 | cornu ammonis layer 3 |
| DG | dentate gyrus |
| DRN | dorsal raphe nucleus |
| DSM-IV | diagnostic and statistical manual of mental disorders fourth edition |
| DSM-V | diagnostic and statistical manual of mental disorders fifth edition |
| DSP-4 | N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride |
| EC | entorhinal cortex |
| EEG | electroencephalography |
| EMG | electromyography |
| FDA | food and drug administration |
| FGF-2 | fibroblast growth factor-2 |
| GCL | granule cell layer |
| HIL | hilus |
| Hz | Herz |
| ILAE | international league against epilepsy |
| ip | intraperitoneal |
| IQR | interquartile range |
| KA | kainic acid |
| LC | locus coeruleus |
| LMEP | laryneal muscle-evoked potential |
| mA | milliampere |
| MDD | major depressive disorder |
| MEP | muscle-evoked potential |
| MST | motor seizure threshold |
| NeuN | neuronal nuclei |
| NG | nodose ganglion |
| NTS | nucleus tractus solitarius |
| NSE | neuron specific enolase |
| PBS | phosphate buffered saline |

| | |
|---|---|
| Pgi | nucleus paragigantocellularis |
| PrH | nucleus prepositus hypoglossi |
| QAT | quinine aversion test |
| SAL | saline |
| SGZ | subgranular zone |
| SPT | saccharin preference test |
| TMS | transcranial magnetic stimulation |
| TrkB | tropomyosin receptor kinase B |
| VNS | vagus nerve stimulation |
| 5-HT | serotonin |

# Outline of the thesis

**Chapter 1** provides a general introduction on epilepsy and depression, two highly prevalent disorders that occur together very frequently. Etiology, diagnostic criteria, classification and treatment options are highlighted. Furthermore, this chapter discusses the use of vagus nerve stimulation (VNS) for the treatment of refractory epilepsy and depression. Several aspects of VNS including the anatomy of the vagus nerve, the implantation procedure, safety and tolerability, stimulation parameters and efficacy are highlighted.

**Chapter 2** describes the research aims and the rationale of this thesis. Furthermore, the animal models and behavioral tests used in this thesis are discussed.

**Chapter 3** comprises three studies. The first study is a proof-of-concept study where the antidepressant potential of VNS is assessed in an animal model for temporal lobe epilepsy and comorbid depression. The second study investigates whether the locus coeruleus plays an important role in the antidepressant-like mechanism of action of VNS. The third literature study resulted in a review on the putative antidepressant mechanism of action of VNS, focusing on two major hypotheses in depression research: the monoaminergic hypothesis and the neural plasticity hypothesis of major depressive disorders.

**Chapter 4** focuses on the optimization of the stimulation parameters and comprises three studies. The first study investigates the effect of various VNS output current intensities on cortical excitability in the motor cortex stimulation rat model. The hypothesis that low output current intensities are sufficient to affect cortical excitability, is tested. The second and third study consist of two translational studies. The goal of these experiments is to determine whether laryngeal motor-evoked muscle potentials or LMEPs can be recorded in a minimally invasive way in chronically VNS-implanted rats and patients. The ultimate goal of these studies is to investigate whether LMEPs can be used to optimize the stimulation parameters and as an indicator of effective delivery of electrical current to the vagus nerve. In a first phase, the technique is optimized in chronically VNS-implanted rats. In a second phase, we aim at translating this technique to clinical practice.

**Chapter 5** starts with the general conclusion by answering the questions listed in the aims in chapter 2. This is followed by the discussion and future perspectives.

# List of publications included in the thesis

Manuscript 1: **Grimonprez A**, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K and Boon P. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. Brain Stimulation 2015; 8: 13-20. **A1 publication, IF: 5.432, Q1 clinical neurology, neurosciences**

Manuscript 2: **Grimonprez A**, Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P and Vonck K. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. Journal of Psychiatric Research 2015; 68: 1-7. **A1 publication, IF: 4.092, Q1 psychiatry**

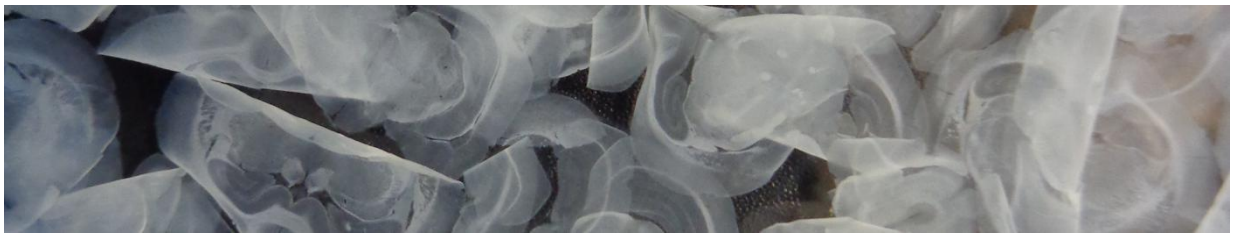
Manuscript 3: **Grimonprez A**, Raedt R, Baeken C, Boon P and Vonck K. The antidepressant mechanism of action of vagus nerve stimulation: evidence from preclinical studies. Neuroscience and Biobehavioral Reviews 2015; 56: 26-34.**Review, IF: 10.284, Q1 behavioral sciences, neurosciences**

Manuscript 4: Mollet L, **Grimonprez A**, Raedt R, Delbeke J, El Tahry R, De Herdt V, Meurs A, Wadman W, Boon P and K Vonck. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. Acta Neurologica Scandinavica 2013; 128: 391-396. **A1 publication, IF: 2.437, Q2 clinical neurology**

Manuscript 5: **Grimonprez A**, De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P and Vonck K. Laryngeal motor-evoked potentials mark vagus nerve activation: a preclinical study. Submitted since May 5$^{th}$ to International Journal of Neural Systems – under review. **A1 publication: IF: 6.056, Q1 computer science, artificial intelligence**

Manuscript 6: **Grimonprez A** and De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P and Vonck K. Laryngeal motor-evoked potentials as an indicator of vagus nerve activation: a clinical pilot trial. In preparation. **A1 publication: in preparation**.

# Chapter 1



Introduction

# Epilepsy

With a worldwide prevalence of approximately 0.5-1.0%, epilepsy is the second most common neurological disorder following neurovascular diseases [1]. The International League against epilepsy (ILAE) defines epilepsy as a chronic neurological condition, characterized by recurrent epileptic seizures. An epileptic seizure manifests itself as a transient occurrence of signs and/or symptoms which may include alterations of consciousness, motor, sensory, autonomic or psychic events and results from abnormal excessive or synchronous neuronal activity in the brain [2]. Two epileptic seizures are separated in time by a period without seizures, which is defined as the interictal period. Although no overt signs of epilepsy may be visible during the interictal period, there might be some abnormalities such as interictal spikes [3].

According to the classification of the ILAE, seizures are divided into two main categories depending on their onset in the brain: partial and generalized seizures. Partial or focal seizures result from an abnormal paroxysmal discharge originating in one of the cerebral hemispheres. The clinical expression and the severity of these seizures is dependent on the region of onset (e.g. motor or visual symptoms when the motor or the visual cortex is involved respectively). These seizures are further subdivided into *simple and complex seizures* referring to the retention and the impairment or complete loss of consciousness respectively. Both types of partial seizures can develop into secondary generalized seizures when the epileptic activity spreads to the contralateral hemisphere. Primary generalized seizures result from abnormal paroxysmal discharges arising in both cerebral hemispheres. These seizures are further subdivided into *tonic-clonic, tonic, clonic, atonic, absence and myoclonic seizures* according to the clinical and electroencephalographic (EEG) characteristics. *Tonic-clonic seizures* are characterized by a sudden tonic contraction of muscles followed by clonic convulsions and subsequent muscle relaxation. During this type of seizures, there is loss of consciousness from seizure onset to the late phase of recovery. As their name suggests, *tonic* and *clonic seizures* only consist of the tonic or clonic phase respectively. *Atonic seizures* on the other hand, are characterized by a sudden loss of muscle tone and subsequently often result in falling. *Absence seizures* are characterized by a sudden interruption of ongoing activities, a blank stare and a sudden end. These seizures are associated with typical bursts of bilateral synchronous spike-wave discharges on the EEG. Children between the age of 4-12 years are most susceptible to this type of seizures. *Myoclonic seizures* present as involuntary single or multiple sudden, brief, shock-like contractions of muscles or muscle groups and are not associated with loss of consciousness [4, 5].

The diagnosis of epilepsy is made when two or more unprovoked seizures have occurred and is primarily based on the medical history of the patient and on EEG recordings. Furthermore, additional investigations including a neuropsychological evaluation, magnetic resonance imaging, single photon emission computed tomography, positron emission tomography and magnetoencephalography can be used to investigate the etiology, to determine the affected brain region and to subclassify the epilepsy syndrome.

## Etiology

In the normal brain, a constant equilibrium is maintained between excitation and inhibition. In an epileptic brain on the other hand, this balance is disturbed and inhibition fails to counteract sudden hyperexcitable and/or synchronous electrical activity. This may result from changes in mechanisms intrinsic to neurons such as changes in conductance of ion channels, second messenger systems and protein expression or from mechanisms extrinsic to neurons such as changes in amounts of neurotransmitters [6]. The potential causes of epilepsy are heterogeneous, ranging from genetic defects, structural abnormalities, metabolic diseases, infections of the central nervous system, neurodegenerative disorders, brain injury, stroke to brain tumors [7]. According to the underlying cause, *symptomatic, idiopathic and cryptogenic epilepsy syndromes* were defined by the ILAE [8]. *Symptomatic epilepsy syndromes* are caused by structural or metabolic abnormalities in the brain, which can either be acquired (e.g. infections or brain trauma), endogenous (e.g. cortical dysplasia) or genetic (e.g. tuberous sclerosis) in origin. *Idiopathic epilepsy syndromes* present without a structural abnormality and are believed to have a strong underlying genetic basis. *Cryptogenic epilepsy syndromes* are epilepsy syndromes with unknown etiology [8]. The ILAE has proposed a new categorization of these epilepsy syndromes based on their etiology. In this new classification, the epilepsy syndromes are divided into *genetic, metabolic/structural and of unknown cause* [2].

## Standard treatment

### Pharmacotherapy - antiepileptic drugs

Antiepileptic drugs are the standard first-line treatment for epilepsy. The mechanism of action of different antiepileptic drugs varies, but the main mechanisms are based on restoring the disturbed excitation/inhibition equilibrium within the brain through (1) blockade of voltage-gated ion channels, (2) stimulation of the inhibitory GABA-ergic system and (3) inhibition of the excitatory glutamatergic

system [9]. Initially, one antiepileptic drug is started at a low dose and is slowly up-titrated. In case no optimal response is achieved due to side effects or ongoing seizures, a second monotherapy is started. When two first-line monotherapy trials fail, the chance to render a patient seizure free with a third monotherapy drops to 5% and a combination of two or more antiepileptic drugs is administered [5]. Common adverse events of antiepileptic drugs are somnolence, dizziness, blurry vision and cognitive problems [10].

**Prognosis and alternative treatments**

Although the majority of epileptic patients experience a significant reduction in seizure frequency with antiepileptic drugs, more than 30% of patients suffer from uncontrolled seizures or intolerable side effects despite an adequate treatment [11]. These patients suffer from **refractory epilepsy,** which is associated with excess injury, mortality, significant cognitive impairment and economic costs [12]. The ILAE defines refractory epilepsy as: "the failure of adequate trials of two tolerated and appropriately chosen and used antiepileptic drug schedules (whether as monotherapy or in combination) to achieve sustained seizure freedom" [13]. For patients suffering from refractory epilepsy, alternative treatments are necessary and include phase 3 trials with newly developed antiepileptic drugs, epilepsy surgery, dietary treatments, immunological treatments and neurostimulation modalities [14].

Phase 3 trials with newly developed antiepileptic drugs

The administration of newly developed antiepileptic drugs leads to 50% seizure frequency reduction and seizure freedom in only 21% and 6% of refractory patients respectively [15]. Furthermore, patients who are included in consecutive phase 3 trials often experience a poor quality of life [16].

Epilepsy surgery

Epilepsy surgery is a neurosurgical procedure where the area of the brain involved in seizure generation is either *resected or disconnected. Resective surgery* consists of the removal of brain tissue that is responsible for provoking habitual seizures in an individual patient (e.g. lobectomy, lesionectomy or hemispherectomy). *Disconnective surgery* involves the disconnection of nerve fibers through which abnormal epileptic activity spreads to the adjacent tissue (e.g. callosotomy, multiple

subpial transections or gamma-knife surgery) [17]. Epilepsy surgery results in seizure freedom in up to 85% of the patients depending on the localization of the seizure focus [18].

Dietary treatments

Dietary treatments involve the *ketogenic and the Atkins diet*. The *ketogenic diet*, which is very high in fat and low in carbohydrates, is thought to simulate the metabolic effects of starvation by forcing the body to use fat as a primary fuel source, thereby creating ketones. These ketones are then used in the brain as an alternative energy source, which is associated with a significant (>90%) seizure reduction and complete seizure freedom in 30% and 10-15% of patients respectively [19]. Despite the substantial seizure reduction, the diet is often discontinued because of side effects such as constipation, sleepiness and nausea. Furthermore, the ketogenic diet is not typically offered to adults due to the significant lifestyle alterations needed for its use. The *Atkins diet* is also based on the intake of fat and the restriction of carbohydrates, but the daily allowed amount of proteins is higher compared to the ketogenic diet. Half of the patients experience a seizure frequency reduction of 50-90%, while 28% of patients even report a seizure frequency reduction of more than 90% [14]. Nevertheless, a substantial part of the patients also stop the Atkins' diet because of inefficacy, side effects and restrictiveness [20, 21].

Immunological treatments

Immune system dysfunction may play a role in epilepsy by triggering or maintaining epileptic seizures. Therefore, immunological treatments may have a beneficial effect on epileptic seizures. In approximately half of patients, seizure frequency can be reduced with 50% [22]. However, this treatment is associated with significant side effects including electrolyte disturbances, glucose intolerance, hypertension, increased susceptibility for infections, osteoporosis, myopathy and cardiomyopathy [14].

Neurostimulation modalities

Neurostimulation is a treatment modality in which electrical pulses are administered directly to or in the neighborhood of nerve tissue in order to manipulate a pathological substrate and to achieve a symptomatic or even curative therapeutic effect. The different types of neurostimulation differ in the

part of the nervous system affected and in the way the stimulation is administered [23] and include repetitive transcranial magnetic stimulation, transcranial direct current stimulation, trigeminal nerve stimulation, deep brain stimulation and (transcutaneous) vagus nerve stimulation. The responder rate for these neurostimulation treatements ranges from 23% to 43%, depending on the type of stimulation and the duration of the treatment [24, 25]. A full discussion on all techniques is beyond the scope of the thesis and is given elsewhere [23, 25, 26]. However, as vagus nerve stimulation is the main topic of this thesis, detailed information on clinical efficacy can be found below (see p. 21).

# Depression

The World Health Organization estimates that by 2020, major depressive disorders (MDD) will become the second largest cause of global disease problems in the world, immediately following ischemic heart disease [27]. The lifetime prevalence for MDD is reported to be as high as 17% and the 12-month prevalence is estimated to be 4-8% [28, 29]. The disease appears to develop independent of ethnicity, education or income and is usually associated with substantial symptom severity and role impairment. The resulting disability and burden do not only affect the individual in terms of decreased productivity, but the level of health care utilization and suicide is also increased [30].

According to the diagnostic and statistical manual of mental disorders (DSM-V) [31] , MDD manifests with a heterogeneous set of symptoms, both at the psychological and behavioral level as well as at the physiological level. A depressive episode requires the presence of one or two of the following core symptoms for at least two weeks: (1) depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feeling sad or empty) or observation made by others (e.g. appears tearful). Note: In children and adolescents, can be irritable mood and (2) markedly diminished interest or pleasure in all, or almost all activities that usually would be enjoyed and this most of the day, nearly every day (anhedonia) (as indicated by either subjective account or observation made by others). In addition, four of the following symptoms must be present (three if both core symptoms are present):

(1) Significant weight gain or weight loss when not dieting (e.g. a change of more than 5% in body weight in a month), or decrease or increase in appetite nearly every day. Note: In children, consider failure to make expected weight gains; (2) insomnia or hypersomnia nearly every day; (3) psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings or restlessness or being slowed down); (4) fatigue or loss of energy every day; (5) feelings of worthlessness or excessive, inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick); (6) diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others) and (7) recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan or a suicide attempt or a specific plan for committing suicide [31, 32].

The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning. Furthermore, the symptoms are not caused by the direct physiological effects of a substance (e.g. a drug of abuse or a medication) or a general medical condition (e.g. hyperthyroidism) and cannot result from bereavement (e.g. after the loss of a loved one) .

The diagnosis and the evaluation of response to treatment is based on clinical observations and the results from depression rating scales. These scales differ according to the observer (self-rating versus observer rating scales), the symptoms investigated, the number of items and the criteria used. The most commonly used depression rating scales are the Hamilton depression Rating scale, the Montgomery-Asberg depression rating scale, and the Beck depression inventory [33].

**Etiology**

Depression is a disease with plenty of risk factors including environmental, genetic and psychological ones. The multifaceted character of depression renders its etiology challenging to study. In this regard, a lot of pathological processes have been identified including overactivity of the hypothalamic-pituitary-adrenal axis, increased neuroinflammation, impaired endogenous opioid function, imbalances in several neurotransmitter systems, reduced neural plasticity and dysfunction of specific brain structures and circuits [34-36]. The abundance of pathological processes has led to the emergence of several hypotheses of MDD, two very important ones being *the monoamine and the neural plasticity hypothesis of MDD. The monoamine hypothesis* postulates that the pathophysiological basis of depression is the deficient activity of the monoamines - mainly serotonin and noradrenaline - in the central nervous system. Indeed, almost all currently available antidepressants are based on enhancing the serotonergic and/or noradrenergic system (see below) [34, 37]. The main assumption in *the neural plasticity hypothesis of MDD* is that reduced neural plasticity plays a major role in the pathophysiology of depression, and that its restoration represents a critical mechanism underlying antidepressant efficacy [35].

## Standard treatment

### Pharmacotherapy – antidepressant drugs

Antidepressant drugs and psychotherapy are the standard treatments for MDD. The mechanism of action of most antidepressant drugs is based on increasing the concentration of serotonin and noradrenaline in the synaptic cleft via (1) inhibition of their reuptake (tricyclic antidepressants, selective serotonin reuptake inhibitors, noradrenaline reuptake inhibitors and serotonin-noradrenaline reuptake inhibitors), (2) antagonism of inhibitory presynaptic autoreceptors and (3) inhibition of monoamine oxidases (monoamine oxidase inhibitors), which are the enzymes for monoamine degradation [34, 37].

### Psychotherapy

The conceptualization of depression as a psychological disorder has inspired the development of various forms of psychotherapy [38]. The two main types of psychotherapy for the treatment of MDD are *cognitive behavioral therapy and interpersonal therapy*. *Cognitive behavioral therapy* attempts to change dysfunctional patterns of thinking in order to prevent the development and maintenance of depressive symptoms [39]. *Interpersonal therapy* assists patients in analyzing their interpersonal relationship modes, correlating their relational states with their mood and learning to use better communication [40].

## Prognosis and alternative treatments

In the majority of patients, depressive symptoms can be effectively treated with pharmacotherapy, psychotherapy or the combination of both interventions [41]. Nevertheless, up to 30% of patients fail to respond to these standard interventions and hence suffer from **refractory depression** [42]. Even more problematic is the fact that the disease tends to recur, with greater than 75% of patients experiencing more than 1 episode in a 10-year period [43, 44]. The lack of success with the standard therapies highlights the importance of optimizing alternative therapies for patients suffering from refractory depression. Similar as in refractory epilepsy, neurostimulation modalities including electroconvulsive therapy, repetitive transcranial magnetic stimulation, transcranial direct current stimulation, trigeminal nerve stimulation, deep brain stimulation and (transcutaneous) vagus nerve stimulation are used for the treatment of refractory depression [38].

# Comorbidity of epilepsy and depression

The risk of developing MDD is approximately five times higher in refractory epilepsy patients than among the general population [45-47]. On the other hand, major depressive episodes and suicide attempts independently increase the risk of developing unprovoked seizures and epilepsy [48, 49]. Traditionally, epilepsy-related depression was believed to result from the psychosocial burden of having a chronic debilitating neurologic disorder and the stigma related to epilepsy, which are still the major reasons why depression remains underdiagnosed in the epileptic population [50]. However, the bidirectional relationship suggests that this comorbidity is more than a psychosocial phenomenon and that the two disorders likely share common pathogenic mechanisms [45, 47, 48, 51]. These mechanisms might include a hyperactive hypothalamic–pituitary–adrenal axis and its neuroanatomic and neuropathologic complications, as well as disturbances in serotonergic, noradrenergic, GABA-ergic and glutamatergic neurotransmitter systems, all of which may be interrelated [52]. Furthermore, increased neuroinflammation [36, 53] and changes in neural plasticity [54, 55] are abnormalities found both in epilepsy and depression and might therefore also be involved in the comorbidity of these disorders.

As depressive symptoms have a more profound impact on an epileptic patient's quality of life than seizure severity or frequency [56-59], it is key to diagnose and treat these symptoms as well. Current pharmacological treatment options for patients suffering both from epilepsy and depression are limited by the fact that antiepileptic drugs can contribute to mood disturbances, while antidepressant drugs can increase seizure susceptibility [60, 61]. The lack of success with current pharmacological interventions for patients suffering both from refractory epilepsy and depression, highlights the importance of further optimizing alternative neuromodulatory treatments. These treatments include repetitive transcranial magnetic stimulation, transcranial direct current stimulation, deep brain stimulation and vagus nerve stimulation (VNS) [25, 38]. As VNS is the main topic of this thesis, this treatment modality will be discussed in detail below.

# VNS for the treatment of epilepsy and depression

## Historical background

The historical basis of peripheral stimulation for the treatment of seizures dates back to the sixteenth and seventeenth century, when physicians described the use of a ligature around the limb in which a seizure commences to arrest its progress [62]. This method was described by the ancient Greek author Pelops for whom this observation was proof that epileptic fits originate in the limb itself. This hypothesis was reviewed in the beginning of the nineteenth century, when Odier and Brown-Sequard showed that ligatures are equally efficacious in arresting seizures caused by organic brain disease e.g. a brain tumor [63]. At the end of this century, Gowers attributed these findings to a raised resistance in the sensory and motor nerve cells corresponding to the limb involved. This would in turn arrest the spread of the discharge. Gowers also reported several other ways by which sensory stimulation could prevent seizures from spreading e.g. pinching of the skin and inhalation of ammonia [64]. Almost a hundred years later, Rajna and Lona demonstrated that afferent sensory stimuli can abort epileptic paroxysms in humans [62, 65]. The vagus nerve became a point of interest among neurologists in the nineteenth century when it was thought that seizures could be aborted by applying pressure to the this nerve via the carotid artery located in the neck [66]. Subsequently, early research in animal models confirmed that electrical stimulation of the vagus nerve decreases the frequency and severity of epileptic seizures [67, 68]. Since then, numerous preclinical studies have confirmed the antiepileptic effect of VNS (reviewed in [69]).

## Indications

VNS was approved for the treatment of refractory epilepsy in 1994 and 1997 in Europe and the United States respectively. Mood improvements in epilepsy patients treated with VNS, irrespective of the effects on seizure frequency [46, 70], provided the initial rational for using VNS for the treatment of refractory depression. Subsequently, VNS was approved in 2001 in Europe and Canada for the treatment of non-psychotic unipolar and bipolar depressed patients that had failed to respond to at least four antidepressant trials. Four years later - in July of 2005 - the treatment was approved for refractory depression by the Food and Drug Administration (FDA) in the United States as well [71-74]. Since the first patient was implanted in 1988 [75], over 100.000 VNS devices have been implanted in more than 75.000 patients worldwide [76]. As the vagus nerve innervates and thereby influences

virtually the whole body (see below), it is not surprising that VNS is currently under investigation for the treatment of several other diseases including chronic heart failure, Alzheimer's disease, pain, tinnitus, obesity and anxiety disorders [77-79].

**Anatomy – efferent and afferent projections**

The tenth cranial nerve or vagus nerve is the longest of the cranial nerves and extends from the brain stem to the abdomen. Its complex anatomical distribution has earned the vagus nerve its name, as vagus is the Latin word for wanderer [80]. The left vagus nerve comprises approximately 100.000 axons, 20% of which are *efferent* (motor) fibers and 80% of which are *afferent* (sensory) fibers. Figure 1 gives a schematic overview of the main efferent and afferent projections.

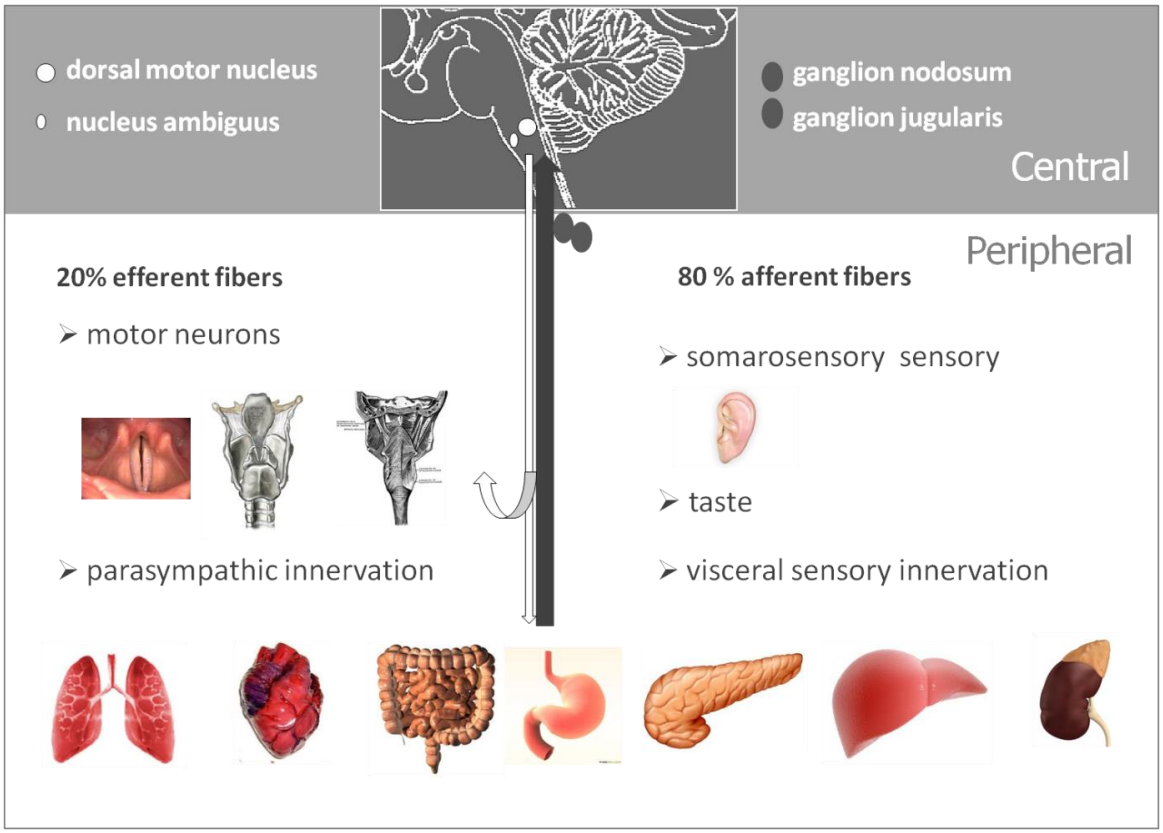



**Figure 1:** Schematic representation of the efferent and afferent projections of the vagus nerve. Adapted from *Vonck et al. 2007* [80].

The *efferent fibers* of the vagus nerve originate from the dorsal motor nucleus and the nucleus ambiguus. A fraction of these fibers of the vagus nerve provides parasympathetic innervation to the lungs and the abdominal viscera, while another fraction contributes to the recurrent laryngeal nerve,

which branches off from the vagus nerve and carries low-threshold vagal motor neurons to the larynx, pharynx and vocal cords. The *afferent fibers* of the vagus nerve originate from the ganglion nodosum and jugularis and convey somatosensory information of the ear, taste information and visceral information to the brain [80, 81]. At the cervical level, the vagus nerve mainly consists of small diameter unmyelinated C-fibers (65–80%) and of a smaller portion (35-20%) of intermediate diameter myelinated B-fibers and large-diameter myelinated A-fibers [62]. As the upstream afferent projections within the brain are considered to be key in the mechanism of action of VNS, these connection will be discussed later in detail (see chapter 3-3).

**Implantation procedure**

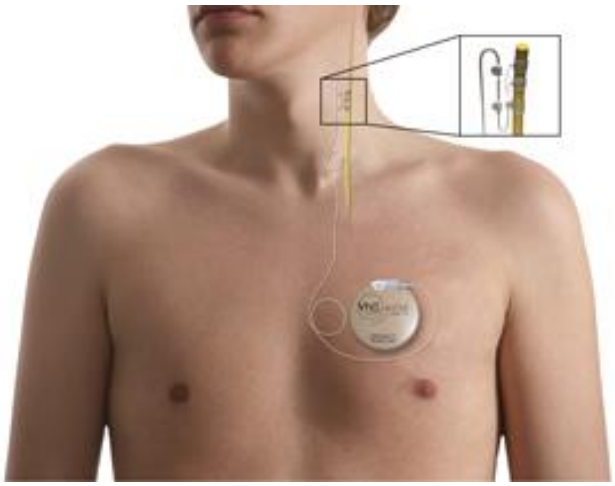


**Figure 2:** VNS generator, lead and bipolar electrode location in chest wall.

VNS is an extracranial form of neurostimulation which consists of electrically stimulating the left vagus nerve through an implanted electrode and a pulse generator (figure 2 [82]). The surgical procedure should be carried out by a neurosurgeon familiar with the surgical approach for carotid endarterectomy because of the location of the vagus nerve in the neck within the carotid sheath, where the vagus nerve is running between the carotid artery and the internal jugular vein [62]. The insertion of the device is usually performed under general anesthesia and involves 2 incisions. The first incision is made at the level of the neck, where the platysmal and subplatysmal cervical fascia are dissected until the carotid sheath is exposed. The vagus nerve is identified within the sheath and at least 2.5 cm of the nerve is exposed. The bipolar electrode consisting of two silicone helical coils, is then wrapped around the vagus nerve, with the cathode and the anode placed rostrally and caudally respectively. A third helical coil is located further caudally to tether the lead to the vagus nerve. Subsequently, the lead from the electrode to the pulse generator is tunneled through the subcutaneous fat layer into the left chest area where the second incision is made. There, a subcutaneous pocket in the anterior chest wall is made for the insertion of the pulse generator [83].

**Stimulation parameters**

Electrical pulses applied in VNS are defined by the following stimulation parameters: *output current, frequency, pulse width and ON/OFF time or duty cycle* [84]. The programming of the stimulation parameters is performed using an external wand that is connected to a handheld computer. The pulse generator is usually turned on 2 weeks postoperatively to allow wound healing. Typically, the *output current* is set at 0.25 mA at the start of the therapy and is gradually ramped-up in steps of 0.25-0.50 mA every 2-4 weeks according to the individual tolerance level of the patient or to a maximum of 3.5 mA. The *frequency and the pulse width* of the stimulation pulse are typically set at 20-30 Hz and 250-500 µs respectively. The standard *duty cycle* is 30 s ON/ 5 min OFF. The choice of intermittent stimulation is based upon safety studies with regard to the stimulation of neural tissue [85], efficacy studies showing that the effect of stimulation outlasts the stimulus duration [67, 86] and the knowledge that intermittent stimulation is associated with a longer battery life. All parameter settings can be modified in order to reach maximum therapeutic efficacy, while minimizing stimulation-related side effects and preserving battery life. Table 1. shows the range of possible settings, the programming steps, the recommended initial values and the typical target values.

| stimulation parameters | programmable range | programming steps | recommended inital values | typical target values |
|---|---|---|---|---|
| output current | 0-3.5 mA | 0.25 mA | 0.25 mA | 1.00-2.00 mA |
| frequency | 1-30 Hz | 1, 2, 5, 10, 15, 20, 25, 30 Hz | 20-30 Hz | 20-30 Hz |
| pulse width | 130-1000 µs | 130, 250, 500, 750, 1000 µs | 250-500 µs | 250-500 µs |
| duty cycle | 10-100% | function of signal ON, OFF times | 10% | 10% |
| signal ON time | 7-60 s | 7, 14, 21, 30, 60 s | 30 s | 30 s |
| signal OFF time | 0.2-180 min | 5–60 min, 5-min steps<br>60–180 min, 30-min steps | 5 min | 5 min |

**Table 1:** Adapted from *Labiner et al. 2007* [87, 88].

The stimulation parameters used currently in clinical practice and in experimental studies are based on what is known to be safe and tolerable and are therefore not evidence-based but rather empirically determined. However, optimizing the stimulation paradigm is an indispensable step towards the achievement of a better clinical outcome. Therefore, the

research presented in chapter 4 of this thesis will aim at optimizing the stimulation parameters.

**Safety and tolerability**

Side effects due to VNS can be subdivided into *surgery- and stimulation-related side effects*. *Surgery-related side effects* are rare but include fluid accumulation at the generator site, incisional infections, vocal cord paresis, lower facial weakness and bradycardia [89]. Rare cases of ventricular asystole have also been reported when the device is tested during the implantation procedure in the operating room. However, no long-term negative outcomes resulted in these cases [84, 90]. Concerning cosmetic adverse events, several generator models have been developed with each successive model having smaller dimensions to improve cosmetic outcome (see figure 3) [91].


**Figure 3:** Different types of VNS generators. The size and volume of the generators have been reduced over time, thereby reducing cosmetic adverse events.

The most frequent *stimulation-related side effects* are tingling sensations in the throat and hoarseness or voice alterations [84, 89]. The tingling sensations in the throat results from the secondary afferent stimulation of the superior laryngeal nerve, which branches off from the vagus nerve superior to the location of the implanted electrode and carries sensory fibers to the laryngeal mucosa. Hoarseness and voice alterations on the other hand, result from the efferent stimulation of the recurrent laryngeal nerve which branches off distally from the location of the electrode and carries motor fibers to the laryngeal muscles [92]. The stimulation-related side effects generally disappear when the stimulation parameters are adapted e.g. when the output current, the frequency or the pulse width are reduced. Therefore, VNS-related side effects are in general mild and short-lived and subsequently, VNS is considered to be a safe and well-tolerated treatment [89]. With regard to other side effects related to the stimulation of vagal efferents, the effect on the heart rate has been a major concern. In this regard, left VNS is preferred over right VNS, owing to the fact that the left vagus nerve has fewer efferent projections into the heart, thereby reducing the risk of cardiac side effects, such as bradycardia [93]. However, the stimulation parameters used to suppress

seizures in patients and experimental animals do not maximally activate the high-threshold, unmyelinated vagal C-fibers [94]. Activation of these C-fibers is necessary to induce bradycardia [95], but not to reduce seizure severity [96]. Indeed, previous studies in non-human primates have demonstrated that right-sided VNS does not induce detectable cardiac effects [68]. Furthermore, it was shown that right-sided VNS is as effective as left-sided VNS in reducing epileptic seizures in rats [97] and pigs [98]. In patients as well, right-sided VNS was shown to be safe and to have antiepileptic effects in subjects that were previously successfully treated with left-sided VNS but had to discontinue the treatment due to nerve injury [99-101]. Therefore, right-sided VNS could be proposed as an alternative treatment for refractory epilepsy. Nevertheless, future trials on bigger patient samples, including electrocardiogram monitoring to detect the presence of possible cardiac side effects, are needed to confirm this hypothesis.

Compared to antiepileptic and antidepressant pharmacological treatments, VNS has several advantages. First of all, non-compliance with drug treatments remains a big problem. A significant portion of patients do not take their medication as prescribed or even do not take it at all [102]. Moreover, most patients will stop the treatment as soon as they are feeling better [103]. As VNS occurs continuously through an implanted device, compliance is not an issue with this therapy. Furthermore, the typical drug-induced side effects such as cognitive impairment and somnolence, are not reported in patients treated with VNS [89]. On the contrary, VNS-induced cognitive improvements and increased alertness have been observed in patients suffering from both refractory epilepsy and depression [104]. Due to the nature of the treatment, VNS therapy can be combined with currently available pharmacologic treatments without the risk of drug interactions. Furthermore, when used as an adjunctive therapy, VNS results in a better control of seizures and depressive symptoms at smaller doses of antiepileptic or antidepressant medications, consequently resulting in decreased dose-dependent side effects of these pharmacological treatments [105]. In contrast to many pharmacological compounds, treatment tolerance does not develop with VNS therapy [80]. Contrary to pharmacological treatments, efficacy tends to increase with a longer duration of the treatment [71, 80, 106, 107]. Another advantage of VNS is the fact that the patient or caregiver is provided with a magnet, which allows additional stimulation when an aura or a seizure occurs [108]. On the other hand the patient can stop the stimulation in situations where it may cause discomfort, e.g. during public speaking [91]. Therefore, the use of this magnet gives the patients a feeling of control over their situation, thereby reducing stress [109]. In contrast to ablative neurosurgical interventions, VNS has the advantage of being reversible as the stimulation can be stopped or the explantation of the device can be easily performed in case the patient wants to

discontinue the treatment. Furthermore, the surgical procedure for a VNS implantation involves a lower risk and fewer complications than for ablative surgery [93, 110].

