“My function’s almost anything, and vagus is my name”

Faculty of Medicin and Health Sciences
Department of Neurology
Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology

Lies Mollet

The role of the locus coeruleus and the hippocampal noradrenergic neurotransmission in the antiepileptic effect of vagus nerve stimulation

Thesis submitted in fulfillment of the requirements
for the degree of doctor (PhD) in Medical Sciences

Onderzoek gefinancieerd met een aspirantenbeurs van het Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-Vlaanderen)

Research funded by a PhD grant of the “Fonds voor Wetenschappelijk Onderzoek” Flanders (FWO-Flanders)

Members of the Jury

Promoter:	**Prof. Dr. Kristl Vonck** Ghent University, Belgium
Co-Promoter:	**Prof. Dr. Robrecht Raedt** Ghent University, Belgium
Examination/reading board:	**Prof. Dr. Hermann Stefan** University of Erlangen, Germany
	Prof. Dr. Lieven Lagae University of Leuven, Belgium
	Prof. Dr. Stefanie Dedeurwaerdere University of Antwerp, Belgium
	Prof. Dr. Stefaan Vandenberghe Ghent University, Belgium
	Prof. Dr. Chris Baeken Ghent University, Belgium
	Prof. Dr. Dirk Van Roost Ghent University, Belgium
	Dr. Evelien Carrette Ghent University, Belgium

Table of contents

List of abbreviations

AED	Antiepileptic Drug
CA	Cornu Ammonis
CAP	Compound Action Potential
CE	Conformité Européenne
CNM	Center for Neurophysiological Monitoring
DbH	Dopamine-β-Hydroxylase
DBS	Deep Brain Stimulation
DRN	Dorsal Raphe Nucleus
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EEG	Electro-Encephalo-Graphy
ILAE	International League Against Epilepsy
FDA	Food and Drug Administration
LC	Locus Coeruleus
LMEP	Larynx Muscle Evoked Potential
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MES	Maximal Electro Shock
MRI	Magnetic Resonance Imaging
MTLE	Mesial Temporal Lobe Epilepsy
MST	Motor Seizure Threshold
NP-Y	Neuropeptide Y
NTS	Nucleus Tractus Solitarius
PET	Positron Emission Tomography
PGi	Nucleus Paragigantocellularis
PrH	Nucleus Prepositus Hypoglossi
PTZ	Pentylenetetrazol

rTMS	repetitive Transcranial Magnetic Stimulation
SE	Status Epilepticus
SPECT	Single Photon Emission Computed Tomography
SSS	Seizure Severity Score
tDCS	transcranial Direct Current Stimulation
TLE	Temporal Lobe Epilepsy
TNS	Trigeminal Nerve Stimulation
TSSS	Total Seizure Severity Score
VNS	Vagus Nerve Stimulation
5-HT	Serotonin

Outline of the thesis

Chapter 1: “General introduction on epilepsy”. This chapter provides a general introduction on epilepsy, refractory epilepsy and its treatment options. The animal models used in this thesis are described.

Chapter 2: “Vagus nerve stimulation for refractory epilepsy”. This chapter provides an introduction on the cervical anatomy and physiology of the vagus nerve, the antiepileptic mechanism of action of vagus nerve stimulation (VNS), the VNS stimulation parameters and the efficacy and safety of the VNS therapy.

Chapter 3: “Vagus nerve stimulation and the central noradrenergic pathway”. This chapter describes the central noradreneregic pathway as an important contributor in the antiepileptic effect of VNS.

Chapter 4: “Rationale and research aims of the thesis”.

Chapter 5: “Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model”. This study addresses the role of noradrenaline in the antiepileptic effect of VNS. VNS-induced changes in hippocampal noradrenaline levels are measured and its potential involvement in the antiepileptic action of VNS is determined in an animal model for limbic seizures.

Chapter 6: “Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability”. This study investigates the effect of various VNS output current intensities on cortical excitability in the motor cortex stimulation rat model. The hypothesis that output current intensities in the lower range are sufficient to affect cortical excitability is evaluated.

Chapter 7: “Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats”. The goal of this study is to determine an objective parameter that can be used as an indicator of effective VNS-induced activation of the vagus nerve in rats.

Chapter 8: “Electrophysiological responses from vagus nerve stimulation in rats”. In this study, stimulation-induced vagus nerve electrophysiological responses are measured using various stimulation parameters in rats, in order to determine a biological marker reflecting true vagal fiber activation when electrical stimulation is applied to the vagus nerve.

Chapter 9: “Conclusion, discussion and future perspectives”.

Chapter 1

General introduction on epilepsy

Definition and epidemiology

According to the definition of the International League Against Epilepsy (ILAE), epilepsy is a chronic neurological condition characterized by the occurrence of recurrent, usually unprovoked epileptic seizures (Fisher et al. 2005). An epileptic seizure represents the signs and symptoms that result from excessive, synchronous, abnormal firing patterns of groups of neurons which are usually, but not necessarily, located in the cerebral cortex (Seino 2006). The clinical manifestation consists of a sudden and transitory abnormal phenomenon which may include alterations of consciousness, motor, sensory, autonomic or psychic events (Engel 2006a). The occurrence of a single seizure does not make a person have epilepsy, but when two or more seizures occur, the diagnosis of epilepsy can be made (Boon et al. 1996).

Epilepsy is the second most common serious neurological disorder following neurovascular diseases, with a worldwide prevalence of approximately 0.5-1% (Sander and Shorvon 1996; Banerjee and Hauser 2008). It is estimated that the annual incidence of new onset epilepsy in the general population is more than 80 per 100,000 (Hirose 2013). Epilepsy affects both sexes and the incidence rate is age related. The highest incidence rate is observed in the first year of life. Incidence is lowest during adult years and increases in the elderly as a result of the higher prevalence of cerebrovascular disorders (Hauser et al. 1996).

Diagnosis and classification

The initial diagnosis of epilepsy is based on clinical symptoms, the medical history and Electro-Encephalo-Graphic (EEG) recordings. A correct diagnosis of epilepsy includes the identification of the epileptic focus in the brain cortex and the type(s) of epileptic seizure(s) the patient is suffering from. Different types of epileptic seizures can occur within a single epilepsy syndrome. Classifying epileptic seizures is challenging, as they are numerous and diverse in their presentation, underlying pathophysiology, age relationships, prevalence and triggering factors (Panayiotopoulos 2007).

The most universally accepted classification of epileptic seizures is the International Classification of Seizures that was developed by the Commission on Classification of the ILAE (Commission on Classification 1981). Epileptic seizures can be divided into two main categories depending on their onset in the brain: those that are partial in onset and those that have a generalized onset. *Partial or*

focal seizures are the result of an abnormal paroxysmal discharge originating in one cerebral hemisphere. They can be subclassified as simple - with retention of consciousness - and complex - with impairment or complete loss of consciousness. The clinical presentation of a seizure depends on the localization of the ictal onset in the brain, as well as the pattern of propagation. Seizures can affect sensory, motor and autonomic function, consciousness, emotional state, memory, cognition and behaviour (Panayiotopoulos 2007). Both simple and complex partial seizures may gradually develop into secondary generalized seizures when epileptic activity spreads to the contralateral hemisphere. *Primary generalized seizures* differ from focal seizures in clinical presentation, aetiology, neuroanatomy and neurobiology. They result from abnormal paroxysmal discharges arising in both cerebral hemispheres and can be subclassified as tonic-clonic seizures, absences and myoclonic seizures. Tonic-clonic seizures are often preceded by an aura and are characterized by an intial tonic and a subsequent clonic phase. During the tonic phase, the patient's muscles stiffen and they lose consciousness. During the subsequent clonic phase, the individual's muscles begin to spasm and jerk. Postictally stupor, confusion, autonomic behaviour and sleep may occur (Zifkin and Dravet 2008). Absence seizures, sometimes referred to as petit mail seizures, are brief genezalized seizures of sudden onset and termination, characterized by loss of consciousness and bursts of spike-wave discharges on the EEG (Panayiotopoulos 2007). Children between 4-12 years old are most susceptible to absences seizures. Myoclonic seizures manifest as involuntary, shock-like and often arrhythmic unidirectional movements that can be focal, multifocal or generalized (Panayiotopoulos 2007). They usually occur without detectable loss of consciousness.

The ILAE Commission on Classification also devised a classification of epilepsy syndromes (Commission on Classification 1989). According to the underlying presumptive cause, epilepsy syndromes can be divided into symptomatic, idiopathic and cryptogenic. *Symptomatic epilepsy syndromes* arise as a result of structural or metabolic abnormalities in the brain, which can be either acquired (e.g. infections, trauma), endogenous (e.g. neoplasm) or genetic (e.g. tuberous sclerosis) in origin. *Idiopathic epilepsy syndromes* are without an identifiable structural abnormality or aetiology, and are thought to be genetic in origin. *Cryptogenic epilepsy syndromes* are presumably symptomatic but currently of unknown aetiology.

The ILAE has recently proposed a new categorization of epilepsy syndromes based on the aetiology (Berg et al. 2010). Instead of the terms symptomatic, idiopathic and cryptogenic, epilepsy syndromes can be divided into genetic, structural/metabolic and of unknown cause. The concept of *genetic epilepsy* is that the epilepsy is, as best as understood, the direct result of a known or presumed genetic defect in which seizures are the core symptom of the disorder. For *structural/metabolic epilepsy*, there is a distinct other structural or metabolic condition or disease that has been

demonstrated to be associated with a substantially increased risk of developing epilepsy in appropriately designed studies. The term *unknown cause* is used to designate that the nature of the underlying cause is as yet unknown.

Once the initial diagnosis of epilepsy is made, additional tools such as long-term video-EEG monitoring, Magnetic Resonance Imaging (MRI), Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) can help to discover the aetiology of the epilepsy, to determine the affected brain region, to (sub)classify the epilepsy syndrome and to determine an appropriate treatment, especially in patients with pharmacoresistant epilepsy.

Pathophysiology

Within the normal brain, a constant equilibrium exists between excitation and inhibition. Epileptic seizures are generally believed to arise as a result of an imbalance between excitation and inhibition (in favour of excitation), making the cortex prone to sudden hyperexcitable and uncontrolled electrical activity. Epileptogenesis refers to a dynamic process of pathological changes that progressively alters neuronal excitability and transforms a normal healthy brain into an epileptic brain (Pitkänen and Lukasiuk 2011). These pathological changes include neurodegeneration, neurogenesis, gliosis, axonal damage or sprouting, dendritic plasticity, blood brain barrier damage and recruitment of inflammatory cells into brain tissue.

Treatment

Uncontrolled epilepsy is associated with excess injury and mortality, and increased adverse psychosocial, behavioural and cognitive consequences, resulting in a low quality of life and an enormous burden of economic costs (Tomson et al. 2004; Wirrell 2006; Cardarelli and Smith 2010). Epilepsy treatment is generally initiated after a second unprovoked seizure. The goal of medical treatment in epilepsy is to achieve seizure freedom without inducing unwanted side-effects. Antiepileptic Drugs (AEDs) are the primary option for the management of epilepsy. Kwan and Brodie have shown that the first AED leads to seizure freedom in 47% of patients with newly diagnosed epilepsy. Thirteen percent of patients are seizure free with the second AED, and only 1% after switching to a third AED (Kwan and Brodie 2001). The ILAE defined pharmacoresistant or refractory epilepsy as “failure of adequate trials of two tolerated, appropriately chosen and used AED schedules, whether as monotherapy or in combination, to achieve sustained seizure freedom” (Kwan et al. 2010). Patients with refractory epilepsy require a thorough diagnostic and therapeutic evaluation in a specialized epilepsy center. For these patients, alternative treatment options include

the administration of newly developed AEDs, epilepsy surgery, gamma knife radiosugery, dietary treatments, immune-based therapies and neurostimulation.

Newly developed AEDs

The least invasive alternative treatment for refractory epilepsy consists of including patients in trails with newly developed AEDs. Administration of newly developed AEDs leads to seizure freedom in only 6% and to 50% seizure frequency reduction in only 21% of refractory patients (Fisher 1993; Beyenburg et al. 2010).

Epilepsy surgery

Epilepsy surgery is an invasive but often curative treatment option that involves the resection or disconnection of the epileptogenic zone, believed to be responsible for seizure occurrence, in order to eliminate seizures (Spencer 2002). Epilepsy surgery is considered when the epileptogenic zone can be identified and is located in a brain area that does not cause a functional deficit when removed. Epilepsy sugery is a successful treatment for patients with focal epilepsy with long-term seizure freedom of 40-75% (Wiebe et al. 2001; Engel et al. 2003; Cohen-Gadol et al. 2006; de Tisi et al. 2011).