**Efficacy and predictors of response**

Epilepsy

The earliest studies demonstrated that one third of patients has a significant improvement in seizure control with a reduction in seizure frequency of at least 50% (responders), one third of patients experiences a worthwhile reduction in seizure frequency between 30% and 50% (partial responders) and the remaining third of patients experiences little or no effect (non-reponders). More recent large series of patients, both in children [111] and in adults [112], with long-term follow-up of over 5 years have been reported and demonstrate that up to 60% of patients become responders. A recent meta-analysis of VNS efficacy in epilepsy revealed that VNS produces an average reduction in seizure frequency of 45%, with 36% seizure reduction at 3-12 months after surgery and 51% seizure reduction after more than one year of therapy. Furthermore, at the last follow-up, seizures were shown to be reduced by 50% or more in approximately 50% of the patients [113]. The meta-analysis revealed that patients with generalized epilepsy and children benefit significantly from VNS despite their exclusion from initial approval of the device. Furthermore, posttraumatic epilepsies and tuberous sclerosis were shown to be positive predictors of a favorable outcome [109, 113]. In a study by Janszky et al., the absence of bilateral interictal epileptiform discharges and the presence of malformations of cortical development were associated with a seizure-free outcome [114]. A recent study by Acros et al. demonstrated that a temporal lobe discharge is an indicator of an early response and that the presence of a lesion indicates a late response. Furthermore, patients with fewer rates of seizures were found to have a better prognosis in the latter study [115].

Depression

As only a limited number of trials on the antidepressant effect of VNS have been performed to date [116], a meta-analysis is not yet available. Open label studies have demonstrated a steadily increasing improvement of depressive symptoms with full benefit after 6 to 12 months, sustained for up to two years (for an overview, see [117, 118]). These studies reported response rates (defined as a > 50% decrease in depression severity) up to 53% and remission rates up to 39% after 3-24 months of treatment [117-119]. Unfortunately, the only blinded sham-controlled clinical trial was inconclusive,

because the output current in some patients had not been adequately ramped-up and these patients did therefore not receive the full therapy [116]. Although responder identification studies are lacking, it was shown that the history of treatment resistance is predictive of VNS outcome. That is, patients who have never received electroconvulsive therapy, are 3.9 times more likely to respond to VNS. Thus, VNS appears to be most effective in patients with low to moderate, but not extreme antidepressant resistance [117, 120].

The comorbidity of epilepsy and depression

Several studies have demonstrated VNS-induced mood improvements in patients suffering from epilepsy and comorbid depressive symptoms [70, 121-123]. Surprisingly, only one study [122] has found an association between seizure reduction and mood improvement. This may indicate additional effects of VNS on mood, independent of improved seizure control and therefore independent of epilepsy. Although the results from these studies are promising, future randomized controlled trials are needed to confirm the hypothesis that VNS has antidepressant effects in patients suffering from epilepsy and comorbid depression.

## Mechanism of action

As for many drugs, the clinical application of VNS preceded the research into its mechanism of action. The initial hypothesis on the mechanism of action was based on the knowledge that the tenth cranial nerve afferent fibers have numerous projections within the central nervous system and that in this way, action potentials generated in vagal afferent fibers have the potential to affect the entire organism [124]. Clues on the mechanism of action of VNS have arisen from electrophysiological studies, functional imaging studies, neuropsychological studies and behavioral studies both in humans [80, 125] and in experimental animals [69, 126]. Despite the abundance of experimental studies, the precise mechanism of action remains to be elucidated. As a full review of all studies is beyond the scope of this thesis and is given elsewhere [69, 91, 126], only a brief summary will be given on the putative mechanism of action VNS.

Epilepsy

As epileptic seizures are characterized by an increase in cortical excitability and synchronous firing of populations of neurons, it was hypothesized that VNS suppresses seizures by reducing cortical

excitability and desynchronizing neuronal activity. Subsequently, experimental animal studies were conducted demonstrating that VNS indeed decreases interictal epileptiform EEG discharges [127-129] and reduces cortical excitability [130, 131]. Furthermore, VNS was shown to induce an increase both in EEG synchronization and desynchronization, depending on the frequency of the stimulation [128, 132]. Interestingly, numerous studies have found that VNS reduces cortical excitability and/or seizures in a wide range of experimental animal models (reviewed in [69]). In these models, seizures are originating from different cortical sites, e.g. from the hippocampus or amygdala in the hippocampal and amygdala kindling model respectively or from the motor cortex in the cortical stimulation model [130, 131, 133-135]. This suggests that the sites at which VNS controls neuronal excitability are widely distributed, which is consistent with the widespread afferent projections of the vagus nerve in the central nervous system. Based both on clinical and experimental animal studies, it is assumed that effective VNS is mediated through the activation of the afferent myelinated A- and B-fibers and not of the small unmyelinated C-fibers [96, 136-139]. These afferent A- and B-fibers project to the nucleus tractus solitarius (NTS). In turn, the NTS has widespread projections to virtually the whole brain, including areas important for epileptogenesis such as the amygdala, the hippocampus and the thalamus [140]. Consistent with this knowledge, functional neuroimaging studies have demonstrated widespread VNS-induced metabolic changes in brain regions implicated in seizure generation including the thalamus, cerebellum, orbitofrontal cortex, limbic system, hypothalamus and medulla (reviewed in [125]). Further work by Naritoku and colleagues [141] examined the molecular biological effects of VNS on multiregional neuronal activities in the brainstem and cerebral cortex. This group found VNS-induced increases in the expression of neuronal c-fos - a marker for increased metabolic activity - in the medullary vagal complex, the locus coeruleus and several thalamic and hypothalamic nuclei. The changes in neuronal activity in these diffuse cortical networks can most likely be explained by the VNS-induced changes in neurotransmitter concentrations. That is, the intracranial effect of VNS may be based on VNS-induced increases in the concentration of the inhibitory neurotransmitter GABA or decreases in the concentration of the excitatory neurotransmitters glutamate and aspartate [142-151]. Furthermore, the mechanism of action of VNS may involve other modulatory neurotransmitters that are known to have antiepileptic effects, such as serotonin and noradrenaline [133]. The relevant anatomy, the effects of VNS on the dorsal raphe nucleus and the locus coeruleus – which are the main sources of serotonin and noradrenaline respectively - and the subsequent release of serotonin and noradreanline in the brain, will be discussed in detail in the review on the putative antidepressant mechanism of action of VNS (see below, chapter 3-3). Apart from the known effects of VNS on GABA, glutamate, aspartate serotonin and noradrenaline, a growing body of evidence suggests that the therapeutic effects of VNS are mediated by acetylcholine release through the activation of the nucleus basalis [152, 153].

To summarize, the exact antiepileptic mechanism of action of VNS remains to be elucidated, but research has demonstrated that stimulation of the left vagus nerve induces inhibitory effects on neuronal excitability in a wide range of cortical structures. These changes could potentially result from the VNS-induced changes in neurotransmitter concentrations including GABA, glutamate, aspartate, serotonin, noradrenaline and acetylcholine. The molecular changes underlying the neurotransmitter-induced alterations in cortical excitability are incompletely understood to date, but a growing body of evidence suggests that neuroplastic [55, 154-157] and neuroimmunomodulatory [53, 158-161] effects might be the missing link.

Depression

As for epilepsy, the exact mechanism of action of VNS for depression remains to be elucidated. A review on the putative antidepressant mechanism of action of VNS can be found in chapter 3-3, a summary of which is given in the following paragraph.

Consistent with the monoamine theory of depression, serotonin and noradrenaline were identified as key players in the antidepressant mechanism of action of VNS [133, 145, 156, 162-167]. However, VNS induces an acute elevation of the monoamines, while its antidepressant effect in patients is only established after long-term treatment. In this regard, a growing body of evidence suggests that neuroplastic changes might be the missing link [154-157, 168]. In other words, we hypothesize that VNS exerts its antidepressant effects through a rapid increase in the concentration of the monoamines, which then enhances neuronal plasticity in the hippocampus. Newborn cells could then functionally integrate and restore disturbed cortico-limbic networks in depressed subjects. Processes such as increased dendritic complexity and the formation of new synapses could further strengthen these networks [155]. The latter processes take several weeks to months to be completed, which provides an explanation for the therapeutic lag.

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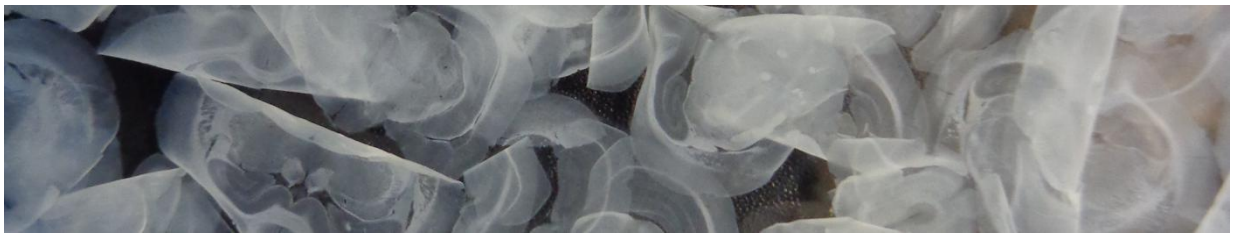
# Chapter 2



Aims and rationale of the thesis

# Aims and rationale of the thesis

VNS is a well-established, safe and effective add-on therapy for patients suffering from refractory epilepsy [1]. Mood improvements in epilepsy patients treated with vagus nerve stimulation (VNS), provided the initial rationale for using VNS for the treatment of refractory depression [2-4]. However, randomized controlled trials confirming the antidepressant effect of VNS in epilepsy patients are lacking. Furthermore, the mechanism of action is still unknown, optimal stimulation parameters remain elusive and about one third of patients do not benefit from the treatment. Therefore, the aim of this thesis is (1) to perform a proof-of-concept study and to unravel the antidepressant mechanism of action of VNS and (2) to optimize the stimulation parameters in order to improve clinical outcome.

**More specifically, this thesis addresses the following research questions:**

**Proof-of-concept and mechanism of action**

1. Does VNS have and antidepressant-like effect in the kainic acid rat model for temporal lobe epilepsy and comorbid anhedonia, the latter being a key symptom of major depression.
2. Does the locus coeruleus play a key role in the antidepressant-like mechanism of action of VNS?

**Optimization of stimulation parameters**

1. Are low VNS output current intensities sufficient to reduce cortical excitability in the motor cortex stimulation rat model?
2. Can VNS-induced laryngeal muscle-evoked potentials or LMEPs be measured in chronically VNS-implanted rats in a minimally invasive way?
3. Can LMEPs be used to determine optimal stimulation parameters, to identify non-responders and to individualize post-operative recovery periods?
4. Can this technique be translated to clinical practice?

## Study 1: The antidepressant potential of VNS in the kainic acid (KA) model for temporal lobe epilepsy and comorbid anhedonia

Depressive disorders are the most common type of psychiatric comorbidity in epileptic patients, especially in individuals suffering from refractory temporal lobe epilepsy. Despite the availability of a variety of both antiepileptic and antidepressant drugs, up to 30% of patients fail to respond adequately to standard medication. Furthermore, current treatment options for patients suffering both from epilepsy and depression are limited by the fact that anticonvulsant drugs can contribute to mood disturbances, while antidepressant drugs can increase seizure susceptibility [5-7]. The lack of success with current pharmacological interventions for patients suffering from both epilepsy and depression, highlights the importance of optimizing non-pharmacological, neuromodulatory treatments such as VNS for this patient population. The initial rationale for using VNS to treat refractory depression was fueled by the observation that VNS induces mood improvements in epilepsy patients, irrespective of the effect on seizure frequency [2-4]. However, there are no randomized controlled trials confirming the antidepressant effect of VNS in epileptic patients. Studies on the antidepressant effect of VNS in a clinical population are confounded by multiple factors, including concomitant antiepileptic drug therapy, psychosocial and intellectual effects. The use of animal models overcomes this problem and is important in unraverling the mechanism of action of VNS for the treatment of epilepsy-related depression. Therefore, we wanted to perform a proof-of-concept study to assess the antidepressant potential of VNS in an animal model for epilepsy and comorbid anhedonia, a key symptom of major depression. For this purpose the effect of VNS on the hedonic state was assessed in the the post status epilepticus KA rat model for temporal lobe epilepsy and comorbid anhedonia, using the saccharin preference and the quinine aversion test.

**The post status epilepticus KA rat model** is a validated model for temporal lobe epilepsy. KA is a potent neuroexcitatory amino acid that acts by activating receptors for glutamate, the principal excitatory neurotransmitter in the central nervous system. When KA is injected systemically in rats, it rapidly produces epileptic seizures, which are characterized by by the following phenomena: stage 1: immobility, eye closure, twitching of vibrissae, facial clonus, wet dog shakes; stage 2: head nodding, chewing, severe facial clonus, wet dog shakes; stage 3: clonus of one forelimb; stage 4: rearing, bilateral forelimb clonus; stage 5: rearing, bilateral forelimb clonus, loss of balance and falling [8-10]. These seizures typically last for several hours without complete recovery in between, and can therefore be considered a status epilepticus. This status epilepticus is followed by a latent period,

during which epileptogenic changes (e.g. hippocampal cell loss, mossy fiber sprouting and dentate gyrus cell dispersion [11, 12]) occur in the brain. These epileptogenic changes result in spontaneous, frequently secondarily generalized seizures [9], thereby closely resembling temporal lobe epilepsy in humans [10]. Furthermore, it was recently shown that KA rats display anhedonia, a key symptom of major depression [13].

In animal research, anhedonia can be assessed using the **saccharin preference test**. This validated test is based on the rewarding properties of sweet substances, such as dilute saccharin solutions. Healthy animals have a strong inherent taste preference towards these sweet solutions, while depressed animals show a significantly reduced saccharin preference. This loss of taste preference reflects a decrease in reward sensitivity, i.e. anhedonia, which can be reversed by an antidepressant treatment [14-18]. In the saccharin preference test, the cage of the animal is supplied with two identical preweighed drinking bottles. One of the bottles contains regular water while the other contains a 0.1% saccharin solution. The animal is presented with the bottles for a certain period of time, typically ranging from 1 hour to 24 hours. At the end of the test, both bottles are removed and reweighed. Saccharin preference is calculated as the volume of the saccharin solution consumed divided by the total fluid volume (saccharin solution plus regular water) consumed and expressed as a percentage [19, 20]. To exclude the possibility that differences in saccharin preference are caused by alterations in taste perception, the **quinine aversion test** can be performed. This test consists of the same procedure, except for the fact that the 0.1% saccharin solution is replaced by a 0.05% quinine solution. Quinine is a bitter tasting substance, which is highly aversive to rats with a normal taste perception [21].

### Study 2: The role of the locus coeruleus in the antidepressant mechanism of action of VNS

Although VNS is already used in clinical practice for the treatment of refractory depression, the antidepressant mechanism of action of this neuromodulatory treatment remains to be elucidated. A better understanding of the mechanism of action is indispensable to identify potential responders prior to surgery and may guide the search for optimal stimulation parameters, finally improving clinical efficacy. Previously, it was shown that VNS has an antidepressant-like effect in the rat forced swim test [22]. The mechanism of action underlying this effect is incompletely understood, but there is a large body of evidence suggesting that the LC – which is the main source of noradrenaline in the brain - might play an important role. Therefore, we wanted to test the hypothesis that the VNS-induced antidepressant-like effect in the forced swim test is mediated through activation of the LC.

For this purpose, LC neurons were lesioned using DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride], a highly selective neurotoxin for the noradrenergic axons originating from the LC.

**The forced swim test** is one of the most commonly used and validated animal models for behavioral despair. In this test, the animal is placed in a water-filled cylinder from which it cannot escape. Initially, the animal will be very mobile in trying to escape, but eventually it gives up and adopts an immobile posture (see figure 1).

**Figure 1:** The left and right side of the figure show mobile, escaping directed and immobile, passive behavior respectively.

This immobile posture is interpreted as the behavioral correlate of despair. The standard test as first described by Porsolt et al. [23], consists of two swimming exposures. The first exposure consists of a 15-minute swim session and the second exposure, which is the actual test phase, is performed 24 hours to 1 week later and consists of a 5-minute swim session of which the mobility and immobility time are assessed. During the 24 hours to 1 week between the two swim sessions, a potential antidepressant manipulation is performed. A reduction in passive immobile behavior in the 5-minute swim session is a validated measure for an antidepressant-like effect of the manipulation, provided it does not increase general locomotor activity, which could generate a false positive result [24]. To assess potential differences in locomotor activity, an **open field test** can be performed.

To rule out the possibility that the effects in the forced swim test are caused by an overall change in locomotor activity, an open field test can be performed. This test is performed in an open field arena with walls to prevent escaping. The floor of the arena is divided into equal squares. During the test, the animal is placed in the center or one of the four corners of the open field arena and is allowed to

explore the apparatus for five minutes (see figure 2). The number of squares crossed with all four paws during the 5-minute trial is a validated measure for the locomotor activity [25].

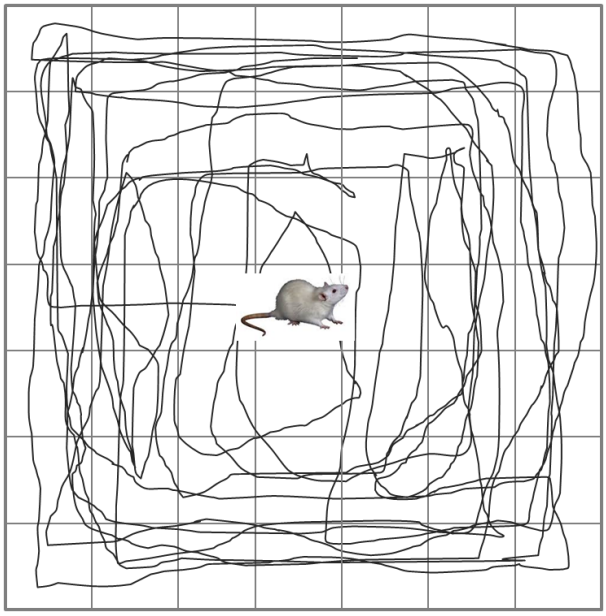


**Figure 2:** Schematic top view of the open field test arena. The trace on the floor of the arena represents the path of the rat during the 5-minute test.

## Study 3: Review on the antidepressant mechanism of action of VNS: evidence from preclinical studies

The third literature study of chapter 3 consist of a review that provides an overview of the preclinical VNS studies in view of two major hypotheses in depression research: the monoaminergic and the neural plasticity hypothesis of MDD.

## Optimization of the stimulation parameters (chapter 4)

### Study 4: Intensity-dependent modulatory effects of VNS on cortical excitability

As stated above, the optimal stimulation parameters for effective VNS are still unknown. However, optimizing the stimulation paradigm is an indispensable step towards the achievement of a better clinical outcome. The stimulation parameters used currently in experimental studies and in clinical practice are based on what is known to be safe and tolerable and are therefore not evidence based but rather empirically determined. As the efficacy of VNS is dependent on the adequate activation of the vagal A- and B-fibers [26-28], the stimulation parameters should be optimized to activate these fibers. The activation threshold of the A- and B-fibers is lower than the output currents used in experimental settings and in clinical practice and therefore we hypothesize that VNS output currents lower than those used today, are sufficient and at least equally effective in reducing cortical excitability as VNS at higher output currents.

This hypothesis was tested in the **motor cortex stimulation rat model**, which is a validated model for cortical excitability. In this model, the effect of a potential seizure-suppressing treatment on cortical excitability can be determined. This is done by applying a ramp-shaped pulse to the motor cortex of awake rats, through implanted epidural electrodes. The stimulation is stopped by the observer when a motor response (retraction of the head and/or forelimb) is elicited. The threshold for eliciting a motor response is a validated measure for cortical excitability. If the threshold increases due to an intervention, this intervention can be considered to decrease cortical excitability, as more current is required to excite the neurons of the motor cortex [29-31] .

### Study 5: Laryngeal motor-evoked potentials as an indicator of effective vagus nerve activation: a preclinical study

Two major problems in VNS therapy are that (1) optimal stimulation parameters are unknown and (2) about one third of patients do not benefit from the treatment (non-responders). It is possible that the vagus nerve of some non-responders is not adequately activated, for multiple reasons such as lead failure, poor electrode contact or nerve damage. To date, there is no tool to test this hypothesis in an experimental set-up. Previous studies from our lab have shown that activation of the Aα-motor fibers of the recurrent laryngeal nerve, as measured by LMEPs, is reflective of the activation of the vagus nerve [32, 33]. Therefore, LMEPs could provide us with valuable information to deduct optimal

stimulation parameters and to identify ineffective stimulation of the vagus nerve leading to non-response. The techniques used in previous studies [32, 33] required invasive surgery or the use of special VNS electrodes for simultaneous stimulation and recording. The aim of the study described in this thesis was to investigate the feasibility and reliability of LMEP recordings using a minimally invasive, easy-to-use tool in a chronic experimental setting.

### Study 6: Laryngeal motor-evoked potentials as an indicator of effective vagus nerve activation: a clinical pilot trial

In clinical practice as well, one third of patients are non-responders and optimal stimulation parameters required to effectively activate the vagus nerve are still unknown [34, 35]. The therapeutically applied stimulation intensity is typically the highest output current tolerated by the individual patient. This is obviously not an evidence-based way to determine the individual, optimal output current for vagal fiber activation. Research should therefore be directed towards finding a non-invasive method that can guide individual titration of the stimulation parameters. Such biomarker for effective delivery of VNS pulses to the nerve could support the choice for individual stimulation parameters in a more rational way. To date, no such technique is available for clinical use in chronically VNS-implanted patients. In a previous study from our lab, it was shown that it is feasible to record VNS-induced laryngeal muscle-evoked potentials or LMEPs in chronically VNS-implanted experimental rats using a non-invasive electromyography technique [36]. LMEPs are indicative of the effective delivery of electrical current to the cervical fibers of the vagus nerve and could subsequently be used to identify non-responders due to ineffective activation of the nerve and to determine individual optimal stimulation parameters to activate the vagal fibers. The aim of the presented study was to translate this technique to clinical practice.

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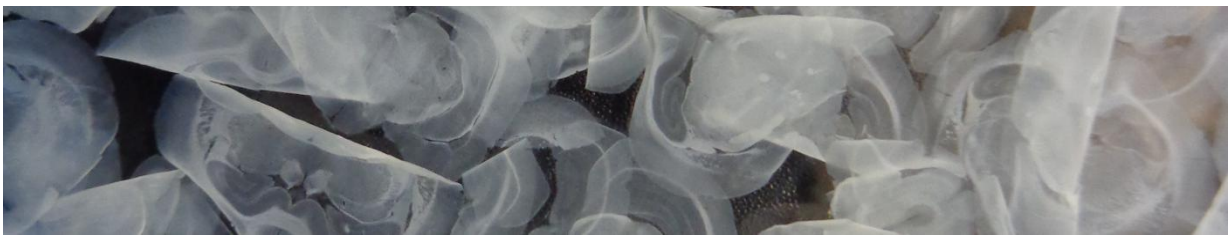
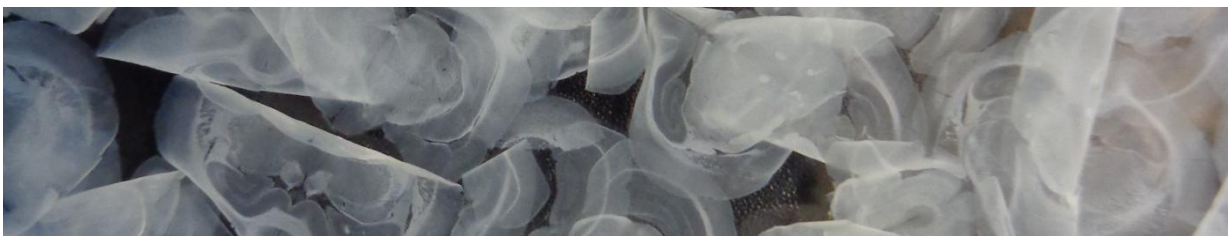
# Chapter 3



Proof-of-concept and mechanism of action

# Chapter 3 – study 1



Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy

## Study 1: Proof-of-concept

Depressive disorders are the most common type of psychiatric comorbidity in epileptic patients, especially in individuals suffering from refractory temporal lobe epilepsy. Despite the availability of a variety of both antiepileptic and antidepressant drugs, up to 30% of patients fail to respond adequately to standard medication. Furthermore, current treatment options for patients suffering both from epilepsy and depression are limited by the fact that anticonvulsant drugs can contribute to mood disturbances, while antidepressant drugs can increase seizure susceptibility. The lack of success with current pharmacological interventions for patients suffering both from epilepsy and depression, highlights the importance of optimizing non-pharmacological, neuromodulatory treatments such as VNS for this patient population. The initial rationale for using VNS to treat refractory depression was fueled by the observation that VNS induces mood improvements in epilepsy patients, irrespective of the effect on seizure frequency. However, there are no randomized controlled trials confirming the antidepressant effect of VNS in epileptic patients. Studies on the antidepressant effect of VNS in a clinical population are confounded by multiple factors, including concomitant antiepileptic drug therapy, psychosocial and intellectual effects. The use of animal models overcomes this problem and is important in unraveling the mechanism of action of VNS for the treatment of epilepsy-related depression. Therefore, a proof-of-concept study was performed to assess the antidepressant potential of VNS in an animal model for epilepsy and comorbid anhedonia, a key symptom of major depression. For this purpose the effect of VNS on the hedonic state was assessed in the the post status epilepticus kainic acid rat model for temporal lobe epilepsy and comorbid anhedonia, using the saccharin preference and the quinine aversion test.

# Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy

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**Abstract**

Background: Depression is the most common psychiatric comorbidity in epilepsy patients. The lack of success with current pharmacological interventions for this patient population, highlights the importance of optimizing non-pharmacological neuromodulatory treatments such as vagus nerve stimulation (VNS). Studies on the antidepressant effect of VNS in epilepsy patients may be confounded by concurrent antiepileptic drug therapy. To date, studies in epilepsy models overcoming this problem are lacking.

Objective: We investigated whether VNS affects anhedonia, a key symptom of major depression, in the kainic acid rat model for temporal lobe epilepsy.

Methods: Anhedonia was assessed in kainic acid (KA) and saline (SAL) injected rats using the saccharin preference test (SPT). To exclude differences in taste perception, the quinine aversion test (QAT) was performed. Both groups were randomly subdivided in a VNS and a SHAM group, yielding 4 experimental arms: KA-VNS, KA-SHAM, SAL-VNS and SAL-SHAM. Both VNS groups received 2 weeks of VNS, while the SHAM groups were not stimulated. Thereafter, the SPT and QAT were repeated.

Results: Saccharin preference was significantly reduced in the KA compared to the SAL rats ($p<0.05$), without differences in quinine aversion. Two weeks of VNS significantly increased the saccharin preference in the KA-VNS group ($p<0.05$), while it had no effect on quinine aversion. No effects of VNS or SHAM were found in the other groups.

Conclusion: The KA rats displayed anhedonia which was significantly decreased by VNS, indicating that this neuromodulatory treatment could likewise diminish depressive symptoms in patients suffering from temporal lobe epilepsy and comorbid depression.

**Keywords:** Vagus nerve stimulation, temporal lobe epilepsy, depression, anhedonia, kainic acid model

## Introduction

Depressive disorders are the most common type of psychiatric comorbidity in patients with epilepsy [1-3], especially in individuals suffering from refractory temporal lobe epilepsy [4-8]. Despite the availability of a variety of both antiepileptic and antidepressant drugs, up to 30% of the patients fail to respond adequately to standard medication [9]. Furthermore, current treatment options for patients suffering both from epilepsy and depression are limited by the fact that anticonvulsant drugs can contribute to mood disturbances, while antidepressant drugs can increase seizure susceptibility [4, 10, 11]. The lack of success with current pharmacological interventions highlights the importance of optimizing non-pharmacological neuromodulatory treatments such as vagus nerve stimulation (VNS).

VNS consists of electrically stimulating the left vagus nerve at the cervical level by means of implanted electrodes and a programmable pulse generator. It is a well-established, safe and effective add-on therapy for the treatment of refractory epilepsy [12]. Clinical trials reported response rates (defined as the fraction of patients with >50% reduction in seizure frequency) of 20-40% in the first year of treatment [13, 14]. This response rate was shown to increase with time [15, 16]. Furthermore, the antiepileptic effect of VNS has been shown in numerous animal models for epilepsy [17-29].

The initial rationale for using VNS for the treatment of refractory depression was based on mood improvements in epilepsy patients treated with VNS, irrespective of the effects of VNS on seizure frequency [5, 30, 31]. The therapeutic effect of chronic VNS for treatment-resistant depression has been assessed in several open-label and long-term clinical studies in depressed patients without epilepsy [32-43]. VNS produced steadily increasing improvement of depressive symptoms with full benefit after 6-12 months and sustained efficacy during 2 years of follow-up [44]. Furthermore, these studies reported response rates (defined as the fraction of patients with >50% decrease in depression severity) of 30-40% and a remission rate of 15-17% after 3-24 months of treatment [45]. Unfortunately the only blinded sham-controlled clinical trial was inconclusive because some patients had not been adequately ramped-up and therefore did not receive the full therapy [32]. As for animal research, it has been shown that both acute [46] and chronic [47] VNS produce antidepressant-like effects in the rat forced swim test model [48].

To date, studies specifically addressing the antidepressant effects of VNS in epilepsy patients are lacking. Furthermore, such studies could be confounded by multiple factors, including antiepileptic drug therapy, psychosocial, socio-economic and intellectual effects [8]. The use of animal models overcomes this problem and may be useful in identifying potential therapies for the treatment of

depression in epileptic patients. This study aims at investigating the antidepressant potential of VNS in the kainic acid model for temporal lobe epilepsy with comorbid anhedonia.

Anhedonia, or the inability to experience pleasure [49], is a key symptom of major depression. Because pleasure is a subjective feeling, the DSM-IV operationally defines anhedonia as a diminished interest or pleasure in response to stimuli that were perceived as rewarding during the premorbid state [50]. In animal research, anhedonia can be assessed using the saccharin or sucrose preference test. This validated test for anhedonia is based on the rewarding properties of sweet substances, such as saccharin or sucrose solutions. Healthy animals have a strong inherent taste preference towards these sweet solutions, while animal models for depression show a significantly reduced saccharin or sucrose preference. This loss of taste preference reflects a decrease in reward sensitivity, i.e. anhedonia, which can be reversed by treatment with antidepressants [51-55]. To exclude the possibility that the reduced saccharin preference in our experiments is caused by a loss of taste due to the kainic acid, the induced status epilepticus or the subsequent neuronal loss, the quinine aversion test was performed.

**Methods**

A schematic overview of the study design is shown in figure A.1. Rats were implanted with a VNS electrode and electroencephalogram (EEG) recording electrodes. After one week of recovery, half of the animals received intraperitoneal injections with kainic acid (KA) to induce status epilepticus. The other half of the animals was injected with matched volumes of saline (SAL). Both the KA group and the SAL group were randomly subdivided in a VNS group and a SHAM group, yielding 4 experimental arms: KA-VNS, KA-SHAM, SAL-VNS and SAL-SHAM. Anhedonia was evaluated 5 weeks after KA or SAL injections using the saccharin preference test. To control for loss of taste due to KA-induced status epilepticus, the quinine aversion test was performed. After this baseline testing, the VNS groups (KA-VNS and SAL-VNS) received 2 weeks of VNS, the SHAM groups (KA-SHAM and SAL-SHAM) were also connected to the set-up but were not stimulated. Subsequently, the saccharin preference test and the quinine aversion test were repeated in all animals. All procedures are discussed in detail below.

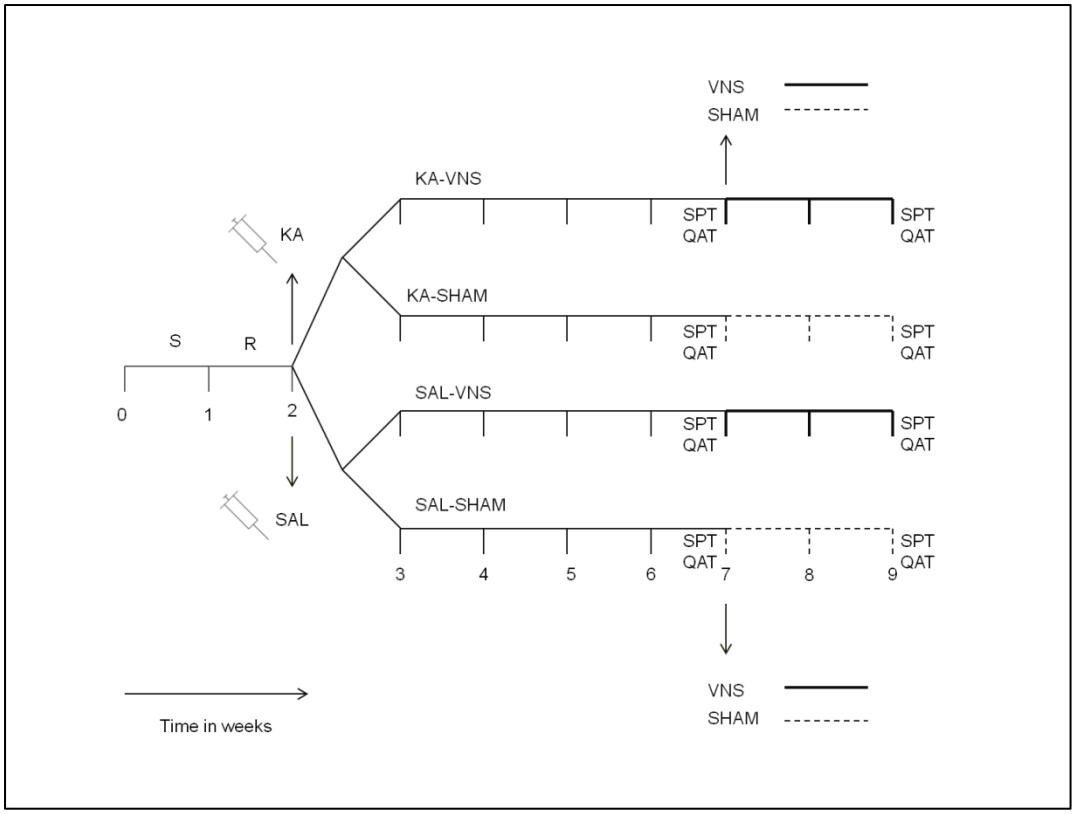


**Figure A.1:** Schematic representation of the study design. S: surgery; R: recovery; SAL: saline; KA: kainic acid; SPT: saccharin preference test; QAT: quinine aversion test; VNS: vagus nerve stimulation.

Animals

Forty-five male Sprague Dawley rats (Harlan, The Netherlands) weighing 250–300 g were used. Animals were treated according to the guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Medical department (ECP 13/33). All animals were kept under environmentally controlled conditions: 12 h light/dark cycles with artificially dimmed light (4-6 Lux in the rat home cages), lights went on at 8:00 AM and off at 8:00 PM. Temperature and relative humidity were kept at 20-23°C and 50% respectively and food and water were provided ad libitum.

Surgery

The animals were anesthetized with a mixture of Isoflurane (5% for induction, 2% for maintenance) and medical $O_2$. To minimize post-operative pain, buprenorphine (0.025 mg/kg) (Schering-Plough, New Jersey, USA) was administrated intramuscularly 30 minutes prior to surgery. An incision was made over the anterior cervical region. The skin and muscles were retracted and the left vagus nerve was carefully dissected from the aortic sheet. Subsequently, the custom-made silicone cuff electrode was wound around the nerve with the anode placed caudally. The leads of the electrodes were tunneled subcutaneously to an incision in the scalp. Details on the construction of the vagal electrode are described elsewhere [56]. Furthermore, rats were implanted with two epidural EEG recording electrodes through the os frontale and an epidural reference electrode through the os occipitale, close to the sutura lambdoidea. A bipolar depth EEG recording electrode consisting of two polyimide coated stainless steel wires (Bilaney, Germany) was stereotactically implanted in the left hippocampus (coordinates relative to bregma: anteroposterior -5.6 mm; mediolateral -4.6 mm; dorsoventral -4.6 mm). The leads of the EEG recording electrodes were assembled together with the leads of the VNS electrode to a connector in a head cap on the skull of the rat using acrylic cement. Xylocaine gel (2%) was applied to the incision wounds to minimize pain. Animals were allowed to recover from surgery during 1 week.

Induction of status epilepticus

One week after surgery, half of the animals were intraperitoneally injected with KA (Tocris bioscience, USA) to induce status epilepticus. KA injections (5 mg/kg/h in a volume of 1.5 ml/kg) were administered until the animal displayed a self-sustained status epilepticus for at least 3 hours,

according to the protocol of Hellier et al. [57]. During status epilepticus, video-EEG monitoring was performed continuously and behavioral seizures were scored according to a modified version of Racine's scale: stage 1: immobility, eye closure, twitching of vibrissae, facial clonus, wet dog shakes; stage 2: head nodding, chewing, severe facial clonus, wet dog shakes; stage 3: clonus of one forelimb; stage 4: rearing, bilateral forelimb clonus; stage 5: rearing, bilateral forelimb clonus, loss of balance and falling [58]. The length of the status epilepticus was defined as the time between the first epileptic spike on the EEG and the moment the EEG spike activity dropped below a frequency of 1 Hz for more than one hour. The SAL group was injected with matched volumes of sterile saline (vehicle).

Saccharin preference test and quinine aversion test

Anhedonia was evaluated using the saccharin preference test. In this test, each cage was supplied with two identical drinking bottles filled with water, to avoid place preference in the rats (habituation phase). On the day of the experiment, the water bottles were replaced by two new bottles with known weight. One of the bottles contained water while the other contained a 0.10% saccharin (Sigma Aldrich, the Netherlands) solution. The location of the bottle of saccharin solution relative to the water bottle was counterbalanced across the rats. The experiment started at 1:00 PM and ran for 20 hours. At the end of the test, both bottles were removed and weighed. Saccharin preference was calculated as the volume of the saccharin solution consumed divided by the total fluid volume (saccharin solution plus regular water) consumed and expressed as a percentage. We assessed saccharin preference over a time period of 20 hours because short term testing (for example 2 hours) can be influenced by many factors that are not related to the hedonic state (e.g. subtle stressors at the moment of the test). Furthermore, longer measurements increase the accuracy, as the error can be very high during a short sampling period. Another reason for test prolongation was to minimize potential neophobic reactions to the taste of the saccharin solution [59]. In order to minimize the influence of metabolic factors, we did not apply food and water deprivation in our experiment.On the next day, the same procedure was repeated with a bitter tasting 0.05% quinine (Sigma Aldrich, The Netherlands) solution.