Gamma knife radiosurgery

In gamma knife radiosurgery, specialized equipment focuses gamma radiation to well-defined small volumes of brain tissue, rendering it less seizure-prone (Romanelli and Anschel 2006). Cortical structures can be targeted with gamma knife radiosurgery with a stereotactic precision, without opening the skull. The precision of gamma knife radiosurgery results in minimal damage to healthy tissue surrounding the epileptogenic zone. Outcome in terms of seizure control is variable, but good safety profiles are reported (Regis et al. 2004; Chang et al. 2010; Romanelli et al. 2012).

Dietary treatments

The ketogenic diet encourages the intake of excessive amounts of fat (80% of daily meal), adequate amounts of protein (15% of daily meal) and low amounts of carbohydrate (5% of daily meal). The aim of the diet is to force the body to find an alternative energy source due to carbohydrate restriction. Stored body fat is metabolized in the liver and as a final product, ketones are released into the circulation and used in the brain as an alternative energy source (Huffman and Kossoff 2006). The presumed correlation between seizure reduction and ketone increase remains unproven. Acute side effects that can occur immediately upon starting the diet include nausea and vomiting, hypoglycemia, excessive ketosis and acidosis. Chronic implications include constipation, renal stones, cardiomyopathy, weight loss, retarded growth, high cholesterol levels, higher chances of infection,

specific vitamin and/or mineral deficiencies and rarely pancreatitis. But the most common reason for discontinuation of the diet is felt to be the “too restrictive nature” of the diet (Dhamija et al. 2013). Long-term prospective studies show a reduction of seizure frequency of more than 90% in one third of the patients who are able to continue the diet (Freeman et al. 1998, 2006). Because of the high chance of side effects, the ketogenic diet is only used for catastrophic childhood epilepsy, such as epileptic encephalopathies (Dhamija et al. 2013).

The Atkins’diet is a variant of the ketogenic diet and was initially developed for the purpose of weight loss (Carrette et al. 2008). The Atkins’ diet also encourages the intake of fat and the restriction of carbohydrates, but the daily allowed amount of protein is higher compared to the ketogenic diet. The Atkins’ diet allows meals containing 60% fat, 30% protein and 10% carbohydrates. Because of strong carbohydrate restriction, patients following the Atkins’ diet also produce ketones. The main reasons why patients stop the Atkins’s diet are inefficacy, side effects and restrictiveness (Kossoff et al. 2008).

Immune-based therapies

Immune system dysfunction may play a role in epilepsy by triggering or maintaining epileptic seizures. Immunoglobulin treatment as a result may have a beneficial effect on epileptic seizures. The suggested mechanism of action of immunoglobulin treatment for refractory epilepsy has been related to (i) the compensation of possible immunoglobulin deficiencies, (ii) the suppression of infections, (iii) the neutralization of pathogenic autoantibodies and (iv) the interference with cytokine production (Villani and Avanzini 2002). Because of the lack of double-blind controlled clinical studies, no definite conclusions can be made concerning efficacy and safety of this approach.

Treatment with corticosteroids is a primary treatment option for children with West Syndrome (Arya et al. 2012). This devastating epilepsy syndrome is characterized by infantile spasms and is poorly responsive to conventional antiepileptic medications. The exact mechanism of action of corticosteroid treatment is unknown, although many putative mechanisms have been reported. Corticosteroids have several regulatory effects on growth of neuroblasts, myelination and metabolism in the developing brain (Hrachovy and Frost 2008). In addition, corticosteroids have been demonstrated to influence important enzymes and growth factors in the developing cerebrum in animals (Molteni et al. 2001) and more than 200 steroid-responsive genes have been identified in the rat hippocampus involved in axonogenesis, synaptogenesis, cell adhesion and signal transduction (Vreugdenhil et al. 2001).

Neurostimulation

Neurostimulation-based treatments for epilepsy have gained considerable interest in the last decade. Electrical pulses are administered directly to or in the vicinity of nervous tissue in order to prevent or suppress seizure occurrence. Various neurostimulation strategies have been developed targeting different parts of the nervous system in an invasive or non-invasive way (Bagary 2011; Bergey 2013; DeGiorgio and Krahl 2013). Among these are Deep Brain Stimulation (DBS), repetitive Transcranial Magnetic Stimulation (rTMS), transcranial Direct Current Stimulation (tDCS), Trigeminal Nerve Stimulation (TNS) and Vagus Nerve Stimulation (VNS).

Deep Brain Stimulation

DBS involves the intracranial implantation of one or more electrodes in a selected brain region. Via an implanted pulse generator and a subcutaneous lead, electrical pulses are sent to specific parts of the brain to interfere with the neural activity of the target site. DBS has clear therapeutic benefits for treatment-resistent movement disorders and is currently being explored for a variety of other neurological diseases, such as refractory epilepsy (Gwinn and Spencer 2004; Vonck et al. 2013). Despite the long history of DBS, its underlying principles and mechanisms remain to be elucidated.

Repetitive Transcranial Magnetic Stimulation

TMS is an extracranial, non-invasive and generally well-tolerated form of cortical stimulation. The basic principle of TMS is the application of short magnetic pulses over the scalp of the patient with the aim of inducing electrical currents in the neurons of the cortex. A typical TMS device consists of a stimulator that generates a strong electrical current, and a coil in which fluctuating electrical currents generate magnetic pulses. If the magnetic pulses are delivered in the proximity of a conductive medium, e.g. the brain, a secondary current in the conductive medium is induced (Kobayashi and Pascual-Leone 2003). Continuing progress on the technical aspects of TMS devices made it possible to deliver multiple pulses within a short time period, i.e. rTMS. rTMS produces effects that outlast the stimulation duration by increasing or decreasing the excitability of neuronal networks. Changes in rTMS frequency and stimulation patterns can result in varying long-term effects. High-frequency stimulation (> 3 Hz) generally results in a synaptic facilitation - an effect that shares similarities with Long-Term Potentiation (LTP), while low-frequency rTMS (≤ 1 Hz) induces a reduction of synaptic efficiency - an effect that shares similarities with Long-Term Depression (LTD) (Fitzgerald et al. 2006; Gersner et al. 2011). Antiepileptic properties of rTMS were previously studied, but results of randomized, double-blind and sham controlled studies show diverting results (Theodore et al. 2002; Cantello et al. 2007). For this reason, rTMS has not yet been widely adopted as a treatment for refractory epilepsy.

Transcranial Direct Current Stimulation

tDCS is a form of neurostimulation in which electrical currents (1-2 mA) are delivered to the scalp via anodal or cathodal electrodes. tDCS selectively modulates cortical excitability and has been shown to affect a range of motor, somatosensory, visual, affective and cognitive functions (Been et al. 2007). Future research will point out whether this treatment can be used as a new neurostimulation treatment for refractory epilepsy.

Trigeminal Nerve Stimulation

TNS is an extracranial form of neurostimulation, in which the superfacial location of trigeminal nerve branches allows for minimally invasive stimulation approaches (DeGiorgio et al. 2009). Animal and human data demonstrate that stimulation of the trigeminal nerve inhibits seizures (Fanselow et al. 2000; DeGiorgio et al. 2011). The presumed antiepileptic mechanism of action involves activation of the reticular-activating system in the brainstem, thereby causing a desynchronization of thalamic and cortical activity and a generalized arousal (Fanselow et al. 2000).

Vagus Nerve Stimulation

VNS is an extracranial form of neurostimulation developed in the 1980s, where the left vagus nerve is stimulated in the neck area by means of a helical stimulation electrode connected to a subclavicularly implanted pulse generator (Ben-Menachem 2002). Following two randomized, double-blind controlled studies showing short-term efficacy and safety of VNS (Ben-Menachem et al. 1994; DeGiorgio et al. 2000), the Food and Drug Administration (FDA, USA) approved this treatment in 1997. Since then, over 60,000 patients have been implanted worldwide (Magdaleno-Madrigal et al. 2014). Despite the established efficacy and safety, the large number of treated patients and more than 20 years of experience with VNS, some specific issues about this treatment remain unresolved. Better comprehension of the antiepileptic mechanism of action of VNS could lead to the identification of certain seizure types and epilepsy syndromes that respond best to VNS and to the identification of responder characteristics. In addition, it may guide the search for a more appropriate choice of stimulation parameters.

A recently developed non-invasive alternative for VNS consists of stimulating the auricular branch of the vagus nerve and is called transcutaneous VNS (t-VNS). A recently performed pilot study of t-VNS for pharmacoresistant epilepsy indicates that t-VNS is safe, effective, well-tolerated and practicable for long-term treatment (Stefan et al. 2012). Based on these first promising clinical data concerning feasibility and safety, the t-VNS device received CE approval in 2012.

Temporal lobe epilepsy

Introduction

The term Temporal Lobe Epilepsy (TLE) is used to designate localization-related epilepsy syndromes of diverse aetiology, originating in the temporal lobe. Two main types can be distinguished: mesial TLE (MTLE) with seizure focus in medial temporal lobe structures (e.g. hippocampal formation) and the much rarer neocortical TLE with seizure focus in the neocortex. We will only describe MTLE.

MTLE is characterized by simple and/or complex focal seizures, with or without secondary generalization. Prior to the onset of their habitual partial seizures, patients commonly have a previous history of an initial precipitating insult such as febrile seizures, head trauma, congenital brain malformation, central nervous system infection, brain tumor or Status Epilepticus (SE) (Tatum 2012). Months or years following the initial neural damage, MTLE is developed (Arzimanoglou et al. 2002). MTLE accounts for approximately 30-35% of all epilepsies, and is the most prevalent type of epilepsy in adults (Panayiotopoulos 2007). More than 30% of MTLE patients are medically refractory (Pascual 2007).

Neuroanatomy of the hippocampal formation

The hippocampal formation is located in the dorsomedial part of the temporal lobe, just posterior to the amygdala (Fig.1). The hippocampal formation plays a major role in higher order brain functions, such as memory, spatial navigation, mood and attention control (Eichenbaum 2004; Squire 2004).

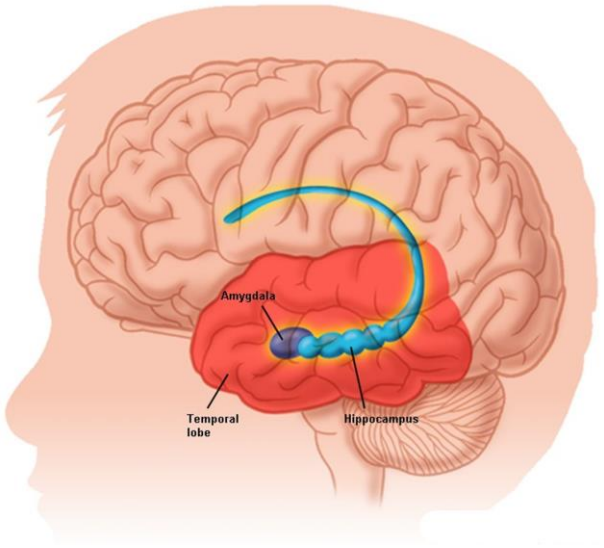


Figure 1| Location of the temporal lobe (red), amygdala (purple) and hippocampal formation (blue) **(adapted from www.brainconnection.com)**.

The hippocampal formation can be divided into several distinct regions: the dentate gyrus, the Cornu Ammonis (CA) fields CA1, CA2, CA3 and CA4/hilus (which underlies the dentate gyrus), and the subiculum (Fig.2). The main input to the hippocampal formation, which includes neocortical, subcortical, limbic and brainstem afferents, originates from the pyramidal cells of the entorhinal cortex and enters the hippocampus via the perforant path, which densely projects to the granule cells of the dentate gyrus and to the apical dendrites of the CA3 cells. Information flow through the hippocampus proceeds from the dentate gyrus to the CA3 via the mossy fiber pathway. The CA3 cells are connected to the CA1 cells via CA2 cells. There are also direct projections from the CA3 to the CA1 cells. The CA1 cells eventually target the subiculum. The cingulum bundle and fornix receive the main output from the hippocampus.

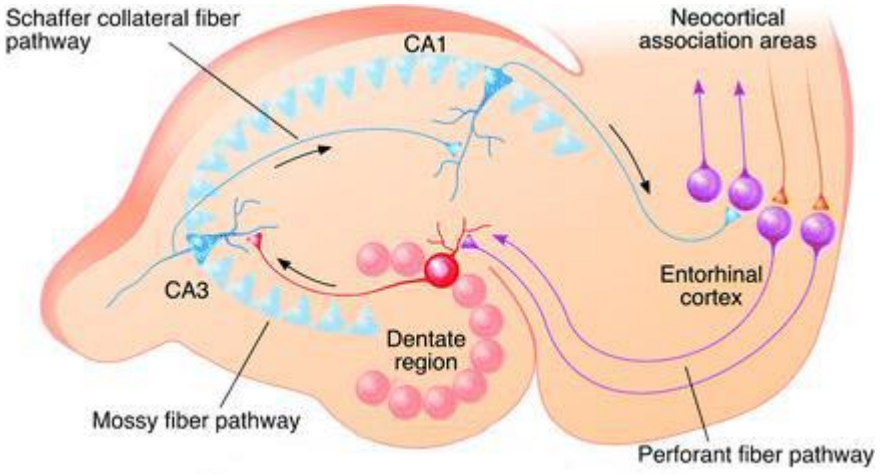


Figure 2| Illustration of the different regions of the hippocampal formation and information flow. Neurons of the dentate gyrus receive input from the entorhinal cortex via the perforant pathway, and extend projections into the CA3 via the mossy fiber pathway. Subsequent information flows to CA1 and the subiculum, from which extra-hippocampal areas are targeted **(adapted from (Lie et al. 2004))**.