Vagus nerve stimulation

The rats were connected to a constant-current stimulator via a spring-covered cable. An electrical swivel allowed the rats to move freely within their cage. The animals in the KA-VNS and the SAL-VNS

group received 2 weeks of VNS with an output current intensity that was gradually ramped up: 0.25 milliampere (mA) during the first 3 days, 0.50 mA during the next 4 days and 1.00 mA during the final 7 days, to minimize stimulus-related side effects. Electrical pulses with a pulse width of 250 µs, were delivered at a frequency of 30 Hz. The stimulator was programmed to deliver VNS for 24 hours per day with a duty cycle of 7 s ON/18 s OFF. This duty cycle was chosen based on previous experiments from our group demonstrating the potency to affect intracerebral neurotransmitter release [17]. The animals in the KA-SHAM and the SAL-SHAM groups were also connected to the stimulation set-up, but the output current was set to 0.00 mA. The impedance of the VNS electrodes was measured daily using a square wave pulse with an amplitude of 1.00 mA. All impedances remained low throughout the experiment (< 10 kOhm). After two weeks of SHAM or VNS, behavioral testing was repeated.

Histology

Animals were euthanized with an intraperitoneal excess dose of sodium pentobarbital (180 mg/kg). The brains were fixed in paraformaldehyde (4%), cryoprotected in 30% sucrose at 4°C, snap-frozen in ice-cold isopentane and subsequently stored at -20°C. Coronal frozen sections (70 µm) were made using a cryostat (Leica). Electrode positions were identified on the brain sections.

Statistics

Nonparametric tests were performed because the data was not normally distributed. All results are expressed as median and interquartile range (between brackets). The duration of the status epilepticus and the dose of KA in the KA groups (KA-SHAM and KA-VNS) and the outcome parameters in the saccharin preference and quinine aversion test at five weeks after KA or SAL injections, were compared using Kruskal-Wallis tests followed by Mann–Whitney U post hoc tests with Bonferonni corrections. To assess the effects of SHAM and VNS treatment on the outcome parameters in the saccharin preference and quinine aversion test, Wilcoxon matched pairs signed ranks tests were used.

Status epilepticus

Sixteen out of twenty-five animals survived the KA-induced status epilepticus. In the surviving animals, the median duration of the status epilepticus was 11.3 h (1.4 h) and the median dosage of KA was 12.5 mg/kg (0.3 mg/kg). There were no differences in the duration of status epilepticus and the dosage of KA between the KA-SHAM and the KA-VNS group: 10.9 h (2.2 h) versus 11.6 h (0.5 h) and 12.5 mg/kg (0.0 mg/kg) versus 12.5 mg/kg (1.25 mg/kg) respectively ($p>0.05$ in both cases). None of the animals in the SAL group developed status epilepticus.

Hedonic outcome five weeks after SAL or KA injection

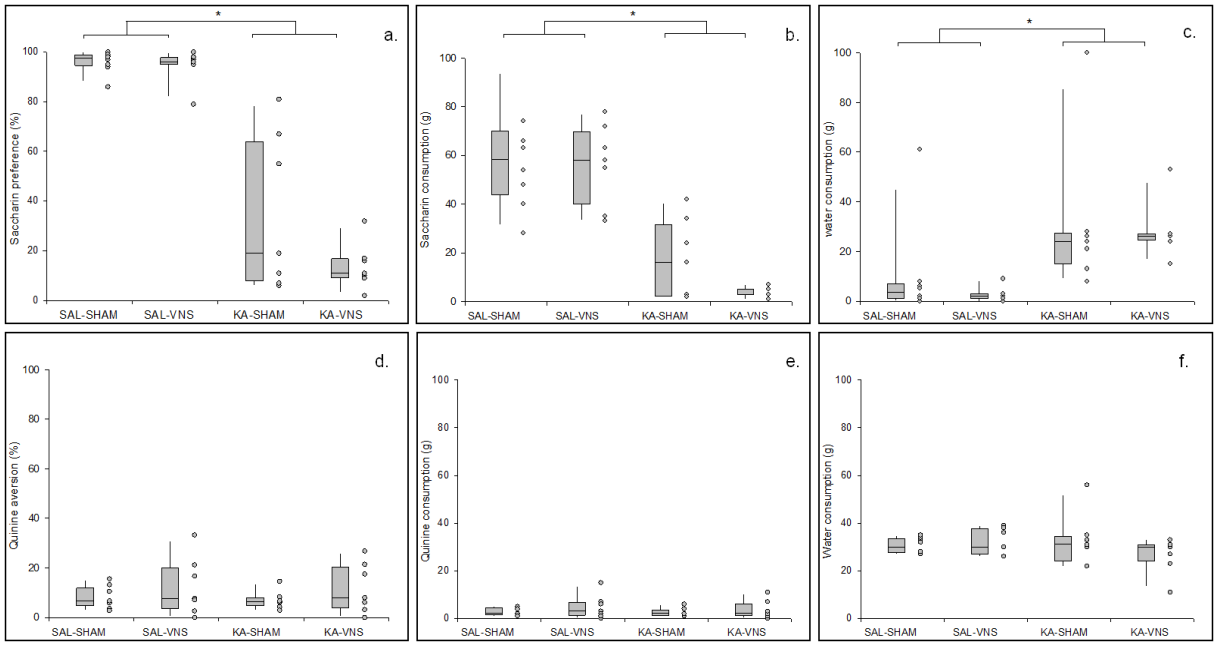


**Figure A.2:** Outcomes in the saccharin preference and quinine aversion test 5 weeks after KA or SAL injections. Panel a, b and c depict the saccharin preference (in %), saccharin consumption (in g) and water consumption (in g) respectively in the saccharin preference test. Panel d, e and f depict the quinine aversion (in %), quinine consumption (in g) and water consumption (in g) respectively in the quinine aversion test. Boxplots depict the median (full line), the interquartile range (box boundaries) and the 5-95$^{th}$ percentile (whiskers). Each dot represents a value of an individual animal (SAL-SHAM:

n = 8, SAL-VNS: n = 7, KA-SHAM: n=7, KA-VNS: n = 7). * p<0.05, Kruskal-Wallis test followed by Mann–Whitney U post hoc tests.

In the saccharin preference test, the KA groups showed a significantly lower saccharin preference compared to the SAL groups: 19.4% (60.0%), 10.3% (7.0%), 97.5% (5.0%) and 97.2% (4.0%) for the KA-SHAM, KA-VNS, SAL-SHAM and SAL-VNS group respectively (p<0.05, see figure A.2.a). This significant reduction in saccharin preference resulted from both a significantly lower saccharin consumption and a significantly higher water consumption in the KA groups compared to the SAL groups (p<0.05, see figure A.2.b and c respectively). Combined, the results from the saccharin preference test show that the KA animals display anhedonia.

No differences in aversion towards quinine were found across the groups: 6.3% (4.0%), 8.0% (18.2%), 6.7% (8.4%) and 7.7% (18.6%) for the KA-SHAM, KA-VNS, SAL-SHAM and SAL-VNS group respectively (p>0.05, see figure A.2.d). This results from an equal consumption of quinine and water in the KA and the SAL groups (p>0.05, see figure A.2.e and f respectively). Combined, the results from the quinine aversion test show that taste perception was not compromised after KA-induced status epilepticus.

<u>Effects of two weeks of VNS or SHAM on the hedonic state</u>

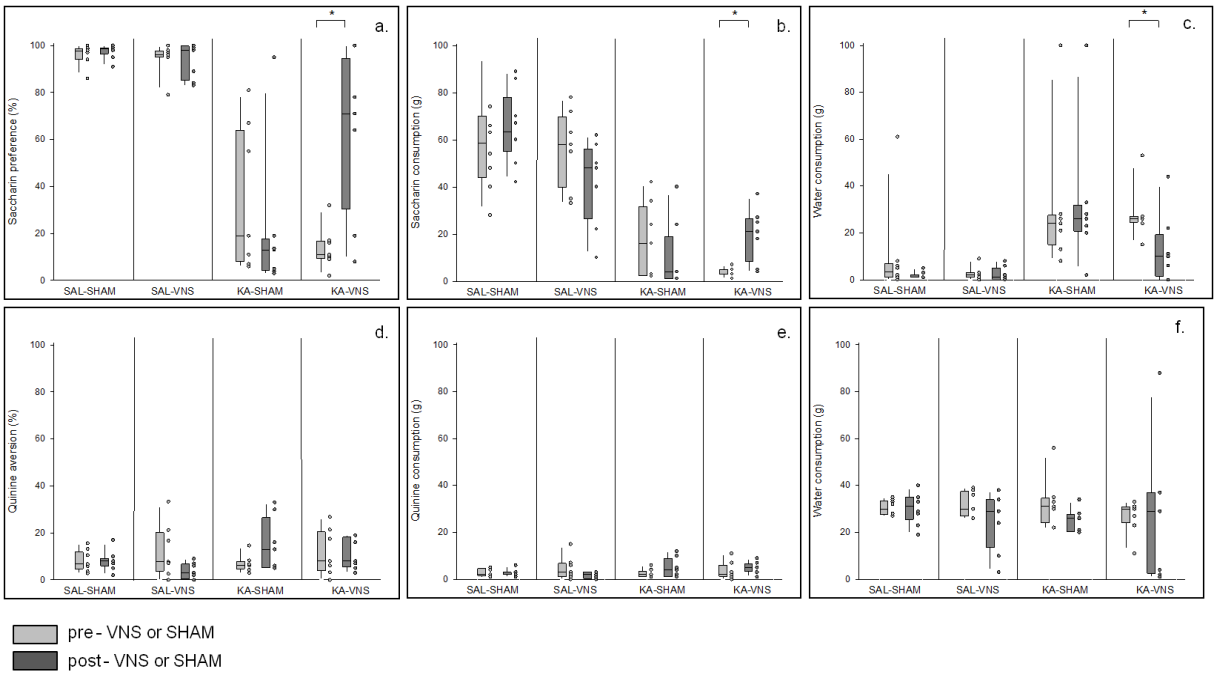

**Figure A.3:** The effect of two weeks of VNS or SHAM treatment in the saccharin preference and quinine aversion test in SAL and KA animals. Panel a, b and c depict the saccharin preference (in %), saccharin consumption (in g) and water consumption (in g) respectively in the saccharin preference test. Panel d, e and f depict the quinine aversion (in %), quinine consumption (in g) and water consumption (in g) respectively in the quinine aversion test. Boxplots depict the median (full line), the interquartile range (box boundaries) and the 5–95th percentile (whiskers). Each dot represents the saccharin preference or quinine aversion of an individual animal (SAL-SHAM group: n = 8; SAL-VNS group:n = 7; KA-SHAM group: n = 7; KA-VNS group: n = 7). *p<0.05, Wilcoxon matched pairs signed ranks test.

Two weeks of VNS significantly increased the saccharin preference in the KA-VNS group: from 10.3% (7.0%) before treatment to 71.1% (81.0%) after treatment (p<0.05, see figure A.3.a). This significant increase in saccharin preference resulted from both a significant increase in saccharin consumption (p<0.05, see figure A.3.b) and a significant reduction in water consumption after two weeks of VNS in the KA-VNS group (p<0.05, see figure A.3.c). Combined, the results from the saccharin preference test show that VNS significantly decreases anhedonia in KA rats.

No differences in saccharin preference were found in the other groups before vs. after VNS or SHAM treatment: 97.5% (5.0%) versus 98.5% (3.0%), 97.2% (4.0%) versus 98.4% (17.0%) and 19.4% (60.0%) versus 12.5% (15.0%), for the SAL-SHAM, SAL-VNS and KA-SHAM group respectively (p>0.05 in all cases, see figure A.3.a). Accordingly, no differences in saccharin consumption and water consumption in the saccharin preference test before vs. after VNS or SHAM treatment were found in these three groups (p>0.05, see figure A.3.b and c respectively).

No differences in quinine aversion were found before vs. after VNS or SHAM treatment: 6.7% (8.4%) versus 7.9% (4.0%), 7.7% (18.6%) versus 2.9% (7.0%), 6.3% (4.0%) versus 13.3% (25.0%) and 8.0% (18.2%) versus 8.0% (14.0%) for the SAL-SHAM, SAL-VNS, KA-SHAM and KA-VNS group respectively (p>0.05, see figure A.3.d). Accordingly, VNS and SHAM treatment did not affect the amount of quinine consumption, nor did it affect the amount of water consumption in the quinine aversion test (p>0.05, see figure A.3.e and f respectively).

**Discussion**

The KA rat model for temporal lobe epilepsy presents anhedonia, as demonstrated by a significant decrease in saccharin preference. The reduced saccharin preference in the KA group was not caused by an altered taste perception, as the aversion towards quinine was unaltered in the KA-treated rats. Two weeks of VNS decreased the anhedonic state in the KA rats, as indicated by a significant increase in saccharin preference. No effects were found in the other groups (KA-SHAM, SAL-VNS and SAL-SHAM). Furthermore, VNS nor SHAM treatment had an effect on taste perception, as shown in the quinine aversion test. Our findings demonstrate the antidepressant effect of VNS in the KA model for temporal lobe epilepsy and comorbid anhedonia.

Anhedonia, or the inability to experience pleasure, is an indicator of clinical depression and is present in the chronic mild stress model, the most widely used and validated animal model for depression [54, 60, 61]. As depressive symptoms are the most common type of psychiatric comorbidity in epilepsy patients, it is not surprising that anhedonia has also been found in rat models for both partial and generalized epilepsy [8, 62, 63]. In accordance with previous studies that demonstrated a significant decrease in saccharin preference in the pilocarpine model for temporal lobe epilepsy [49, 64] and a significant decrease in sucrose preference in the KA model for temporal lobe epilepsy [65], we found a significant decrease in saccharin preference in KA-treated rats. This makes the model suitable for investigating the potential antidepressant effects of VNS.

The frequent co-occurrence of epileptic seizures and anhedonia in chronic epilepsy models suggest that these symptoms may share common underlying pathological mechanisms. Although the specific mechanisms have not been identified to date, emerging evidence shows that there is a remarkable overlap in the abnormalities found in epilepsy and depression models, the most important ones being (i) imbalances in neurotransmitter systems [1], (ii) increased neuroinflammation [66, 67] and (iii) changes in hippocampal neurogenesis [68, 69].

It has been shown that the noradrenergic [70-72], serotonergic [15, 71-79], dopaminergic [65, 76, 80-86], GABAergic [87-90] and glutamatergic [88, 91] neurotransmitter systems are disturbed both in the KA model for temporal lobe epilepsy and the chronic mild stress model for anhedonia. Increased neuroinflammatory markers such as altered levels of cytokines (i.e. IL-1α, IL-1β, IL-2 and TNF-α) have also been found in both models [75, 92, 93], providing another putative underlying mechanism for the anhedonia we observed in the KA model. Furthermore, significantly reduced or excessively increased progenitor proliferation in the granule cell layer of the hippocampus have been found in the chronic mild stress model for anhedonia [91, 94, 95] and the KA model for temporal lobe epilepsy [96-98] respectively. These changes contribute to disturbing neural networks important in the

pathophysiology of epileptic seizures and anhedonia. Therefore, it is likely that these changes further contribute to the development of the anhedonic state in the KA model.

The exact mechanism of action of VNS remains to be elucidated, but VNS research has demonstrated potential in affecting all three of the above described brain abnormalities that may play a role in the common pathophysiological basis of epilepsy and depression. Previous research has demonstrated that VNS may exert its antidepressant and antiepileptic effects through correcting dysfunctional neurotransmitter systems. Electrophysiological [99-104] and neurochemical [17, 105-108] studies have demonstrated that VNS enhances the noradrenergic and serotonergic neurotransmission through activation of the locus coeruleus and the dorsal raphe nucleus, which are the two main sources of brain noradrenaline and serotonin respectively [109, 110]. In this study, antidepressant effects were found using a relatively intense stimulation protocol. Roosevelt and co-workers had previously reported a bilateral increase in noradrenaline levels in the cortex (39%) and the hippocampus (28%) in response to one hour of VNS using a stimulation protocol typically applied in clinical settings to control seizures (20 Hz, 1.00 mA, 500 μs, 30 s ON / 10 min OFF) [105]. In a previous experiment by our group, a more than two-fold higher increase (69%) in extracellular hippocampal noradrenaline was achieved by using a more intensive stimulation protocol: 30 Hz, 1.00 mA, 250 μsec, 7 sec ON / 18 sec OFF [17], resulting in the delivery of a higher load of electrical pulses to the vagus nerve. The duty cycle used in our experiments (7 s ON / 18 s OFF) is referred to as 'rapid cycling' in clinical practice, and is sometimes used to treat patients in whom VNS with the standard duty cycle of 30 s ON / 10 min OFF, has no significant therapeutic effect [111, 112]. It remains to be demonstrated which duty cycles are optimal to achieve clinical efficacy while conserving battery life.

Although VNS research has focused on the noradrenergic and serotonergic neurotransmission in the central nervous system, it should be noted that stimulation of the vagus nerve most likely induces a much more complex cascade of both central and peripheral neurochemical changes. Indeed, VNS was shown to affect the glutamatergic, GABAergic, dopaminergic and cholinergic systems [101, 113-121]. Strong evidence suggests that VNS-induced neuroplastic changes are mediated by acetylcholine through activation of the nucleus basalis [122, 123]. Furthermore, VNS also has proven anti-inflammatory effects, which may further contribute to the antidepressant and antiepileptic effects of VNS [124-133]. Interestingly, a growing body of evidence suggests that VNS could produce its effects through increasing hippocampal neurogenesis [134-137], thereby creating newborn cells which can functionally integrate and re-establish normal network activity. This hypothesis is supported by the fact that the time lag of the therapeutic effects of VNS corresponds to the time needed for the integration of newborn cells in the hippocampal granule cell layer into existing circuits.

## Conclusion

The intraperitoneal KA rat model for temporal lobe epilepsy presents anhedonia which is significantly decreased by two weeks of VNS treatment. These results provide evidence for the antidepressant effect of VNS in the KA model for temporal lobe epilepsy and indicate that VNS could likewise diminish depressive symptoms in patients suffering from temporal lobe epilepsy and comorbid depression. The promising results encourage further studies in this model in order to gain a better understanding of the underlying mechanism of action of VNS. This mechanism of action most likely results from a complex interplay between several mechanisms that underlie the pathophysiology of epilepsy and depression, i.e. the correction of dysfunctional neurotransmitter circuits, the induction of anti-inflammatory effects and the promotion of neurogenesis.

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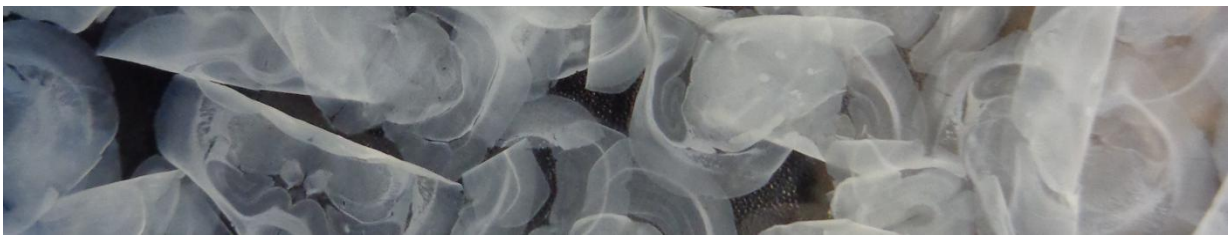
# Chapter 3 - study 2



The antidepressant-like effect of vagus nerve stimulation is mediated through the activation of the locus coeruleus

## Study 2: Mechanism of action

Although VNS is already used in clinical practice for the treatment of refractory depression, the antidepressant mechanism of action of this neuromodulatory treatment remains to be elucidated. A better understanding of the mechanism of action is indispensable to identify potential responders prior to surgery and may guide the search for optimal stimulation parameters, finally improving clinical efficacy. Previously, it was shown that VNS has an antidepressant-like effect in the rat forced swim test. The mechanism of action underlying this effect is incompletely understood, but there is a large body of evidence suggesting that the locus coeruleus (LC) – which is the main source of noradrenaline in the brain - might play an important role. Therefore, the hypothesis that the VNS-induced antidepressant-like effect in the forced swim test is mediated through activation of the LC, was tested. For this purpose, LC neurons were lesioned using DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride], a highly selective neurotoxin for the noradrenergic axons originating from the LC.

# The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus

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**Abstract**

It has been shown that vagus nerve stimulation (VNS) has an antidepressant-like effect in the forced swim test. The mechanism of action underlying this effect is incompletely understood, but there is evidence suggesting that the locus coeruleus (LC) may play an important role. In this study, noradrenergic LC neurons were selectively lesioned to test their involvement in the antidepressant-like effect of VNS in the forced swim test.

Forced swim test behavior was assessed in rats that were subjected to VNS or sham treatment. In half of the VNS-treated animals, the noradrenergic neurons from the LC were lesioned using the selective neurotoxin DSP-4 [*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride], yielding three experimental arms: sham, VNS and DSP-4-VNS (n = 8 per group). Furthermore, the open field test was performed to evaluate locomotor activity. A dopamine-β-hydroxylase immunostaining was performed to confirm lesioning of noradrenergic LC neurons.

VNS significantly reduced the percentage of immobility time in the forced swim test compared to sham treatment (median: 56%, interquartile range: 41% vs. median: 75%, interquartile range: 12%). This antidepressant-like effect of VNS could not be demonstrated in the DSP-4-VNS group (median: 79%, interquartile range: 33%). Locomotor activity in the open field test was not different between the three treatment arms. The absence of hippocampal dopamine-β-hydroxylase immunostaining in the DSP-4-treated rats confirmed the lesioning of noradrenergic neurons originating from the brainstem LC.

The results of this study demonstrate that the noradrenergic neurons from the LC play an important role in the antidepressant-like effect of VNS.

**Keywords:** Vagus nerve stimulation, depression, forced swim test, locus coeruleus, noradrenaline

## Introduction

Major depressive disorders are highly prevalent, widely distributed in the population and usually associated with substantial symptom severity and role impairment [1]. While depressive symptoms can be effectively treated with antidepressant drugs or psychotherapy in the majority of patients, up to 20% of patients fail to respond to standard interventions [2]. These drug-refractory patients are candidates for treatment with neurostimulation therapies such as vagus nerve stimulation (VNS).

VNS is an extracranial neurostimulation technique, where the cervical region of the left vagus nerve is stimulated by means of a helical electrode, connected to a subclavicularly-implanted pulse generator. It is a well-established, safe and effective add-on therapy for refractory epilepsy [3]. The initial rationale for using VNS for the treatment of refractory depression was based on mood improvements observed in epilepsy patients treated with VNS, irrespective of the effects of VNS on seizure frequency [4, 5]. Several clinical studies have subsequently confirmed the therapeutic efficacy of VNS for treatment resistant depression [6-15], but the mechanism of action is still unknown.

The forced swim test is one of the most commonly used and validated experimental assays to assess depression-like behavior in rodents. During the test, animals are placed in a cylinder filled with water from which they cannot escape. Mobile escape-related behavior (defined as forepaw movements along the side of the cylinder and swimming throughout the cylinder) and immobile passive behavior (defined as the lack of whole body movement, except for small efforts to keep the head above water) are scored blindly on videotaped images of the test. A reduction in immobile passive behavior is reflective of an antidepressant-like effect of the investigated intervention [16, 17]. To rule out the possibility that the effects in the forced swim test are caused by an overall change in locomotor activity, an open field test can be performed. Krahl et al. showed that VNS produces an antidepressant-like effect in the forced swim test in rats with the same efficacy as electroconvulsive shock therapy and the tricyclic antidepressant desipramine [18].

Since the 1960s there has been a strong emphasis on the role of noradrenaline both in the pathogenesis of depressive disorders and in the mechanism of action of antidepressants [19, 20]. This largely results from the fact that many of the first generation antidepressants, the tricyclics, increase the synaptic concentration of noradrenaline [21]. There is extensive evidence demonstrating that VNS also enhances the noradrenergic neurotransmission through the activation of the LC [22-30], which is the main source of cortical noradrenaline [31]. Therefore, we investigated the hypothesis that the VNS-induced antidepressant-like effect in the forced swim test is mediated through activation of the LC and subsequent release of noradrenaline. For this purpose LC neurons

were lesioned using DSP-4, a highly selective neurotoxin for the noradrenergic axons originating from the LC [32, 33].

**Methods and materials**

A schematic overview of the study design is shown in figure 1. All procedures are described in detail below.

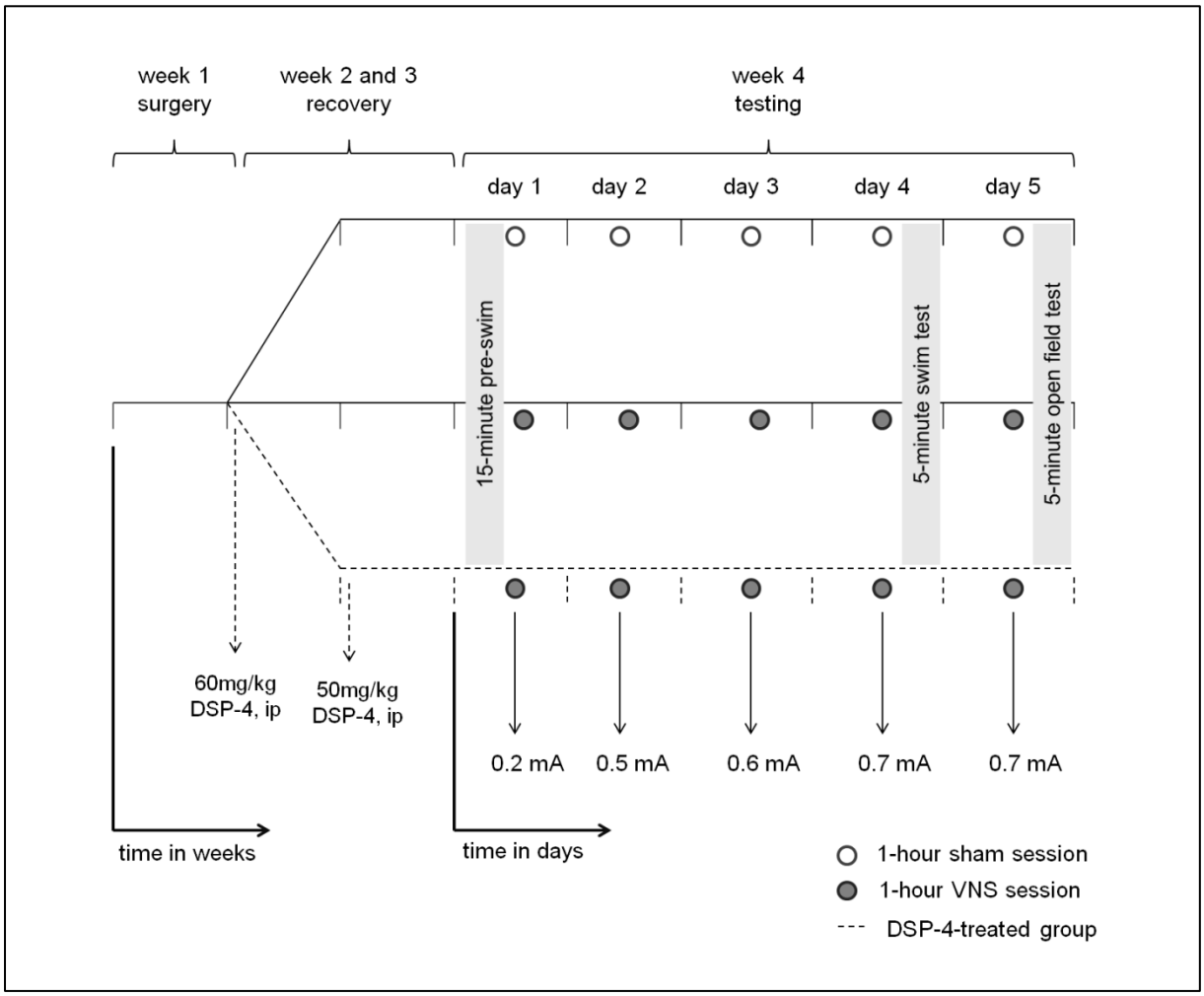


**Figure 1:** Schematic representation of the study design. DSP-4, [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride]; ip, intraperitoneal; mA, milliampere; VNS; vagus nerve stimulation.

Animals

Male rats (Harlan, The Netherlands) weighing 250–300 g were used. As in the study of Krahl, Wistar Kyoto rats were chosen because they are known to be sensitive to the depressogenic effects of the forced swim test [18]. Animals were treated according to the guidelines approved by the European

ethics committee (decree 86/609/EEC). The study protocol was approved by the animal experimental ethical committee of Ghent University medical department (ECP 13/33). All animals were kept under environmentally controlled conditions: 12 h light/dark cycles with artificially dimmed light, temperature and relative humidity at 20-23°C and 40-60% respectively. Food and water were provided ad libitum.

Surgery

The animals were anesthetized with a mixture of medical $O_2$ and Isoflurane (5% for induction, 2% for maintenance). A dose of Buprenorphine (0.025 mg/kg, subcutaneously) was administered preoperatively. The skin of the ventral cervical region was shaved and disinfected and a midline incision was made. The skin and muscles were retracted and the left vagus nerve was carefully dissected from the aortic sheet. Subsequently, a custom-made bipolar silicone cuff electrode was wrapped around the nerve with the anode placed caudally. The leads of the electrode were tunneled subcutaneously up to an incision in the scalp and attached to the skull using anchor screws and acrylic cement. Xylocaine and Neobacitracine gel were applied to the incision wounds in order to minimize pain and reduce the risk of postoperative infections respectively. Furthermore, Metacam (1 mg/kg, subcutaneously) was given to the animals postoperatively and every 24 hours after surgery for two days. The animals were assigned at random to one of the three treatment groups (sham, VNS or DSP-4-VNS, n = 8 per group) and were allowed to recover from surgery for two weeks.

DSP-4 injections

Rats in the DSP-4-VNS group were injected with DSP-4 twice. The first injection (60 mg/kg, ip, dissolved in a volume of 1 ml of sterile saline) was given on the first day of the recovery period, the second injection (50 mg/kg, ip, dissolved in a volume of 1 ml of sterile saline) was given on the seventh day of the recovery period (i.e. 14 and 7 days before the start of the forced swim test [34, 35]).

Forced swim test

The forced swim test procedure was performed as described previously [18]. For all swimming procedures, the rats were placed in a glass cylinder (diameter: 26 cm; height: 65 cm) filled with tap

water (26.0-26.5°C) to a height of 40 cm. The animals could not support themselves by touching the bottom of the cylinder with the hind paws or tail. The test consisted of two sessions, following an established and validated method [18, 36]. The first session (day 1 of the testing week), called the pre-swim, consisted of placing the rats in the water for 15 minutes. The second session, the 5-minute swim test, took place three days after the pre-swim (day 4 of the testing week) [18]. The 5-minute swim test was videotaped and analyzed off-line by two independent investigators blinded for the group or treatment (AG and CB). In 5-s epochs, the investigators judged whether the rat was immobile or mobile. The number of epochs with immobile behavior was divided by the total number of epochs for each rat (60) and multiplied by 100 to determine the percentage of immobility time, as previously described by Krahl et al. [18]. After the swim sessions, the rats were removed from the cylinder and dried with paper towels. Between each swim session, the cylinder was washed with a soap solution and refilled with fresh water.

Vagus nerve stimulation

Animals in the VNS and the DSP-4-VNS group received one hour of VNS therapy on four consecutive days. The first session was administered immediately after the 15-minute pre-swim on day 1 of the testing week. In order to minimize stimulation-related side effects, the stimulator output current was initially low and ramped up every day in the following incremental steps: 0.20 milliampere (mA), 0.50 mA, 0.60 mA and 0.70 mA on day 1, 2, 3 and 4 of the testing week respectively. The stimulus consisted of electrical pulses with a duration of 250 µs, delivered at a frequency of 30 Hz and a duty cycle of 7 s ON / 18 s OFF. This duty cycle was chosen based on previous experiments from our group demonstrating its antidepressant potential and the potency to affect intracerebral noradrenaline release [27, 37]. Animals in the sham group were also connected to the stimulation set-up, but the output current was set at 0.00 mA. On day 4 of the testing week, VNS or sham sessions were immediately followed by the 5-minute forced swim test. On day 5 of the testing week, the animals received an additional VNS session at 0.70 mA or a sham session at 0.00 mA, which was immediately followed by the open field test. The impedance of the VNS electrodes was measured daily and remained low throughout the experiment (< 10 kOhm).

Open field test

An open field test was performed after the fifth and last VNS or sham session (on day 5 of the testing week). This test was performed to assess spontaneous locomotor activity. The animals were placed

individually in the center of a wooden box (dimensions: 1 m (l) x 1 m (w) x 0.4 m (h)) with white walls and a white floor. On the floor of the wooden box, 49 equal squares (dimensions: 0.14 m x 0.14 m) were marked with black lines. The rats were allowed to explore the open field for 5 minutes. Sessions were videotaped and the number of squares crossed with four paws during the 5-minute trial was assessed offline by two independent investigators blinded for the group and treatment (AG and CB). After each test, the arena was cleaned thoroughly with a soap solution, rinsed with fresh water and dried with paper towels.

Histology

Animals were deeply anesthetized with an overdose of sodium pentobarbital (180 mg/kg, ip) and transcardially perfused with ice-cold paraformaldehyde (4%, pH 7.4). The brains were post-fixed in paraformaldehyde (4%, pH 7.4) for 24 hours and subsequently cryoprotected in 10%-20%-30% sucrose at 4°C, snap-frozen in ice-cold isopentane and stored at -20°C. Coronal frozen sections (40 µm) were made using a cryostat (Leica). The sections were washed twice for five minutes in phosphate buffered saline (PBS) and subsequently incubated with 0.6% hydrogen peroxide for 30 minutes at room temperature. After washing twice for five minutes in PBS, the sections were incubated with PBS containing 3% Donkey serum and 0.25% Triton-X for 30 minutes. The sections were then incubated with the primary anti-dopamine-β-hydroxylase antibody (1:1000, Merck Millipore, MAB308) diluted in PBS for one hour at room temperature and subsequently overnight at 4 °C. Next day, the sections were washed twice during five minutes in PBS, followed by incubation with biotinylated donkey-anti-mouse antibody (1:1000, Jackson ImmunoResearch lab) diluted in PBS for two hours at room temperature. After two rinses in PBS, dopamine-β-hydroxylase in the sections was detected with the avidin–biotin conjugate (Vectastain ABC kit, Vector Laboratories) and revealed with 3,3-diaminobenzidine (DAB; brown precipitate). The slices were mounted on glass slides and cover slipped using Entellan.

Statistical analysis

Statistical analyses were performed using SPSS 21. Nonparametric tests were used because the data was not normally distributed. All results are expressed as median and interquartile range. To compare immobility in the forced swim test and locomotor activity in the open field test between the three groups, Kruskal–Wallis tests were used followed by Mann–Whitney U post hoc tests with adjusted p-values after Bonferroni correction. A Spearman correlation test was used to assess

possible correlations between the immobility in the forced swim test and the locomotor activity in the open field test. Graphs were drawn in Sigmaplot 11.0. Statistical significance was set at $p<0.05$.

**Results**

Forced swim test and open field test

The fraction of immobility in the forced swim test was significantly lower in the VNS group (median: 56%, interquartile range: 41%) compared to the sham group (median: 75%, interquartile range: 12%) ($p<0.05$). This antidepressant-like effect of VNS was abolished in the DSP-4-VNS group (median: 79%, interquartile range: 33%), reflected by a level of immobility similar to the sham group ($p>0.05$) and significantly higher than the VNS group ($p<0.05$) (see figure 2.A).

There were no significant differences in locomotor activity in the open field test between animals of the three groups; median: 59 squares, interquartile range: 55 squares, median: 52 squares, interquartile range: 68 squares and median 30 squares, interquartile range: 55 squares for the sham, VNS and DSP-4-VNS group respectively ($p>0.05$) (see figure 2.B). No correlation was found between the immobility in the forced swim test and the locomotion in the open field test (Spearman correlation coefficient= -0.0826, $p>0.5$, see figure 2.C).

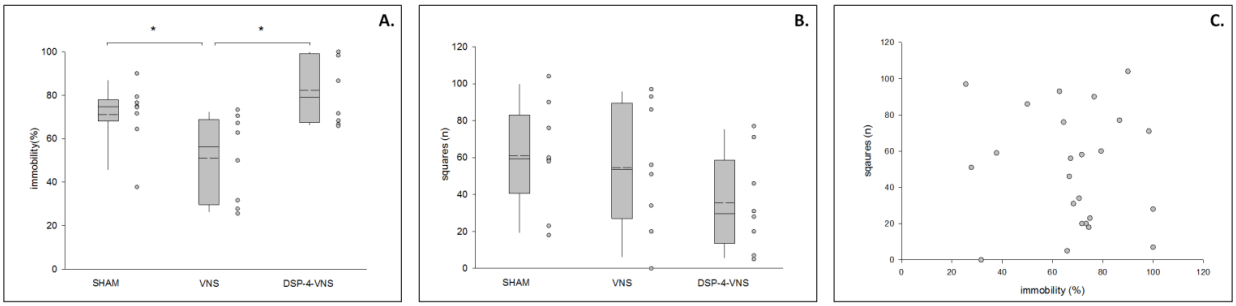


**Figure 2:** Results of the forced swim test and the open field test. **A.** Immobility in the forced swim test (in %). **B.** Locomotor activity in the open field test (in amount of squares crossed). **C.** Immobility in the forced swim test versus locomotor activity in the open field test. Boxplots depict the median (full line), the mean (dashed line), the interquartile range (box boundaries) and the 5-95$^{th}$ percentile (whiskers). Each dot represents a value of an individual animal (n = 8 per group). * $p<0.05$, Kruskal–Wallis tests, followed by Mann–Whitney U post hoc tests with adjusted p-levels after Bonferroni correction. DSP-4, [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride]; VNS, vagus nerve stimulation.

Histology

To validate the efficacy of the DSP-4 effect on the noradrenergic neurons, an immunostaining was performed using antibodies against the membrane-bound enzyme dopamine-β-hydroxylase. This enzyme converts dopamine to noradrenaline and is a specific marker for noradrenergic nerve terminals [38]. The lesion in the DSP-4-treated group was confirmed as the absence of dopamine-β-hydroxylase-immunostained noradrenergic axons in the hippocampus (see figure 3). The hippocampus was chosen because the LC provides the sole source of noradrenaline for this structure [32].

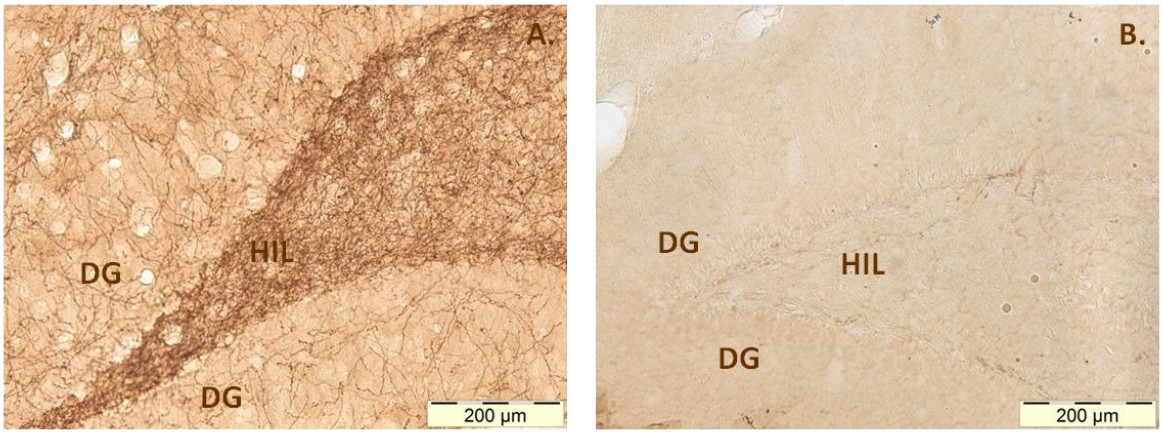


**Figure 3:** Microscopic images of the dopamine-β-hydroxylase staining. **A.** Microscopic image of dopamine-β-hydroxylase-immunostained noradrenergic axons in the hippocampus of a naive rat and **B.** the absence of staining in a DSP-4-treated rat. DG, dentate gyrus; DSP-4, [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride]; HIL, hilus.

**Discussion**

In this study, we demonstrated that VNS has an antidepressant-like effect based on a significant reduction of the immobility time in the forced swim test. These findings are congruent with the results of a previous study by Krahl et al. [18]. In the present study, the antidepressant-like effect of VNS was completely abolished when the noradrenergic neurons arising from the LC were eliminated using the selective noradrenergic neurotoxin DSP-4. To rule out the possibility that the effects in the forced swim test were caused by an overall change in locomotor activity, the animals were tested in an open field. No significant difference in locomotor activity was found between groups. Furthermore, the immobility in the forced swim test did not correlate with the locomotor activity in the open field test, ruling out a mere locomotor effect as an explanation for the observed forced swim test results. Our findings demonstrate a key role for the noradrenergic LC neurons in the antidepressant-like mechanism of action of VNS.

The LC and its neurotransmitter noradrenaline, are convincingly involved in the treatment of depression [20, 39]. On the one hand, direct electrical stimulation of the LC has been shown to produce an antidepressant-like effect in the forced swim test [40]. On the other hand, lesioning studies have shown that an intact LC is required to observe a therapeutic effect of antidepressant drugs [40-42]. Moreover, it was demonstrated that various classes of antidepressants influence the discharge rate of LC neurons [43-46]. It is well-known that VNS also influences the LC through the vagal afferent fibers. These fibers have their cell bodies in the nodose ganglion and predominantly project to the nucleus tractus solitarius. In turn, the neurons from the nucleus tractus solitarius project to the LC and support the VNS-induced increases in LC firing rate [22-25]. Furthermore, VNS was shown to significantly increase the percentage of LC neurons firing in bursts [22-24, 47], a firing mode that leads to greater release of noradrenaline compared to single pulses [29]. Moreover, chronic stimulation of the vagus nerve was shown to increase the number of spikes per burst and the burst length, contributing further to the increase in mean firing rate of the noradrenergic LC neurons [22, 24]. The results from these electrophysiological studies are consistent with several studies that have demonstrated VNS-induced increases in noradrenaline in brain structures involved in mood regulation, including the basolateral amygdala [30], the prefrontal cortex [28, 47] and the hippocampus [26, 27]. In the present study, a relatively intense duty cycle (7 s ON / 18 OFF) was used. This duty cycle is referred to as ‘rapid cycling’ in clinical practice, and is sometimes used to treat patients in whom VNS with the standard duty cycle (30 s ON / 300 s OFF), has no significant therapeutic effect [48, 49]. We have decided to use rapid cycling in this experiment for two reasons. First, we recently demonstrated that rapid cycling VNS has antidepressant effects in the kainic acid

model for temporal lobe epilepsy and comorbid depression [37]. Second, Roosevelt and colleagues have previously reported a bilateral increase in noradrenaline levels in the hippocampus (28% above baseline) in response to one hour of VNS, using the standard duty cycle [26]. However, in an experiment by our own group, a more than two-fold higher increase (69% above baseline) in extracellular hippocampal noradrenaline was achieved in response to one hour of rapid cycling VNS [27]. This large body of evidence on the VNS-induced enhancement of the LC noradrenergic system, combined with the findings of the present study and the established role of the LC and noradrenaline in the therapeutic effect of many antidepressants [20, 39, 40, 42], strongly supports the hypothesis that the antidepressant effect of VNS is mediated through the activation of the LC and subsequent release of noradrenaline.

In previous LC lesioning studies, it was demonstrated that an intact LC is also required for the antiepileptic and the antinociceptive effects of VNS [50-53]. Considering the loss of therapeutic efficacy of VNS for several disorders after LC destruction, it could be hypothesized that the LC functions as a gateway structure, by primarily releasing noradrenaline which can then trigger other mechanisms important in several conditions, including depression, epilepsy and pain [50-53]. A hypothesis to consider for depression is the enhancement of other neurotransmitter systems such as the serotonergic [22, 47, 54] and dopaminergic [23, 55] neurotransmission, two monoaminergic systems which are implied in the pathophysiology and the treatment of depression [56, 57]. While noradrenergic system activation is already present after one hour of VNS [22, 24, 47], it was demonstrated that the effect of VNS on the serotonergic and dopaminergic neurons only appears after chronic stimulation (2 weeks of VNS). Indeed, it was shown that serotonin is implied in the antidepressant-like effect of chronic VNS (2 weeks of continuous stimulation) [58]. Manta et al. showed that the effect of VNS on serotonergic neuronal firing is indirect and mediated by the noradrenergic LC neurons through the enhanced activation of the excitatory α1-adrenoreceptors located on dorsal raphe nucleus serotonergic cell bodies [22]. Furthermore, it was shown that direct LC stimulation elicits burst firing of the dopaminergic ventral tegmental neurons through the excitatory α1-adrenoreceptors as well [59-61]. Consequently, it can be hypothesized that the effect of VNS on the dopaminergic system is also indirectly mediated through the activation of the LC noradrenergic system.

Another plausible hypothesis is that VNS produces its antidepressant-like effect through increasing neuroplasticity in the hippocampus, a limbic structure involved in mood regulation. The term "neuroplasticity" encompasses an array of mechanisms, from the birth, survival, migration, and integration of new neurons (or neurogenesis), to neurite outgrowth, synaptogenesis and the

modulation of mature synapses [62]. The rationale for this hypothesis originates from the knowledge that stressful events such as forced swimming in rodents, lead to a significant reduction in hippocampal neuroplasticity, while the mode of action of several antidepressants involves increasing hippocampal neuroplasticity [63]. Airan et al. even showed that antidepressant efficacy in the forced swim test requires intact hippocampal neurogenesis [64]. A growing body of evidence suggests that VNS could produce its antidepressant effect through increasing hippocampal neuroplasticity as well [28, 65-68]. As the hippocampus is rich in LC noradrenergic innervation [69] and noradrenaline has proven neuroplastic effects [70-72], it is tempting to hypothesize that these effects of VNS are also indirectly mediated though the activation of the LC and subsequent release of noradrenaline in the hippocampus.

In conclusion, this study demonstrates the key role of the LC in the antidepressant-like mechanism of action of VNS. Despite this, further research is required to unravel the upstream mechanisms by which the VNS-induced activation of the LC exerts its antidepressant effect. Hypotheses to consider are the enhancement of neurotransmitter systems involved in depression and/or the upregulation of hippocampal neuroplasticity.

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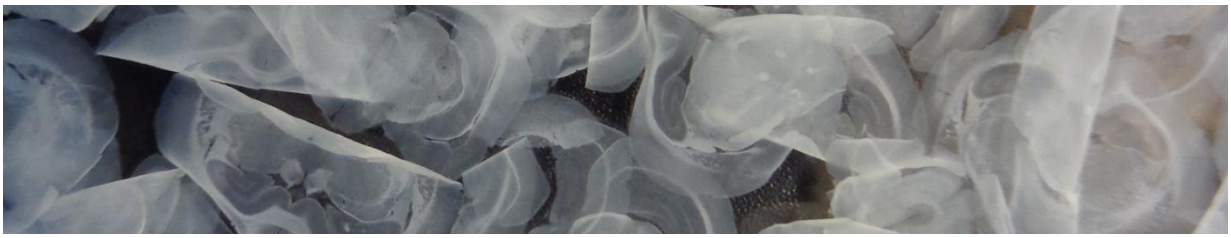
# Chapter 3 - study 3



The antidepressant mechanism of action of vagus nerve stimulation: evidence from preclinical studies

## Study 3: Review on the antidepressant mechanism of action of VNS

The third literature study of chapter 3 consist of a review that provides an overview of the preclinical VNS studies in view of two major hypotheses in depression research: the monoaminergic and the neural plasticity hypothesis of major depression.

# The antidepressant mechanism of action of vagus nerve stimulation: evidence from preclinical studies

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**Abstract**

Vagus nerve stimulation (VNS) is a proposed neuromodulatory treatment for medically refractory major depression. Although VNS is already used in clinical practice, the underlying mechanism of action remains unknown. The present review provides an overview of the preclinical VNS studies in view of two major hypotheses in depression research: the monoaminergic and the neural plasticity hypothesis of depression.

**Keywords:** vagus nerve stimulation, major depressive disorder, the monoaminergic hypothesis of major depressive disorder, the neural plasticity hypothesis of major depressive disorder.

## Introduction

The World Health Organization estimates that by 2020, major depressive disorders (MDD) will become the second largest cause of global disease problems in the world, only behind ischemic heart disease [1]. The lifetime prevalence for MDD is reported to be as high as 17% and the 12-month prevalence is estimated to be 4-8% [2, 3]. Despite the availability of a variety of antidepressant agents and improved tolerance of new antidepressant medications, up to 20% of patients fail to respond adequately to standard antidepressant treatments [4]. This relative lack of efficacy significantly interferes with the psychosocial functioning and quality of life of refractory patients. In addition, it is well-recognized that the failure to reach full clinical remission after antidepressant treatment involves a high risk of relapse and recurrence in patients suffering from MDD [5]. The lack of success with current pharmacological interventions, highlights the importance of optimizing non-pharmacological treatments for refractory patients.

Among other neuromodulation modalities for refractory MDD, vagus nerve stimulation (VNS) is the electrical stimulation of the left vagus nerve at the cervical level, by means of implanted electrodes and a programmable pulse generator. It is also a well-established, safe and effective add-on therapy for refractory epilepsy [6]. The initial rationale for using VNS for the treatment of refractory depression, resulted from mood improvements in epilepsy patients treated with VNS, irrespective of the presence or absence of beneficial effects on seizure frequency [7-9]. A recent study from our laboratory confirmed the antidepressant effect of VNS in the kainic acid rat model for temporal lobe epilepsy and comorbid depression [10]. Of interest, MDDs are the most common type of psychiatric comorbidity in patients suffering from refractory epilepsy [11-13].

The therapeutic effect of chronic VNS for treatment resistant depression has been assessed in several clinical studies [14-28]. VNS demonstrated steadily increasing improvement of depressive symptoms with full benefit after 6 to12 months, sustained for up to 2 years. These studies reported response rates of 30-40% and remission rates of 15-17% after 3 to 24 months of treatment [29]. Furthermore, a recent meta-analysis comparing 'VNS with treatment as usual' (n = 1035) versus 'treatment as usual alone' (n = 425), revealed that 'VNS with treatment as usual' results in greater response and remission rates that are more likely to persist in the long-term [30].

Although VNS has proven to be effective in reducing depressive symptoms in several clinical trials, the optimal stimulation parameters and the mechanism of action remain elusive. A retrospective analysis by Muller et al., revealed that VNS at low-strength/high-frequency stimulation parameters is effective in reducing depressive symptoms, while VNS at high-strength/low-frequency stimulation

parameters is not [26]. Furthermore, a randomized, double-blind, multicenter VNS dosing study by Aaronson et al., compared the safety and effectiveness of different stimulation parameters, i.e. low, medium or high dose VNS. The results from the study showed that VNS induces significant, durable antidepressant effects, irrespective of the applied stimulation parameters. However, higher electrical dose parameters were shown to be associated with response durability [25]. Concerning the mechanism of action, functional brain imaging studies in humans have demonstrated that VNS causes immediate and longer-term changes in brain regions implicated in neuropsychiatric disorders. The regions affected by VNS include the thalamus, cerebellum, prefrontal cortex, limbic system, hypothalamus and medulla [27, 28, 31].

Further unraveling the antidepressant mechanism of action of VNS may support the optimization of stimulation parameters and the identification of biomarkers to predict therapeutic response. The present review discusses the putative antidepressant mechanisms of VNS, in the context of two major hypotheses in MDD research: the monoaminergic and the neural plasticity hypothesis of MDD.

## VNS and the monoaminergic hypothesis of MDD

The last 50 years of depression research have been dominated by the monoaminergic hypothesis. The main assumption in this hypothesis is that depression is caused by an impairment of central monoaminergic functioning. Monoamines are neurotransmitters containing one amino group that is connected to an aromatic ring by a two-carbon chain [32]. These neurotransmitters affect a wide range of normal brain functions related to mood control, such as sleep, motivation and hedonic state [33]. Decreased activity of the monoamines, due to decreased availability, impaired postsynaptic receptors and/or reduced sub-cellular messenger activity, is a pivotal pathogenic mechanism of depressive disorders and represents the main target for the development of antidepressant therapy [11, 33, 34]. Almost all currently available antidepressant drugs that reverse depressive symptoms are based on enhancing the monoaminergic neurotransmission, primarily the noradrenergic and/or serotonergic system. Most antidepressant agents increase the concentration of noradrenaline and/or serotonin in the synaptic cleft via 1) reuptake inhibition, 2) antagonism of inhibitory presynaptic autoreceptors or 3) inhibition of monoamine oxidases, which are the enzymes for monoamine degradation [33, 35]. Research on the antidepressant actions of drugs has mainly focused on the locus coeruleus (LC) and the dorsal raphe nucleus (DRN) due to their role in noradrenaline and serotonin release respectively. In the next paragraphs, we will describe how VNS can theoretically enhance the noradrenergic and serotonergic neurotransmission in the brain areas important in mood regulation such as the prefrontal cortex, the amygdala and the hippocampus. This will be based on

the neuroanatomical connections from the vagus nerve to the LC and the DRN and on evidence from experimental animal studies.

The vagus nerve is best known for its efferent parasympathetic actions, such as autonomic control and regulation of the heart and the gastrointestinal system [36]. However, the nerve comprises approximately 80% afferent fibers, carrying information from the body to the brain (see figure 1, for a detailed review on the anatomy of the vagus nerve, see [37]). These fibers have their cell bodies in the nodose ganglion and predominantly project to the nucleus tractus solitarius, an important gateway nucleus for many primary afferents from cardiovascular, respiratory, gastrointestinal and other visceral sensory receptors [38]. In turn, the neurons of the nucleus tractus solitarius project to the LC through three disynaptic pathways: (i) GABAergic inhibitory neurons localized in the nucleus prepositus hypoglossi, acting primarily on the $GABA_A$ receptor subtypes in the LC neurons [39, 40], (ii)

neurons localized in the nucleus paragigantocellularis containing excitatory amino acids [41, 42] and (iii) inhibitory GABAergic interneurons surrounding the LC [43].

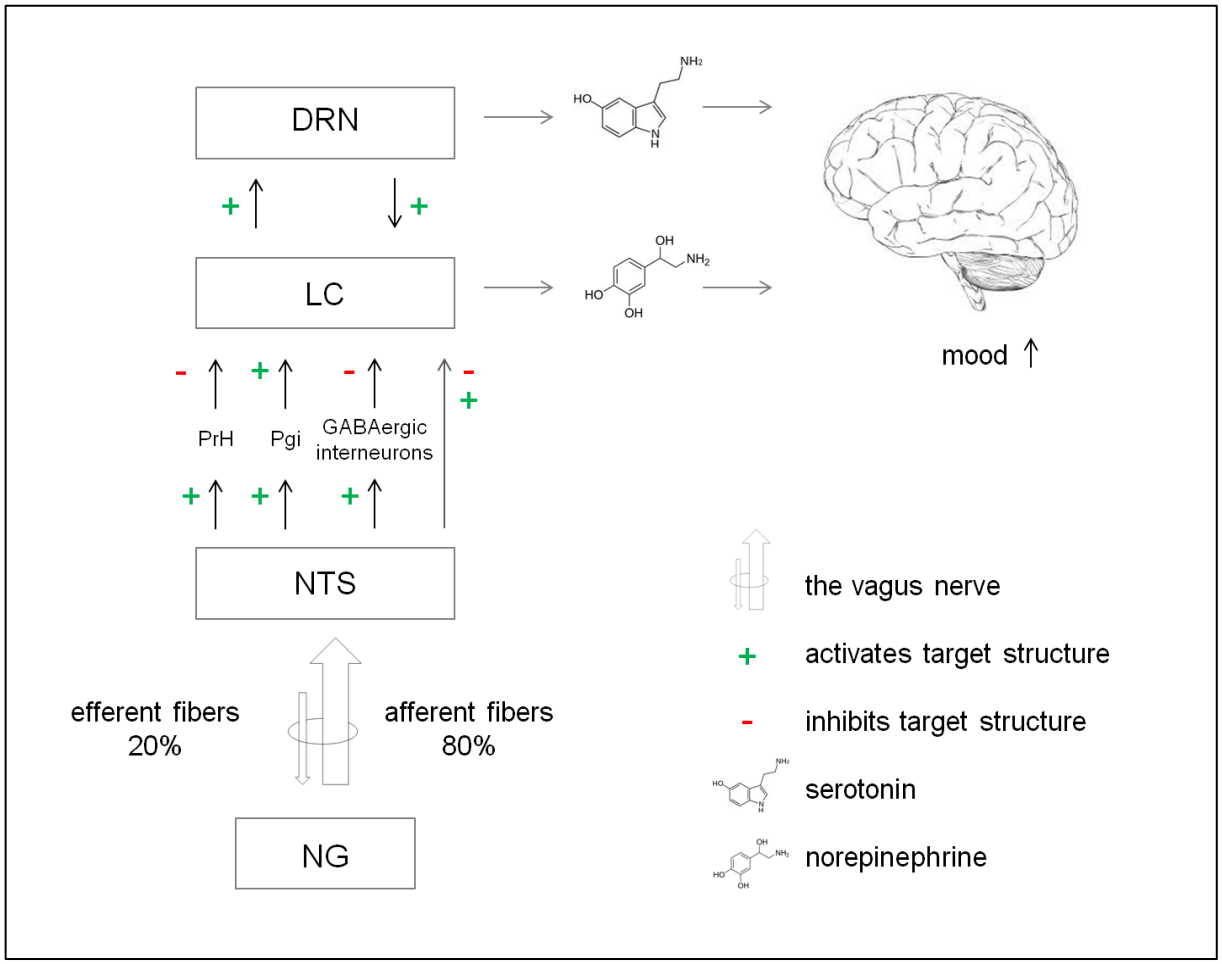

**Figure 1:** The afferent projections of the vagus nerve. Nodose ganglion: NG, nucleus tractus solitarius: NTS, locus coeruleus: LC, nucleus prepositus hypoglossi: PrH, nucleus paragigantocellularis: Pgi, dorsal raphe nucleus: DRN.

The nucleus tractus solitarius afferents to the nucleus prepositus hypoglossi, the nucleus paragigantocellularis and the GABAergic interneurons are mainly glutamatergic [42, 44, 45]. Next to these disynaptic pathways, the nucleus tractus solitarius neurons also sends both excitatory and inhibitory monosynaptic projections to the LC [42, 45]. The latter pontine nuclues is the main source of noradrenaline in the central nervous system and provides widespread noradrenergic innervation of virtually the entire brain [37, 46]. These anatomical projections provide a pathway to the brain supporting the VNS-induced increases in the concentration of noradrenaline in structures important for mood regulation, including the amygdala, the prefrontal cortex and the hippocampus [47-51]. These structures are part of a corticolimbic circuit that is known to be disturbed in patients suffering from MDD [52]. Hassert and colleagues were the first to show an increase in noradrenaline concentration in the basolateral amygdala in rats (98% above baseline levels) [51]. In the study of Roosevelt et al., VNS significantly increased noradrenaline concentrations both in the cortex (39% above baseline levels) and the hippocampus (28% above baseline levels) [48]. Raedt et al., found even higher VNS-induced increases in hippocampal noradrenaline (69% above baseline levels) [47]. A similar increase in noradrenaline concentration was found by Follesa et al. in the medial prefrontal cortex (70% above baseline levels) [49]. Recently, Manta et al. have found a significant increase in noradrenaline in the prefrontal cortex (58% above baseline levels) and the hippocampus (14% above baseline levels) as well [50]. Moreover, Landau et al. recently demonstrated that VNS decreases $\alpha_2$-adrenoceptor binding, further supporting the increased noradrenaline release in response to this treatment [53]. These neurochemical effects of VNS fit the results from electrophysiological studies in rats which have shown that VNS increases the firing rate of noradrenergic LC neurons [40, 54-56]. Furthermore, VNS significantly increases the percentage of LC neurons firing in bursts [40, 50, 54, 55], a firing mode that leads to greater release of noradrenaline compared to single pulses [57]. Long-term stimulation of the vagus nerve was shown to increase both the number of spikes per burst and the burst length, contributing further to the increase in the mean firing rate of LC noradrenergic neurons [40, 54]. Manta et al. suggested that the effects of VNS on the LC are mediated through a greater facilitation of the excitatory pathways from the nucleus tractus solitarius to the LC compared to the inhibitory pathways [40]. In this hypothesis, it is assumed that VNS activates the nucleus tractus solitarius, which is indirectly supported by the recent finding that transcutaneous VNS increases the firing rate of the nucleus tractus solitarius neurons [58]. The decreased synaptic

efficacy observed in the nucleus tractus solitarius after vagotomy [59], provides further indirect evidence for this hypothesis. On the other hand, it has been shown that VNS at a frequency of 20 Hz evokes synaptic depression in the nucleus tractus solitarius neurons [60, 61], thereby suppressing the primarily inhibitory input from the nucleus prepositus hypoglossi and the GABAergic interneurons to the LC [43]. This results in a disinhibition of the LC neurons, leading to a subsequent increased firing rate and release of noradrenaline in the brain.

Another important connection in the context of the monoaminergic hypothesis of MDD is the excitatory projection from the LC to the DRN [62], which is the major source of serotonin in the brain [63]. It has been shown that both exogenous noradrenergic agonists and endogenously released noradrenaline activate the excitatory $\alpha_1$-adrenoreceptors on the cell bodies of the serotonergic neurons in the DRN and consequently increase the firing rate of these neurons [40, 54, 64]. Furthermore, studies have found that administration of an $\alpha_1$-adrenoreceptor antagonist reduces the firing activity of serotonergic DRN neurons [40, 65, 66]. Consistent with this knowledge, it was shown that VNS also enhances the firing rate of the serotonergic neurons of the DRN, but only after 14 days of stimulation [40, 54]. Furthermore, a selective lesion of the noradrenergic LC neurons prevents the excitatory action of VNS on the serotonergic DRN neurons [40]. Based on these findings, Manta et al. suggested that the effect of VNS on the serotonergic neurons of the DRN is indirect and secondarily mediated through its robust effect on the noradrenergic neurons from the LC [40]. This hypothesis is further supported by the observation that VNS produces an acute activation of the LC neurons as revealed by an increase in c-fos, a nuclear protein which is expressed under conditions of high neuronal activity [67, 68], while only a delayed activation of the neurons in the DRN is shown by an increase in delta FosB after chronic treatment [67, 69]. The DRN in turn, also innervates the LC [62, 70], creating the opportunity for cross-modulation between these two brainstem nuclei.

To date, behavioral and mechanistic studies assessing the antidepressant actions of VNS with clinically relevant stimulation parameters in validated animal models for depression are scarce. In this regard, the forced swim test is one of the most commonly used experimental set-ups for assessing antidepressant-like behavior in rodents. The test involves the scoring of active mobile or passive immobile behavior when rodents are forced to swim in a cylinder from which they cannot escape. A reduction in passive behavior is reflective of an antidepressant-like effect of the therapy [71, 72]. Krahl et al. showed that short-term VNS (4 consecutive days, 30 minutes per day) produces an antidepressant-like effect in the forced swim test in rats with the same efficacy as electroconvulsive shock therapy and the tricyclic antidepressant desipramine [73]. Since the impact of VNS on the serotonergic neurons of the DRN is estimated to be minor after short-term stimulation [40, 54], this antidepressant-like effect was most likely mediated through increased noradrenergic

signaling of the LC neurons. This hypothesis was recently tested in our laboratory. Using a selective noradrenergic neurotoxin for the LC neurons, we confirmed the key role of the LC in the antidepressant-like mechanism of action of VNS [74].

The question remains how VNS-induced noradrenaline and serotonin increases produce an antidepressant effect in subjects suffering from medically resistant depression, while drug-induced noradrenaline and serotonin increases remain without an effect in this patient population. Dorr et al. suggested that antidepressant agents and VNS enhance the noradrenergic and serotonergic neurotransmission in a different way [40]. Antidepressant drugs that increase the noradrenaline and/or serotonin concentrations, initially decrease the firing rate of the LC and/or the DRN neurons, due to binding of the neurotransmitters to the somatodendritic autoreceptors on these neurons [75]. Long-term treatment restores the firing activity back to baseline due to desensitization of these receptors while keeping high synaptic availability of noradrenaline and/or serotonin. Antidepressant treatments thus increase the efficacy of the noradrenergic and/or serotonergic neurotransmission via an increase in neurotransmitter release and altered sensitivity of inhibitory autoreceptors (reviewed in [76]). VNS on the other hand, enhances the noradrenergic and serotonergic neurotransmission by inducing an increase in the firing rate of both the LC and the DRN neurons above their baseline activity [40, 55]. Moreover, VNS represents the first antidepressant treatment able to induce increased firing activity of both serotonergic and noradrenergic neurons [54]. Dorr et al. have found that both the serotonergic 5-$HT_{1A}$ and the noradrenergic $\alpha_1$-somatodendritic autoreceptors are fully functional after long-term treatment with VNS [54]. The increase in firing rate is therefore not a result of a desensitization of the autoreceptors but rather of a distinct mechanism. A possibility considered by the authors is that VNS increases the release of noradrenaline and serotonin in the terminal regions such as the hippocampus and the medial prefrontal cortex, but not in the vicinity of the LC and the DRN [54]. Therefore, VNS probably exerts antidepressant activity through alternative mechanisms compared to conventional drugs. This may provide an explanation why VNS proves to be beneficial for patients with treatment resistant MDD [54].

Although monoamines were convincingly shown to play a major role in the pathophysiology of MDD and its treatment, the monoamine hypothesis is incomplete and does not fully explain some important clinical observations. First, monoamine depletion in healthy individuals does not consistently produce depressive symptoms [77-79]. Secondly, antidepressant treatments produce a fast elevation of monoamine concentrations, while the antidepressant effects of these treatments are only established after chronic treatment in patients [80]. In this regard, growing body of evidence suggests that neuronal plasticity might be the missing link.

**Vagus nerve stimulation and the neural plasticity hypothesis of MDD**

The neural plasticity hypothesis of depression postulates that reduced neural plasticity plays a major role in the pathophysiology of MDD, and that its restoration may represent a critical mechanism underlying antidepressant efficacy. The term "neural plasticity" encompasses an array of mechanisms, from the birth, survival, migration and integration of new neurons to neurite outgrowth, synaptogenesis and the modulation of mature synapses [81]. The rationale for this hypothesis originates from the observation that stress - which is the main cause of depression - can lead to significant atrophy, cell loss and changes in synaptic strength in limbic brain structures that are involved in mood regulation both in humans and in animals [82, 83]. In this regard, the subgranular layer of the hippocampal dentate gyrus, which is one of the only two regions where neurogenesis takes place in the adult brain, is of particular importance [84]. Neurogenesis is the process by which fully functional neurons are generated from neural stem and progenitor cells [85] (for a schematic overview see figure 2).

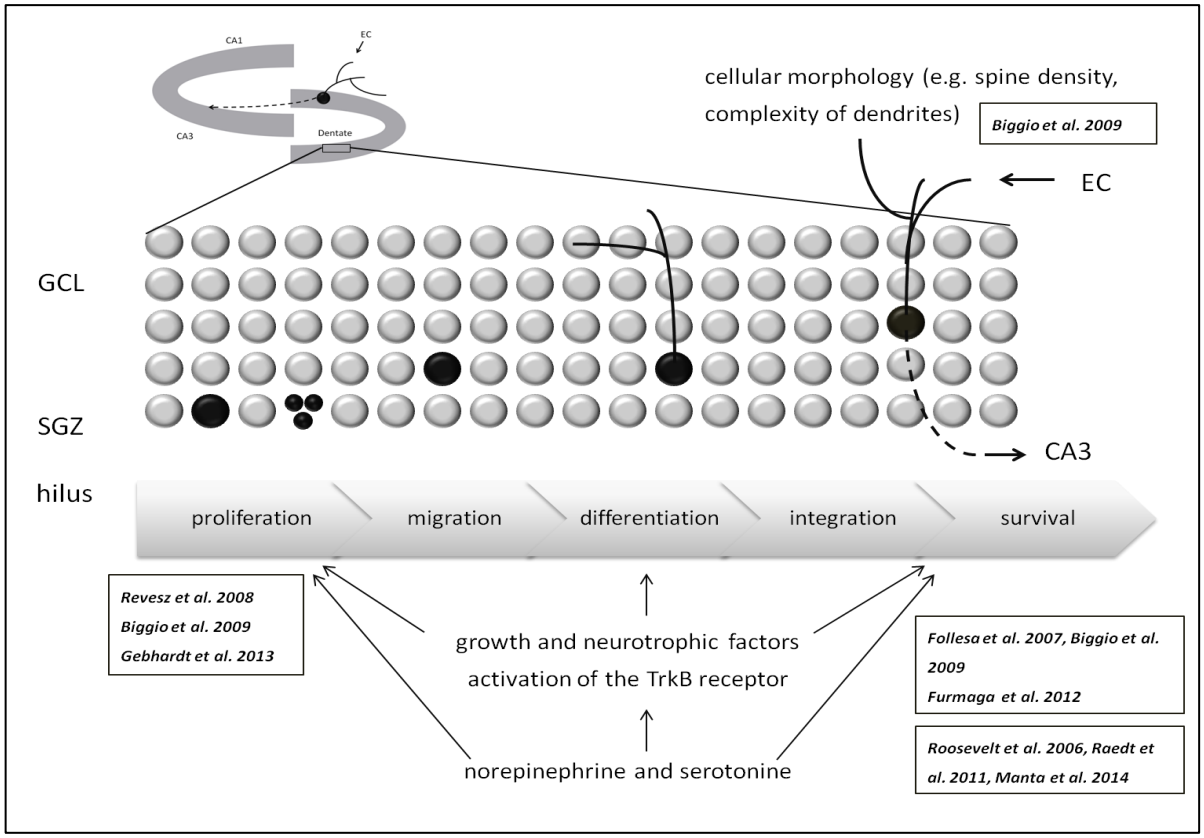


**Figure 2:** Schematic overview of neural plasticity in the hippocampus. Subgranular zone: SGZ, granule cell layer: GCL, cornu ammonis layer 3: CA3, entorhinal cortex: EC, tropomyosin receptor kinase B:TrkB, mature granule cells are depicted in gray, proliferating granule cells are depicted in black. The boxes show the studies in which influences of VNS were shown.

This process takes place in the subgranular zone of the dentate gyrus, situated between the granule cell layer and the hilus. Neural progenitor cells proliferate and give rise to new cells that migrate into the granule cell layer and differentiate into mature granule cells. These new granule cells then integrate functionally into the existing granule cell layer by sending their axons to the pyramidal CA3 neurons (mossy fiber pathway) and receiving input from the entorhinal cortex (perforant fiber pathway) [85]. It requires about seven weeks for newborn cells to become functionally indistinguishable from the older granule cell population [86, 87]. Several studies have reported hippocampal volume loss in patients suffering from MDD [88, 89]. Moreover, these hippocampal volume changes were shown to be correlated with the duration of the illness [88] and were reversed or prevented by chronic antidepressant treatment in MDD patients [90]. In animal models for depression, stress-induced suppression of hippocampal neurogenesis was shown to be normalized by chronic antidepressant treatment [83, 91-93]. These findings support the hypothesis that increased neurogenesis in the hippocampus may be a mechanism by which antidepressant treatments overcome the stress-induced atrophy and loss of hippocampal neurons. Increases in hippocampal neurogenesis can result from increased proliferation of neural stem/progenitors cells, from enhanced survival of newborn neural progenitors or neurons or from a combination of both [94, 95]. Morphological cellular changes such as up- or down regulation of synapse formation and spine density or extension and retraction of dendrites, further contribute to altered neural plasticity in the hippocampus [85]. Although this part of VNS research is still in its infancy, a growing body of evidence suggests that stimulation of the vagus nerve could produce its antidepressant effect through increasing neural plasticity by influencing these processes (table 1 gives an overview of the relevant studies).

| | stimulation parameters | short-term stimulation | effect | long-term stimulation | effect | behavioral test | effect |
|---|---|---|---|---|---|---|---|
| **Revesz et al. 2008** | 0.25, 0.75, or 1.5 mA, 20 Hz, 250 µs and 30 s on/5 min off | 48 h | ↑ hippocamapal progenitor proliferation, but only in the 0.75 mA group<br>no effect on cell survival | / | / | / | / |
| **Biggio et al. 2009** | 1.5 mA, 30 Hz, 500 µs,<br>30 s on/5 min off | 3 h | ↑ progenitor proliferation<br><br>↑ dendritic complexity (↑ number of intersections) | 1 month | no effect on progenitor proliferation<br><br>↑ BDNF expression in CA3 of the hippocampus<br><br>↑ dendritic complexity (↑ number of intersections and length of denrites) | forced swim test<br>+ elevated plus maze | no effect |
| **Follesa et al. 2007** | 2 mA, 30 Hz, 500 µs, 30 s on/5 min off | 3 h | ↑ expression of BDNF and bFGF in the hippocampus and the cerebral cortex<br><br>↑ concentration of norepinephrine in the prefrontal cortex | / | / | / | / |
| **Gebhardt et al. 2013** | 2 mA, 30 Hz, 500 µs, 30 s on/ 5 min off | / | / | 3 weeks | ↑ progenitor profileration | one-way active avoidance learning | restorative effect on coginitive deficits |
| **Furmaga et al. 2013** | 0.25 mA, 20 Hz, 250 µs,<br>30 s on/5 min off | 2h | phosphorylation of TrkB in the hippocampus | 2 weeks | phosphorylation of TrkB in the hippocampus | / | / |

**Table 1:** Overview of the different stimulation paradigms used and the main results of studies on the effects of VNS on plasticity. Brain derived neurotrophic factor: BDNF, basic fibroblast growth factor: bFGF, cornu ammonis layer 3: CA3, Herz: Hz, milliampere: mA, tropomyosin receptor kinase B: TrkB.

The effect of VNS on progenitor proliferation

Revesz et al. have shown that short-term VNS in adult rats produces a significant 50% increase in hippocampal progenitor proliferation compared to SHAM stimulation. An inverted U-shaped dose-dependent response to VNS was observed: moderate stimulation (0.75 mA) vs. high (1.5 mA) and low (0.5 mA) stimulation produced a significant and non-significant increase in progenitor proliferation compared to SHAM stimulation respectively [95]. Interestingly, this type of VNS dose dependency has previously been reported in experiments concerning learning and memory in rats [96-98] as well as in humans [99]. The authors suggest that the lower progenitor proliferation at the highest stimulus intensity (1.5 mA) results from a stress-induced decrease in hippocampal progenitor proliferation [95].

Biggio et al. found an increased progenitor proliferation using short-term (but not long-term) VNS with an output current of 1.5 mA [100]. The same study also assessed the effects of long-term VNS in the forced swim test and the elevated plus maze, to determine the behavioral correlates of despair

and anxiety respectively. However, the observed changes in the hippocampal neurons were not associated with evident behavioral alterations characteristic of an antidepressant or anxiolytic action [100]. In a recent study of Gebhardt et al., long-term VNS increased the number of newborn cells in the hippocampus of bulbectomized rats, which is a validated animal model for depression [101]. In this study, a restorative effect of VNS on the disturbed one-way active avoidance learning in the bulbectomized rats was found [101].