The intrinsic connectivity of the hippocampal formation is more complicated than the simple input - trisynaptic circuit - output scheme. One single hippocampal neuron typically projects to several other neurons in its target area (Andersen et al. 2007). Hippocampal connectivity is further refined by the presence of (mainly inhibitory) interneurons in all areas of the hippocampal formation. These interneurons receive input from neurons which are located in the same hippocampal region, as well as in other (typically more proximal) hippocampal regions (Amaral et al. 2007). In summary, a complex network of serial and parallel excitatory and inhibitory connections is formed, in which every neuron influences the state of many other neurons, and is itself under control of many neurons.

There is still a lot of debate about the mechanisms underlying the development and intractability of MTLE, but it is generally accepted that the hippocampal formation plays a crucial role. One of the reasons for this is the fact that surgical removal of the hippocampal formation in MTLE patients leads to seizure freedom in 70% of cases (Engel 1992; Foldvary et al. 2000; Spencer 2002). Also depth EEG recordings and neuroimaging studies show that seizures in MTLE typically originate in the hippocampal formation (King and Spencer 1995; Vossler et al. 2004). Histological studies on tissue, obtained surgically from patients with intractable and unilateral MTLE, reveal specific changes which could underlie hyperexcitability of the removed structures. In 70% of cases, the distinctive neuropathological feature of MTLE is hippocampal sclerosis (Burton 1988; de Lanerolle and Lee 2005).

Hippocampal sclerosis

Hippocampal sclerosis is the most common type of neuropathological lesion identified in temporal lobectomy series in MTLE patients. Hippocampal sclerosis is frequently the result of a previous SE, encephalitis or an ischemic insult (French et al. 1993). However, also the seizures themselves can cause or aggravate hippocampal sclerosis (Sutula and Pitkänen 2001). Hippocampal sclerosis is characterized by selective neuronal loss and astrocytic gliosis in the CA1, CA3 and CA4 subfields, with relatively sparing of CA2 pyramidal cells and dentate granule cells (Burton 1988). Neuronal cell loss involves both glutamatergic excitatory neurons and GABAergic inhibitory interneurons. In response to this, disorganization and altered connectivity of hippocampal neurons are commonly observed - the axons of the mossy fibers that lost their targets innervate towards regions that they normally do not innervate. This process is called mossy fiber sprouting and is a presumed mechanism of increased excitability of the hippocampal circuit (Brandt et al. 2003). Mossy fiber sprouting was first observed in hippocampal tissue resected during epilepsy surgery in humans, but is also a typical feature of chronic animal models of MTLE (Scheibel et al. 1974; Cavazos et al. 1991; Mello et al. 1993; Mazarati et al. 2002).

Animal models of epilepsy

Introduction

Because of the ethical and experimental limitations inherent to studies in humans, animal models have been and still are essential to the study of human disease processes, including epilepsy. In order to better understand the mechanisms involved in seizure-initiation, epileptogenesis and spontaneous seizures, different animal models, that replicate some features of epileptic seizures and syndromes in

humans, have been developed. These models are valuable tools in the ongoing search for new treatments. It should however be pointed out that animal models are never more than an approximation of the disease process they emulate, and data derived from experimental animal studies should be interpreted with this caveat in mind. This is especially true for *in vitro* animal models, as these studies are usually carried out on tissue samples which have been physically removed from the rest of the brain, thereby severely compromising neuronal connectivity.

Different types of *in vivo* animal models of epilepsy exist. They can be subdivided into two main categories: (i) acute epilepsy models, which do not necessarily indicate the presence of an epileptic disorder and (ii) chronic epilepsy models, which are associated with permanent "epileptogenic" disturbances (Engel 2006b).

In acute animal models, seizures are evoked by audiogenic, electrical or chemical stimuli. These models are preferably used as an initial screening test for antiseizure potency of newly developed treatment strategies. Two of the most frequently used acute models are the Maximal Electro Shock (MES) model and the Pentylenetetrazol (PTZ) model. The MES model, in which bilateral transauricular or corneal electrodes are used, is used to search for compounds with activity against generalized tonic-clonic seizures (Walker et al. 2002). The PTZ model involves systemic injection of the convulsant and is used to discover drugs with efficacy against non-convulsive absence or myoclonic seizures (Löscher 2002).

Chronic epilepsy models more closely resemble the pathophysiology and epileptic state of human epilepsy (Mascott et al. 1994; Löscher 2002). Many chronic epilepsy models were created specifically to reproduce specific types of human epilepsy, particularly the most common form, i.e. MTLE (Wieser 2004). Some chronic epilepsy models are genetically predetermined to express spontaneous limbic seizures. One example is the Ihara epileptic rat, displaying neuronal microdysgenesis and gliosis in the hippocampus (Arai et al. 2003). In previously healthy rodents, spontaneous limbic seizures can be provoked by different types of insults such as brain infarction (Kelly et al. 2001), traumatic brain damage (Kharatishvili et al. 2006), febrile seizures (Dube et al. 2006), kindling (Goddard 1983) and SE. These chronic animal models of MTLE cover three phases: (i) the initial precipitating insult, (ii) a period of epileptogenesis during which molecular and structural changes occur and (iii) chronic epilepsy characterized by the occurrence of spontaneous, recurrent seizures (Morimito et al. 2004).

While they are clearly more suited to the study of many - especially chronic - aspects of MTLE compared to acute models, chronic models have a number of practical disadvantages. Because of the latency period between the initial epileptogenic insult and the occurrence of spontaneous seizures,

and because they require long periods of EEG and video monitoring, chronic models are more labor intensive and technically demanding compared to acute models. Also, the timing of seizures in these models is unpredictable, which makes them less suitable for certain purposes.

On the other hand, the most important shortcoming of acute seizure models is that seizures do not arise as a result of a pre-existing abnormality in the brain, but are artificially induced in normal brain tissue. A second shortcoming of acute seizure models is that they cannot be used to study chronic processes, such as epileptogenesis. Nevertheless, acute seizure models have proven to be very productive. Since seizures in acute models can be reliably induced at a time of the researcher's choosing, precise measurements can be performed at the moment of seizure induction and during seizure activity. Because of their relative ease of use and reliability, acute models are the first models used to screen potential antiepileptic compounds.

As a full review of all acute and chronic animal models of epilepsy is beyond the scope of this thesis, only the animal models used in this thesis will be described.

The motor cortex stimulation model

The motor cortex stimulation model is an acute, fast-screening animal model in which the threshold for evoking focal, motor seizures is determined by electrical stimulation of the motor cortex in unanaesthetized rats (Voskuyl et al. 1989; Liebetanz et al. 2006). Cortical stimulation typically is performed using a ramp-shaped pulse train with biphasic, rectangular pulses with increasing amplitude. The cortical stimulation train is interrupted when the first symptoms of a focal seizure are detected on visual inspection. The clinical expression of a focal seizure is typically a forelimb clonus. The motor seizure threshold (MST) is defined as the current intensity corresponding to the first clinical symptoms of a focal seizure. Compounds are considered to have an antiseizure effect when increasing the MST.

The intrahippocampal pilocarpine model of acute limbic seizures

The intrahippocampal pilocarpine model is an acute animal model of limbic seizures with or without secondary generalization (Millan et al. 1993; Smolders et al. 1997a,b,c), in which intrahippocampal microdialysis is used both as a tool for local drug delivery and as a sampling technique.

Microdialysis

Microdialysis is an invasive technique that is widely used in neuroscience research both as a drug delivery and sampling tool. A microdialysis probe is implanted in the tissue of interest and

continuously perfused with an aqueous solution that closely resembles the (ionic) composition of the surrounding tissue fluid. Not only the brain but various peripheral tissue types can be targeted.

The main design of the microdialysis probe is illustrated in figure 3 and resembles a concentric tube where the perfusion fluid enters through the inner tube, flows to the distal end, exits the inner tube and enters the space between the inner tube and the outer semipermeable membrane. This is where the "dialysis" takes place - small molecules can cross the semipermeable membrane by passive diffusion between the extracellular tissue fluid and the perfusate. The direction of the analyte flow is determined by the respective concentration gradient and allows the usage of microdialyses probes as a local delivery as well as a sampling tool. The dialysate leaving the probe is collected at certain time intervals for analysis.

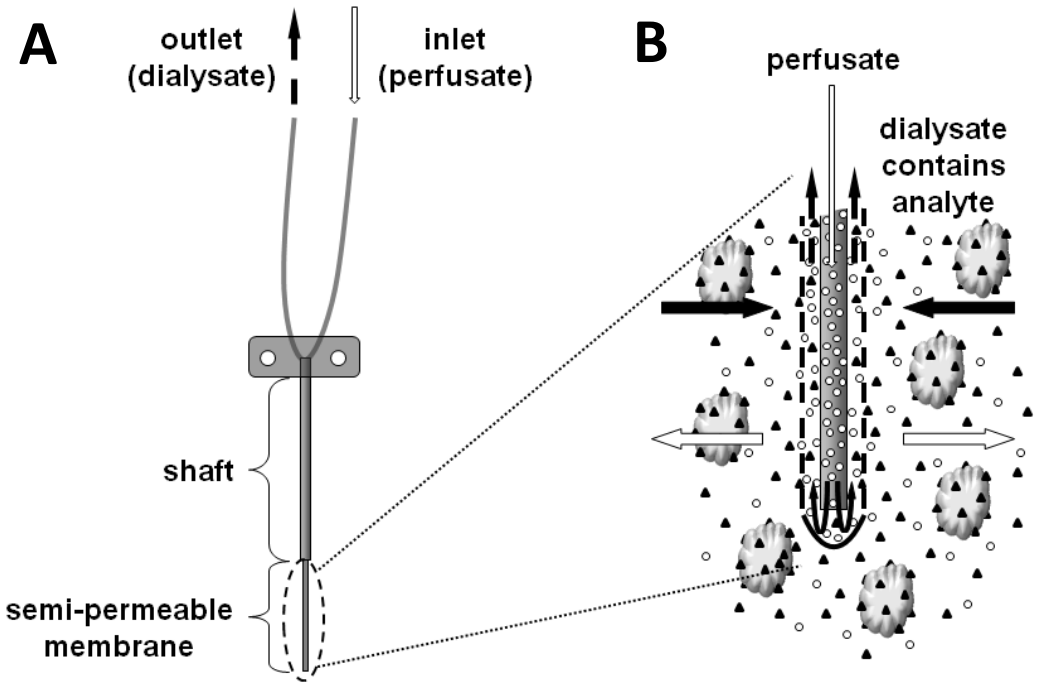


Figure 3| A: The main design of the microdialysis probe consists of (i) an inlet, via which the perfusion fluid enters the probe, (ii) a semi-permeable membrane, through which diffusion of molecules takes place between the perfusion fluid and the extracellular tissue fluid, and (iii) an outlet, via which the microdialysate is collected in a microvial to be analyzed. **B:** Detailed illustration of the microdialysis concentric tube **(adapted from Wikipedia)**.

The intrahippocampal pilocarpine model of acute limbic seizures

Limbic seizures are evoked in awake, freely moving animals by intrahippocampal perfusion of the non-selective muscarinic receptor agonist pilocarpine via the microdialysis probe. Pilocarpine-induced seizures are initiated via muscarinic receptors and maintained by NMDA receptor activation

(Maslanski et al. 1994; Smolders et al. 1997a,b,c). Video-EEG monitoring is used to assess the pilocarpine-induced seizure activity. Behavioural changes indicative of limbic seizure activity are rated on a seizure severity scale based on Racine's scale, which was adapted to include all behavioural changes observed in focal limbic seizure models: (0) normal, non-epileptic activity; (1): mouth and facial movements, hyperactivity, grooming, sniffing, scratching, wet dog shakes; (2) head nodding, staring, tremor; (3) forelimb clonus, forelimb extension; (4) rearing, salivating, tonic-clonic activity; (5) falling, status epilepticus. Typically, for each twenty minute interval following initiation of pilocarpine perfusion, the highest seizure severity score (SSS) is retained. Total seizure severity score (TSSS) is then calculated as the sum of the SSSs and used as a measure for seizure severity throughout the experiment. Hippocampal and cortical EEG recordings are used to determine the latency to occurrence of the first epileptiform activity (spikes) and the total duration of the epileptiform activity after the start of pilocarpine perfusion. Compounds, administered systemically or perfused simultaneously with pilocarpine through the microdialysis probe, are considered to have an antiepileptic effect when decreasing the TSSS, increasing the latency to occurrence of the first epileptiform activity and/or decreasing the total duration of the epileptiform activity.