There are some contradictions among the results of the different studies which can most likely be attributed to methodological differences, such as the stimulation paradigm used and the definitions of 'short-term' and 'long-term' stimulation. Apart from the results found in the different studies discussed above, table 1 also gives an overview of the different stimulation paradigms used.

The effect of VNS on cell survival and differentiation

In the study of Revesz et al., VNS had no effects on cell survival, suggesting that the effects of VNS on hippocampal progenitors are merely proliferative in nature [95]. However, it was suggested by others that VNS promotes the survival of the newborn cells generated in the early phases of stimulation, rather than increasing cell proliferation indefinitely [100]. This suggestion is based on the observation that VNS robustly increases the expression of neurotrophic and growth factors such as brain derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) [49, 100]. These factors are known to promote the differentiation and survival of neurons [102-105], thereby increasing neuroplasticity. It is well known that the levels of these factors are reduced in mood disorder [106-108] and that antidepressants induce their upregulation [109-111]. Biggio et al. showed that long-term VNS also induces long-lasting increases in the amount of BDNF immunoreactivity and the number of BDNF-positive cells both in the cell bodies and fibers of hippocampal neurons [100]. Likewise, Follesa et al. found that short-term VNS significantly increases the expression of BDNF and bFGF in the hippocampus and cerebral cortex [49]. An alternative way by which antidepressants enhance BDNF functioning is by increasing its signaling through phosphorylation and subsequent activation of its receptor, i.e. the tropomyosin receptor kinase B (TrkB) receptor [109, 112-114]. Moreover, Saarelainen et al. have found that signaling via the TrkB receptor is required for inducing a behavioral response typically induced by antidepressants [112, 114]. Furmaga et al. have shown that both long-term and short-term VNS in rats phosphorylate and thus activate the TrkB receptor [112].

The effect of VNS on cellular morphology

A growing body of evidence suggests that mechanisms also other than cell proliferation, differentiation and survival may be responsible for structural remodeling of the hippocampal formation under circumstances of chronic stress or depression [115]. It has even been suggested that volumetric changes in the hippocampus of depressed subjects result from reduced dendritic complexity and not from the ablation of hippocampal neurogenesis [116]. At a cellular level, these changes can occur in the form of up- or down regulation of synapse formation and spine density or extension and retraction of dendrites [82, 117-119]. Interestingly, it was shown by Biggio et al. that both short-term and long-term VNS have an effect on the dendritic morphology of hippocampal neurons [100]. In particular, both types of VNS significantly increase the complexity of the hippocampal dendrites by increasing the number of intersections. Moreover, the length of the dendrites that project into the molecular layer of the hippocampus is significantly greater in rats subjected to long-term VNS than in those subjected to sham surgery [100].

Although it is clear that VNS increases neuronal plasticity, it is problematic that only two animal studies have tried to correlate VNS-induced changes in neuroplasticity to antidepressant-like effects in behavioral testing paradigms. Only the study by Gebhardt et al. [101] found an association between the increased progenitor proliferation and the restorative effects on cognition. An association does not prove the causal relationship between the observed phenomena and therefore the results should be interpreted with caution. That is, the possibility exists that the neuroplastic effects of VNS are merely an epiphenomenon of other more important processes leading to sustained antidepressant effects. The dissociation between the presence of neuroplastic effects and the lack of behavioral effects in the study of Biggio et al. [100], supports this hypothesis. Alternatively, this dissociation could result from the fact that preclinical studies are of insufficient duration to investigate long-term effects of VNS. Clinical studies have demonstrated that VNS-induced therapeutic effects are increasingly observed after several months of treatment [17]. A plausible hypothesis for this gradual increase in efficacy could be that it requires several weeks to months for newborn cells to become functionally indistinguishable from mature, fully functional hippocampal granule cells [86, 87] and subsequently to restore dysfunctional networks in depressed subjects. Current models of MDD hypothesize a dysregulation of several interconnected structures in the frontal and limbic circuitry. Key structures in this network do not only include the hippocampus, but also upstream structures such as the prefrontal cortex (medial, orbital and dorsolateral), amygdala, insular cortex, cingulate cortex, striatum, dorsal thalamus, and hypothalamus [120]. Functional imaging studies in depressed patients treated chronically with VNS, have demonstrated

that antidepressant response may be associated with gradual changes in the cerebral metabolic activity of these upstream structures [27, 31]. Therefore neuroplastic changes in the hippocampus may represent a first, early but indispensible step towards achieving therapeutic efficacy.

Another shortcoming of the preclinical studies performed so far, is that none of them have investigated whether the newborn progenitor cells differentiate into mature neurons and integrate functionally into the hippocampal network. Therefore, future studies should determine the phenotype of the newborn cells using specific markers for mature neurons, such as NeuN (neuronal nuclei) or NSE (neuron specific enolase). Furthermore, connectivity studies should be performed to confirm the hypothesis that the newborn neurons restore the disturbed cortico-limbic networks in depressed subjects.

**The monoamine and the neural plasticity hypothesis of MDD meet again**

Albeit that the neural plasticity hypothesis of MDD has directed research away from the monoamines and towards the putative role of plasticity in the adult brain, both hypotheses might be more intertwined than it seems at first sight. The hippocampus is rich in noradrenergic and serotonergic innervation [121] and changes in these monoamine concentrations in the central nervous system have been shown to affect hippocampal plasticity. On the one hand, selective noradrenergic depletion decreases the number of proliferating progenitors in the dentate gyrus of the adult rat [122]. On the other hand, chronic monoaminergic antidepressant treatments, including serotonin and noradrenaline selective reuptake inhibitors and monoamine oxidase inhibitors increase neural plasticity in the rodent hippocampus [85]. Blockage of noradrenergic autoreceptors, which increases noradrenaline levels in the brain, also enhances the survival of progenitor cells in the hippocampus [123]. The neuroplastic effects of the monoamines could be mediated directly through binding to their receptors in the hippocampus, most likely to the β3-adrenergic [124] and 5-$HT_{1A}$ receptors [125, 126] for noradrenaline and serotonin receptively. How the β3-adrenergic and 5-$HT_{1A}$ serotonergic receptor induce the proliferation of neural precursors is currently unknown. However, it is well-known that these receptors are seven transmembrane proteins coupled to heterotrimeric G-proteins that can activate intracellular second messenger cascades. Given that binding of noradrenaline and serotonin to these receptors up-regulates the cAMP cascade [85] and that increases in the intracellular levels of cAMP regulate the proliferation of hippocampal precursors in vivo [127], it is possible that the receptor-driven activation of neural precursors may also utilize this cAMP-mediated signaling mechanism [124]. In addition, monoamines could also indirectly influence hippocampal

plasticity via the alteration of growth and neurotrophic factors. Duman et al. suggested that the effects of noradrenaline and serotonin on hippocampal plasticity are produced through the upregulation of BDNF gene expression of neurons containing noradrenergic and serotonergic receptors [85]. Furthermore, noradrenergic and serotonergic neurons also contain fibroblast growth factor-2 (FGF-2) [128], which is known to enhance adult hippocampal neurogenesis [129]. However, it has not yet been shown that FGF-2 is released from the noradrenergic or sertonergic neurons when the vagus nerve is stimulated. It is also worth mentioning that noradrenaline application in cultured hippocampal neurons is able to induce TrkB phosphorylation and downstream signaling via G-coupled receptor transactivation of TrkB [130], providing an additional link between the monoamine hypothesis and the neural plasticity hypothesis of depression. Altogether, these findings support the hypothesis that the observed increase in hippocampal plasticity after VNS could be mediated in part by changes in hippocampal noradrenergic and serotonergic activity.

## Conclusion

The present review provides an overview of the available preclinical VNS studies in view of two major hypotheses in depression research: the monoaminergic and the neural plasticity hypothesis of MDD. Consistent with the monoamine theory of depression, noradrenaline and serotonin were identified as key players in the antidepressant mechanism of action of VNS. VNS induces an acute elevation of the monoamine levels, while its antidepressant effect in patients is established typically after several weeks or months of treatment. In this regard, a growing body of evidence suggests that neuroplastic changes might be the missing link. In other words, we hypothesize that VNS exerts its antidepressant effects through a rapid increases in the concentration of the monoamines, which then enhance neuronal plasticity in the hippocampus. Newborn cells could then functionally integrate and restore the disturbed cortico-limbic networks in depressed patients. Furthermore, processes such as increased dendritic complexity and the formation of new synapses could further strengthen these networks. Indeed, current models of MDD hypothesize a dysregulation of several interconnected structures in the frontal an limbic circuitry, in which the hippocampus plays a major role. Other key structures in this network include the prefrontal cortex, amygdala, insular cortex, cingulate cortex, striatum, dorsal thalamus and hypothalamus. The fact that it requires several weeks to months for newborn cells to become functionally indistinguishable from mature, fully functional hippocampal granule cells [86, 87] and subsequently to restore the dysfunctional networks in depressed subjects, could provide an explanation for the therapeutic lag of VNS in the treatment of depression. Furthermore, other neuromodulatory changes requiring time to establish - including changes in receptor sensitivity within these networks - could be responsible for the therapeutic lag. Importantly, all evidence discussed in this review originates from preclinical animal studies. Future studies addressing the mechanism of action of VNS in humans will be required to confirm these hypotheses.

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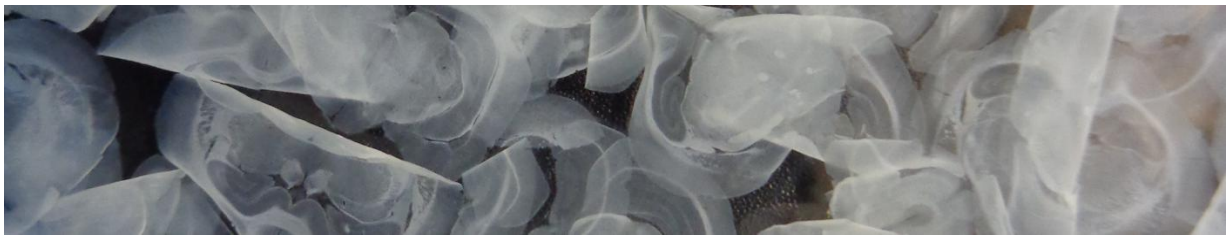
# Chapter 4

Optimization of the stimulation parameters

# Chapter 4 - study 4



Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability

## Study 4: Optimization of the stimulation parameters

The optimal stimulation parameters for effective VNS are still unknown. However, optimizing the stimulation paradigm is an indispensable step towards the achievement of a better clinical outcome. The stimulation parameters used currently in experimental studies and in clinical practice are based on what is known to be safe and tolerable and are therefore not evidence based but rather empirically determined. As the efficacy of VNS is dependent on the adequate activation of the vagal A- and B-fibers, the stimulation parameters should be optimized to activate these fibers. The activation threshold of the A- and B-fibers is lower than the output currents used in experimental settings and in clinical practice and therefore we hypothesize that VNS output currents lower than those used today, are sufficient and at least equally effective in reducing cortical excitability as VNS at higher output currents. This hypothesis was tested in the motor cortex stimulation rat model.

# Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability

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**Abstract**

**Objectives:** Vagus nerve stimulation (VNS) is an effective treatment for refractory epilepsy. It remains unknown whether VNS efficacy is dependent on output current intensity. The present study investigated the effect of various VNS output current intensities on cortical excitability in the motor cortex stimulation rat model. The hypothesis was that output current intensities in the lower range are sufficient to significantly affect cortical excitability.

**Material and methods:** VNS at 4 output current intensities (0.00 mA, 0.25 mA, 0.50 mA and 1.00 mA) was randomly administered in rats (n = 15) on 4 consecutive days. Per output current intensity, the animals underwent 5 one-hour periods: (1) baseline, (2) VNS1, (3) wash-out1, (4) VNS2 and (5) wash-out2. After each one-hour period, the motor seizure threshold (MST) was measured and compared to baseline (i.e. $\Delta MST_{baseline}$, $\Delta MST_{VNS1}$, $\Delta MST_{wash\text{-}out1}$, $\Delta MST_{VNS2}$ and $\Delta MST_{wash\text{-}out2}$). Finally, the mean $\Delta MST_{baseline}$, mean $\Delta MST_{wash\text{-}out1}$, mean $\Delta MST_{wash\text{-}out2}$ and mean $\Delta MST_{VNS}$ per VNS output current intensity were calculated.

**Results:** No differences were found between the mean $\Delta MST_{baseline}$, mean $\Delta MST_{wash\text{-}out1}$ and mean $\Delta MST_{wash\text{-}out2}$ within each VNS output current intensity. The mean $\Delta MST_{VNS}$ at 0.00 mA, 0.25 mA, 0.50 mA and 1.00 mA was 15.3 ± 14.6 µA, 101.8 ± 23.5 µA, 108.1 ± 24.4 µA and 85.7 ± 18.1 µA respectively. The mean $\Delta MST_{VNS}$ at 0.25 mA, 0.50 mA and 1.00 mA were significantly larger compared to the mean $\Delta MST_{VNS}$ at 0.00 mA (p=0.002 for 0.25 mA; p=0.001 for 0.50 mA; p=0.011 for 1.00 mA).

**Conclusions:** This study confirms efficacy of VNS in the motor cortex stimulation rat model and indicates that, of the output current intensities tested, 0.25 mA is sufficient to decrease cortical excitability and higher output current intensities may not be required.

## Introduction

Vagus nerve stimulation (VNS) is an efficacious and widely applied neurostimulation modality for patients with medically or surgically refractory epilepsy [1, 2]. The left vagus nerve is stimulated in the neck area by means of a helical stimulation electrode connected to a subclavicular implanted pulse generator. The clinically available stimulation parameters include output current intensity (range: 0.25 - 3.50 mA), frequency (range: 20 - 30 Hz), pulse width (range: 250 - 500 µsec) and duty cycle (range ON time (sec) / OFF time (min): 30/5, 30/3, 30/1.8, 30/1.1, 21/0.8, 14/0.5) which can all be modified in order to reach maximum therapeutic efficacy [3]. It has been demonstrated that VNS has both an acute effect on seizures, i.e. it is able to interrupt ongoing seizure activity, as well as having a more chronic seizure preventative effect following long-term treatment [2, 4, 5].

The antiepileptic mechanism of VNS remains incompletely understood. Previous experimental research showed that VNS exerts its antiepileptic effect by stimulating the afferent fibers of the vagus nerve [6-8]. The afferent fibers originate from the nodose and jugular ganglion and primarily project to the nucleus of the solitary tract (NTS). The NTS in turn has widespread projections to numerous areas in the brain including the locus coeruleus (LC), which is the major brain source of noradrenaline, and important areas for epileptogenesis such as the amygdala and the thalamus. Furthermore the NTS, LC and thalamus have many diffuse cortical connections. Different neurochemical and neuromodulatory changes affecting cortical excitability seem to play a role in the mode of action of the acute and chronic effects of VNS [9-12].

One clinical drawback of current VNS therapy is the variable therapeutic outcome [13-15]. Currently, VNS is successful in about half of treated patients [16]. It is routine clinical practice to uptitrate output current intensity in order to reach seizure control over several weeks/months. So far, analysis of large patient series have not demonstrated a correlation between output current intensities and seizure control. Several experimental studies in animals and humans using functional imaging and c-fos however, do suggest that lower output current intensities are sufficient to induce significant intracerebral effects [17-19].

A study by De Herdt et al. showed efficacy of acute VNS in the motor cortex stimulation rat model using an output current intensity of 0.75 mA [11]. In this rat model, the threshold for evoking focal, motor seizures is determined by electrical stimulation of the motor cortex in unanaesthetized rats [20, 21]. VNS significantly increased the threshold for evoking focal, motor seizures.

The present study investigated the effect of various VNS output current intensities on cortical excitability in the motor cortex stimulation rat model. The hypothesis was that output current intensities in the lower range are sufficient to significantly affect cortical excitability.

Animals

Fifteen male Wistar rats (Harlan, The Netherlands) weighing 250 - 275 g were used. Animals were treated according to the guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital (ECP 08/47). All animals were kept under environmentally controlled conditions (12h light/dark cycles, 20-23°C and 50% relative humidity) with food and water intake ad libitum.

Surgery

Rats were anesthetized with Isoflurane (induction: 5%; maintenance: 1 - 2%). An incision was made over the left anterior cervical region. The left cervical vagus nerve was carefully dissected from the aortic sheet and a custom-made silicone spiral cuff electrode with two platinum contacts (3 $mm^2$ area each, with 1 mm space between them) was implanted around the vagus nerve with the anode placed caudally and the cathode placed rostrally. The cuff electrode leads were tunneled under the skin over the back of the neck towards an incision made over the skull. Animals were then placed in a stereotactic frame (Bilaney Consultants, Düsseldorf, Germany), the skull was exposed and eight holes were drilled to insert electrodes and anchor screws. For stimulation of the motor cortex, two epidural stainless steel screw electrodes were stereotactically positioned over the motor area of the left and right frontal cortex (coordinates relative to bregma: dorsoventral -1.0 mm; mediolateral ± 3.0 mm). Four epidural stainless steel screw electrodes were implanted bilaterally on the parietal cortex; three of them were used for electroencephalogram (EEG) recording, the fourth was used as a reference/ground electrode. Two anchor screws were implanted bilaterally on the parietal cortex. The leads of the epidural electrodes and the leads of the cuff electrode were assembled in a head cap on the skull of the rat using acrylic cement. To minimize post-operative pain, Buprenorphine (Temgesic®, 0.03 mg/kg) was subcutaneously administered and a 2% Xylocaine gel was applied to the incision wounds. Animals were allowed to recover from surgery under an infrared lamp. Correct positioning of the cuff electrode around the left vagus nerve was verified post-mortem.

EEG monitoring, cortical stimulation and VNS

One week after surgery, rats were placed in neuromonitoring cages. Rats were connected via an electrical swivel (Plastics One, Roanoke, USA) to (i) a custom-made digital EEG monitoring system for

EEG recording, which was used to confirm the focal character of the induced seizures and (ii) two external constant-current stimulators (DS4, Digitimer Ltd., Hertfordshire, England) for cortical stimulation and for delivering VNS. Rats were allowed to move freely in their cages.

Cortical stimulation

Cortical stimulation was performed using a ramp-shaped pulse train with biphasic, rectangular pulses (1000 µs, 50 Hz) with increasing amplitude (0 - 10 mA). The maximum duration of the cortical stimulation train was 150 s (i.e. 1.3 µA increments every pulse). The cortical stimulation train was interrupted when the first symptoms of a focal seizure were detected on visual inspection. The clinical expression of a focal seizure was typically a forelimb clonus. The motor seizure threshold (MST) was then defined as the current intensity corresponding to the first clinical symptoms of a focal seizure.

VNS

The effect of one hour of VNS (30 Hz, 250 µsec, 30 sec ON / 1.8 min OFF) at 4 different output current intensities (0.00 mA, 0.25 mA, 0.50 mA and 1.00 mA) on the MST was evaluated. These VNS parameters are typically used in clinical practice.

Experimental design

The experimental design is represented in figure 1 and detailed below. VNS was administered in each rat on 4 consecutive days. On each day, VNS was given at one of the 4 output current intensities (see higher) in a random order. Per VNS output current intensity, the animals underwent 5 one-hour periods: (1) baseline, (2) VNS 1, (3) wash-out 1, (4) VNS 2 and (5) wash-out 2 (on any given day, VNS1 and VNS2 represent the same VNS intensity). Immediately after each one-hour period ended, the MST was measured (i.e $MST_{baseline}$, $MST_{VNS1}$, $MST_{wash\text{-}out1}$, $MST_{VNS2}$ and $MST_{wash\text{-}out2}$). Prior to baseline, the impedance between the two vagus nerve electrode contacts was measured.

Part 1: Outlasting effect of VNS

Per rat and per VNS output current intensity, the $\Delta MST_{baseline}$ (i.e. $MST_{baseline}$ minus $MST_{baseline}$), $\Delta MST_{wash\text{-}out1}$ (i.e. $MST_{wash\text{-}out1}$ minus $MST_{baseline}$) and $\Delta MST_{wash\text{-}out2}$ (i.e. $MST_{wash\text{-}out2}$ minus $MST_{baseline}$) were calculated. Finally, the mean $\Delta MST_{baseline}$, mean $\Delta MST_{wash\text{-}out1}$ and mean $\Delta MST_{wash\text{-}out2}$ per VNS output current intensity were calculated.

Part 2: Effect of various VNS output current intensities on the MST

Per rat and per VNS output current intensity, the $\Delta MST_{VNS1}$ (i.e. $MST_{VNS1}$ minus $MST_{baseline}$) and $\Delta MST_{VNS2}$ (i.e. $MST_{VNS2}$ minus $MST_{baseline}$) were calculated. Finally, the mean $\Delta MST_{VNS}$ per VNS output current intensity was calculated as the mean of all $\Delta MST_{VNS1}$ and $\Delta MST_{VNS2}$ values.

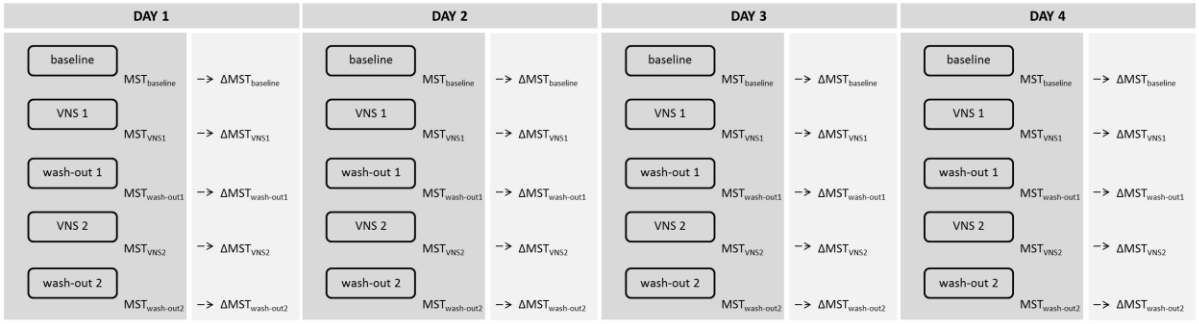


**Figure 1.** Schematic representation of the experimental design. Vagus nerve stimulation (VNS) at four output current intensities was administered randomly in each rat on four consecutive days. Per output current intensity, five-one-hour periods were conducted and the motor seizure threshold (MST) was determined after each period. ΔMST values were obtained by comparing the MST values with the baseline MST value.

Statistical analysis

A generalized linear mixed model and post-hoc Bonferroni for multiple comparisons was used to 1) compare the mean $\Delta MST_{wash\text{-}out1}$ and the mean $\Delta MST_{wash\text{-}out2}$ with the mean $\Delta MST_{baseline}$ within each VNS output current intensity and 2) to compare the mean $\Delta MST_{VNS}$ at 0.25 mA, 0.50 mA and 1.00 mA with the mean $\Delta MST_{VNS}$ at 0.00 mA. Statistical analyses were performed using SPSS 20 for Windows. Data are expressed as mean ± standard error of the mean. The significance level for demonstrating differences was set at $\alpha = 0.05$.

**Results**

The impedance between the vagus nerve electrode contacts showed normal values in all rats during all experiments (1 - 4 kOhm). Within each VNS output current intensity, no differences were found between the mean $\Delta MST_{baseline}$, the mean $\Delta MST_{wash\text{-}out1}$ and the mean $\Delta MST_{wash\text{-}out2}$, showing that VNS-induced changes in MST were transient and returned to baseline in the inter-stimulus periods.

The mean $\Delta MST_{VNS}$ as a function of VNS output current intensity is plotted in figure 2. The mean $\Delta MST_{VNS}$ at 0.00 mA, 0.25 mA, 0.50 mA and 1.00 mA was 15.3 ± 14.6 µA, 101.8 ± 23.5 µA, 108.1 ± 24.4 µA and 85.7 ± 18.1 µA respectively. The mean $\Delta MST_{VNS}$ at 0.25 mA, 0.50 mA and 1.00 mA were significantly larger compared to the mean $\Delta MST_{VNS}$ at 0.00 mA ($p = 0.002$ for 0.25 mA; $p = 0.001$ for 0.50 mA and $p = 0.011$ for 1.00 mA).

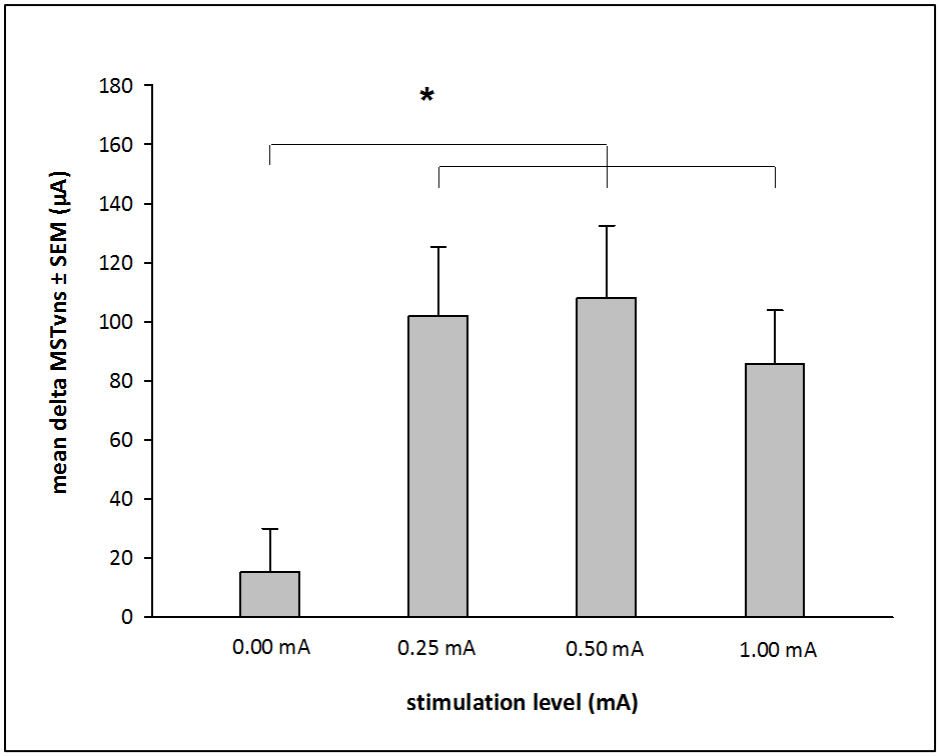


**Figure 2.** Effect of various vagus nerve stimulation (VNS) output current intensities on the motor seizure threshold (MST). The mean $\Delta MST_{VNS}$ (SEM) is plotted as a function of the VNS output current intensity.

No electro-encephalographic, epileptiform discharges were observed in the parietal cortical areas during any epileptic seizure, indicating that the elicited epileptiform activity was restricted to the motor cortex. Furthermore, no afterdischarges were observed on the EEG after cessation of cortical stimulation.

**Discussion**

The main findings of our study in the motor cortex stimulation rat model are that (i) VNS at 0.25 mA, 0.50 mA and 1.00 mA significantly increases the threshold for evoking focal, motor seizures compared to stimulation at 0.00 mA and (ii) effects of one hour VNS are no longer present one hour later.

A previous study by our group showed that acute VNS at 0.75 mA in the motor cortex stimulation rat model is effective in decreasing cortical excitability [11]. The findings of De Herdt et al. and our findings are in agreement with the reported direct and indirect acute effects of VNS on cortical excitability in preclinical and clinical experiments [7, 9, 22].

In our study, significant effects of acute VNS on cortical excitability in rats were already observed at 0.25 mA. Also in other types of preclinical and clinical research, significant effects of low-intensity, acute VNS were found. Acute VNS at 0.25 mA in conscious rats increased staining for c-fos, an indirect marker of neuronal activity, in the NTS and many regions that receive its projections [19]. In a functional neuroimaging study by our group, acute VNS, using an output current intensity of 0.25 mA, induced significant cerebral blood flow changes in the human brain, particularly in the thalamus and the limbic system [17]. These findings were confirmed in a human imaging study by Vonck et al. [18].

Our observation, together with the observation of De Herdt et al. [11], that VNS at 0.25 mA, 0.50 mA, 0.75 mA and 1.00 mA in rats significantly increased the MST supports the theory that vagal afferent fibers with low-to-moderate activation thresholds (i.e. A- and B-fibers) may be responsible for the antiepileptic effect of VNS. The vagus nerve contains three types of fibers (A-, B- and C-fibers), distinguished by their diameter and conduction velocity. In rats, recruited at the lowest threshold (0.02-0.20 mA) are the large, myelinated A-fibers. At thresholds of 0.04-0.60 mA, smaller, myelinated B-fibers are recruited. C-fibers are small, unmyelinated fibers with the highest stimulation threshold of above 2.00 mA [23, 24]. Initially, it was thought that the antiepileptic effect of VNS was directly related to the extent of C-fiber activation [6]. This theory was discarded after Krahl et al. demonstrated seizure suppression in rats even following selective destruction of C-fibers using capsaicin [8]. Furthermore, the group of Bunch concluded that therapeutically effective stimulation levels are below the threshold for C-fiber activation [24]. In an electrophysiological study by Evans et al., a C-fiber response was identified in 4 out of 8 patients using therapeutic VNS parameters [25]. However, because the C-fiber response (i) was apparent in 2 of the 4 patients only with 2.00 or 3.00 mA stimulation (which is at the upper limit of intensities used clinically) and (ii) was not measured

consistently, the authors concluded that C-fiber activation is probably not necessary for the antiepileptic effect of VNS.

Additional support that low-to-moderate output current intensities are sufficient to reduce seizure activity comes from a study of Woodbury and Woodbury, in which VNS at 0.20-0.50 mA already reduced chemically-induced seizures in rats [6]. In vivo intracellular recordings in the temporal association cortex in rats showed that stimulus intensities that predominantly activate myelinated vagal fibers (≤ 0.20 mA) were already effective in reducing the excitability of pyramidal neurons [26]. Our low effective current values are even more impressive considering that the authors above used a 500 µsec pulse width, which, according to the classical strength-duration relationship and according to Takaya et al. [27], is expected to require about half the current to yield the same effect as a 250 µs pulse. Lower output current intensities also seemed to be effective in the antidepressant activity of VNS in rats [28], in the effect of VNS on recognition memory in rats and humans [29, 30] and in the effect of VNS on human tolerance for pain [31].

A modeling study on the neurophysiology of the human vagus nerve suggested an output current level between 0.75 and 1.75 mA to reach optimal seizure control [32]. A direct comparison with the results of our study is not possible due to a large number of factors including: 1) experimental rats versus humans; 2) much smaller diameter of rat vagus nerve; 3) different electrodes (cuff versus helicoidal); 4) the model does not take surgical neurotrauma into account; 5) structural irregularities such as the presence of different nerve vessels modify thresholds but are not modeled; 6) tissue conductivities and geometry have a significant influence but are only rough approximations in a model. Both the study of Helmers et al. and our study however give insight in appropriate ways to optimize the therapeutic output current intensity and save battery-life. Our study in particular suggests that output current intensity quickly reaches a saturation level in therapeutic effectiveness and that higher output current intensities are not required to reach significant effects on cortical excitability. This idea may be extrapolated to human clinical practice in future clinical trial design. Due to the lack of prospective clinical trials comparing the effects of lower and higher output current intensities on seizure control, it is, even with the knowledge of our study, too early to defend convincingly the benefit of lower stimulation currents, although it would save battery life and decrease adverse events. However, it is worthwhile to extrapolate the implications of our findings to human VNS therapy. Combination of VNS and recording of vagal nerve compound action potentials could help to decide at what level this change in strategy should be applied in individual patients [33].

Taken together, this study confirms efficacy of VNS in the motor cortex stimulation rat model, and indicates that, of the VNS output current intensities tested, 0.25 mA is sufficient to decrease cortical excitability and higher output current intensities may not be required. Further research is needed to determine if even lower output current intensities are sufficient. Preliminary results in our rats, using vagal compound action potential recordings with single VNS pulses and a short pulse width, indicate that fiber recruitment may reach a saturation level at output current intensities lower than 0.25 mA.

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*contribution of the candidate: Annelies Grimonprez did the practical work of this study, as a part of her master thesis.

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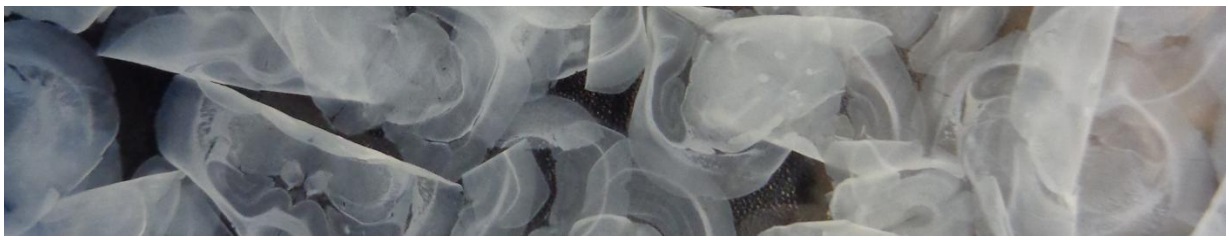
# Chapter 4 – Study 5



Laryngeal motor-evoked potentials mark vagus nerve activation: a preclinical study

## Study 5: Optimization of the stimulation parameters

Two major problems in VNS therapy are that (1) optimal stimulation parameters are unknown and (2) about one third of patients do not benefit from the treatment (non-responders). It is possible that the vagus nerve of some non-responders is not adequately activated, for multiple reasons such as lead failure, poor electrode contact or nerve damage. To date, there is no tool to test this hypothesis in an experimental setting nor in clinical practice. Previous studies from our lab have shown that the activation of the Aα-motor fibers of the recurrent laryngeal nerve, as measured by laryngeal motor-evoked potentials or LMEPs, is reflective of the activation of the vagus nerve. Therefore, LMEPs could provide us with valuable information to deduct optimal stimulation parameters and to identify ineffective stimulation of the vagus nerve leading to non-response. The techniques used in previous studies required invasive surgery or the use of special VNS electrodes for simultaneous stimulation and recording. The aim of the following study was to investigate the feasibility and reliability of LMEP recordings using a minimally invasive, easy-to-use tool in a chronic experimental setting.

# Laryngeal motor-evoked potentials mark vagus nerve activation: a preclinical study

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**Abstract**

Vagus nerve stimulation (VNS) is a treatment for refractory epilepsy and depression. Previous studies using invasive recording electrodes showed that VNS induces laryngeal motor-evoked potentials (LMEPs) through the co-activation of the recurrent laryngeal nerve and subsequent contractions of the laryngeal muscles. The present study investigates the feasibility of recording LMEPs in chronically VNS-implanted rats, using a minimally-invasive technique, to assess effective current delivery to the nerve and to determine optimal VNS output currents for vagal fiber activation.

Three weeks after VNS electrode implantation, signals were recorded using an electromyography electrode in the proximity of the laryngeal muscles and a reference electrode on the skull. The VNS output current was gradually ramped up from 0.10 mA to 1.00 mA in 0.10 mA steps.

In 13/27 rats, typical LMEPs were recorded at low VNS output currents (median 0.30 mA, IQR 0.20 - 0.30 mA). In 11/27 rats, significantly higher output currents were required to evoke electrophysiological responses (median 0.70 mA, IQR 0.50 - 0.70 mA, $p<0.001$). The latencies of these responses deviated significantly from LMEPs ($p<0.05$). In 3/27 rats, no electrophysiological responses to simulation were recorded.

Minimally-invasive LMEP recordings are feasible to assess effective current delivery to the vagus nerve. Furthermore, our results suggest that low output currents are sufficient to activate vagal fibers.

**Keywords**

Vagus nerve stimulation, stimulation parameters, responders, electromyography, laryngeal motor-evoked potentials

## Introduction

Vagus nerve stimulation (VNS) consists of the electrical stimulation of the left vagus nerve at the cervical level by means of an implantable electrode and a programmable pulse generator. This neuromodulation technique has been used since 1988 as an add-on therapy for the treatment of refractory epilepsy [1, 2]. Despite the abundant experience with VNS, one third of patients do not benefit from the treatment and optimal stimulation parameters remain elusive [2, 3]. More recently, VNS has also been approved for refractory depression[4-13] and is currently under investigation for the treatment of several other conditions including chronic heart failure, Alzheimer's disease, pain, tinnitus, obesity and anxiety disorders [14-16]. Considering the chronic nature of these diseases and the fact that the use of VNS for these indications is still in the experimental phase, long-term preclinical VNS studies are required. Effective delivery of electrical current to the vagus nerve is a prerequisite to obtain reliable experimental results. To date, there is no technique available in a chronic experimental setting to assess whether the electrical current is effectively delivered to the nerve and the vagal fibers are subsequently effectively activated.

The recurrent laryngeal nerve branches off from the vagus nerve at the level of the aortic arch, ascends next to the trachea and carries low threshold vagal Aα-motor fibers to the larynx, the pharynx and the vocal cords. Effective delivery of current to the vagus nerve at the cervical level, co-activates the recurrent laryngeal nerve and induces contractions of the laryngeal muscles and the vocal cords. In this context, we have previously recorded VNS-induced far field laryngeal motor-evoked potentials or LMEPs in an acute intra-operative setting using invasive electrodes [17]. Nerve lesions and neuromuscular blocking agents confirmed that these electrophysiological responses were LMEPs, induced by the co-activation of the Aα-motor fibers of the recurrent laryngeal nerve. These LMEPs were characterized by an initial negative peak wave with a latency of 2.88 ± 0.27 ms after the stimulation pulse. Based on these results, a 95% confidence interval for LMEP latencies can be calculated (2.34 ms - 3.42 ms), which can be used to differentiate between LMEPs and muscle-evoked potentials or MEPs resulting from other sources such as direct muscle activation due to current spill-over. This study, aiming at a future chronic and clinical applicability has minimized invasiveness and exploits 'clinical VNS' stimulation parameters at the cost of stronger artifacts and limited verifications. Our LMEP source identification is based on replication and congruency of previously obtained results from our lab, where experiments were conducted to clearly identify the recorded potential sources [17].

To allow for chronic VNS studies, a less invasive, reliable technique for LMEP recordings is required to exclude rats where electrical current delivery to the vagus nerve is doubtful, to identify the optimal

moment for initiation of the experiment and to determine the optimal stimulation output current to activate the relevant fibers without inducing stimulation-related side effects. The aim of this study was therefore to investigate the feasibility and reliability of LMEP recordings by acute testing after recovery from VNS electrode implantation, using a minimally invasive commonly available electromyography (EMG) technique.

**Methods**

Animals

Male Wistar rats (n=27, Harlan, The Netherlands) weighing 200 – 250 g were used. Animals were treated according to the guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University medical department. All animals were kept under environmentally controlled conditions: 12 hour light/dark cycles, temperature and relative humidity were kept at 20 - 23°C and 50% respectively. Food and water were provided ad libitum.

Surgery

The animals were anesthetized with a mixture of Isoflurane (5% for induction, 2% for maintenance) and medical $O_2$. A lack of hind leg withdrawal upon toe-pinching was used a criterion to ensure that the animal was fully anesthetized. Body temperature was maintained using a heating pad. A dose of Buprenorphine (0.025 mg/kg, subcutaneously) was administered preoperatively. For the VNS electrode placement, the skin of the ventral cervical region was shaved and disinfected. An incision was made over the anterior cervical region. The skin and muscles were retracted and the left vagus nerve was carefully dissected from the aortic sheet. Subsequently, a custom-made bipolar silicone cuff electrode was wrapped around the nerve. The leads of the electrode were tunneled subcutaneously to an incision in the scalp and were fixed to the skull using anchor screws and acrylic cement. Xylocaine gel and Neobacitracine gel were applied to the incision wounds to minimize pain and to reduce the risk for postoperative infections respectively. Metacam (1 mg/kg, subcutaneously) was given to the animals postoperatively and every 24 hours after surgery for 2 days. Animals were allowed to recover from surgery for 2 - 3 weeks.

Recording and stimulation

The animals were anesthetized with a mixture of Isoflurane (5% for induction, 2% for maintenance) and medical $O_2$. A lack of hind leg withdrawal upon toe-pinching was used a criterion to ensure that the animal was fully anesthetized. Body temperature was maintained using a heating pad. The impedance of the VNS electrode was verified before each recording session and remained low in all animals (< 10 kOhm). For placement of the EMG recording electrode, the skin of the ventral cervical region was shaved and disinfected. A small skin incision of approximately 2 mm was made over the

left cervical region to facilitate the subcutaneous insertion of the tip of an EMG electrode in the proximity of the laryngeal muscles (figure 1). This EMG electrode consisted of a custom-made monopolar needle electrode, made from an 18 gauge needle of which the tip was blunted and bended to obtain a non-traumatic hook electrode. Signals were recorded referred to a distant epidural electrode which consisted of a standard stainless steel skull screw. Signals were recorded using a distant epidural electrode on the skull as a reference. The VNS electrode was connected to a constant current stimulator and the left vagus nerve was stimulated with biphasic, charge-balanced square-wave pulses of 250 µs per phase. The intensity of the stimulation pulses was gradually ramped up from 0.1 to 1.0 mA in incremental steps of 0.1 mA. For each intensity, 20 sweeps were recorded to improve the signal to noise ratio and to assess the reproducibility of the signals. There was a 1-second interval between every sweep. At this frequency (1 Hz), no fatigue of the muscles is expected [18]. Each recorded sweep covered 1000 ms including a 100 ms pre-stimulus period. The zero time for MEP latency measurements was defined as the end of the stimulus duration of 250 µs. For each intensity, two sets of recordings were performed. For one set, the negative (cathodal) phase was given first at the proximal electrode contact. For the other set, the stimulation polarity was inversed, i.e. the negative phase was given first at the distal electrode contact. The succession order of these two types of stimuli was randomized. Signals were amplified using a High Performance AC Preamplifier Model P511 (Grass Technologies). The amplifier gain was set at 2000, the sampling rate was 100 kHz, with 16 bit resolution. The filters -3 dB bandpass limits were set at 3 Hz and 3 kHz. The data was digitized using a National Instruments acquisition board (NI USB 6259) and stored locally on a personal computer. Data acquisition and subsequent analysis was performed with Matlab 2007a (the MathWorks, Natick, Massachusetts).

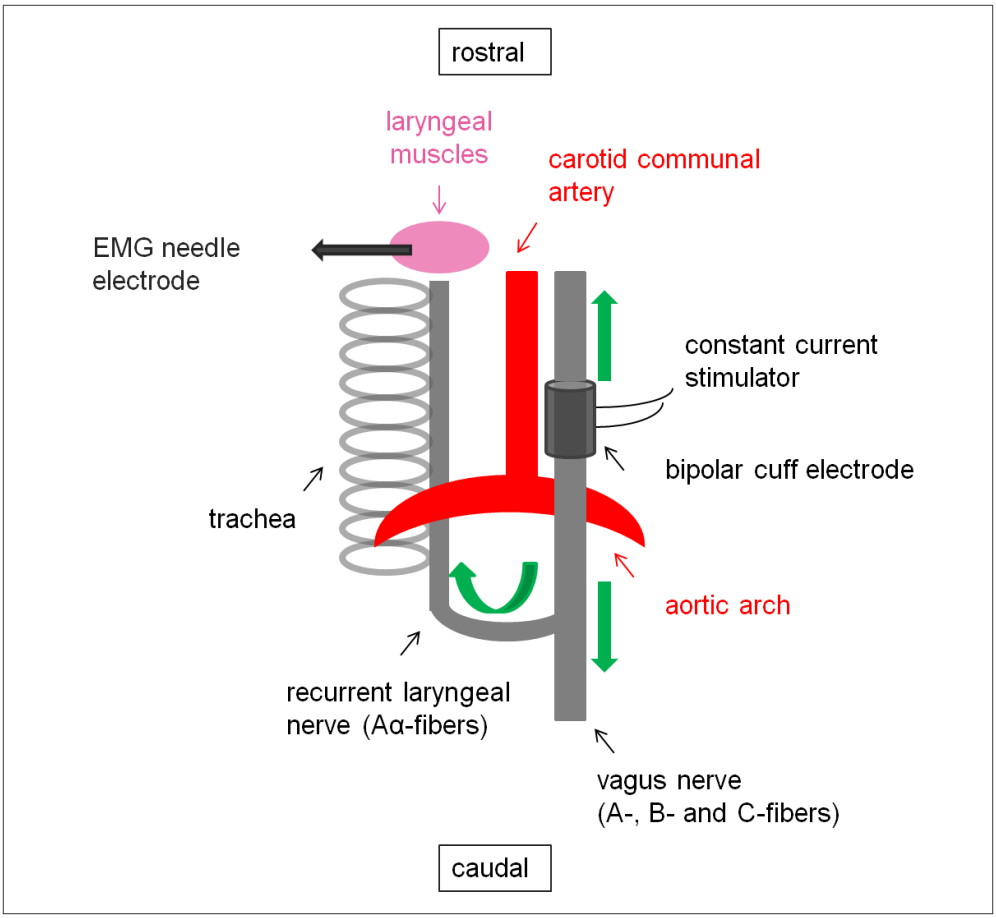


**Figure 1:** Schematic representation of the anatomy of the left cervical vagus nerve and the experimental setup for the recording of VNS-induced signals.

Definition of variables and statistical analysis

A VNS-induced MEP was defined as the initial negative peak wave appearing after the stimulation artefact, which had the same shape and polarity despite stimulation polarity inversion and was reproducibly recorded in all 20 sweeps at a specific stimulation output current. The latency was defined as the time between the onset of the stimulation pulse and the initial negative peak wave of the MEP in the averaged trace. The threshold stimulation current was defined as the lowest current at which a MEP could clearly and reproducibly be identified visually on the EMG recordings. For a MEP to be defined as an LMEP, the latency of the negative peak wave needed to be within the expected range, i.e. the 95% confidence interval for LMEP latencies (2.34 ms - 3.42 ms, as determined in a previous study from our lab), using both stimulation polarities. Statistical analyses were performed using Sigmaplot. The threshold stimulation currents to evoke LMEPs versus MEPs

were compared. As this data was not normally distributed, a non-parametric Mann–Whitney U test was used and results were expressed as median and interquartile range (IQR). Furthermore, the threshold stimulation currents to evoke LMEPs were compared between both stimulation polarities, using a non-parametric Wilcoxon Signed Rank Test. Again, results were expressed as median and IQR. The latency of the LMEPs at the threshold stimulation current was compared between both stimulation polarities using a paired t-test. As this data was normally distributed, the results were expressed as mean ± standard deviation. Statistical significance was considered at $p<0.05$.

**Results**

When the initial negative phase of the VNS pulse was given at the proximal electrode contact, VNS-induced MEPs were reproducibly recorded in 24/27 rats. Figure 2 shows a typical VNS-induced MEP, recorded using both stimulation polarities. The overlay of 20 sweeps demonstrates the low variability and high reproducibility of the signals and suggests that the recording of 5 to 10 sweeps is sufficient to assess reproducibility in future experiments. The MEPs were characterized by a negative peak wave with a mean latency of 2.76 ± 1.25 ms. The threshold stimulation current for evoking MEPs ranged from 0.1 mA to 0.8 mA. In 13/24 rats, MEPs were identified as LMEPs, as their latency at the threshold output current was within the expected interval for LMEP latencies. In the other 11/24 cases, the latency of the MEP was significantly higher (in 4/24 rats) or lower (in 7/27 rats) than expected for LMEPs ($p<0.05$). The threshold stimulation current to evoke LMEPs was significantly lower than the threshold stimulation current to evoke MEPs: median 0.3 mA, IQR 0.2 - 0.3 mA versus median 0.7 mA, IQR 0.5 - 0.7 mA for LMEPs and MEPs respectively ($p<0.001$). In 3/24 cases, no response to VNS was recorded.

When the initial negative phase of the VNS pulse was given at the distal electrode contact (reversed stimulation polarity), the stimulation artifact was indeed reversed while the shape and polarity of the MEP remained unchanged, thereby confirming that these MEPs were not merely a part of the stimulation artifact. With this reverted stimulus polarity, VNS-induced MEPs were reproducibly recorded in the same 24/27 rats. The MEPs were characterized by a negative peak wave with a mean latency of 2.81 ± 1.18 ms. The results for the threshold stimulation current and the fraction of rats displaying MEPs, LMEPs and no recordable signals were the same as when the negative phase of the VNS pulse was given at the proximal electrode contact. Table 1 shows the individual results. Once a MEP was recorded at the threshold stimulation current, it remained present for all the following incremental stimulation currents. At higher stimulation currents however, additional high amplitude components became visible on the EMG recordings, potentially due to co-activation of cervical muscles as suggested by visible contractions. The threshold stimulation current to evoke LMEPs did not differ significantly between both stimulation polarities: median 0.3 mA, IQR 0.2 - 0.3 mA versus median 0.3 mA, IQR 0.2 - 0.3 mA for the initial negative phase of the pulse at the proximal and the distal electrode contact respectively, $p>0.05$. Furthermore, the latency at the threshold stimulation current to evoke LMEPs did not differ significantly between both stimulation polarities: 2.80 ± 0.23 ms versus 2.78 ± 0.23 ms for the initial negative phase of the VNS pulse at the proximal and the distal electrode contact respectively, $p>0.05$.

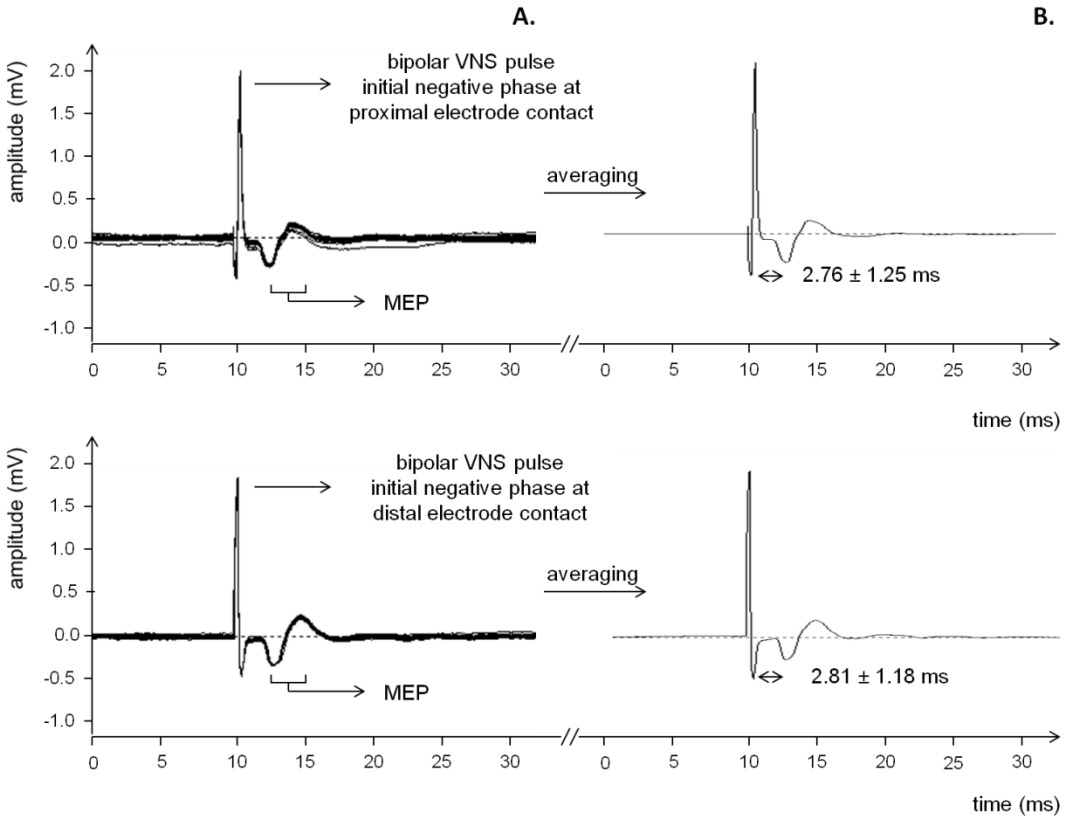


**Figure 2:** Example of a typical VNS-induced MEP. A. The overlap of 20 sweeps. B. The averaged signal. The upper and lower panel of the figure show VNS-induced MEPs when the initial negative phase of the VNS pulse is given at the proximal or distal electrode contact respectively. The bipolar stimulation artifact of the VNS pulse is observed at 100 ms. This is followed by a MEP that is characterized by an initial negative peak wave at 2.76 ± 1.25 ms or 2.81 ± 1.18 ms after the pulse artifact when the initial negative phase of the VNS pulse was given at the proximal or distal electrode contact respectively.

| | latency of the initial negative peak wave of the muscle evoked potential (ms) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| output current | 0.1 mA | 0.2 mA | 0.3 mA | 0.4 mA | 0.5 mA | 0.6 mA | 0.7 mA | 0.8 mA | 0.9 mA | 1.0 mA | threshold stimulation current (mA) | latency at the threshold stimulation current (ms) |
| RAT 1 | 2.77 | 2.63 | 2.63 | 2.58 | 2.65 | 2.67 | 2.56 | 2.68 | 2.56 | 2.50 | 0.1 | 2.77 |
| RAT 1 | 2.82 | 2.48 | 2.44 | 2.44 | 2.39 | 2.45 | 2.45 | 2.45 | 2.45 | 2.52 | 0.1 | 2.82 |
| RAT 2 | - | 4.07 | 4.07 | 4.02 | 3.94 | 3.98 | 4.15 | 5.38 | 5.28 | 5.12 | 0.2 | 4.07 |
| RAT 2 | - | 4.33 | 3.94 | 3.86 | 3.86 | 3.86 | 3.94 | 5.19 | 5.15 | 5.02 | 0.2 | 4.33 |
| RAT 3 | - | 2.75 | 2.75 | 2.71 | 2.71 | 2.75 | 2.67 | 2.68 | 2.54 | 2.54 | 0.2 | 2.75 |
| RAT 3 | - | 2.69 | 2.65 | 2.65 | 2.65 | 2.65 | 2.61 | 2.74 | 2.73 | 2.73 | 0.2 | 2.69 |
| RAT 4 | - | 2.82 | 2.65 | 2.61 | 2.61 | 2.61 | 2.60 | 2.65 | 2.65 | 3.21 | 0.2 | 2.82 |
| RAT 4 | - | 2.86 | 2.77 | 2.68 | 2.59 | 2.77 | 2.77 | 2.77 | 3.58 | 3.49 | 0.2 | 2.86 |
| RAT 5 | - | 2.69 | 2.46 | 2.35 | 2.31 | 2.31 | 2.28 | 2.35 | 2.31 | 2.35 | 0.2 | 2.69 |
| RAT 5 | - | 2.42 | 2.36 | 2.25 | 2.32 | 2.29 | 2.29 | 2.20 | 2.25 | 2.29 | 0.2 | 2.42 |
| RAT 6 | - | 2.40 | 2.30 | 2.60 | 2.50 | 2.30 | 2.50 | 2.30 | 2.30 | 2.50 | 0.2 | 2.40 |
| RAT 6 | | 2.48 | 2.5 | 2.47 | 2.63 | 2.50 | 2.33 | 2.50 | 2.47 | 2.36 | 0.2 | 2.48 |
| RAT 7 | - | 2.62 | 2.54 | 2.50 | 2.63 | 2.11 | 2.59 | 2.54 | 2.55 | 2.63 | 0.2 | 2.62 |
| RAT 7 | - | 2.52 | 2.54 | 2.54 | 2.46 | 2.96 | 2.92 | 2.88 | 2.92 | 2.88 | 0.2 | 2.52 |
| RAT 8 | - | - | 2.90 | 2.90 | 2.70 | 2.70 | 2.60 | 2.60 | 2.40 | 2.30 | 0.3 | 2.90 |
| RAT 8 | - | - | 3.19 | 3.10 | 3.11 | 3.15 | 3.15 | 3.19 | 3.19 | 3.15 | 0.3 | 3.19 |
| RAT 9 | - | - | 2.63 | 2.46 | 2.10 | 2.04 | 1.90 | 1.89 | 1.80 | 1.80 | 0.3 | 2.63 |
| RAT 9 | - | - | 2.84 | 3.07 | 2.99 | 3.92 | 4.00 | 3.92 | 3.83 | 3.83 | 0.3 | 2.84 |
| RAT 10 | - | - | 2.97 | 2.97 | 2.97 | 2.97 | 2.97 | 3.02 | 3.06 | 3.11 | 0.3 | 2.97 |
| RAT 10 | - | - | 2.67 | 2.84 | 3.42 | 3.38 | 3.38 | 3.21 | 3.13 | 3.00 | 0.3 | 2.67 |
| RAT 11 | - | - | 2.72 | 2.68 | 2.72 | 2.36 | 2.54 | 2.54 | 2.54 | 2.54 | 0.3 | 2.72 |
| RAT 11 | - | - | - | 2.68 | 2.5 | 2.50 | 2.41 | 2.41 | 2.50 | 2.5 | 0.4 | 2.68 |
| RAT 12 | - | - | - | 3.05 | 3.00 | 3.01 | 3.02 | 3.03 | 3.04 | 3.05 | 0.4 | 3.05 |
| RAT 12 | - | - | 3.10 | 3.13 | 3.06 | 3.23 | 3.20 | 3.10 | 3.17 | 3.07 | 0.3 | 3.10 |
| RAT 13 | - | - | - | - | - | - | 2.67 | 2.67 | 2.63 | 2.63 | 0.7 | 2.67 |
| RAT 13 | - | - | 2.92 | 2.77 | 2.76 | 2.71 | 2.79 | 2.79 | 2.75 | 2.84 | 0.3 | 2.92 |
| RAT 14 | - | - | - | 6.45 | 6.36 | 6.28 | 6.36 | 6.62 | 6.54 | 6.71 | 0.4 | 6.45 |
| RAT 14 | - | - | - | 6.13 | 6.06 | 6.21 | 6.06 | 5.98 | 6.39 | 7.32 | 0.4 | 6.13 |
| RAT 15 | - | - | - | - | 1.40 | 1.36 | 1.44 | 1.40 | 1.24 | 1.31 | 0.5 | 1.40 |
| RAT 15 | - | - | - | - | - | - | 1.18 | 1.15 | 1.04 | 1.04 | 0.7 | 1.18 |
| RAT 16 | - | - | - | - | 1.15 | 1.10 | 1.10 | 1.02 | 1.02 | 1.06 | 0.5 | 1.15 |
| RAT 16 | - | - | - | - | 1.45 | 1.40 | 1.36 | 1.31 | 1.36 | 1.36 | 0.5 | 1.45 |
| RAT 17 | - | - | - | - | 3.34 | 3.3 | 3.34 | 3.38 | 3.38 | 3.38 | 0.5 | 3.34 |
| RAT 17 | - | - | - | - | 2.97 | 2.76 | 2.76 | 2.89 | 3.02 | 3.06 | 0.5 | 2.97 |
| RAT 18 | - | - | - | - | 1.26 | 1.04 | 1.04 | 1.03 | 1.00 | 1.00 | 0.5 | 1.26 |
| RAT 18 | - | - | - | - | - | - | 1.97 | 1.96 | 1.91 | 1.31 | 0.7 | 1.97 |
| RAT 19 | - | - | - | - | - | 1.68 | 1.63 | 1.59 | 1.59 | 1.51 | 0.6 | 1.68 |
| RAT 19 | - | - | - | - | 1.98 | 1.94 | 1.81 | 1.81 | 1.81 | 1.81 | 0.5 | 1.98 |
| RAT 20 | - | - | - | - | - | - | 1.54 | 2.39 | 2.35 | 2.31 | 0.7 | 1.54 |
| RAT 20 | - | - | - | - | - | - | - | 1.27 | 1.96 | 2.08 | 0.8 | 1.27 |
| RAT 21 | - | - | - | - | - | - | 1.75 | 1.45 | 1.27 | 1.23 | 0.7 | 1.75 |
| RAT 21 | - | - | - | - | | - | 2.41 | 2.46 | 2.41 | 2.37 | 0.7 | 2.41 |
| RAT 22 | - | - | - | - | - | - | 1.68 | 1.77 | 1.66 | 0.99 | 0.7 | 1.68 |
| RAT 22 | - | - | - | - | - | - | 1.55 | 1.47 | 1.42 | 1.38 | 0.7 | 1.55 |
| RAT 23 | - | - | - | - | - | - | - | 3.25 | 3.17 | 3.13 | 0.8 | 3.25 |
| RAT 23 | - | - | - | - | - | - | 3.47 | 3.40 | 3.40 | 3.36 | 0.7 | 3.47 |
| RAT 24 | - | - | - | - | - | - | - | 5.61 | 3.90 | 4.11 | 0.8 | 5.61 |
| RAT 24 | - | - | - | - | - | - | - | 5.22 | 3.77 | 3.68 | 0.8 | 5.55 |
| RAT 25 | - | - | - | - | - | - | - | - | - | - | - | - |
| RAT 25 | - | - | - | - | - | - | - | - | - | - | - | - |
| RAT 26 | - | - | - | - | - | - | - | - | - | - | - | - |
| RAT 26 | - | - | - | - | - | - | - | - | - | - | - | - |
| RAT 27 | - | - | - | - | - | - | - | - | - | - | - | - |
| RAT 27 | - | - | - | - | - | - | - | - | - | - | - | - |
| ratio of rats with MEPs | 1/27 | 7/27 | 13/27 | 14/27 | 19/27 | 19/27 | 23/27 | 24/27 | 24/27 | 24/27 | | latencies within the 95% CI for LMEPs |
| ratio of rats with LMEPs | 1/27 | 6/27 | 12/27 | 12/27 | 13/27 | 13/27 | 13/27 | 13/27 | 13/27 | 13/27 | | |

**Table 1:** Individual latencies (ms) of the negative peak wave of the MEP for all stimulation currents. The rats are arranged in the table according to increasing threshold stimulation currents. For each rat, the latency values are given for both stimulation polarities; the upper and the lower line show the latency values for the stimulation polarity where the initial negative phase was given at the proximal or distal electrode contact respectively. Each value is the average of 20 recordings. A dash (-) indicates the absence of a MEP. Dotted boxes indicate that a MEP was recorded using one stimulation polarity but not the other. The second last column shows the threshold stimulation current. The last column repeats the latency of the MEP at the threshold stimulation current. Gray shades in this column depict latencies that lay within the previously established 95% confidence interval for LMEP latencies.

**Discussion**

Our results demonstrate that it is feasible to record VNS-induced MEPs using a minimally invasive, easy-to-use EMG tool. MEPs were reliably recorded in the majority (24/27) of our chronically VNS-implanted rats. However, in less than half of the rats (13/27), the MEPs were identified to be LMEPs, resulting from the co-activation of the recurrent laryngeal nerve and the subsequent contraction of the laryngeal muscles. In this case, the latency of the MEP is expected to be within the previously established 95% confidence interval for LMEP latencies[17, 19]. LMEPs occurred at low VNS output currents, i.e. median 0.3 mA, IQR 0.2 - 0.3 mA .

Significantly higher latencies of the MEPs were recorded in 4/27 rats and could be due to an incomplete recovery of the vagus nerve after surgery. Indeed, it has been shown previously that the implantation of cuff electrodes causes an initial loss of myelination, with a subsequent regeneration of the myelin sheath over several weeks to months[20]. In animals with more damage to the myelin sheath, a longer postoperative recovery period before initiating a VNS experiment is warranted. In a previous study from our lab using dedicated electrodes for simultaneous stimulation and recording, 13/21 rats displayed a recordable LMEP 2 - 4 weeks after surgery. In the remaining rats, a post-surgical recovery period up to 7 weeks was required before an LMEP could be recorded. Once an LMEP was present, it remained stable during the entire follow-up period of two months [19]. It would be very interesting to use the presented non-invasive method to follow-up LMEP stability over time in future experiments. The total absence of a MEP in 3/27 rats, could likewise result from a dysfunctional nerve. Consequently, rats with longer latency LMEPs or without LMEPs should have longer recovery periods or should be excluded from the experiment, as the adequate delivery of current to the nerve is doubtful in these animals, while this is obviously a prerequisite to obtain reliable experimental results.

The significantly lower MEP latency in 7/27 rats most likely results from electrical current spillover and subsequent direct muscle activation[14]. Such direct muscle activation occurred at higher stimulation intensities (≥ 0.5 mA) and was easily identified as the appearance of high amplitude MEPs on the EMG recordings and as the visual observation of cervical muscle twitches. This VNS-induced contraction of the cervical muscles at higher VNS output currents occurred in 18/27 tested rats and most likely results in the frequently observed stimulation-related side effects in awake animals, e.g. head nodding or scratching the neck at the implantation side during the ON-phase of the stimulation. These stimulation-related side effects may cause stress and discomfort in the animals and may consequently confound the results of preclinical VNS studies.

Consistent with our findings, there is a large body of evidence both in animals and humans suggesting that low or moderate VNS output currents are sufficient and optimal to achieve therapeutic effects [21-28]. This hypothesis is further supported considering the anatomy of the vagus nerve. The nerve consists of A-, B- and C-fibers. The A- and B-fibers are myelinated and have a large and intermediate diameter respectively. The C-fibers are unmyelinated and have a small diameter. Subsequently, the A-, B- and C-fibers have a low (< 0.2 mA), intermediate (< 0.4 mA) and high (> 2.0 mA) threshold for activation respectively[24]. The earliest animal studies suggested that the antiepileptic potential of VNS was directly related to the fraction of vagal afferent C-fibers stimulated[22, 29]. However, Krahl et al. demonstrated that the effect of VNS on PTZ-induced seizures is still present following selective destruction of the C-fibers using capsaicin in rats[30]. Based on this and other studies[31-34], it is conceivable that the A- and B-fibers are responsible for the therapeutic effects of VNS and subsequently, low and intermediate output currents should be sufficient to evoke therapeutic responses. Apart from the fiber characteristics and the VNS output current, nerve fiber activation during stimulation depends on other important factors. Fibers located closer to the perimeter of the nerve and thereby closer to the VNS electrode are exposed to a stronger electric field and are easier to excite compared to fibers located deeper in the nerve. Also, fibrous tissue encapsulation at the site of the electrode can increase resistance, altering the electric field and resulting in increased voltage requirements for fiber excitation[35, 36]. These factors suggest individual variations and therefore, one optimal VNS output current cannot be defined. This is reflected in our results by the fact that the output currents required to evoke LMEPs are variable and range from 0.1 to 0.3 mA, and even to 0.5 mA in one animal displaying LMEPs.

LMEP recordings represent an indirect monitoring tool in the sense that it records the activity of the low threshold Aα-motor fibers, while the therapeutic effects of VNS are believed to be mediated by higher threshold A- and B-fibers. Nevertheless, it is well-known that physiological features such as fiber diameter, activation sensitivity to electrical stimulation, action potential conduction velocity and function are all closely related[37]. When the threshold to one specific fiber type can be reliably recorded, it can be used as an indicator to determine the expected threshold for all other fibers. The threshold stimulation strength to produce LMEPs is thus directly related to the required therapeutic VNS stimulus strength. The value of the ratio between both stimulus strengths still has to be established and will be part of our future work. However, given that the variation in activation sensitivity with fibre diameter is rather limited in the large diameter range[38], we expect to find a ratio value close to unity between the LMEP threshold stimulation current and efficient therapeutic VNS output current.

LMEPs result from efferent stimulation, while the therapeutic effects of VNS are believed to be mediated through the activation of the afferent fibers. Both in experimental studies and in clinical practice, one is trying to selectively stimulate the afferent fibers by administering the initial negative phase at the proximal electrode contact. It is hoped that anodal block, i.e. a conduction block due to hyperpolarization of the nerve, at the level of the distal electrode contact, will occur. Consequently, this would limit efferent effects of VNS, e.g. hoarseness, cardiac and pulmonary side effects. However, this study shows that LMEPs are recorded at the same output current and have the same latency irrespective of the stimulation polarity configuration and thus, that anodal block does not occur. In practice, anodal block is indeed only observed using high output currents and long pulses[39, 40], while it is not the case using clinically relevant stimulation parameters[41-43].

In conclusion, the main findings of this study are that VNS-induced LMEPs are reproducibly recorded in about half of the animals 2 - 3 weeks after surgery and that these LMEPs occurred at low stimulation intensities. At higher stimulation intensities, the MEPs had significantly higher or lower latencies than expected for an LMEP, pointing at nerve damage and direct muscle activation due to current spill-over respectively. These results suggest that low stimulation currents are sufficient to achieve therapeutic effects, while high stimulation currents can lead to significant stimulation-related side effects. Apart from reducing stimulation-related side effects, stimulating the vagus nerve with a lower output current density would be beneficial in the sense that it would save battery life, thereby postponing the surgical procedure and costs for battery replacement. Furthermore, we suggest that this minimally invasive, easily-applicable method is a valid tool in an experimental setting to identify non-responders due to ineffective activation of the vagus nerve and to determine individual post-operative recovery periods.

Future steps in this research field should focus on the optimization of this technique in awake animals, the relationship between Aα- and other A- and B-fiber activation thresholds, the correlation of LMEP recordings with therapeutic responses and the translation of this technique to clinical practice. Although VNS is well-established in clinical practice, one third of patients are classified as non-responders [2, 3]. Potential causes for non-response that could be further investigated using LMEP recordings are lead failure, a poor nerve-electrode contact due to scar tissue and nerve damage such as demyelination. Optimal stimulation parameters required for effective activation of the pertinent vagus nerve components are still unknown. For this purpose as well, LMEP recordings in a clinical population could optimize the therapeutic approach with VNS.

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# Chapter 4 - study 6

Laryngeal motor-evoked potentials as an indicator of vagus nerve activation: a clinical pilot trial

In clinical practice, one third of patients are non-responders and optimal stimulation parameters required to effectively activate the vagus nerve are still unknown. The therapeutically applied stimulation intensity is typically the highest output current tolerated by the individual patient. This is obviously not an evidence-based way to determine the individual, optimal output current for vagal fiber activation. Research should therefore be directed towards finding a non-invasive method that can guide individual titration of the stimulation parameters. Such biomarker for effective delivery of VNS pulses to the nerve could support the choice for individual stimulation parameters in a more rational way. To date, no such technique is available for clinical use in chronically VNS-implanted patients. In a previous study from our lab, it was shown that it is feasible to record VNS-induced laryngeal muscle-evoked potentials or LMEPs in chronically VNS-implanted experimental rats using a non-invasive electromyography technique. LMEPs are indicative of the effective delivery of electrical current to the cervical fibers of the vagus nerve and could subsequently be used to identify non-responders due to ineffective activation of the nerve and to determine individual optimal stimulation parameters to activate the vagal fibers. The aim of the presented study was to translate this technique to clinical practice.

# Laryngeal motor-evoked potentials as an indicator of vagus nerve activation: a clinical pilot trial

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In preparation

**Abstract**

**Rationale:** Vagus nerve stimulation (VNS) is an adjunctive therapy for patients with medically refractory epilepsy and depression. However, one third of patients do not benefit from the treatment and optimal stimulation parameters remain elusive. To date, a non-invasive technique to assess whether the vagus nerve is effectively activated by the applied stimulation parameters is lacking. We have recently demonstrated in a preclinical setting that VNS-induced laryngeal motor-evoked potentials (LMEPs) can serve as indicators of effective activation of the cervical vagal fibers. The aim of this study is to investigate the feasibility and applicability of recording VNS-induced LMEPs in patients treated with VNS.

**Methods:** To record LMEPs, six surface EMG electrodes were placed in the cervical region of the patients (n = 2) according to 3 perpendicular axes around the larynx. The VNS parameters were set at a pulse width of 250 µs, a frequency of 10 Hz and a duty cycle of 7 s ON / 18 s OFF. The VNS output current was gradually ramped down from the patients' usual output current to 0.00 mA, in steps of 0.25 mA. Subsequently, the output current was gradually ramped up again to the patient's usual settings. For each output current, the responses to two 7-second VNS trains were recorded. LMEP amplitude and latency were analyzed by two independent investigators.

**Results:** VNS-induced LMEPs were recorded in both patients with high reproducibility. The LMEPs were already observed at low output currents (0.25 - 0.50 mA) and the amplitude of the signals reached a plateau at 0.75 - 1.00 mA. The LMEP latency remained constant for each stimulation intensity. LMEP amplitude and latency were very reproducibly assessed across stimulation trains and for the two observers.

**Conclusion:** Our study demonstrates that it is feasible to record VNS-induced LMEPs in a reproducible, easy and non-invasive way in chronically VNS-implanted patients. Furthermore, our results suggest that output currents of 0.75 – 1.00 mA are sufficient to activate the Aα-motor fibers of the vagus nerve. VNS-induced LMEPs may help neurologists to choose the optimal stimulation parameters in a more objective way and to identify non-responders due to ineffective stimulation of the vagus nerve.

## Introduction

Vagus nerve stimulation (VNS) is an effective treatment for patients suffering from refractory epilepsy and depression. A small pulse generator is surgically implanted subcutaneously in the left thoracic area and delivers intermittent electrical pulses via an electrode that is partially wrapped around the left vagus nerve in the mid-cervical region. The afferent signals are further processed in the nucleus tractus solitarius and relayed to various regions of the brain [1]. Although VNS is well-established in clinical practice, there are still some drawbacks associated with the treatment. One third of patients do not benefit from the therapy and are classified as non-responders [2, 3]. Potential causes for non-response are a poor electrode contact due to gliotic tissue and nerve damage such as demyelination. Optimal stimulation parameters required to effectively activate the vagus nerve are still unknown. In clinical practice, the therapeutically applied stimulation intensity is typically the highest output current tolerated by the individual patient. A biomarker for effective delivery of VNS pulses to the nerve could support epileptologists in their choice for individual stimulation parameters in a more rational way. To date, no such technique is available for clinical use in chronically-implanted patients receiving VNS treatment.

Recently, we have shown that it is feasible to record VNS-induced laryngeal muscle-evoked potentials or LMEPs in chronically implanted experimental rats using a non-invasive electromyography (EMG) technique [4]. These LMEPs result from contractions of the laryngeal muscles which are induced by the co-activation of the Aα-motor fibers of the recurrent laryngeal nerve, which branches off from the vagus nerve at the level of the aortic arch. LMEPs are thus indicative of the effective delivery of electrical current to the cervical fibers of the vagus nerve and could subsequently be used to identify non-responders due to ineffective activation of the nerve and to determine optimal stimulation parameters. In humans, VNS-induced LMEPs have also been recorded using acute intra-operative vocal cord EMG, endotracheal tubes with electrodes and laryngoscopy [5-7]. The intra-operative measurement of LMEPs may provide a marker for effective activation of the vagal fibers by VNS, but remains invasive, prolongs the implantation procedure and provides no information on the effective activation of the vagal fibers in the chronic phase of VNS treatment. Therefore, the aim of the present study was to evaluate whether it is feasible to reliably record VNS-induced LMEPs in chronically implanted awake patients, using a non-invasive, easy-to-use EMG tool.

## Methods

### Patients

To date, two patients (1 male, 1 female, mean age 37 years), suffering from refractory epilepsy and implanted with a VNS device (Cyberonics, Houston, TX, USA) were recruited for the study. The study took place in the Reference Center for Refractory Epilepsy at Ghent University Hospital, Ghent, Belgium. Patients were included in the study if they met the following criteria: 1) at least 18 months of treatment with VNS for refractory epilepsy; 2) older than 18 years and 3) a VNS electrode impedance below 10 kOhms. The main clinical characteristics of the patients and their usual VNS parameters are summarized in table 1. The study was approved by the ethics committee of Ghent University Hospital. After a full description of the procedure was provided and explained, both patients gave written informed consent.

| patient ID | sex (M/F) | age (years) | length (cm) | response | implanted since (year) | electrode impedance (kOmh) | VNS parameters | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | output current (mA) | frequency (Hz) | pulse width (µs) | ON time (sec) | OFF time (min) |
| Patient 1 | M | 50 | 174 | reponder | 2004 | 2 | 2.25 | 15 | 250 | 30 | 10 |
| Patient 2 | F | 23 | 163 | non-responder | 2011 | 2 | 1.25 | 20 | 500 | 30 | 10 |

**Table 1:** The main clinical characteristics of the patients and their usual VNS parameters. Response was defined as a seizure frequency reduction of ≥ 50%.