Besides local drug delivery, microdialysis in the intrahippocampal pilocarpine model is also used to monitor compound-related or seizure-related biochemical changes concomitantly as the site of seizure induction. The collected dialysates from the intrahippocampal extracellular space can be analysed for a wide range of endogenous substances such as amino acids, monoamines, histamine, neuropeptides, hormones, ions, cyclic nucleotides, oxidative stress components and metabolic markers. As such, microdialysis in the intrahippocampal pilocarpine model is an elegant tool to elucidate the mechanism of action of compounds against limbic seizures and to gain insights into neuronal circuits involved in the generation, spread and control of pilocarpine-induced seizures. Increases in extracellular glutamate, GABA, dopamine and serotonin have consistently been observed during pilocarpine-induced limbic seizures (Smolders et al. 1997a,b,c, 2002, 2004; Khan et al. 1999, 2000; Lindekens et al. 2000; Meurs et al. 2006; Stragier et al. 2006).

References

Amaral DG, Lavenex P. Hippocampal Neuroanatomy. In: The Hippocampus Book (Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J, eds) 2007. Oxford, U.K.: Oxford University Press.

Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J. The Hippocampus Book, 1st Edition 2007. Oxford, U.K.: Oxford University Press.

Arai M, Amano S, Ryo A, Hada A, Wakatsuki T, Shuda M, Kondoh N, Yamamoto M. Identification of epilepsy-related genes by gene expression profiling in the hippocampus of genetically epileptic rat. Brain Res Mol Brain Res 2003;118(1-2):147-151.

Arya R, Shinnar S, Glauser TA. Corticosteroids for the treatment of infantile spasms: a systematic review. J Child Neurol 2012;27(10):1284-1288.

Arzimanoglou A, Hirsch E, Nehlig A, Castelnau P, Gressens P, Pereira de Vasconcelos A. Epilepsy and neuroprotection: an illustrated review. Epileptic Disord 2002;4(3):173-182.

Bagary M. Epilepsy, consciousness and neurostimulation. Behav Neurol 2011;24(1):75-81.

Banerjee PN, Hauser WA. Incidence and prevalence. In Epilepsy: a comprehensive textbook, 2nd Edition (Engel J, Pedley TA, eds.) 2008. Philadelphia, USA: Lippincot Williams & Wilkins.

Been G, Ngo TT, Miller SM, Fitzgerald PB. The use of tDCS and CVS as methods of non-invasive brain stimulation. Brain Res Rev 2007;56(2):346-361.

Ben-Menachem E, Mañon-Espaillat R, Ristanovic R, Wilder B, Stefan H, Mirza W, tarver W, Wernicke J. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 1994;35(3):616-626.

Ben-Menachem E. Vagus-nerve stimulation for the treatment of epilepsy. Lancet Neurol 2002;1(8):477-482.

Berg A, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshé SL, Nordli D, Plouin P, Scheffer IE. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 2010;51(4):676-685.

Bergey GK. Neurostimulation in the treatment of epilepsy. Exp Neurol 2013;224:87-95.

Beyenburg S, Stavem K, Schmidt D. Placebo-corrected efficacy of modern antiepileptic drugs for refractory epilepsy: systematic review and meta-analysis. Epilepsia 2010;51(1):7-26.

Boon P. De Deyn P, Hauman H, Mol L, Schmedding E, Vlietinck R, Willaert B. Epidemiologie van epileptische toevallen in Vlaanderen. Tijdschrift voor Geneeskunde 1996;52:47-52.

Brandt C, Glien M, Potschka H, Volk H, Loscher W. Epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats. Epilepsy Res 2003;55(1-2):83-103.

Burton CJ. The neuropathology of temporal lobe epilepsy 1988. Oxford, U.K.: Oxford University Press (Maudsley Monographs).

Cantello R, Rossi S, Varrasi C, Ulivelli M, Civardi C, Bartalini S, Vatti G, Cincotta M, Borgheresi A, Zaccara G, Quartarone A, Crupi D, Laganà A, Inghilleri M, Giallonardo AT, Berardelli A, Pacifici L, Ferreri F, Tombini M, Gilio F, Quarato P, Conte A, Manganotti P, Bongiovanni LG, Monaco F, Ferrante D, Rossini PM. Slow repetitive TMS for drug-resistant epilepsy: clinical and EEG findings of a placebo-controlled trial. Epilepsia 2007;48(2):366-374.

Cardarelli WJ, Smith BJ. The burden of epilepsy to patients and payers. Am J Manag Care 2010;16(S12):S331-S336.

Carrette E, Vonck K, De Herdt V, Dewaele I, Raedt R, Goossens L, Van Zandijcke M, Wadman W, Thadani V, Boon P. A pilot trial with modified Atkins' diet in adult patients with refractory epilepsy. Clin Neurol Neurosurg 2008;110(8):797-803.

Cavazos JE, Golarai G, Sutula TP. Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. J Neurosci 1991;11(9):2795-2803.

Chang EF, Quigg M, Oh MC, Dillon WP, Ward MM, Laxer KD, Broshek DK, Barbaro NM. Epilepsy Radiosurgery Study Group. Predictors of efficacy after SRS for medial temporal lobe epilepsy. Neurology 2010;74(2):165-172.

Cohen-Gadol AA, Wilhelmi BG, Collignon F, White JB, Britton JW, Cambier DM, Christianson TJ, March WR, Meyer FB, Cascino GD. Long-term outcome of epilepsy surgery among 399 patients with nonlesional seizure foci including mesial temporal lobe sclerosis. J Neurosurg 2006;104(4):513-524.

Commission on Classification. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the Commission on Classification and Terminology of the International Leage Against Epilepsy. Epilepsia 1981;22(4):489-501.

Commission on Classification. Proposal for revised classification of epilepsies and epileptic syndromes. From the Commission on Classification and Terminology of the International Leage Against Epilepsy. Epilepsia 1989;30(4):389-399.

DeGiorgio CM, Schachter SC, Handforth A, Salinsky M, Thompson J, Uthman B, Reed R, Collins S, Tecoma E, Morris GL, Vaughn B, Naritoku DK, Henry T, Labar D, Gilmartin R, Labiner D, Osorio I, Ristanovic R, Jones J, Murphy J, Ney G, Wheless J, Lewis P, Heck C. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia 2000;41(9):1195-2200.

DeGiorgio CM, Murray D, Markovic D, Whitehurst T. Trigeminal nerve stimulation for epilepsy: long-term feasibility and efficacy. Neurology 2009;72(10):936-938.

DeGiorgio CM, Fanselow EE, Schrader LM, Cook IA. Trigeminal nerve stimulation: seminal animal and human studies for epilepsy and depression. Neurosrug Clin N Am 2011;22(4):449-456.

DeGiorgio CM, Krahl SE. Neurostimulation for drug-resistant epilepsy. Continuum (Minneap Minn) 2013;19(3):743-755.

de Lanerolle NC, Lee TS. New facets of the neuropathology and molecular profile of human temporal lobe epilepsy. Epilepsy Behav 2005;7(2):190-203.

de Tisi J, Bell GS, Peacock JL, McEvoy AW, Harkness WF, Sander JW, Duncan JS. The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: a cohort study. Lancet 2011;378(9800):1388-1395.

Dhamija R, Eckert S, Wirrell E. Ketogenic diet. Can J Neurol Sci 2013;40(2):158-167.

Dube C, Richichi C, Bender RA, Chung G, Litt B, Baram TZ. Temporal lobe epilepsy after experimental prolonged febrile seizures: prospective analysis. Brain 2006;129(4):911-922.

Eichenbaum H. Hippocampus: cognitive processes and neural representations that underlie declarative memory. Neuron 2004;44(1):109-120.

Engel J Jr. Update on surgical treatment of the epilepsies. Clin Exp Neurol 1992;29:32-48.

Engel J Jr, Wiebe S, French J, Sperling M, Williamson P, Spencer D, Gumnit R, Zahn C, Westbrook E, Enos B. Practice parameter: temporal lobe and localized neocortical resections for epilepsy – Report of the quality standards subcommittee of the American Academy of Neurology, in association with the American Epilepsy Society and the American Association of Neurological Surgeons. Neurology 2003;60(4):538-547.

Engel J Jr. Report of the ILAE classification core group. Epilepsia 2006a;47(9):1558-1568.

Engel J Jr. What should be modelled? In: Models of Seizures (Pitkänen A, Schwartskroin P, Moshe S, eds) 2006b. Burlington, MA, U.S.A.; Elsevier Academic Press.

Fanselow EE, Reid AP, Nicolelis MA. Reduction of pentylenetetrazole-induced seizure activity in awake rats by seizure-triggered trigeminal nerve stimulation. J Neurosci 2000;20(21):8160-8168.

Fisher RS. Emerging antiepileptic drugs. Neurology 1993;43(11S5):S12-S20.

Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, Engel J Jr. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 2005;46(4):470-472.

Fitzgerald PB, Benitez J, De Castella A, Daskalakis ZJ, Brown TL, Kulkarni J. A randomized, controlled trial of sequential bilateral repetitive transcranial magnetic stimulation for treatment-resistant depression. Am J Psychiatry 2006;163(1):88-94.

Foldvary N, Nashold B, Mascha E, Thompson EA, Lee N, McNamara JO, Lewis DV, Luther JS, Friedman AH, Radtke RA. Seizure outcome after temporal lobectomy for temporal lobe epilepsy: A Kaplan-Meier survival analysis. Neurology 2000;54(3):630-634.

Freeman JM, Vining EP, Pillas DJ, Pyzik PL, Casey JC, Kelly L, and Millicent. The Efficacy of the ketogenic diet-1998: a prospective evaluation of intervention in 150 children. Pediatrics 1998;102(6):1358-1363.

Freeman J, Veggiotti P, Lanzi G, Tagliabue A, Perucca E. The ketogenic diet: from molecular mechanisms to clinical effects. Epilepsy Res 2006;68(2):145-180.

French JA, Williamson PD, Thadani VM, Darcey TM, Mattson RH, Spencer SS, Spencer DD. Characteristics of medial temporal lobe epilepsy: I. Results of history and physical examination. Ann Neurol 1993;34(6):774-780.

Gersner R, Kravetz E, Feil J, Pell G, Zangen A. Long-term effects of repetitive transcranial magnetic stimulation on markers for neuroplasticity: differential outcomes in anesthetized and awake animals. J Neurosci 2011;31(20):7521-7526.

Goddard GV. The kindling model of epilepsy. Trends in Neurosci 1983;6:275-279.

Gwinn RP, Spencer DD. Fighting fire with fire: brain stimulation for the treatment of epilepsy. Clin Neurosci Res 2004;4(1-2):95-105.

Hauser WA, Annegers JF, Rocca WA. Descriptive epidemiology of epilepsy: contributions of population-based studies from Rochester, Minnesota. Mayo Clin Proc 1996;71(6):576-586.

Hirose G. An overview of epilepsy: its history, classification, pathophysiology and management. Brain Nerve 2013;65(5):509-520.

Hrachovy RA, Frost JD Jr. Adrenocorticotrophic hormone and steroids. In: Epilepsy: A Comprehensive Textbook (Engel J Jr, Pedley TA, eds) 2008. Philadelphia, PA: Lippincott, Williams & Wilkins.

Huffman J, Kossoff EH. State of the ketogenic diet(s) in epilepsy. Curr Neurol and Neurosci Rep 2006;6(4):332-340.

Kelly KM, Kharlamov A, Hentosz TM, Kharlamova EA, Williamson JM, Bertram EH, III, Kapur J, Armstrong DM. Photothrombotic brain infarction results in seizure activity in aging Fischer 344 and Sprague Dawley rats. Epilepsy Res 2001;47(3):189-203.

Khan GM, Smolders I, Lindekens H, Manil J, Ebinger G, Michotte Y. Effects of diazepam on extracellular brain neurotransmitters in pilocarpine-induced seizures in rats. Eur J Pharmacol 1999;373(2-3):153-161.

Khan GM, Smolders I, Ebinger G, Michotte Y. Anticonvulsant effect and neurotransmitter modulation of focal and systemic 2-chloroadenosine against the development of pilocarpine-induced seizures. Neuropharmacology 2000;39(12):2418-2432.

Kharatishvili I, Nissinen JP, McIntosh TK, Pitkänen A. A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. Neuroscience 2006;140(2):685-97.

King D, Spencer S. Invasive electroencephalography in mesial temporal lobe epilepsy. J Clin Neurophysiol 1995;12(1):32-45.

Kobayashi M, Pascual-Leone A. Transcranial magnetic stimulation in neurology. Lancet Neurol 2003;2(3):145-156.