### VNS-induced LMEP recordings

At the beginning of the study, the VNS electrode impedance was checked and the body length of the patient was measured. Subsequently, 8 surface electrodes were placed on the skin of the patient (see figure 1). Six Ag/AgCl (Kendall H92SG ECG electrodes, Ø 35 mm) recording electrodes were placed in the neck according to 3 perpendicular axes around the larynx. Electrode pair 1a-1b, 2a-2b and 3a-3b were placed according to the sagittal, horizontal and vertical axes respectively. Furthermore, a grounding electrode (G1) and a common reference electrode (rb) were placed on the forehead and the sternum respectively. EMG was recorded using Micromed System Plus (Micromed, Mogliano, Italy).

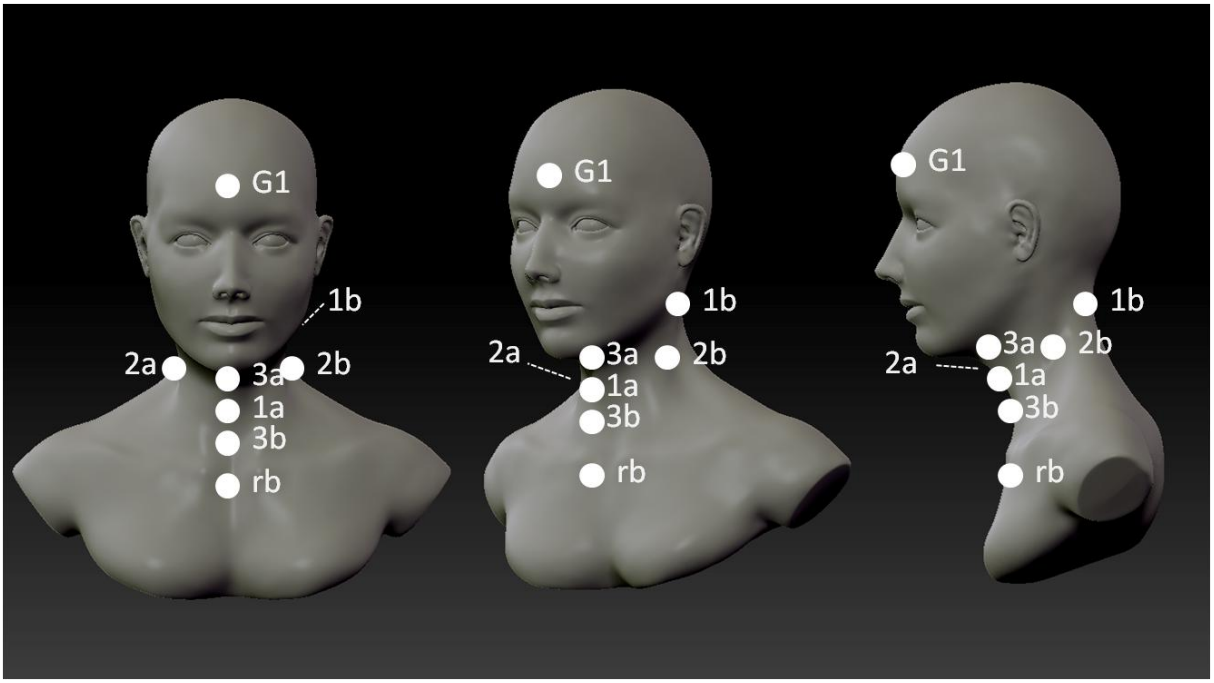


**Figure 1:** Schematic representation of the electrode placement.

For the duration of the experiment, the frequency of the VNS pulse was set at 10 Hz in order to avoid overlap of the stimulation artifacts and the electrophysiological responses. Furthermore, the pulse width and the duty cycle were set at 250 µs and 7 s ON / 18 s OFF respectively in all patients to assure reproducibility and to limit the time of the procedure respectively. Using the handheld computer, the stimulation output current was gradually ramped down from the usual output current (2.25 mA and 1.25 mA for patient 1 and 2 respectively) to 0.00 mA, in steps of 0.25 mA. For each intensity, the response to 2 stimulation trains was recorded to assess the reproducibility of the signals (see figure 2). Subsequently, the stimulation output current was gradually ramped up again from 0.00 mA to the usual output current, in incremental steps of 0.25 mA. Again, two trains per stimulation intensity were recorded (not shown in the figure). The signals were digitized online with a sampling frequency rate of 1024 Hz, an antialiasing filter of 250 Hz, a gain of 50 dB and a resolution of 16 bits. The signals were analyzed offline using BrainVision Analyzer 2.0. Two stimulation trains per output current level and five pulses per stimulation train were analyzed and this was done in duplo by two independent investigators. Apart from the fact that the pulses could not be disturbed by the electrocardiogram, the pulses were chosen at random. One investigator analyzed the signals from the ramp down condition, the other investigator analyzed the signals from the ramp up condition. Therefore, 20 pulses per output current were analyzed in total. The results were compared to check reproducibility. After the experiment, the pulse generator was programmed back to the usual stimulation parameters of the patient.

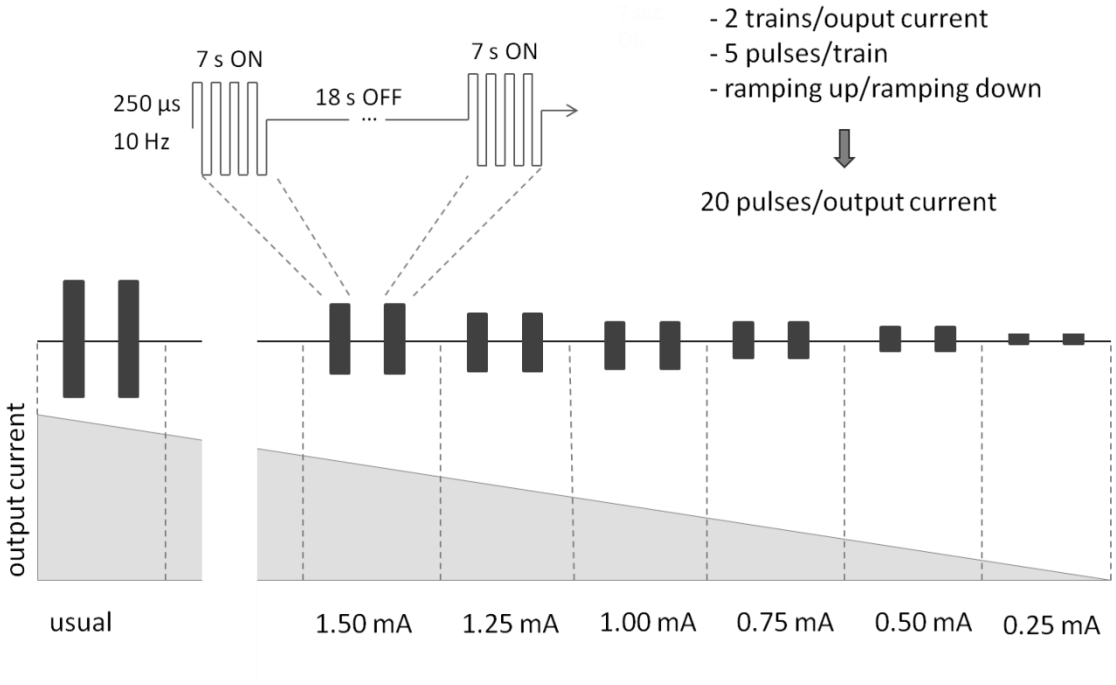


**Figure 2:** Schematic representation of the ramping down part of the protocol.

Definition of variables

An LMEP was defined as the initial positive peak that was reproducibly recorded after each stimulation artifact. The threshold stimulation current was defined as the output current at which an LMEP could be reproducibly identified in the recorded EMG signals. The latency was defined as the time between the negative peak of the stimulation pulse and the peak of the first positive deflection of the LMEP above noise level. The amplitude of the LMEP was defined as the difference in amplitude between the first positive and the following negative peak of the LMEP. Input-output curves were drawn for the amplitude of the LMEPs and the amplitude of the stimulation artifact. All graphs were drawn with Sigmaplot.

## Results

LMEPs were very reproducibly recorded in both patients (figure 3, upper part panel A and B). Although LMEPs were clearly identified in all three derivations (1a-1b, 2a-2b and 3a-3b), they had the biggest amplitude in 3a-3b and 1a-1b for patient 1 and 2 respectively. In patient 1, LMEPs were recorded at the lowest output current (0.25 mA) and their amplitude increased until it reached a plateau at an output current of 0.75 - 1.00 mA (figure 3, lower part panel A). In patient 2, LMEPs were recorded from 0.50 mA onwards and the amplitude of these LMEPs also reached a plateau at an output current of 0.75 - 1.00 mA (figure 3, lower part panel B). The latency of the LMEPs was 9.76 ms and 8.60 ms for patient 1 and 2 respectively. LMEP amplitude could be assessed very reproducibly between the two observers working respectively on the ramping up and ramping down data (see figure 3, lower part panel A and B). The amplitude of the stimulation artifact kept on increasing with the intensity of the output current (figure 4), while the LMEP amplitude reached a plateau around 0.75 - 1.00 mA in both patients. This finding provides strong evidence that the recorded LMEP signals are not merely part of the stimulation artifact but are true electrophysiological responses.

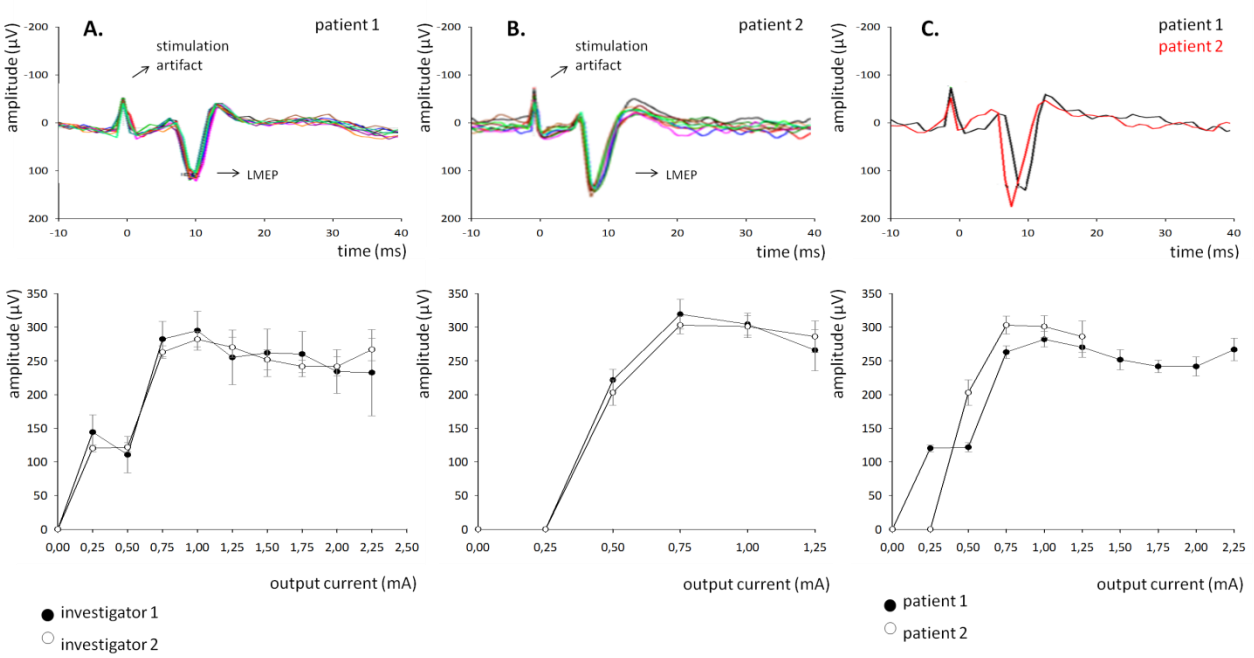


**Figure 3:** Panel A, B and C show the results for patient 1, patient 2 and the overlap of patient 1 and 2 respectively. In the upper part of panel A and B, the overlay of 10 LMEP recordings at an output current of 1.00 mA is shown for patient 1 and 2 respectively. The stimulation artifact is seen at 0 ms, followed by an LMEP at 9.76 ms and 8.60 ms for patient 1 and 2 respectively. The lower part of panel

A and B depict the amplitude of the LMEP in function of the output current for patient 1 and 2 respectively. For both patients, the overlay of the analysis of the two independent investigators is shown. The upper part of panel C shows the overlay of the averaged signal of patient 1 and 2. The lower part of panel C depicts the overlay of the amplitude in function of the output current of patient 1 and 2.

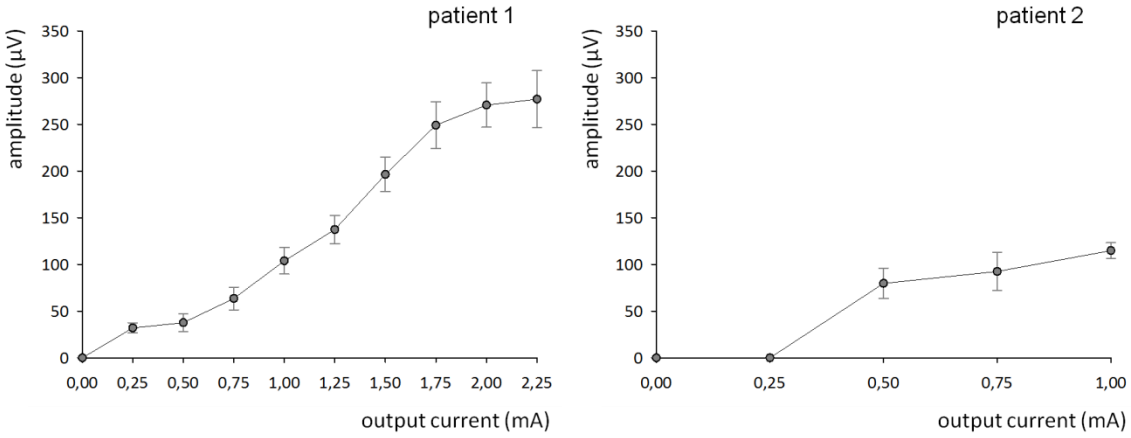


**Figure 4:** Amplitude of the stimulation artifact in function of the output current for both patients.

**Discussion**

The results from this pilot trial show that LMEPs can be reproducibly recorded in patients chronically treated with VNS, using a non-invasive EMG approach. These LMEPs result from contractions of the laryngeal muscles which are induced by the co-activation of the Aα-motor fibers of the recurrent laryngeal nerve upon VNS. In a recent study by Kim et al., LMEPs were recorded upon direct stimulation of the recurrent laryngeal nerve, using invasive stimulation and recording needle electrodes. The mean latency of the responses was 1.98 ± 0.26 ms when the stimulation electrode was placed 3 cm below the lower margin of the cricoid cartilage [8]. Considering a conduction delay of around 1 ms for neuromuscular transmission at the end plate [9], the conduction velocity in that study can be estimated at 31 m/s. In the present clinical pilot trial, the mean LMEP latency was 9.18 ± 0.82 ms. Based on the knowledge that the mean length of the left recurrent laryngeal nerve in humans is 13.7 cm [10], that the VNS electrode is implanted approximately 8 cm above the clavicle [11] and that the recurrent laryngeal nerve branches off from the vagus nerve approximately 5 cm under the clavicle, a total estimated distance of 26.7 cm can be calculated from the VNS electrode to the laryngeal muscles. This distance value, the 1-ms conduction delay and the mean LMEP latency of 9.18 ± 0.82 ms obtained in our study, lead to an approximate conduction velocity of about 33 m/s. The very similar conduction velocities estimated from the study of Kim et al. [8] and the present clinical pilot trial, support the hypothesis that the signals recorded are of the same origin, i.e. the activation of the Aα-motor fibers of the recurrent laryngeal nerve and subsequent contraction of the laryngeal muscles. Obviously, the conduction velocities calculated here are merely approximations and future studies should assess the true conduction velocity of the fibers by recording signals from two locations on the laryngeal nerve at a known distance from each other.

In clinical practice, the therapeutically used stimulation intensity is typically the highest output current tolerated, with a median value of 1.75 mA (ranging from 0.75 mA to 3.50 mA) after long-term treatment [12]. However, as the efficacy of VNS is dependent on the adequate activation of the low (< 0.20 mA) and moderate (< 0.40 mA) threshold vagal A- and B-fibers [13-15], the currently used stimulation output currents are probably much higher than what is required to achieve therapeutic effects. In both patients included in the present study, LMEPs, reflecting Aα-fiber activation, were already recorded at low VNS output currents, i.e. 0.25 and 0.50 mA in patient 1 and 2 respectively. These results are consistent with the findings from the invasive study by Ardesch et al., where the effect of VNS on the vocal folds was already present at VNS output currents of 0.25 - 0.50 mA, without necessarily causing VNS-induced hoarseness [7]. In the present study, the amplitude of the

LMEPs increased until it reached a plateau at an output current of 0.75 - 1.00 mA in both patients. The lack of further LMEP amplitude increase at higher output currents, most likely reflects the fact no additional Aα-fibers are recruited at higher VNS output currents and subsequently suggests that stimulating the vagus nerve at higher output currents will not results in an additional therapeutic effect. However, it must be acknowledged that one general optimal stimulation current cannot be defined. That is, individual thresholds for the activation of vagal A- and B-fibers will vary as a result from differences in the anatomy of the vagus nerve, i.e. the relative position of fibers within the nerve, electrode contact position and local tissue gliosis. It was shown by Helmers et al. that the presence of 110 µm of fibrotic tissue can decrease fiber activation by 50% [1]. Furthermore, LMEP recordings may underestimate the VNS output current required to achieve therapeutic efficacy as it records the activity of the low threshold Aα-motor fibers, while the therapeutic effects of VNS are believed to be mediated by the slightly higher threshold A- and B-fibers. Nevertheless, it is known from physiological studies in all nerves and in all species that physiological features such as fiber diameter, activation sensitivity to electrical stimulation and action potential conduction velocity are all closely related [16]. If the threshold to one specific type of fiber can be measured, it can be used as a correction coefficient to determine the expected threshold for all other fibers. Given that the variation in threshold intensity with fiber diameter is rather limited in the large diameter range [17], we expect to find a ratio value close to unity between the LMEP threshold stimulation current and efficient therapeutic VNS parameters. Therefore, we suggest that the presented non-invasive LMEP recording technique could be used to guide individual titration of the stimulation parameters.

Another important problem in VNS therapy is the high non-responder rate and the lack of explanation for this fact. It is possible that the vagus nerve of some non-responders is not adequately activated, for multiple reasons such as lead failure, poor electrode contact or nerve damage. Even general conditions such as diabetes can lead to neuropathy of the vagus nerve [18]. A temporary paresis of the vocal cords after VNS surgery has previously been described in humans [19-21]. This indicates that the surgical procedure and implantation of the electrode can cause nerve damage such as demyelination and a transient failure of the vagus nerve, hence requiring a recovery period before becoming functional again [22]. To allow recovery of the nerve, a delay of two weeks is usually foreseen between the implantation of the system and the start of VNS. The duration of this delay is determined rather empirically, while an individualized approach, based on the recovery of the nerve could be beneficial. The absence of an LMEP at reasonable stimulation currents or a prolonged latency of the LMEP could indicate incomplete recovery of the nerve. In that case, a further rest period might be beneficial for the nerve and more easily accepted then unsuccessful trials. Future

studies should include LMEP recordings starting immediately after surgery, to follow-up the recovery of the nerve.

Furthermore, we suggest that the lack of LMEPs could be used to identify non-response resulting from inefficient activation of the vagus nerve. Of course, non-response could also result from the heterogeneity of the underlying pathophysiological mechanisms or the variability in characteristics of more central structures in the neural pathway involved in VNS. In this regard, genetic differences in neurotransmitter systems could be an example of hypotheses to consider. Therefore, the presence of LMEPs is not an identifier of response nor is it a biomarker for the anti-epileptic or antidepressant efficacy of VNS but rather an indication that the nerve is effectively stimulated, which is an essential step to achieve therapeutic response. This is reflected in our data by the fact that both patients display LMEPs while only one of them is a responder. Non-response in the other patient despite the proper activation of the vagus nerve as reflected by the presence of LMEPs, could result from upstream problems such as an impaired noradrenergic signaling.

In conclusion, the results from this pilot trial show that VNS-induced LMEPs can be recorded very reproducibly in chronically VNS-implanted patients using a non-invasive EMG approach. Furthermore, our results suggest that low to moderate output currents are sufficient to activate the vagal fibers and therefore to achieve therapeutic responses. We hypothesize that LMEPs could be used for the identification of ineffective stimulation leading to non-response and for the individualization of post-operative recovery delays. However, long-term longitudinal studies in a bigger sample of patients are needed to confirm these hypotheses.

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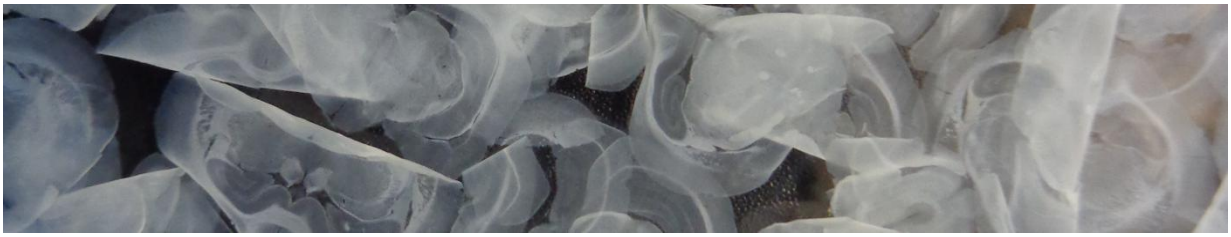
# Chapter 5



Conclusions, discussion and future perspectives

# Conclusions

## Proof-of-concept and mechanism of action

From this thesis, we can conclude that:

1. **Chapter 3 – Study 1|** The kainic acid (KA) rat model for temporal lobe epilepsy presents anhedonia, as demonstrated by a significant decrease in saccharin preference. The reduced saccharin preference in the KA-treated rats was not caused by an altered taste perception, as the aversion towards quinine was unaltered. Two weeks of VNS decreased the anhedonic state in the KA rats, as indicated by a significant increase in saccharin preference. No effects were found in the other groups (KA-SHAM, SAL-VNS and SAL-SHAM). Furthermore, VNS nor SHAM treatment had an effect on taste perception, as shown in the quinine aversion test. **Our findings demonstrate the antidepressant-like effect of VNS in the KA rat model for temporal lobe epilepsy and comorbid anhedonia.**

2. **Chapter 3 – Study 2|** We confirmed that VNS has an antidepressant-like effect based on a significant reduction of the immobility time in the rat forced swim test. The antidepressant-like effect of VNS was completely abolished when the noradrenergic axons arising from the locus coeruleus (LC) were eliminated using the selective noradrenergic neurotoxin DSP-4. To rule out the possibility that the effects in the forced swim test were caused by an overall change in locomotor activity, the animals were tested in an open field. No significant differences in locomotor activity were found between groups. Furthermore, the immobility in the forced swim test did not correlate with the locomotor activity in the open field test, ruling out a mere locomotor effect as an explanation for the observed forced swim test results. **Our results confirm the key role of the noradrenergic LC neurons in the antidepressant-like mechanism of action of VNS**.

From this thesis, we can conclude that:

1. **Chapter 4 – Study 4|** VNS is effective in reducing cortical excitability in the motor cortex stimulation rat model. **A low VNS output current (0.25 mA) is sufficient to reduce cortical excitability**, while higher output currents (0.50 mA and 1.00 mA) do not result in an additional therapeutic effect and may therefore not be required. These results suggest that the effect of VNS on cortical excitability is mediated through the low and moderate threshold A- and B-fibers and not through the high threshold C-fibers.
2. **Chapter 4 – Study 5|**VNS-induced LMEPs can be recorded reproducibly in chronically VNS-implanted experimental **rats**, using a minimally invasive, easy-to-implement electromyography (EMG) technique.
3. **Chapter 4 – Study 5|**In experimental rats, LMEPs occurred at low stimulation intensities (median 0.2 mA, IQR 0.2 - 0.3 mA). At higher stimulation intensities (median 0.7 mA, IQR 0.5 - 0.7 mA), the muscle-evoked potentials had significantly higher or lower latencies than expected for an LMEP, pointing at nerve damage and direct muscle activation due to current spill-over respectively. These results suggest that **low stimulation currents are sufficient to activate the vagus nerve, while higher stimulation currents can lead to significant stimulation-related side effects. Furthermore, we suggest that this technique can be used to identify non-response due to ineffective activation of the vagus nerve and to determine the individual post-operative recovery periods.**
4. **Chapter 4 – Study 6|** VNS-induced LMEPs were recorded in two chronically VNS-implanted **patients** with very high reproducibility.
5. **Chapter 4 – Study 6|** These LMEPs were already present at low output currents (0.25 - 0.50 mA) and the amplitude of the signals reached a plateau around 0.75 - 1.00 mA. These results suggest that **low to moderate output currents are sufficient to activate the vagal fibers in humans.** VNS-induced LMEPs may help neurologists to **choose the optimal stimulation parameters** in a more objective way and to **identify non-responders** due to ineffective stimulation of the vagus nerve.

# Discussion and future perspectives

## Proof-of-concept and mechanism of action

The promising results from the proof-of-concept study encourage further studies in the KA rat model for temporal lobe epilepsy and comorbid depression. First, studies should focus on identifying the pathophysiological processes underlying this comorbidity. The frequent co-occurrence of epileptic seizures and depressive symptoms in chronic epilepsy models, suggests that these symptoms may share common underlying pathophysiological mechanisms. Although the specific mechanisms have not been identified to date, emerging evidence shows that there is a remarkable overlap in the abnormalities found in epilepsy and depression models, the two most important ones being imbalances in neurotransmitter systems [1] and changes in neural plasticity [2, 3]. Therefore, future studies should include neurochemical experiments in order to characterize potential abnormalities in neurotransmitter balances and the investigation of the role of aberrant neural plasticity. In a second phase, experimental studies should focus on unraveling the mechanism of action of VNS in animal models for temporal lobe epilepsy and comorbid depressive symptoms. This mechanism of action most likely results from a complex interplay between the different mechanisms that underlie the pathophysiology of both diseases, i.e. the correction of dysfunctional neurotransmitter circuits and the promotion of neural plasticity.

As the LC and its neurotransmitter noradrenaline are convincingly involved in the treatment of depression [4, 5] and VNS-induced noradrenaline release from the LC has been shown in several experiments [6-8], we hypothesized that the antidepressant-like effect of VNS in the KA model for temporal lobe epilepsy and comorbid depression would be abolished when the LC axons are lesioned using the very selective noradrenergic neurotoxin DSP-4. However, in a pilot trial we found that lesioning the LC axons in KA-treated animals is a lethal intervention. As noradrenaline also has potent antiepileptic effects [9], we hypothesize that our KA-treated animals developed a refractory status epilepticus after DSP-4 injection, eventually leading to their death. Consistent with this hypothesis, it has previously been shown that DSP-4 potentiates pilocarpine- [10], iron- [11] and bicuculline-induced seizures [12]. Although this was not the focus of this thesis, it would be interesting to setup an experiment involving video-EEG monitoring to test this hypothesis in the future.

To assess whether the LC plays a key role in the antidepressant-like mechanism of action of VNS, we searched literature for an alternative model to test our hypothesis in. Such an animal model was found in the publication of Krahl et al. were it was shown that VNS is effective in the rat forced swim test, which is a validated test for behavioral despair [13]. The abolishment of the antidepressant-like effect of VNS after lesioning the noradrenergic LC axons, confirmed the hypothesis that this effect is mediated through the activation of this brainstem nucleus and the subsequent release of noradrenaline.

In previous LC lesioning studies, it was demonstrated that an intact LC is required for the antiepileptic and the antinociceptive effects of VNS [14-17]. Considering the total loss of therapeutic efficacy of VNS for several disorders after the selective destruction of LC axons, we hypothesize that the LC functions as a gateway structure, by primarily releasing noradrenaline which can then trigger other mechanisms important in several conditions. A hypothesis to consider for depression is the enhancement of other neurotransmitter systems such as the serotonergic and the dopaminergic neurotransmission, two monoaminergic systems which are implied in the pathophysiology and the treatment of depression [18, 19] and have been shown to be enhanced by VNS [20-24]. It was previously demonstrated that the enhancing effect of VNS on serotonergic neuronal firing is indirectly mediated by the noradrenergic LC neurons through the enhanced activation of the excitatory $\alpha_1$-adrenoreceptors located on dorsal raphe nucleus serotonergic cell bodies [20]. It would be interesting to investigate in future experiments, whether the effect of VNS on the dopaminergic system is also indirectly mediated through the enhanced noradrenaline release from the LC. In a next step, experiments using selective agonists and antagonists for the different adrenergic receptor subtypes ($\alpha_{1}$-, $\alpha_{2}$-, β-receptors), could provide valuable information on which receptor subtypes are involved in this effect. Previous studies showed that direct LC stimulation elicits burst firing of the dopaminergic ventral tegmental neurons through the excitatory $\alpha_1$-adrenoreceptors [25-27], suggesting that VNS could indeed also activate the dopaminergic system via binding to these receptors. Furthermore, studies using selective serotonergic and dopaminergic neurotoxins should be performed to assess the individual roles of these neurotransmitter systems in the antidepressant-like effect of VNS.

Another plausible hypothesis is that VNS produces its antidepressant-like effect through increasing neural plasticity, especially in the hippocampus, a structure important in mood regulation. As the hippocampus is rich in noradrenergic LC innervation [28] and noradrenaline has proven neuroplastic effects [29-31], it is tempting to hypothesize that these effects of VNS are also indirectly mediated though the activation of the LC and the subsequent release of noradrenaline in the hippocampus.

The rationale for the neural plasticity hypothesis of depression originates from the knowledge that stressful events, such as forced swimming in rodents, lead to a significant reduction in neural plasticity, while several antidepressants increase neural plasticity [32]. A growing body of evidence suggests that VNS also produces its antidepressant-like effect through increasing neural plasticity [8, 33-36]. However, only two experimental animal studies have correlated VNS-induced changes in neural plasticity to antidepressant-like effects in behavioral testing paradigms. Only the study of Gebhardt et al. found an association between the VNS-induced increased progenitor proliferation and its restorative effects on cognition [35]. However, as an association does not imply a causative relationship between the observed phenomena, the results should be interpreted with caution. That is, the possibility exists that the neuroplastic effects of VNS are merely an epiphenomenon of other, more important processes leading to sustained antidepressant effects. The dissociation between the presence of neuroplastic effects and the lack of behavioral effects in the study of Biggio et al. [34], supports this hypothesis. Consequently, future studies should focus on investigating the potential causal relationship between VNS-induced neuroplastic changes and antidepressant-like effects, for example using x-ray irradiation techniques to ablate hippocampal cell proliferation. Another shortcoming of the studies performed so far, is that none of them have investigated whether the newborn progenitor cells differentiate into mature neurons and integrate functionally into the cortico-limbic networks important in depression. Therefore, future studies should determine the phenotype of the newborn cells using specific markers for mature neurons, such as NeuN (neuronal nuclei) or NSE (neuron specific enolase). Furthermore, connectivity studies should be performed to confirm the hypothesis that the newborn neurons restore the disturbed cortico-limbic networks in depressed subjects.

Animal models are indispensable for investigating the etiology of diseases, as well as for developing and optimizing therapeutics for these diseases. However, it must be acknowledged that all animal models have limitations. For example, animals cannot observe feelings of sadness, guilt or suicidal thoughts, depressive symptoms mainly limited to humans [37, 38]. Therefore, it is indispensable to assess whether our findings translate to human subjects treated with VNS. In a previous study by Harden et al., it was found that VNS induces mood improvements in epilepsy patients [39] supporting the findings from our preclinical study. However, there are multiple factors confounding the study of Harden et al. First, this study was not randomized nor placebo-controlled. Consequently, patients in the VNS group were different from patients in the control group with respect to both baseline seizure frequency and mood scale scores. Second, there might have been a placebo effect in the VNS group, simply resulting from the fact that patients in this group chose to be proactive in treating their

epilepsy. Third, patients in the VNS group were seen much more frequently for the adjustment of VNS settings compared to control patients. It is therefore possible that non-specific effects of physician contact accounted for the observed mood improvements. Lastly, although it is well-known that antiepileptic drugs can contribute to mood disturbances [40], drug levels were not systematically measured during this study [39].

To assess whether VNS has a true antidepressant effect in epilepsy patients suffering from comorbid depression, a randomized sham-controlled, double-blind clinical trial should be conducted. First, all patients should be implanted with a VNS device. Subsequently, patients should be randomized in a matched VNS- or sham-treated group. The output current of the patients in the VNS group should be gradually ramped-up, while it should be kept at 0.00 mA in the patients of the sham group. However, patients in both groups should be approached in exactly the same way. That is, they should be seen by a physician as frequently in both groups and similarly as in the VNS group, the physician should make adjustments to the parameters settings of the patients in the sham group, albeit sham adjustments. Furthermore, concomitant drug therapy should be closely monitored and matched. To minimize or even avoid the confounding influences of interactions between different medication groups, anti-epileptic and antidepressant drugs could be down-titrated to a stable monotherapy regime prior to the start of the study. Naturally, for ethical reasons, the output current of the patients in the sham group should be ramped-up after the initial sham-controlled phase of the study. If the results from such clinical trial confirm the results from our preclinical study, VNS could be suggested as a standard treatment for patients suffering from comorbid epilepsy and depression.

Furthermore, clinical studies assessing the role of the LC and noradrenaline in the antidepressant mechanism of action of VNS should be conducted. As lesioning the LC in patients is not feasible for obvious ethical reasons, confirming the causal relationship between the VNS-induced activation of the LC and the antidepressant effect of VNS is impossible in a clinical population. This stresses the importance of the use of animal models for this purpose. To date, non-invasive techniques to directly measure noradrenaline in the human brain are lacking. However, an increase in noradrenaline can be indirectly evaluated through parameters which are modulated by the central noradrenergic signalization, such as the pupil diameter or the P300 component of event-related potentials. A recent study from our lab showed that VNS induces a significant increase of the P300 amplitude at the parietal midline electrode, in VNS responders only. This finding suggests that the VNS-induced activation of the noradrenergic LC system is associated with the therapeutic response to VNS in patients with epilepsy [41]. It would be very interesting to assess in a future study, whether this relationship between the VNS-induced effect on the P300 and the therapeutic response also exists for depressive patients treated with VNS.

**Optimization of the stimulation parameters**

Electrical pulses applied in VNS therapy are defined by the following stimulation parameters: frequency (Hz), pulse width (μs), ON/OFF time or duty cycle (s, min) and output current (mA) [42]. The parameters currently applied in clinical practice and in experimental animal studies are based on a limited number of studies showing their safety and tolerability. Agnew et al. demonstrated that stimulation of the vagus nerve in rats with a low frequency (20 Hz), induces less damage compared to VNS with high frequencies (50 – 100 Hz) [43]. A pulse width of 250 μs instead of 500 μs was shown to increase tolerability in patients treated with VNS [44]. The use of a duty cycle saves battery life and reduces stimulation-related nerve damage [45]. Furthermore, it has been demonstrated that the effect of the stimulation outlasts the stimulus duration [46-48]. In clinical practice, the therapeutically used output current is typically the highest stimulation intensity tolerated. The output currents used in preclinical experimental studies are determined rather empirically and vary considerably between experiments. The lack of evidence-based data for VNS output currents in combination with the lack of efficacy in a substantial number of VNS-treated subjects, demonstrates the need for studies optimizing the VNS output current both in clinical practice and in preclinical experimental studies.

In a previous study from our laboratory by De Herdt et al., it was shown that 1 hour of VNS at an output current of 0.75 mA is effective in lowering cortical excitablity in the motor cortex stimulation rat model [49]. This model allows repeated testing of the threshold for motor seizures in a standardized manner by means of applying a ramp-shaped stimulation pulse train with increasing intensity to the motor cortex [49, 50]. In the experiment presented in this thesis, we used this model to further finetune and investigate the dose-response relationship by measuring the effects of different VNS output currents on cortical excitability, i.e. 0.25, 0.50 and 1.00 mA. We found that VNS at 0.25 mA is sufficient to significantly decrease cortical excitability, while higher output currents (0.50 and 1.00 mA) do not result in an additional therapeutic effect. Our results suggest that the output current intensities may quickly reach a saturation level in terms of therapeutic effectiveness and that higher output current intensities are not required to reach significant effects on cortical excitability. Considering the previous study from our lab by De Herdt et al. [49], the VNS-induced reduction in cortical excitability in the motor cortex stimulation rat model has proven to be very reproducible. Therefore, future VNS studies in this model are warranted. Follow-up studies should assess whether even lower output currents (< 0.25 mA) are sufficient to reduce cortical excitability.

Other stimulation parameter settings including the stimulation frequency, pulse width and duty cycle could be optimized using this model.