Kossoff EH, Rowley H, Sinha SR, Vining EPG. A prospective study of modified Atkins diet for intractable epilepsy in adults. Epilepsia 2008;49(2):316-319.

Kwan P, Brodie MJ. Effectiveness of first antiepileptic drug. Epilepsia 2001;42(10):1255-1260.

Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Hauser WA, Mathern G, Moshé SL, Perucca E, Wiebe S, French J. Definition of drug resistant epilepsy: consenses proposal by ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia 2010;51(6):1069-1077.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 2004;44:399-421.

Liebetanz D, Klinker F, Hering D, Koch R, Nitsche MA, Potschka H, Löscher W, Paulus W, Tergau F. Anticonvulsant effects of transcardial direct-current stimulation (tDCS) in the rat cortical ramp model of focal epilepsy. Epilepsia 2006;47(7):1216–1224.

Lindekens H, Smolders I, Khan GM, Bialer M, Ebinger G, Michotte Y. In vivo study of the effect of valpromide and valnoctamide in the pilocarpine rat model of focal epilepsy. Pharm Res 2000;17(11):1408-1413.

Löscher W. Animal models of epilepsy for the development of antiepileptogenic and disease-modifying drugs. A comparison of the pharmacology of kindling and post-status epilepticus models of temporal lobe epilepsy. Epilepsy Res 2002;50(1-2):105-123.

Magdaleno-Madrigal VM, Valdes-Cruz A, Martinez-Vargas D, Almazan-Alvarado S, Fernandez-Mas R. Effect of vagus nerve stimulation on electrical kindling in different stages of seizure severity in freely moving cats. Epilepsy Res 2014;108(1):81-89.

Mascott CR, Gotman J, Beaudet A. Automated EEG monitoring in defining a chronic epilepsy model. Epilepsia 1994;35(4):895-902.

Maslanski JA, Powelt R, Deirmengiant C, Patelt J. Assessment of the muscarinic receptor subtypes involved in pilocarpine-induced seizures in mice. Neurosci Lett 1994;168(1-2):225-228.

Mazarati A, Bragin A, Baldwin R, Shin D, Wilson C, Sankar R, Naylor D, Engel J, Wasterlain CG. Epileptogenesis after self-sustaining status epilepticus. Epilepsia 2002;43(S5):S74-S80.

Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, Finch DM. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. Epilepsia 1993;34(6):985-995.

Meurs A, Clinckers R, Ebinger G, Michotte Y, Smolders I. Substantia nigra is an anticonvulsant site of action of topiramate in the focal pilocarpine model of limbic seizures. Epilepsia 2006;47(9):1519-1535.

Millan MH, Chapman AG, Meldrum BS. Extracellular amino acid levels in hippocampus during pilocarpine-induced seizures. Epilepsy Res 1993;14(2):139-148.

Molteni R, Fumagalli F, Magnaghi V, Roceri M, Gennarelli M, Racagni G, Melcangi RC, Riva MA. Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. Brain Res Rev 2001;37(1-3):249-258.

Morimito K, Fahnestock M, Racine RJ. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 2004;73(1):1-60.

Panayiotopoulos CP. A clinical guide to epileptic syndromes and their treatment, 2nd Edition 2007. London: Spinger.

Pascual MR. Temporal lobe epilepsy: clinical semiology and neurophysiological studies. Semin Ultrasound CT MR 2007;28(6):416-423.

Pitkänen A, Lukasiuk K. Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol 2011;10(2):173-186.

Regis J, Rey M, Bartolomei F, Vladyka V, Liscak R, Schrottner O, Pendl G. Gamma knife surgery in mesial temporal lobe epilepsy: a prospective multicenter study. Epilepsia 2004;45(5):504-15.

Romanelli P, Anschel DJ. Radiosurgery for epilepsy. Lancet Neurol 2006;5(7):613-620.

Romanelli P, Striano P, Barbarisi M, Coppola G, Anschel DJ. Non-resective surgery and radiosurgery for treatment of drug-resistant epilepsy. Epilepsy Res 2012;99(3):193-201.

Sander JW, Shorvon SD. Epidemiology of the epilepsies. J Neurol Neurosurg Psychiatry 1996;61(5):433-443.

Scheibel ME, Crandall PH, Scheibel AB. The hippocampal-dentate complex in temporal lobe epilepsy. A Golgi study. Epilepsia 1974;15(1):55-80.

Seino M. Classification criteria of epileptic seizures and syndromes. Epilepsy Res 2006;70(S1):S27-S33.

Smolders I, Van Belle K, Ebinger G, Michotte Y. Hippocampal and cerebellar extracellular amino acids during pilocarpine-induced seizures in freely moving rats. Eur J Pharmacol 1997a;319(1):21-29.

Smolders I, Khan GM, Manil J, Ebinger G, Michotte Y. NMDA receptor-mediated pilocarpine-induced seizures: characterization in freely moving rats by microdialysis. Br J Pharmacol 1997b;121(6):1171-1179.

Smolders I, Bogaert L, Ebinger G, Michotte Y. Muscarinic modulation of striatal dopamine, glutamate, and GABA release, as measured with in vivo microdialysis. J Neurochem 1997c;68(5):1942-1948.

Smolders I, Bortolotto ZA, Clarke VR, Warre R, Khan GM, O'Neill MJ, Ornstein PL, Bleakman D, Ogden A, Weiss B, Stables JP, Ho KH, Ebinger G, Collingridge GL, Lodge D, Michotte Y. Antagonists of GLU(K5)-containing kainite receptors prevent pilocarpine-induced limbic seizures. Nat Neurosci 2002;5(8):796-804.

Smolders I, Lindekens H, Clinckers R, Meurs A, O'Neill MJ, Lodge D, Ebinger G, Michotte Y. In vivo modulation of extracellular hippocampal glutamate and GABA levels and limbic seizures by group I and II metabotropic glutamate receptor ligands. J Neurochem 2004;88(5):1068-1077.

Spencer SS. When should temporal-lobe epilepsy be treated surgically? Lancet Neurol 2002;1(6):375-382.

Squire LR. Memory systems of the brain: a brief history and current perspective. Neurobiol Learn Mem 2004;82(3):171-177.

Stefan H, Kreiselmeyer G, Kerling F, Kurzbuch K, Rauch C, Heers M, Kasper BS, Hammen T, Rzonsa M, Pauli E,Ellrich J, Graf W, Hopfengärtner R. Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: a proof of concept trial. Epilepsia 2012;53(7):e115-8.

Stragier B, Clinckers R, Meurs A, De Bundel D, Sarre S, Ebinger G, Michotte Y, Smolders I. Involvement of the somatostatin-2 receptor in the anti-convulsant effect of angiotensin IV against pilocarpine-induced limbic seizures in rats. J Neurochem 2006;98(4):1100-1113.

Sutula TP, Pitkänen A. More evidence for seizure-induced neuron loss: is hippocampal sclerosis both cause and effect of epilepsy? Neurology 2001;57(2):169-170.

Tatum WO. Mesial temporal lobe epilepsy. J Clin Neurophysiol 2012;29(5):356-365.

Theodore WH, Hunter K, Chen R, Vega-Bermudez F, Boroojerdi B, Reeves-Tyer P, Werhahn K, Kelley KR, Cohen L. Transcranial magnetic stimulation for the treatment of seizures: a controlled study. Neurology 2002;59(4):560-562.

Tomson T, Beghi E, Sundqvist A, Johannessen SI. Medical risks in epilepsy: a review with focus on physical injuries, mortality, traffic accidents and their prevention. Epilepsy Res 2004;60(1):1-16.

Villani F, Avanzini G. The use of immunoglobulins in the treatment of human epilepsy. Neurol Sci 2002;23(Suppl1):S33-S37.

Vonck K, Sprengers M, Carrette E, Dauwe I, Miatton M, Meurs A, Goossens L, De Herdt V, Achten R, Thiery E, Raedt R, Van Roost D, Boon P. A decade of experience with deep brain stimulation for patients with refractory medial temporal lobe epilepsy. Int J Neural Syst 2013;23(1): epub ahead of print.

Voskuyl RA, Dingemanse J, Danhof M. Determination of the threshold for convulsions by direct cortical stimulation. Epilepsy Res 1989;3(2):120–129.

Vossler DG, Kraemer DL, Haltiner AM, Rostad SW, Kjos BO, Davis BJ, Morgan JD, Caylor LM. Intracranial EEG in temporal lobe epilepsy: location of seizure onset relates to degree of mesiobasal limbic epilepsy. Epilepsia 2004;45(5):497-503.

Vreugdenhil E, de Kloet ER, Schaaf M, Datson NA. Genetic dissection of corticosterone receptor function in the rat hippocampus. Eur Neuropsychopharmacol 2001;11(6):423-430.

Walker MC, White HS, Sander JW. Disease modification in partial epilepsy. Brain 2002;125(Pt 9):1937-1950.

Wiebe S, Blume, Girvin JP, Eliasziw M. A randomized, controlled trial of surgery for temporal-lobe epilepsy. N Engl J Med 2001;345(5):311-318.

Wieser HG. Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. Epilepsia 2004;45(6):695-714.

Wirrell EC. Epilepsy-related injuries. Epilepsia 2006;47(S1):S453-S468.

Zifkin BJ, Dravet C. Generalized tonic-clonic seizures. In: Epilepsy: a comprehensive textbook, 2nd Edition (Engel J, Pedley TA, eds) 2008. Philadelphia, USA: Lippincot Williams & Wilkins.

Chapter 2

Vagus nerve stimulation for refractory epilepsy

Cervical anatomy, brainstem and brain hemisphere projections of the vagus nerve

The vagus nerve, or tenth cranial nerve, is the longest of the cranial nerves and a major component of the parasympathetic nervous system. The various branches of the vagus nerve mediate important visceral reflexes such as vomiting, coughing, swallowing and control of blood pressure and heart rate. Heart rate is mostly influenced by the right vagus nerve that has dense projections primarily to the atria of the heart (Saper et al. 1990). Sensory impulses from receptors in peripheral organs are transported via sensory vagal fibers to the central nervous system. The signals are processed within the central nervous system and postganglionic vagal efferent fibers are activated to target the appropriate effector organ. These autonomic reflexes occur unconsciously and guarantee normal daily functioning. The parasympathetic nervous system uses acetylcholine as its neurotransmitter and is considered to be protective, defensive and relaxing in its actions (Andrews and Lawes 1992).

Figure 4 gives a schematic overview of the vagus nerve at the cervical level, its projection to the brainstem Nucleus Tractus Solitarius (NTS) and its target organs. At the cervical level, the vagus nerve is a mixed cranial nerve consisting of both afferent sensory fibers, transferring information from the internal organs to the central nervous system, and efferent motor fibers, providing innervation of the laryngeal and pharyngeal muscles (via the recurrent laryngeal nerve that branches off from the vagus nerve at the level of the aortic arch) and parasympathetic outflow to the heart, lungs and abdominal organs. The afferent fibers far outnumber the efferent fibers and comprise approximately 65-80% of all cervical vagal fibers (Foley and DuBois 1937; Agostini et al. 1957; Paintal 1973). Nerve fibers can be subdivided into α, β, γ and δ types according to a decreasing fiber diameter (Erlanger and Gasser 1937). Alternatively, a classification in type ‘A’, ‘B’ and ‘C’ based on the conduction velocity and myelination has been proposed. Approximately 90% of the afferent and 70% of the efferent cervical vagal fibers are small, unmyelinated, high-threshold C-type fibers and a smaller portion are intermediate diameter, myelinated B-type fibers and large diameter, myelinated, low-threshold A-type fibers (Asala and Bower 1986). The link between fiber diameter, conduction velocity and the various components of action potentials has always been the basis for nerve fiber classification (Erlanger and Gasser 1937). However, due to the slightly different fiber contents in various nerves and species, and taking into account the physiological functions, a more complex classification is currently in use: Aα, Aβ, Aγ, Aδ, B and C (Manzano 2008). The fiber diameter and conduction velocity

of the different fiber types are summarized in table 1. Aα fibers subserve large motor functions and proprioception, Aβ fibers small motor movements, touch and pressure sensations, Aγ fibers muscle tone and reflexes, Aδ fibers temperature and sharp pain transmission, B fibers preganglionic autonomic control and C fibers postganglionic autonomic control, and nociception, temperature, pain, touch and pressure sensations.

Table 1| Fiber diameter (µm) and conduction velocity (m/s) of the different fiber types at the cervical level of the vagus nerve.

	Aα	**Aβ**	**Aγ**	**Aδ**	**B**	**C**
Fiber diameter (µm)	12-22	5-12	2-8	1-5	< 3	0.1-1.3
Conduction velocity (m/s)	70-120	30-70	15-30	5-30	3–15	0.6–2.0

Each nerve fiber is wrapped in a protective fibrous tissue sheath, the endoneurium. Different nerve fibers are bundled in fascicles, again surrounded by a protective fibrous tissue sheath, the perineurium. Several fascicles are bundled together with blood vessels within another sheath, the epineurium. At the cervical level, the vagus nerve is characterized by structural heterogeneity. Both the afferent and efferent A, B and C fibers are randomly organized within the fascicles and the fascicles are randomly organized within the nerve bundles (Krasteva et al. 2003; Vuckovic et al. 2008). Furthermore, the organization of the nerve fibers and fascicles at the cervical level of the vagus nerve is different from person to person.

The cell bodies of the efferent fibers are located in the dorsal motor nucleus and the nucleus ambiguous in the brainstem (Fig.4). Upon leaving the medulla, the efferent fibers extend through the jugular foramen and pass into the carotid sheath between the internal carotid artery and the internal jugular vein to provide innervation of their target organs. The afferent fibers run alongside the efferent fibers in the carotid sheet and have their cell bodies in the small jugular and the much larger nodose ganglion, located inside the tympano-occipital fissure and protruding peripherally from the jugular foramen, respectively. The afferent fibers pass through the jugular foramen and the tympano-occipital fissure and enter the lateral medulla as intracranial fiber bundles. Subsequently, they continue rostrocaudally as the tractus solitarius and terminate bilaterally in the rostral and caudal NTS (Kiernan 2009; Krahl and Clark 2012), a nuclear formation located in the dorsal lower brainstem (Koutcherov et al. 2004). The projections of the vagal afferent fibers to the NTS are predominantly ipsilateral (Norman and Bower 1982; Bohotin et al. 2003). The medial, ventral and lateral parts of the NTS predominantly receive input from visceral afferent A and B fibers (Kalia and

Sullivan 1982), whereas the caudal part of the NTS is a target for aortic, carotid sinus, cardiac, pharyngeal and pulmonary afferent B and C fibers (Andresen and Kunze 1994; Deuchars et al. 2000; Corbett et al. 2005; Bailey et al. 2006; Kubin et al. 2006).

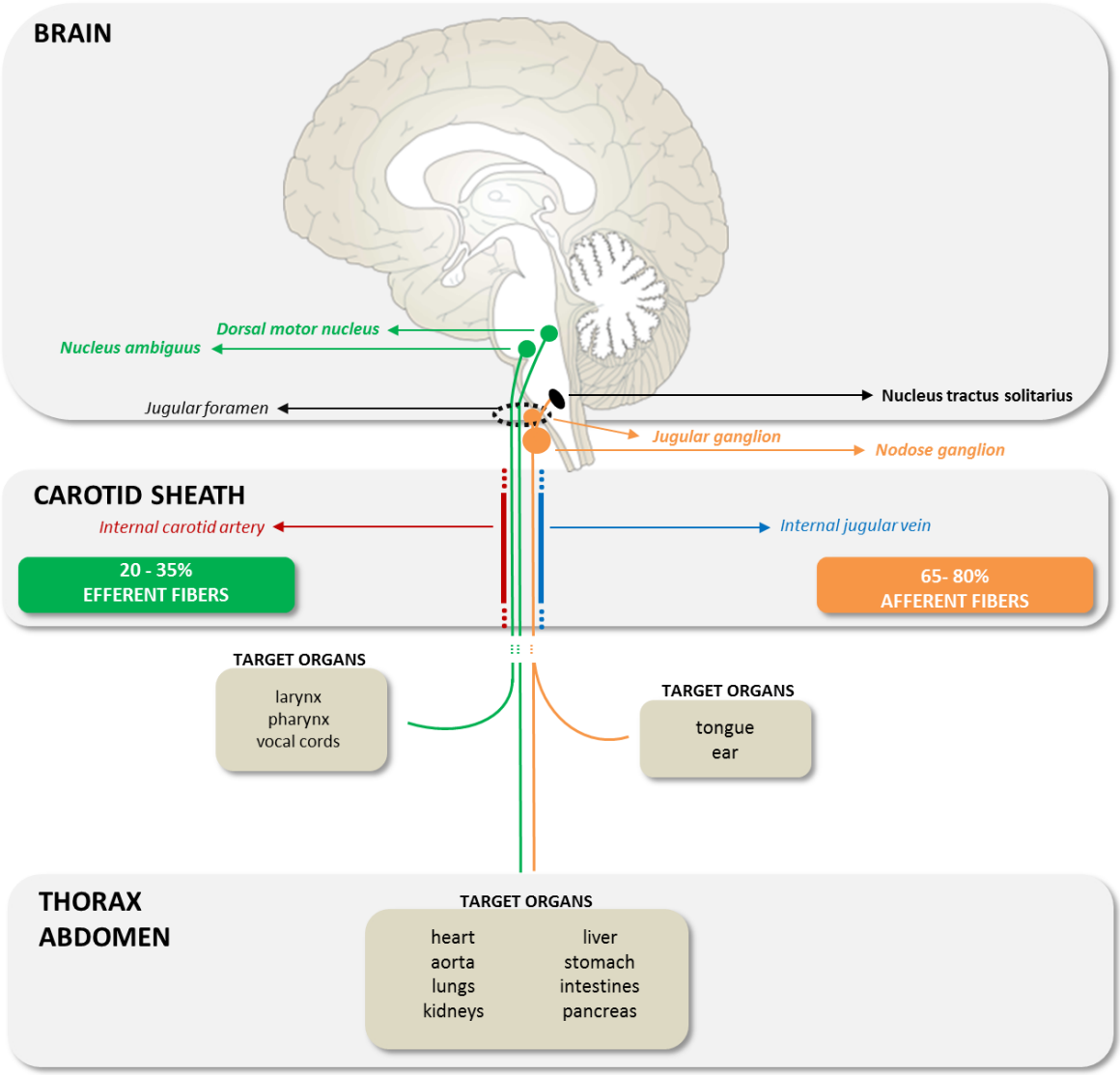


Figure 4| Schematic overview of the vagus nerve at the cervical level, its projection to the brainstem nucleus tractus solitarius and its target organs **(adapted from (Amaral 1999))**.

The NTS is connected to a large number of brainstem and intracerebral structures (Fig.5). The NTS sends out short monosynaptic projections to many bulbo-ponto-mesencephalic structures, such as the hypoglossal and trigeminal brainstem nuclei, the parabrachial nucleus, the reticular formation, the Dorsal Raphe Nucleus (DRN), the Nucleus Paragigantocellularis (PGi), the perifascicular area of the Nucleus Prepositus Hypoglossi (PrH), and the respiratory and cardiovascular centers (Bystrzycka and Nail 1985). The noradrenergic brainstem nucleus, the Locus Coeruleus (LC), receives both

monosynaptic and disynaptic projections from the NTS (Aston-Jones et al. 1991; Van Bockstaele et al. 1999). The disynaptic NTS-LC projections pass through the PGi and PrH. The caudal part of the NTS specifically projects to the nucleus ambiguous and the dorsal motor nucleus of the vagus nerve, areas where cardiac vagal preganglionic neurons are primarily located (Andresen and Kunze 1994; Deuchars et al. 2000; Corbett et al. 2005; Bailey et al. 2006; Kubin et al. 2006). Forebrain and limbic structures also receive monosynaptic NTS projections, in addition to the cerebellum, hypothalamus, thalamus and amygdala (Barnes et al. 2003).

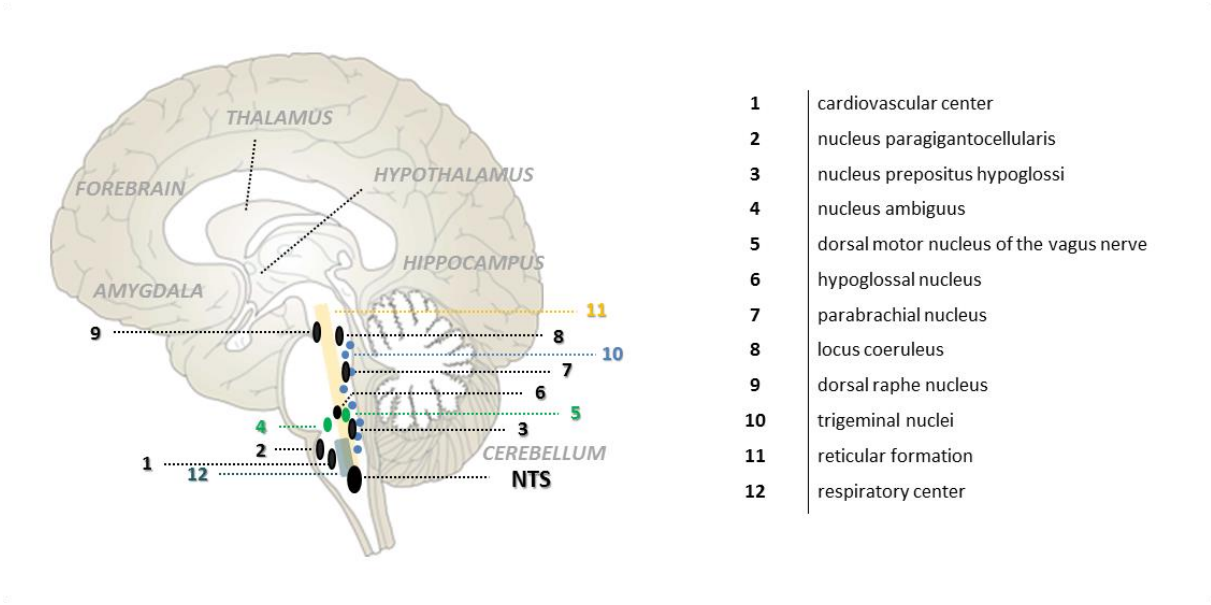


Figure 5| Schematic overview of the nucleus tractus solitarius (NTS) and its brainstem and intracerebral projection structures. The nuclei containing cell bodies of the efferent vagal fibers are presented in green **(adapted from (Amaral 1999))**.

Mechanism of action

In VNS therapy for refractory epilepsy, a bipolar, helical stimulation electrode is implanted around the left vagus nerve at the cervical level. Electrical pulses are administered to the afferent and efferent fibers of the vagus nerve via a subclavicularly implanted pulse generator and a subcutaneous lead (Fig.6).

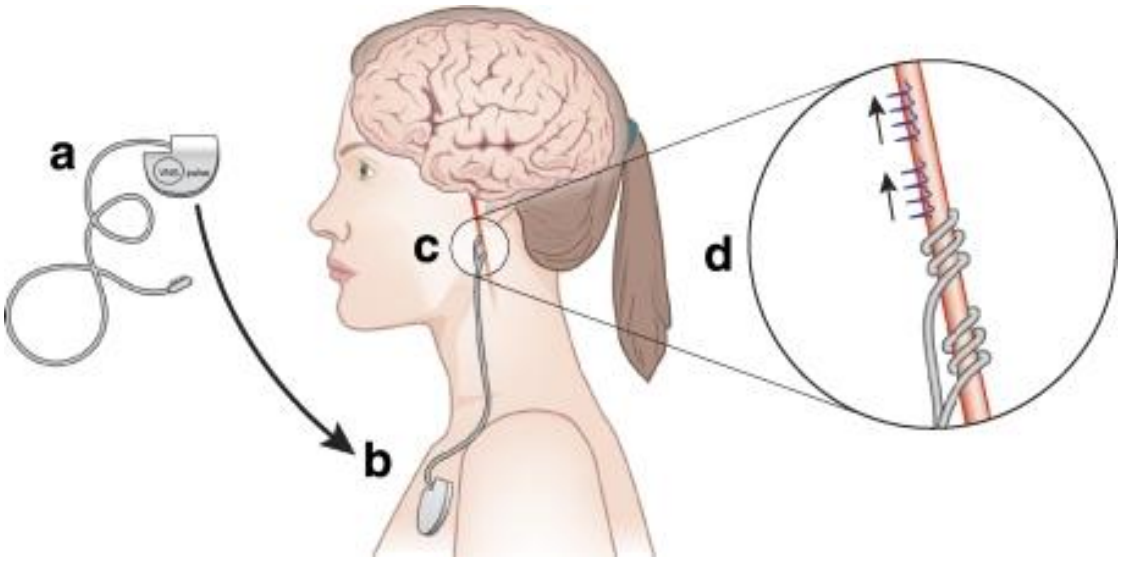


Figure 6| A: A programmable pulse generator is connected to a helical stimulation electrode via a lead. **B:** The programmable pulse generator is subcutaneously implanted at the level of the left clavicle. **C:** The bipolar, helical stimulation electrode is implanted around the left vagus nerve at the cervical level. **D:** Electrical pulses of sufficient strength induce action potentials in the vagus nerve fibers **(adapted from (George and Aston-Jones 2010))**.

As for many AEDs, clinical application of VNS preceded the research into its antiepileptic mechanism of action. Following a limited number of animal experiments in cats, dogs and monkeys, investigating safety and efficacy, the first human trial was performed in 1990 (Uthman et al. 1990). The basic hypothesis on the mechanism of action was based on the knowledge that the tenth cranial nerve afferents have numerous projections within the central nervous system and that in this way action potentials generated in vagal afferents have the potential to affect the entire organism (Berthoud and Neuhuber 2000). To date, the precise mechanism of action of VNS for refractory epilepsy remains to be elucidated.