The results from our preclinical study raise the hypothesis that low VNS output current intensities could be equally sufficient in reducing cortical excitability in humans. However, a direct translation of these output currents to clinical practice is hampered by several factors, including the much smaller diameter of the rat vagus nerve compared to the human nerve and the use of different electrode types, i.e. cuff versus helical electrodes in rats and humans respectively. Therefore, a clinical trial should be performed to assess the effect of different VNS output currents on cortical excitability. Similarly as in the motor cortex stimulation rat model, cortical excitability of the motor cortex can be assessed in humans. However, instead of using electrical currents as is being done in rats, transcranial magnetic stimulation (TMS) can be applied to assess cortical excitability in humans. TMS stimuli are delivered through a coil placed over selected scalp locations overlying the primary motor cortex. These stimuli mainly activate pyramidal neurons transsynaptically, which produces indirect waves descending along the corticospinal fibers. Applied over the motor cortex, these discharges produce a twitch in the corresponding muscles. This muscle activity, referred to as a motor-evoked potential (MEP), can be recorded using electromyography (EMG) from many muscles, including the small muscles of the hand [51, 52]. Future prospective clinical studies using TMS and comparing high versus low VNS output current intensities are required to confirm the hypothesis that low output currents are sufficient to reduce cortical excitability, eventually leading to antiepileptic effects in humans.

Additional evidence supporting the hypothesis that low output currents are sufficient to activate the vagus nerve, was found in the LMEP studies presented in this thesis. Effective delivery of current to the vagus nerve at the cervical level, co-activates the Aα-fibers of the recurrent laryngeal nerve and induces contractions of the laryngeal muscles and the vocal cords. In this context, a previous study from our laboratory has recorded LMEPs in rats in an acute intra-operative setting using invasive electrodes [53]. For chronic VNS studies, a less invasive, reliable technique for LMEP recordings is required (i) to determine the optimal stimulation output current to activate the relevant fibers, (ii) to exclude rats where electrical current delivery to the vagus nerve is doubtful and (iii) to identify the optimal moment for initiation of the experiment. Therefore, we aimed at investigating in a chronic experimental setting, the feasibility and reliability of LMEP recordings using a minimally invasive, easy-to-use EMG tool. In a second phase, we aimed at translating this technique to clinical practice.

We found that VNS-induced LMEPs can be reproducibly recorded in chronically VNS-implanted experimental rats and that these LMEPs occur at low stimulation intensities (median 0.2 mA, IQR:

0.2-0.3 mA). At significantly higher stimulation intensities (median 0.7 mA, IQR 0.5-0.7 mA, p<0.001), the responses had significantly higher or lower latencies than expected for an LMEP, pointing at nerve damage and direct muscle activation due to current spill-over respectively. In chronically VNS-implanted patients as well, LMEPs were recorded with very high reproducibility. These LMEPs were already present at low output currents (0.25 - 0.50 mA) and the amplitude of the signals reached a plateau around 0.75 - 1.00 mA. Combined, these results again support the hypothesis that low to moderate stimulation currents are sufficient to activate the vagal fibers. There is a growing body of evidence, both from preclinical and clinical studies that further supports this hypothesis. Cunningham et al. found that VNS at 0.25 mA in rats significantly increases staining for c-fos, an indirect marker for neuronal activity, in the nucleus tractus solitarius and many other regions that receive projections from the vagus nerve [54]. Furthermore, Woodbury and Woodbury showed that VNS at 0.20 - 0.50 mA reduces chemically-induced seizures in rats [55]. Moreover, in vivo intracellular recordings in the temporal association cortex of rats, showed that VNS output currents of 0.20 mA or less, are effective in reducing the excitability of pyramidal neurons [56]. Interestingly, it was shown that VNS at 0.25 mA and 0.30 mA significantly increases the firing rate of the serotonergic neurons of the dorsal raphe nucleus [20] and of the noradrenergic neurons of the LC [57] respectively. In clinical studies as well, it was shown that low VNS output currents are sufficient to achieve intracerebral effects. In functional neuroimaging studies by our own group, acute VNS at an output current of 0.25 mA, induced significant cerebral blood flow changes, particularly in the thalamus and the limbic system [58, 59]. Furthermore, it was shown that VNS at low output current intensities is effective in enhancing recognition memory in humans [60] and in the effect on human tolerance for pain [61]. Moreover, analyses of large patient series have not demonstrated a positive correlation between output current intensities and seizure control. More specifically, it was shown by Bunch et al. that many VNS-treated patients respond to VNS at low output currents (< 1.00 mA) while higher output currents (≥ 1.00 mA) do not necessarily lead to a greater reduction in seizure frequency. On the other hand, 20% of the initial non-responders from that study became responders when the VNS output current was increased [62]. However, the possibility exists that this improvement did not result from the increase in output current, but rather from neuromodulatory changes that require time to establish. A retrospective study by Labar et al. evaluated the effect of output current on seizure frequency in 269 patients who had not changed their antiepileptic medication over a 1-year period. Output currents were classified as low (0.25 - 1.00 mA), medium (1.25 - 2.00 mA) or high (≥ 2.25 mA). Patients receiving VNS at high output currents experienced a smaller reduction in seizure frequency (median 38%) than those with low (median 64%) or medium (median 61%) output currents. The results from this study again suggest that low and moderate output currents are sufficient or even beneficial to achieve therapeutic effects [63]. However, this study could be confounded by the fact

that initial responders are less likely to undergo an increase in output current compared to non-responders. Therefore, future randomized controlled clinical trials of sufficient duration (e.g. 1 year of follow-up), comparing high versus low to moderate output current intensities are required to confirm the hypothesis that low to moderate output currents are sufficient or even beneficial in exerting therapeutic effects in humans.

This large body of evidence both in animals and humans suggesting that low or moderate VNS output currents might be sufficient to achieve therapeutic effects, is consistent with the knowledge on the anatomy of the vagus nerve. The nerve contains three types of fibers, i.e. A-, B- and C-fibers, distinguished by their diameter and conduction velocity. The A- and B-fibers are myelinated and have a large and intermediate diameter respectively. The C-fibers are unmyelinated and have a small diameter. Subsequently, the A-, B- and C-fibers have a low (< 0.20 mA), intermediate (< 0.40 mA) and high (> 2.00 mA) threshold for activation respectively [57]. The earliest animal studies suggested that the antiepileptic potential of VNS was directly related to the fraction of vagal afferent C-fibers stimulated [55, 64]. However, the theory supporting C-fiber involvement was discarded after Krahl et al. demonstrated VNS-induced seizure suppression in rats following selective C-fiber destruction [65]. Based on this and other studies [6, 46, 62, 66], it is conceivable that the A- and B-fibers are responsible for the therapeutic effects of VNS and subsequently, low and intermediate output currents should be sufficient to evoke therapeutic responses. Apart from reducing stimulation-related side effects, stimulating the vagus nerve with a lower output current density would be beneficial in the sense that it would save battery life, thereby postponing the surgical procedure and costs for battery replacement.

However, it must be acknowledged that one general optimal stimulation current cannot be defined. This is reflected in our results by the fact that the output currents required to evoke LMEPs in rats are variable and range from 0.10 to 0.30 mA, and even to 0.50 mA in one animal. Individual thresholds for the activation of vagal A- and B-fibers will vary as a result from differences in the anatomy of the vagus nerve, i.e. fibers located closer to the perimeter of the nerve and thus closer to the VNS electrode are exposed to a stronger electrical field and are easier to excite compared to fibers located deeper in the nerve. Also, fibrous tissue encapsulation at the site of the electrode can increase resistance, altering the electric field and resulting in increased voltage requirements for fiber excitation [67, 68]. It was shown by Helmers et al. that the presence of 110 µm of fibrotic tissue can decrease fiber activation by 50% [68]. Furthermore, LMEP recordings may underestimate the VNS output current required to achieve therapeutic efficacy as it reflects the activity of the low threshold Aα-motor fibers, while the therapeutic effects of VNS are believed to be mediated by higher threshold A- and B-fibers. Nevertheless, it is well-known that physiological features such as

fiber diameter, activation sensitivity to electrical stimulation, action potential conduction velocity and function are all closely related [69]. When the threshold to one specific fiber type can be reliably recorded, it can be used as an indicator to determine the expected threshold for all other fibers. The threshold stimulation strength to produce LMEPs is thus directly related to the required therapeutic VNS stimulus strength. The value of the ratio between both stimulus strengths still has to be established, for example by correlating LMEP recordings with intracerebral effects, i.e. unit recordings from the LC. However, given that the variation in activation sensitivity with fibre diameter is rather limited in the large diameter range [70], we expect to find a ratio value close to unity between the LMEP threshold stimulation current and efficient therapeutic VNS output currents. Therefore, we suggest that the presented non-invasive LMEP recording technique could be used to guide individual titration of the stimulation parameters.

Furthermore, we suggest that this minimally invasive, easily-applicable method could be a valid tool to identify non-responders due to ineffective activation of the vagus nerve and to determine individual post-operative recovery periods. It is possible that the vagus nerve of some non-responders is not adequately activated, for multiple reasons such as lead failure, poor electrode contact or nerve damage. It has previously been shown that the implantation of cuff electrodes in rabbits causes an initial loss of myelination, with a subsequent regeneration of the myelin sheath over several weeks to months [71]. In animals with more severe damage to the myelin sheath, a longer postoperative recovery period before initiating VNS is warranted. However, the usually applied recovery delays in experimental studies are determined rather empirically and are typically the same for all animals in one study. In a previous study from our lab, 13/21 rats displayed a recordable LMEP 2 to 4 weeks after surgery. In the remaining rats, a post-surgical recovery period of up to 7 weeks was required before an LMEP could be recorded [72]. In the presented preclinical LMEP trial, significantly higher latencies of the LMEPs were recorded in 4/27 rats, which suggests an incomplete recovery of the vagus nerve after surgery in these animals. Furthermore, the total absence of an LMEP in 3/27 rats, could likewise result from a dysfunctional nerve. Consequently, rats with longer latency LMEPs or without LMEPs should be excluded from the experiment, as the adequate delivery of current to the nerve is doubtful in these animals, while this is obviously a prerequisite to obtain reliable experimental results. In clinical practice as well, a temporary paresis of the vocal cords after VNS surgery [73-75] indicates that the surgical procedure and implantation of the electrode can cause nerve damage and a transient failure of the vagus nerve, hence requiring a recovery period before becoming functional again [72]. To allow recovery of the nerve, a delay of two weeks is usually foreseen between the implantation of the system and the start of VNS. Again, the duration of this delay is determined rather empirically, while an individualized approach, based

on the recovery of the nerve could be beneficial. The absence of an LMEP at reasonable stimulation currents or a prolonged latency of the LMEP could indicate incomplete recovery of the nerve. In that case, a further rest period might be beneficial for the nerve and more easily accepted then unsuccessful trials. Of course, non-response could also result from the heterogeneity of the underlying pathophysiological mechanisms or the variability in characteristics of more central structures in the neural pathway involved in VNS. In this regard, genetic differences in neurotransmitter systems could be an example of hypotheses to consider. Therefore, the presence of LMEPs is not an identifier of response nor is it a biomarker for the anti-epileptic or antidepressant efficacy of VNS but rather an indication that the nerve is effectively stimulated, which is an essential step to achieve therapeutic response. This is reflected in our data by the fact that both patients display LMEPs while only one of them is a responder. Non-response in the other patient despite the proper activation of the vagus nerve as reflected by the presence of LMEPs, could result from upstream problems such as an impaired noradrenergic signaling.

In conclusion, VNS-induced LMEPs can be recorded very reproducibly both in chronically VNS-implanted rats and patients using a non-invasive EMG approach. Furthermore, our results suggest that low to moderate output currents are sufficient to activate the vagal fibers. Moreover, LMEPs could be used for the identification of ineffective stimulation leading to non-response and for the individualization of post-operative recovery delays.

Future studies should focus on the optimization of this technique in awake animal models for depression, epilepsy or the comorbidity of both disorders, as there might be an effect of general anesthetics on axonal excitability [76]; the expansion of our sample size in the clinical epilepsy pilot trial; a clinical trial in a patient population suffering from refractory depression; the identification of the ratio between the output current threshold for Aα- and other A- and B-fiber activation and the correlation of LMEP recordings with therapeutic responses both in epileptic and depressed experimental animals and human subjects.

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# Summary

Epilepsy and depression are two highly prevalent disorders, that frequently occur together. Despite the availability of an abundance of both antiepileptic and antidepressant drugs, up to one third of patients fail to respond adequately to standard medication and are classified as refractory. Furthermore, current treatment options for patients suffering from both epilepsy and depression are limited by the fact that antiepileptic drugs can contribute to mood disturbances, while antidepressant drugs can increase seizure susceptibility. The lack of success with current pharmacological interventions for patients suffering from refractory epilepsy, depression or the combination of both diseases, highlights the importance of optimizing non-pharmacological, neuromodulatory treatments such as vagus nerve stimulation (VNS) for this patient population.

VNS is an extracranial form of neurostimulation which consists of stimulating the left vagus nerve in the neck through an implanted electrode and a pulse generator. Since the first patient was implanted with a VNS device in 1988, more than 100.000 patients worldwide have been treated with VNS. Although VNS appears to be an efficacious and safe treatment, some unresolved questions remain to counteract its full therapeutic potential. First, mood improvements in epilepsy patients treated with VNS, irrespective of the effects on seizure frequency, provided the initial rational for using VNS for the treatment of refractory depression. However, randomized controlled trials confirming the antidepressant effect of VNS in epileptic subjects are lacking. Furthermore, the mechanism of action is still unknown, optimal stimulation parameters remain elusive and about one third of patients do not benefit from the treatment (non-responders). Therefore, the research presented in this thesis focused on assessing the antidepressant potential of VNS in an animal model for temporal lobe epilepsy and comorbid depression, unraveling its antidepressant mechanism of action and optimizing the stimulation parameters, which could all contribute to improving clinical outcome in the long run.

The first experimental study consisted of a proof-of-concept experiment in which the antidepressant effect of VNS was confirmed in the post status epilepticus kainic acid rat model for temporal lobe epilepsy and comorbid anhedonia, the latter being a key symptom of major depression. Future clinical studies should be performed to assess whether our findings can be translated to patients. Confirmation of the pre-clinical results in patients could lead to the choice of VNS as a preferred treatment for patients suffering from comorbid epilepsy and depression.

The second study was designed to unravel the antidepressant mechanism of action of VNS. Since the 1960s, there has been a strong emphasis on the role of the locus coeruleus (LC) and its neurotransmitter noradrenaline in the mechanism of action of several antidepressants. There is extensive evidence demonstrating that like antidepressants, VNS enhances the noradrenergic neurotransmission through the activation of the LC. Therefore, we tested the hypothesis that the VNS-induced antidepressant effect in the rat forced swim test is mediated through the activation of the LC. For this purpose, the noradrenergic LC neurons were lesioned using the highly selective neurotoxin DSP-4. The complete abolishment of the antidepressant effect of VNS in DSP-4-treated rats, confirmed the key role of the LC in the antidepressant mechanism of action of VNS. Future studies are warranted to unravel the upstream mechanisms by which the VNS-induced activation of the LC exerts its antidepressant effect. Hypotheses to consider are the upregulation of hippocampal neuroplasticity and the secondary enhancement of other neurotransmitter systems involved in depression, i.e. the serotonergic and dopaminergic neurotransmission.

With regards to the stimulation parameters, we found that VNS at an output current of 0.25 mA is sufficient to decrease cortical excitablity in rodents, while higher output currents (0.50 and 1.00 mA) do not result in an additional therapeutic effect. Further evidence supporting the hypothesis that low to moderate output currents are sufficient to activate the vagus nerve and produce therapeutic effects, came from the laryngeal motor-evoked potential or LMEP studies. VNS-induced LMEPs – reflecting effective activation of the vagal fibers – were recorded very reproducibly both in chronically VNS-implanted rats and patients using a non-invasive electromyography approach. In rats, these LMEPs occurred at low stimulation intensities (median 0.20 mA, IQR 0.20 - 0.30 mA). At higher stimulation intensities (median 0.70 mA, IQR 0.50 - 0.70 mA), the responses had significantly higher or lower latencies than expected for an LMEP, pointing at nerve damage and direct muscle activation due to current spill-over respectively. In a pilot trial in patients, LMEPs were already recorded at low output currents (0.25 - 0.50 mA). Consistent with our findings, there is a growing body of evidence, both in animals and in humans, suggesting that low to moderate output currents are sufficient to achieve intracerebral effects. However, future prospective studies comparing low versus high output current intensities are required to confirm this hypothesis. Furthermore, we suggest that LMEP recordings could be used for the identification of ineffective stimulation of the vagus nerve, leading to non-response. To test this hypothesis, future studies should correlate LMEP recordings with therapeutic responses both in experimental animals and in human subjects.

# Samenvatting

Epilepsie en depressie zijn twee aandoeningen met een hoge prevalentie, die bovendien frequent samen voorkomen. Ondanks het correct gebruik van anti-epileptica en antidepressiva, blijft één derde van de patiënten epileptische aanvallen en/of depressieve symptomen ervaren. Deze patiënten lijden aan moeilijk behandelbare of refractaire epilepsie of depressie. Bovendien zijn de behandelingsmodaliteiten die vandaag beschikbaar zijn voor patiënten die zowel lijden aan epilepsie als depressie, beperkt door het feit dat anti-epileptica een negatieve invloed kunnen hebben op het gemoed, terwijl antidepressiva de gevoeligheid voor epileptische aanvallen kunnen verhogen. Het gebrek aan succes met de beschikbare farmacologische behandelingen voor patiënten die lijden aan refractaire epilepsie, depressie of de comorbiditeit van beide aandoeningen, onderstreept het belang van het optimaliseren van niet-farmacologische, neuromodulatoire behandelingen zoals nervus vagus stimulatie (NVS).

NVS is een extracraniële vorm van neurostimulatie waarbij de linker nervus vagus in de nek gestimuleerd wordt via een geïmplanteerde elektrode en een pulsgenerator. De eerste NVS implantatie bij een epilepsiepatiënt vond plaats in 1988. Sindsdien worden wereldwijd reeds meer dan 100.000 patiënten behandeld met deze therapie. Ondanks de bewezen doeltreffendheid en veiligheid van NVS, dienen nog een aantal aspecten rond deze therapie opgehelderd te worden. Zo merkte men op dat NVS in epilepsiepatiënten een positief effect heeft op het gemoed, onafhankelijk van het effect op de epileptische aanvallen. Deze bevinding heeft geleid tot het gebruik van NVS voor de behandeling van refractaire depressie. Tot op heden zijn er echter nog geen gerandomiseerde, gecontroleerde studies voor handen die aantonen dat NVS daadwerkelijk een antidepressief effect heeft in epilepsiepatiënten. Bovendien zijn het werkingsmechanisme en de optimale stimulatieparameters nog steeds niet gekend. Daarenboven ervaart één derde van de patiënten geen therapeutisch voordeel van de behandeling (non-responders). Dit proefschrift richt zich op het onderzoeken van het antidepressief effect van NVS in een diermodel voor epilepsie en comorbide depressie, het ontrafelen van het antidepressief werkingsmechanisme en het optimaliseren van de stimulatieparameters, met als uiteindelijk doel het verbeteren van de klinische uitkomst.

In de eerste experimentele studie werd bevestigd dat NVS een antidepressief effect heeft in het kainaat rat model voor temporale kwab epilepsie en comorbide anhedonie, een kernsymptoom van majeure depressie. Prospectieve klinische studies zijn nodig om na te gaan of deze bevinding vertaald

kan worden naar patiënten. De bevestiging van de preklinische resultaten in patiënten zou ertoe kunnen leiden dat NVS als standaard behandeling wordt gebruikt voor patiënten die lijden aan de comorbiditeit van epilepsie en depressie.

De tweede studie van dit proefschrift onderzocht het antidepressief werkingsmechanisme van NVS. Sinds de jaren '60 ligt een sterke nadruk op de rol van de locus coeruleus (LC) en zijn voornaamste neurotransmitter noradrenaline, in het werkingsmechanisme van verschillende antidepressiva. Er zijn sterke aanwijzingen dat de LC en noradrenaline ook een belangrijke rol spelen in het werkingsmechanisme van NVS. Daarom werd nagegaan of het antidepressief effect van NVS in de "rat forced swim test" gemedieerd wordt door de activatie van de LC. Om dit te onderzoeken werd een selectieve laesie gemaakt in de noradrenerge neuronen van de LC met behulp van het selectief neurotoxine DSP-4. Het volledig verdwijnen van het antidepressief effect van NVS in DSP-4-behandelde dieren, bevestigt de rol van de LC in het werkingsmechanisme van NVS. Toekomstige studies zijn nodig om te onderzoeken welke mechanismes upstream van de NVS-geïnduceerde LC activatie verantwoordelijk zijn voor het antidepressief effect van NVS. Plausibele hypotheses zijn de opregulatie van hippocampale neuroplasticiteit en van andere neurotransmitters die belangrijk zijn in de pathofysiologie en de behandeling van depressie, bvb. serotonine en dopamine.

Met betrekking tot de stimulatieparameters, kon besloten worden dat NVS aan een intensiteit van 0.25 mA volstaat om de corticale exciteerbaarheid significant te verlagen in het motor cortex stimulatie rat model, terwijl hogere stimulatie intensiteiten (0.50 en 1.00 mA) niet resulteren in een additioneel therapeutisch effect. Verdere evidentie dat lage tot matige NVS-intensiteiten voldoende zijn om de nervus vagus te activeren en therapeutische effecten te bekomen, komt van de "laryngeal motor-evoked potential of LMEP" studies. NVS-geïnduceerde LMEPs – die de efficiënte activatie van de vagale vezels reflecteren – werden op een zeer reproduceerbare, niet-invasieve manier gemeten zowel in ratten als in patiënten. In ratten werden deze LMEPs reeds gemeten bij zeer lage stimulatie intensiteiten (mediaan 0.20 mA, IQR 0.20 - 0.30 mA). Bij hogere stimulatie intensiteiten (mediaan 0.70, IQR 0.50 - 0.70 mA), werden de responsen gekarakteriseerd door significant hogere of lagere latenties dan men zou verwachten voor een LMEP. Deze afwijkende latenties wijzen respectievelijk op zenuwschade en directe spieractivatie door lekstroom. Ook in een klinische piloot studie werden LMEPs gemeten bij lage NVS-intensiteiten (0.25 – 0.50 mA). Deze bevindingen worden ondersteund door verschillende dierexperimentele en humane studies die suggereren dat lage tot matige NVS-intensiteiten voldoende zijn voor het bekomen van positieve effecten in de hersenen. Om deze hypothese te bevestigen zijn prospectieve klinische studies nodig die het effect van lage versus hoge NVS-intensiteiten vergelijken. De resultaten van de gepresenteerde LMEP-studies suggereren dat

LMEP-metingen in de toekomst zouden kunnen gebruikt worden voor het identificeren van inefficiënte activatie van de nervus vagus, wat leidt tot non-response. Om deze hypothese te testen zijn studies verreist die LMEP-metingen correleren met de therapeutische respons van NVS, zowel in proefdieren als in patiënten.

# Résumé

L'épilepsie et la dépression sont des maladies très prévalentes, qui peuvent fréquemment se présenter simultanément. Malgré la disponibilité des traitements antiépileptiques et antidépresseurs, un tiers des patients continuent à avoir des crises d'épilepsie ou des symptômes dépressifs. Ces patients sont considérés 'réfractaires au traitement'. En outre, les médicaments utilisés dans la prise en charge de chacune de ces pathologies peuvent avoir une influence négative l'un sur l'autre: les médicaments antiépileptiques peuvent aggraver la dépression, tandis que les antidépresseurs peuvent diminuer le seuil épileptogène. Vu l'efficacité limitée des traitements pharmacologiques sur les patients souffrant d'épilepsie et/ou de dépression réfractaire, il est important d'optimiser d'autres modalités thérapeutiques non-pharmacologiques. En particulier la stimulation du nerf vague (SNV), afin d'améliorer la qualité de la prise en charge de ces maladies.

La SNV est une forme de neurostimulation extra-crânienne, qui consiste à stimuler le nerf vague au niveau cervical, au moyen d'une électrode de stimulation reliée à un générateur d'impulsions. Le premier implant humain a été réalisé en 1988. De nos jours, plus de 100.000 patients bénéficient de ce traitement dans le monde. Malgré l'efficacité et la sécurité établie de la SNV, certaines questions spécifiques au sujet de ce traitement restent non résolues. Tout d'abord, l'amélioration de la qualité de vie des patients épileptiques traités avec la SNV, indépendamment des effets sur la fréquence les crises, a fourni la justification de l'utilisation de cette technique pour le traitement de la dépression réfractaire. Toutefois, des études randomisées contrôlées confirmant l'effet antidépresseur de la SNV chez les sujets épileptiques, ne sont pas encore disponibles. En outre, le mécanisme d'action et les paramètres d'une stimulation optimale ne sont pas encore définis. En effet, environ un tiers des patients ne réagissent pas au traitement ('non-responders'). Cette thèse a pour but d'évaluer le potentiel antidépresseur de la SNV dans un modèle animal présentant une pathologie comorbide reflétant l'épilepsie et la dépression, de comprendre le mécanisme impliqué dans l'action antidépressive et d'optimiser les paramètres de stimulation afin de contribuer à l'amélioration des résultats cliniques.

Dans notre première étude expérimentale, l'effet antidépresseur de la SNV a été confirmé dans le modèle de rat à acide kaïnique pour la comorbidité épilepsie et anhédonie, celle-ci étant un symptôme de la dépression majeure. Cependant, des essais cliniques devront être effectués afin de

déterminer si ces résultats peuvent être utilisés dans la prise en charge des patients. Si cela devait être le cas, la SNV pourrait être proposée comme traitement standard pour les patients souffrant d'épilepsie et de dépression de façon comorbide.

La deuxième étude avait pour but de mieux comprendre le mécanisme d'action de la SNV. Depuis les années 1960, de nombreuses recherches ont été effectuées, évaluant le rôle du locus coeruleus (LC) et son neurotransmetteur la noradrénaline, dans le mécanisme d'action de plusieurs antidépresseurs. En effet, plusieurs données de la littérature soutiennent qu'au même titre que les antidépresseurs, la SNV améliore la neurotransmission noradrénergique grâce à l'activation du LC. Par conséquent, nous avons testé l'hypothèse que l'effet antidépresseur de la SNV dans le 'rat forced swim test' est médié par l'activation du LC. Dans ce cadre, les neurones noradrénergiques du LC ont été lésés en utilisant le DSP-4, une neurotoxine hautement sélective. Dans les rats traités avec le DSP-4, l'effet antidépresseur de la SNV était supprimé, confirmant le rôle très important du LC dans le mécanisme d'action antidépresseur de la SNV. Des études complémentaires sont nécessaires afin de comprendre les mécanismes d'action, par lesquels l'activation du LC induite par la SNV, exerce son effet antidépresseur. Plusieurs hypothèses peuvent être émises comme l'augmentation de la neuroplasticité hippocampique et le renforcement secondaire d'autres neurotransmetteurs impliqués dans la dépression, par exemple la neurotransmission sérotoninergique ou dopaminergique.

En ce qui concerne les paramètres de stimulation, nous avons constaté que la SNV à 0.25 mA est suffisante pour diminuer l'excitabilité corticale chez les rats, tandis que les intensités de courant plus élevées (0.50 et 1.00 mA) n'avaient aucun effet thérapeutique supplémentaire. Par ailleurs, des études dans lesquelles on a enregistré des potentiels laryngés évoqués moteur (ou des LMEPs) soutiennent l'hypothèse que des courants faibles à modérés sont suffisants pour activer le nerf vague et produire des effets thérapeutiques. Les LMEPs, qui reflètent l'activation efficace des fibres vagales, ont été enregistrés de manière reproductible chez les rats et les patients en utilisant une approche d'électromyographie non-invasive. Chez les rats, ces LMEPs étaient enregistrés à des intensités de stimulation faibles (médiane 0.20 mA, IQR 0.20 – 0.30 mA). A des intensités de stimulation plus hautes (médiane 0.70 mA, IQR 0.50 - 0.70 mA), les réponses avaient des latences significativement plus élevées ou plus basses que prévu pour un LMEP, témoignant respectivement de lésions du nerf vague ou de l'activation musculaire directe. Ces résultats expérimentaux sont soutenus par un essai clinique pilote, ou les LMEPs ont pu être enregistrés à des intensités faibles

(0.25 – 0.50 mA). Nos résultats concordent avec ceux déjà réalisés tant sur les modèles animaux que chez les humains, ce qui soutient l'hypothèse que des intensités de stimulation faibles à modérés sont suffisantes pour obtenir un effet intracérébral. Pour confirmer cette hypothèse, des essais cliniques prospectifs comparant différentes intensités seront nécessaires. En outre, nous suggérons que les enregistrements d'LMEPs pourraient être utilisés afin d'identifier la stimulation inefficace du nerf vague, qui pourrait résulter en une non-réponse. Pour tester cette hypothèse, des futures études devront corréler l'enregistrement d'LMEPs à des réponses thérapeutiques dans des modèles animaux et chez des sujets humains.

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me that I could make it if I didn't give up… and you were right! I appreciate your bubbly personality, your empathy, your determination, your problem solving way of thinking and so much more. Grazzi għal kollox!

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Gent, 2015

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# Curriculum vitae

## Personalia

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## Graduate studies

2006-2011: Master in Biomedical Sciences – Neurosciences – Ghent University

Graduated with greatest distinction

Master thesis: Evaluation of vagus nerve stimulation in the motor cortex stimulation rat model

Selected for the 'EOS scriptieprijs'

Publications in international peer-reviewed journals (A1)

**Grimonprez A**, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K and Boon P. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. Brain Stimulation 2015; 8: 13-20. **A1 publication, IF: 5.432, Q1 clinical neurology, neurosciences**

**Grimonprez A**, Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P and Vonck K. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. Journal of Psychiatric Research 2015; 68: 1-7. **A1 publication, IF: 4.092, Q1 psychiatry**

**Grimonprez A**, Raedt R, Baeken C, Boon P and Vonck K. The antidepressant mechanism of action of vagus nerve stimulation: evidence from preclinical studies. Neuroscience and Biobehavioral Reviews 2015; 56: 26-34.**Review, IF: 10.284, Q1 behavioral sciences, neurosciences**

Mollet L, **Grimonprez A**, Raedt R, Delbeke J, El Tahry R, De Herdt V, Meurs A, Wadman W, Boon P and K Vonck. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. Acta Neurologica Scandinavica 2013; 128: 391-396. **A1 publication, IF: 2.437, Q2 clinical neurology**

**Grimonprez A**, De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P and Vonck K. Laryngeal motor-evoked potentials mark vagus nerve activation: a preclinical study. Submitted to International Journal of Neural Systems. **A1 publication: IF: 6.056, Q1 computer science, artificial intelligence**

**Grimonprez A** and De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P and Vonck K. Laryngeal motor-evoked potentials as an indicator of vagus nerve activation: a clinical pilot trial. In preparation. **A1 publication: in preparation**.

Mollet L, Raedt R, Delbeke J, El Tahry R, **Grimonprez A,** Dauwe I, DeHerdt V, Meurs A, Wadman W, Boon P and K Vonck. Electrophysiological responses from vagus nerve stimulation in rats. International Journal of Neural Systems 2013; 23(6): 1350027. **A1 publication: IF: 6.056, Q1 computer science, artificial intelligence**

Larsen LE, Van Mierlo P, Wadman W, Delbeke J, **Grimonprez A**, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K and Raedt R. Modulation of hippocampal activity by vagus nerve stimulation in freely moving rats. Submitted to Brain Stimulation **A1 publication, IF: 5.432, Q1 clinical neurology, neurosciences**

Abstracts in international journals (C3)

**Grimonprez A**, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K, and Boon P. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. Belgian Brain Council, Luik, Belgium. Frontiers in human neuroscience.

**Grimonprez A**, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K, and Boon P. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. 11$^{th}$ European congress on Epileptology, Stockholm, Sweden. Epilepsia 2014; 55: 228-229.

**Grimonprez A**, De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P and Vonck K. Dose-dependent laryngeal muscle-evoked potentials as an indicator of effective vagus nerve activation. Belgian Brain Council, Luik, Belgium. Frontiers in human neuroscience.

Larsen LE, Van Mierlo P, Wadman W, Delbeke J, **Grimonprez A**, Mollet L, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K and Raedt R. Vagus nerve stimulation decreases hippocampal and prefrontal EEG power in freely moving rats: a biomarker for effective stimulation? Frontiers in human Neuroscience.

## Participation in international congresses en meetings

1. Science day. Belgium, Het Pand Ghent, March 2010.
2. 12th annual international clinical symposium Kempenhaeghe. The Netherlands, Kempenhaeghe Heeze, March 2010.
3. 13th annual international clinical symposium Kempenhaeghe. The Netherlands, Kempenhaeghe Heeze, March 2011.
4. Electrophysiology study day, MRP Neuroscience. Belgium, Het Pand Ghent, October 2011.
5. Stimulating (the) brain: neuromodulation and cognitive neuroscience, Institute for Neuroscience. Belgium, Het Pand Ghent, December 2011.
6. IUAP Meeting: Molecular and cellular mechanisms of electrical excitability. Belgium, Het Pand Ghent, December 2011.
7. Wetenschappelijke NVS-vergadering. The Netherlands, Heeze, September 2012.
8. Epilepsy workshop. Belgium, Het Pand Ghent, October 2012.
9. SWO midwintermeeting. The Netherlands, AMC Amsterdam, February 2012.
10. 14th annual international clinical symposium Kempenhaeghe. The Netherlands, Kempenhaeghe Heeze, March 2013.
11. Knowledge for Growth. Belgium, ICC Ghent, May 2013.
12. SWO midwintermeeting. The Netherlands, AMC Amsterdam, February 2014.
13. 15th annual international clinical symposium Kempenhaeghe. The Netherlands, Kempenhaeghe Heeze, March 2014.
14. Studenten onderzoekssymposium. Belgium, UZ Ghent, April, 29th 2014.
15. European Congress on Epileptology, Sweden, Stockholm, June-July 2014.
16. Symposium on affect and cognition, Belgium, Het Pand Ghent, September, 17th 2014.
17. Belgian Brain Council. Belgium, ICC Ghent, October, 4th 2014.
18. VNS avond, The Netherlands, Kempenhaeghe Heeze, October 9th 2014.
19. Belgian society of physiology and pharmacology, autumn meeting, Brussels, October 2014
20. VNS Pediatric Educational Seminar, Rotterdam, November 6-7th 2014.
21. Science day. Belgium, Het Pand Ghent, March 2015.
22. SWO midwintermeeting. The Netherlands, AMC Amsterdam, March 2015.
23. 16th annual international clinical symposium Kempenhaeghe. The Netherlands, Kempenhaeghe Heeze, March 2015.
24. PhD Day. Belgium, Zebrastraat Ghent, April 2015.

## Oral presentations

1. Datablitssession: Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. Epilepsy workshop, Belgium, Het Pand Ghent, October 2012.
2. Datablitssession: Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. SWO midwintermeeting, The Netherlands, AMC Amsterdam, February 2012.
3. Staff meeting: Vagus nerve stimulation for the treatment of refractory depression and epilepsy. Belgium, UZ Ghent, neurology department, March, 2013.
4. Nervus vagus stimulatie meeting: Nervus vagus stimulatie voor de behandeling van stemmingsstoornissen. Kempenhaeghe, Heeze, October, 2014.
5. International peer review: Vagus nerve stimultion for the treatment of refractory epilepsy and depression. UZ Ghent, neurology department, February 2015.
6. Platform presentation Science day: The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. Het Pand, Ghent, March 2015.

## Poster presentations

Mollet L, **Grimonprez A**, Raedt R, Delbeke J, El Tahry R, De Herdt V, Meurs A, Wadman W, Vonck K, Boon P. Modulation of cortical excitability by vagus nerve stimulation in the cortical stimulation model. SWO Midwintermeeting, The Netherlands, AMC Amsterdam, February 2012.

**Grimonprez A,** Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. Kempenhaege Epilepsy symposium, The Netherlands, Heeze, March 2012.

**Grimonprez A**, Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. Belgian Brain Council, Belgium, Luik, October 2012.

**Grimonprez A**, Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. Knowledge for Growth, Belgium, ICC Ghent, May 2013.

**Grimonprez A,** Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. Student onderzoekssymposium, Belgium, UZ Ghent, April 2014.

**Grimonprez A,** Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. Vagus nerve stimulation has antidepressant potential in the kainic acid model for temporal lobe epilepsy. European Congress on Epileptology, Sweden, Stockholm, June-July 2014.

**Grimonprez A**, De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P, Vonck K. Dose-dependent laryngeal muscle evoked potentials as an indicator of effective vagus nerve stimulation. Belgian Brain Council, Belgium, Ghent, October 2014.

**Grimonprez A**, De Taeye L, Delbeke J, Larsen LE, Raedt R, Boon P, Vonck K. Dose-dependent laryngeal muscle evoked potentials as an indicator of effective vagus nerve stimulation. Belgian society of physiology and pharmacology, autumn meeting, Brussels, October 2014.

**Grimonprez A**, Raedt R, Portelli J, Dauwe I, Larsen LE, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. Kempenhaege Epilepsy symposium, The Netherlands, Heeze, March 2015.

## Awards

Master thesis: shortlist EOS scriptieprijs

Belgian Brain congress 2014: including 10 best posters

## Teaching and students

- Hands-on epilepsy workshop on EEG and design of electrodes, 1° Master Students Biomedical Sciences
- Preparation research internship – Lise Troch (2012-2013).
- Introduction course on epilepsy for master students Biotechnology.
- Master thesis Lise Troch: The role of the locus coeruleus in the antidepressant effect of vagus nerve stimulation (2013-2014)
- Internship Charlotte Bouckaert: Research on the mechanism of action of vagus nerve stimulation (2014-2015)
- Introduction course on (pre)clinical research, for Biomedical master students
- Lesson on epilepsy research at Ghent University, for Biology students

## Courses and varia

- ICH-GCP qualification training course and examination, international survey of regulatory requirements concerning clinical research
- Course in laboratory animal science
- Basis statistics course in SPSS 19 (January 2011)
- Advanced Epilepsy course (2012)
- Advanced statistics course in SPSS 20 (September 2012)
- Course on power and sample size calculation (November 2012)

- Course on Advanced academic English, writing skills. Belgium, UZ Ghent (February-may 2013)
- Course on species specific animal welfare in the lab. Ghent, Belgium, Faculty of Veterinary Medicine Ghent University (May 2014)
- Scientific services, reviewer for scientific publication
- Member of the animal welfare cell of the Ghent University Ethics committee