Research on the mechanism of action of VNS occurs at different levels. The role of the different fiber types that constitute the vagus nerve at the cervical level in the antiepileptic effect of VNS requires further investigation. A next step is to identify the potential role of central nervous system structures that are located on the anatomical pathways from the cervical part of the vagus nerve up to the cortex. Within these central nervous system structures, the identification of specific neurotransmitter systems involved in the antiepileptic effect of VNS is an essential step in VNS research. Over the last few years, researchers have dedicated increasing attention to the role of the immune system in epilepsy and to the hypothesis that VNS could interfere with this process. The vagus nerve indeed plays a critical role in the signalization and modulation of inflammatory processes

(Borovikova et al. 2000; Hosoi et al. 2000) and this could thus represent a new modality in the mechanism of action of VNS for epilepsy.

Stimulation parameters

The vagus nerve is stimulated with biphasic, charge-balanced pulses. Programmable parameters of the pulse generator are output current, frequency, pulse width and ON/OFF times (Labiner and Ahern 2007) (Fig.7).

The *output current* of the pulse generator ranges between 0 and 3.5 mA. The output current is typically set at 0.25 mA at the start of the VNS therapy, and increased in 0.25-0.5 mA steps every 2-4 weeks as patient tolerability permits. The *frequency* of the pulse generator ranges from 1 to 30 Hz and is typically set at 30 Hz. The *pulse width* setting affects the level of output current required to stimulate the vagus nerve, and higher output currents may be needed for shorter pulse widths (Heck et al. 2002). The pulse width of the pulse generator ranges between 130 and 1000 μs. Typical values are 250 μs and 500 μs. Stimulation is administered intermittently, with alternating periods of programmed signal ON and OFF times. *Signal ON time* values range between 7-60 s. For *signal OFF times*, the range is 0.2-180 min. The choice of intermittent stimulation is based upon safety studies with regard to stimulation of neural tissue (Agnew and McCreery 1990), upon efficacy studies showing that the effect of stimulation outlasts the stimulus duration (Zabara 1992; Takaya et al. 1996; Santiago-Rodriguez et al. 2006; Carrette et al. 2007) and upon the knowledge that intermittent stimulation is associated with a longer battery life. Stimulation with an ON time longer than OFF time has resulted in degenerative nerve damage in laboratory animals (Agnew and McCreery 1990). An ON/OFF time of 30 s/5 min is typically used.

All output parameters of the pulse generator can be modified in order to reach maximum therapeutic efficacy, while minimizing treatment-emergent side effects and preserving battery life (Heck et al. 2002).

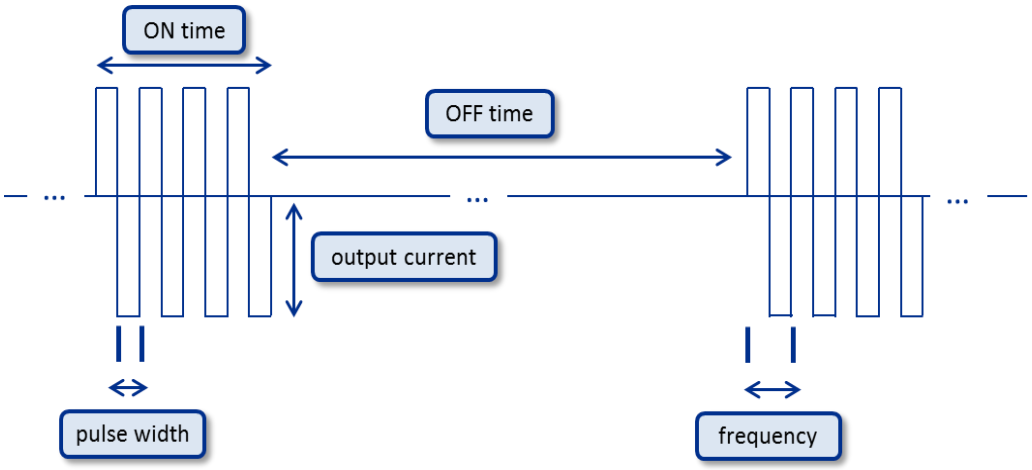


Figure 7| Pulse generator output parameters.

The stimulation parameters currently used in clinical practice are not evidence based and vary considerably. There is incomplete understanding of the neurobiological effects of different VNS parameters and there is a clear need for identification of the most optimal VNS parameter settings. Several preclinical and clinical studies suggest that lower current density might be sufficient to achieve clinical efficacy. These data may be relevant for VNS patients because these "new" parameters could minimize or prevent side effects and increase the battery life of the pulse generator.

Clinical efficacy

Following promising animal studies on the efficacy of VNS for epilepsy (Zabara 1992; McLachlan 1993), clinical trials were initiated in the early ninetees. Initial results from multicenter, single-blind pilot clinical trials (phase-1 trials EO1 and EO2) in a small group of patients (n=14) with refractory complex partial seizures showed a seizure reduction of at least 50% in 9/14 patients treated for 3-22 months (Uthman et al. 1990; Penry and Dean 1990; Wilder et al. 1991). It was noticed that a reduction in seizure frequency, duration and intensity lagged 4-8 weeks after initiation of VNS treatment (Uhman et al. 1990). Three years later, Uthman and coworkers published the long-term results from the EO1 and EO2 studies (Uthman et al. 1993). VNS treatment for 14-35 months resulted in a mean seizure reduction of 46%. Five out of 14 patients had a seizure reduction of at least 50%.

In the meantime, two prospective, multicenter, randomized, double-blind clinical studies (EO3 and EO5) were initiated (Handforth et al. 1998). In these studies, large patient groups (EO3: n=114, EO5: n=196) were divided in a "high" (1.3 mA, 30 Hz, 500 µsec, 30 s ON/5 min OFF) and "low" (1.3 mA, 1

Hz, 130 µsec, 30 s ON/3 h OFF) stimulation group in terms of frequency, pulse width and duty cycle. The parameters in the "high" stimulation group were those believed to be efficacious based on previous animal data. In both studies, patients enrolled in the "high" stimulation group experienced seizure reducing effects ranging between 24-28%. Patients in the "low" stimulation group had a seizure reduction of maximal 15%.

The results of the EO3 and EO5 studies led to a FDA approval and were followed by open label extension trials, demonstrating that the antiepileptic effects of VNS clearly increased over time to values between 35-44% after 2 years of follow-up (Holder et al. 1992; George et al. 1994; Salinsky et al. 1996; Morris et al. 1999; DeGiorgio et al. 2000). In the following years, a growing amount of clinical data confirmed efficacy of VNS. In a retrospective study by our group, an overall seizure frequency reduction of 51% was observed in 138 patients with a minimal follow-up of 1 year (De Herdt et al. 2007). VNS, ranging from 10 days to 11 years, in a consecutive series of 436 adults and children with treatment-resistent epilepsy led to ≥ 90% seizure frequency reduction in 90 patients (22,5%), ≥ 75% seizure control in 162 patients (40,5%), ≥ 50% improvement in 255 patients (63,8%) and < 50% improvement in 145 patients (36,3%) (Elliott et al. 2011a). The same group analyzed the efficacy of VNS over time in 65 patients with refractory epilepsy: the overall seizure frequency reduction improved from 35,7% after 1 year of follow-up to 75,5% after 8 years, eventually followed by a stabilization (Elliott et al. 2011b). Recently, Englot et al. performed the first meta-analysis of VNS efficacy in epilepsy, identifying 74 clinical studies with 3321 patients suffering from intractable epilepsy (Englot et al. 2011). Mean seizure frequency reduction increased from 36% at 3-12 months following surgery to 51% after > 1 year of VNS therapy. The data of the 65 refractory epilepsy patients in the study of Elliott and coworkers were not included in this meta-analysis (Elliott et al. 2011b).

Vonck et al. and Boon et al. concluded that the mean VNS-induced seizure frequency reduction ranges between 25-55% with a large inter-patient variability in efficacy (Vonck et al. 2003; Boon et al. 2007). Efficacy has a tendency to improve with longer duration of treatment. Treatment with VNS (especially long-term treatment) reduces seizures with ≥ 50% in 50% of patients. In about 30% of patients, there is little or no effect. In the other 20% of patients, seizure frequency reduction ranges between 30-50%. It is still unclear why some patients do and others do not respond to the VNS therapy.

Safety, side effects and tolerability

Acute side effects

Perioperative complications occur infrequently. Fluid accumulation at the level of the subclavicularly implanted pulse generator occurs in 1-2% of patients. Postoperative infections occur in 3-6% of patients. These side effects are usually treatable with oral antibiotics (Ben-Menachem 2001).

Ventricular asystole has been observed during perioperative testing of the VNS device, even in patients without any history of cardiac dysfunction (Lanska 2000; Ali et al. 2004; Ardesch et al. 2007). As the left vagus nerve contains less sinoatrial fibers compared to the right vagus nerve, VNS is typically administered to the left vagus nerve, in order to provoke less cardiac side effects. In case of severe postoperative infections, intraoperative bleeding in the approach of the left vagus nerve or mechanical dysfunction due to fibrosis and high impedance of the electrode-nerve interface, the VNS device should be explanted. Implantation of the right vagus nerve could be a good alternative, as several studies report no cardiac side effects and similar antiepileptic effects of left and right VNS (McGregor et al. 2005; Spuck et al. 2008; Navas et al. 2010).

Side effects related to long-term use and tolerability

The most common side effects are coughing, throat pain and hoarseness. These side-effects are stimulus-related, dose-dependent, tend to improve over time and are due to secondary stimulation of the recurrent laryngeal nerve, which carries Aα motor fibers to the laryngeal muscles (Morris and Mueller 1999; Banzett et al. 1999; Ben-Menachem 2001). Less often reported side effects are dyspnea and vomiting (Uthman et al. 1993). Psychiatric side effects have been described. In patients with pre-existing psychiatric disorders decreased sedation and increased alertness may manifest itself as psychosis (Blumer et al. 2001; De Herdt et al. 2003). Unwanted side effects are easy to control by reducing the output current.

VNS at therapeutic levels does not cause central nervous system side effects, such as tiredness, psychomotor slowing, irritation and nervousness, which are all common in AED treatment (Ben-Menachem 2001). VNS also has no effect on the heart rate and gastrointestinal system, and does not affect AED serum levels. A notable increase of the perceived well-being, arousal and attention during VNS treatment is frequently reported (Sherman et al. 2008; Helmers et al. 2012). Relative contra-indications include progressive neurologic or systemic diseases, cardiac arrhythmia, asthma, chronic obstructive pulmonary disease, active peptic ulcer and insulin dependent diabetes mellitus (Elliot 2009).

References

Agnew WF, McCreery DB. Considerations for safety with chronically implanted nerve electrodes. Epilepsia 1990;31(S2):S27-S32.

Agostini E, Chinnock JE, Daly MD, Murray JG. Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. J Physiol 1957;135(1):182-205.

Ali II, Pirzada NA, Kanjwal Y, Wannamaker B, Medhkour A, Koltz MT, Vaughn BV. Complete heart block with ventricular asystole during left vagus nerve stimulation in epilepsy. Epilepsy Behav 2004;5(5):768-771.

Amaral DG. The anatomical organization of the central nervous system. In: Principles of neural science (Kandel ER, Schwartz JH, Jessel TM, eds) 1999. New York: McGraw-Hill.

Andresen MC, Kunze DL. Nucleus tractus solitarius: gateway to neural circulatory control. Annu Rev Physiol 1994;56:93-116.

Andrews P, Lawer I. A protective role for vagal afferents, a hypothesis. In: Ritter S, Ritter RC, Barnes CD (eds.) Neuroanatomy and physiology of abdominal vagal afferents. CRC Press, 1992, Boca Raton, FL, 221 pp.

Asala SA, Bower AJ. An electron microscope study of vagus nerve composition in the ferret. Anat Embryol (Berl) 1986;175(2):247-253.

Aston-Jones G, Shipley MT, Chouvet G, Ennis M, Van Bockstaele E, Pieribone V, Shiekhattar R, Akaoka H, Drolet G, Astier B. Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog Brain Res 1991;88:47-75.

Ardesch JJ, Buschman HP, van der Burgh PH, Wagener-Schimmel LJ, Van Der AA HE, Hageman G. Cardiac responses of vagus nerve stimulation: intraoperative bradycardia and subsequent chronic stimulation. Clin Neurol Neurosurg 2007;109(10):849-852.

Bailey TW, Hermes SM, Andresen MC, Aicher SA. Cranial visceral afferent pathways through the nucleus of the solitary tract to caudal ventrolateral medulla or paraventricular hypothalamus: target-specific synaptic reliability and convergence patterns. J Neuroscience 2006;26(46):11893-11902.

Banzett RB, Guz A, Paydarfar D, Shea SA, Schachter SC, Lansing RW. Caradiorespiratory variables and sensation during stimulation of the left vagus in patients with epilepsy. Epilepsy Res 1999;35(1):1-11.

Barnes A, Duncan R, Chisholm JA, Lindsay K, Patterson J, Wyper D. Investigation into the mechanisms of vagus nerve stimulation for the treatment of intractable epilepsy, suing 99mTc-HMPAO SPET brain images. Eur J Nucl Med Mol Imaging 2003;30(2):301-305.

Ben-Menachem E. Vagus nerve stimulation, side effects, and long-term safety. J Clin Neurophysiol 2001;18(5):415-418.

Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. Auton Neurosci 2000;85(1-3):1-17.

Blumer D, Davies K, Alexander A, Margan S. Major psychiatric disorders subsequent to treating epilepsy by vagus nerve stimulation. Epilepsy Behav 2001;2(5):466-472.

Bohotin C, Scholsem M, Multon S, Martin D, Bohotin V, Schoenen J. Vagus nerve stimulation in awake rats reduces formalin-induced nociceptive behaviour and fos-immunoreactivity in trigeminal nucleus caudalis. Pain 2003;101(1-2):3-12.

Boon P, De Herdt V, Vonck K, Van Roost D. Clinical experience with vagus nerve stimulation and deep brain stimulation in epilepsy. Acta Neurochir Suppl 2007;97(Pt2):273-280.

Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina G, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 2000;405(6785):458-462.

Bystrzycka EK, Nail BS. Brain stem nuclei associated with respiratory, cardiovascular, and other autonomic functions. In: Hindbrain and spinal cord (Paxinos G, ed) 1985. New York, USA: Academic Press.

Carrette E, De Herdt V, Manders J. Correlation of interictal spikes and long-term efficacy in patients with refractory epilepsy treated with vagus nerve stimulation. Epilepsia 2007;48:195-196.

Castle M, Comoli A, Loewy AD. Autonomic brainstem nuclei are linked to the hippocampus. Neuroscience 2005;134(2):657-669.

Corbett EK, Sinfield JK, McWilliam PN, Deuchars J, Batten TF. Differential expression of vesicular glutamate transporters by vagal afferent terminals in rat nucleus of the solitary tract: projections from the heart preferentially express vesicular glutamatetransporter1. Neuroscience 2005;135(1):133-145.

DeGiorgio CM, Schachter SC, Handforth A, Salinsky M, Thompson J, Uthman B, Reed R, Collins S, Tecoma E, Morris GL, Vaughn B, Naritoku DK, Henry T, Labar D, Gilmartin R, Labiner D, Osorio I, Ristanovic R, Jones J, Murphy J, Ney G, Wheless J, Lewis P, Heck C. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia 2000;41(9):1195-2200.

De Herdt V, Boon P, Vonck K, Goossens L, Nieuwenhuis L, Paemeleire K, Meire V, Michielsen G, Dewaele F, Baert E, Van Roost D. Are psychotic symptoms related to vagus nerve stimulation in epilepsy patients? Acta Neurol Belg 2003;103(3):170-175.

De Herdt V, Boon P, Ceulemans B, Hauman H, Lagae L, Legros B, Sadzot B, Van Bogaert P, van Rijckevorsel K, Verhelst H, Vonck K. Vagus nerve stimulation for refractory epilepsy: a Belgian multicenter study. Eur J Paediatr Neurol 2007;11(5):261-269.

Deuchars J, Li YW, Kasparov S, Paton JF. Morphological and electrophysiological properties of neurons in the dorsal vagal complex of the rat activated by arterial baroreceptors. J Comp Neurol 2000;417(2):233-249.

Elliot SK, Peckham PH, Rezai AR. Neuromodulation, 1ste Edition. 2009.London, U.K.: Elsevier.

Elliott RE, Morsi A, Kalhorn SP, Maracus J, Sellin J, Kang M, Silverberg A, Rivera E, Geller E, Carlson C, Devinsky O, Doyle WK. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: long-term outcomes and predictors of response. Epilepsy Behav 2011a;20(1):57-63.

Elliott RE, Morsi A, Tanweer O, Grobelny B, Geller E, Carlson C, Devinsky O, Doyle WK. Efficacy of vagus nerve stimulation over time: review of 65 consecutive patients with treatment-resistant epilepsy treated with VNS > 10 years. Epilepsy Behav 2011b;20(3):478-483.

Englot DJ, Chang EF, Auguste KI. Vagus nerve stimulation for epilepsy: a meta-analysis of efficacy and predictors of response. J Neurosurg 2011;115(6):1248-1255.

Erlanger J, Gasser HS. Electrical signs of nervous activity. 1937. Philadelphia, USA: University of Pennsylvania Press.

Foley JO, DuBois FS. Quantitative studies of the vagus nerve in the cat. I. The ratio of sensory and motor fibers. J Comp Neurol 1937;67(1):49-97.

George R, Salinsky M, Kuzniecky R, Rosenfeld W, Bergen D, Tarver WB, Wernicke JF. Vagus nerve stimulation for treatment of partial seizures: 3. Long-term follow-up on first 67 patients exiting a controlled study. First International Vagus Nerve Stimulation Study Group. Epilepsia 1994;35(3):637-643.

George MS, Aston-Jones G. Noninvasive techniques for probing neurocircuitry and treating illness: vagus nerve stimulation (VNS), transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS). Neuropsychopharmacology 2010;35(1):301-316.

Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, Henry TR, Collins SD, Vaughn BV, Gilmartin RC, Labar DR, Morris GL, Salinsky MC, Osorio I, Ristanovic RK, Labiner DM, Jones JC, Murphy JV, Ney GC, Wheless JW. Vagus nerve stimulation therapy for partial onset seizures: a randomized active control trial. Neurology 1998;51(1):48-55.

Heck C, Helmers SL, DeGiorgio CM. Vagus nerve stimulation therapy, epilepsy, and device parameters, scientific basis and recommendations to use. Neurology 2002;59(6S4):S31-S37.

Helmers SL, Duh MS, Guérin A, Sarda SP, Samuelson TM, Bunker MT, Olin BD, Jackson SD, Faught E. Clinical outcomes, quality of life, and costs associated with implantation ofvagus nerve stimulation therapy in pediatric patients with drug-resistant epilepsy. Eur J Paediatr Neurol 2012;16(5):449-458.

Holder LK, Wernicke JF, Tarver WB. Treatment of refractory partial seizures: preliminary results of a controlled study. Pacing Clin Electrophysiol 1992;15(10Pt2):1557-1571.

Hopkins DA, Holstege G. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp Brain Res 1978;32(4):529-547.

Hosoi T, Okuma Y, Nomura Y. Electrical stimulation of afferent vagus nerve induces IL-1 beta expression in the brain and activates HPA axis. Am J Physiol Regul Integr Comp Physiol 2000;279(1):R141-R147.

Jean A. The nucleus tractus solitarius: neuroanatomic, neurochemical and functional aspects. Arch Int Physiol Biochim Biophys 1991;99(5):A3-52.

Kalia M, Sullivan JM. Brainstem projections of sensory and motor components of the vagus nerve in the rat. J Comp Neurol 1982;211(3):248-265.

Kiernan JA. The human nervous system: an anatomical viewpoint (9th ed.). Lippinocott Williams & Wilkins, Philadelphia 2009.

Koutcherov Y, Mai JK, Ashwell KW, Paxinos G. Organisation of the human dorsomedial hypothalamic nucleus. Neuroreport 2004;15(1):107-111.

Krahl SE, Clark KB. Vagus nerve stimulation for epilepsy: a review of central mechanisms. Surg Neurol Int 2012;3(S4):S255-S259.

Krasteva VT, Papazov SP, Daskalov IK. Peripheral nerve magnetic stimulation: influence of tissue non-homogeneity. Biomed Eng Online 2003;2(19):1-14.

Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR. Central pathways of pulmonary and lower airway vagal afferents. J Appl Physiol 2006;101(2):618-627.

Labiner DM, Ahern GL. Vagus nerve stimulation therapy in depression and epilepsy: therapeutic parameter settings. Acta Neurol Scand 2007;115:23-33.

Lanska DJ. Ventricular asystole during vagus nerve stimulation for epilepsy in humans. Neurology 2000;54(3):775.

Manzano GM, Giuliano LMP, Nobrega JAM. A brief historical note on the classification of nerve fibers. Arg Neuropsiquiatr 2008;66(1):117-119.

McGregor A, Wheless J; Baumgartner J, Bettis D. Right-sided vagus nerve stimulation – Response. Epilepsia 2005;46(1):1152-1153.

McLachlan RS. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. Epilepsia 1993;34(5):918-923.

Morris GL, Mueller WM. Long-term treatment with vagus nerve stimulation in refractory epilepsy patients. The Vagus Nerve Stimulation Study Group E01-E05. Neurology 1999;53(8):1731-1735.

Naritoku D, Mikels JA. Vagus nerve stimulation attenuates electrically kindled seizures. Epilepsia 1996;37:75.

Navas M, Navarrette EG, Pascual JM, Carrasco R, Nunoz JA, Shakur SF, Pastor J, Sola RG. Treatment of refractory epilepsy in adult patients with right-sided vagus nerve stimulation. Epilepsy Res 2010;90(1-2):1-7.

Norman P, Bower AJ. An Autoradiographic study of the brainstem projections of vagal visceral afferent fibres in the domestic hen. J Anat 1982;134(Pt3):583-589.

Paintal AS. Vagal sensory receptors and their reflex effects. Physiol Rev 1973;53(1):159-227.

Penry JK, Dean JC. Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. Epilepsia 1990;31(S2):S40-S43.

Ricardo JA, Koh ET. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala and other forebrain structures in the rat. Brain Res 1978;153(1):1-26.

Salinsky MC, Uthman BM, Ristanovic RK, Wernicke JF, Tarver WB. Vagus nerve stimulation for the treatment of medically intractable seizures. Results of a 1-year open-extension trial. Vagus Nerve Stimulation Study Group. Arch Neurol 1996;53(11):1176-1180.

Santiago-Rodriguez E, Alonso-Vanegas M, Cardenas-Morales L, Haarmony T, Bernardino M, Fernandez-Bouzas A. Effects of two different cycles of vagus nerve stimulation on interictal epileptiform discharges. Seizure 2006;15(8):615-620.

Saper CB, Kibbe MR, Hurley KM, Spencer S, Holmes HR, Leahy KM, Needleman P. Brain natriuretic peptide-like immunoreactive innervation of the cardiovascular and cerebrovascular systems in the rat. Circ Res 1990;67(6):1345-1354.

Sherman EM, Connolly MB, Slick DJ, Eyrl KL, Steinbok P, Farrell K. Quality of life and seizure outcome after vagus nerve stimulation in children with intractable epilepsy. J Child Neurol 2008;23(9):991-998.

Spuck S, Nowak G, Renneberg A, Tronnier V, Sperner J. Right-sided vagus nerve stimulation in humans: an effective therapy? Epilepsy Res 2008;82(2-3):232-234.

Takaya M, Terry WJ, Naritoku DK. Vagus nerve stimulation induces a sustained anticonvulsant effect. Epilepsia 1996;37(11):1111-1116.

Uthman BM, Wilder BJ, Hammond EJ, Reid SA. Efficacy and safety of vagus nerve stimulation in patients with complex partial seizures. Epilepsia 1990;31(S2):S44-S50.

Uthman BM, Wilder BJ, Penry JK, Dean C, Ramsay RE, Reid SA, Hammond EJ, Tarver WB, Wernicke JF. Treatment of epilepsy by stimulation of the vagus nerve. Neurology 1993;43(7):1338-1345.

Van Bockstaele EJ, Peoples J, Telegan P. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. J Comp Neurol 1999;412(3):410-428.

Vonck K, Boon P, Goossens L, Dedeurwaerdere S, Claeys P, Gossiaux F, Van Hese P, De Smedt T, Raedt R, Achten E, Deblaere K, Thieleman A, Vandemaele P, Thiery E, Vingerhoets G, Miatton M, Caemaert J,Van Roost D, Baert E, Michielsen G, Dewaele F, Van Laere K, Thadani V, Robertson D, Williamson P. Neurostimulation for refractory epilepsy. Acta Neurol Belg 2003;103(4):213-217.

Vuckovic A, Tosato M, Struijk JJ. A comparative study of three techniques for diameter selective fiber activation in the vagal nerve: anodal block, depolarizing prepulses and slowly rising pulses. J Neural Eng 2008;5(3):275-286.

Wilder BJ, Uthman BM, Hammond EJ. Vagal stimulation for control of complex partial seizures in medically refractory epileptic patients. Pacing Clin Electrophysiol 1991;14(1):108-115.

Zabara J. Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia 1992;33(6):1005-1012.

Chapter 3

Vagus nerve stimulation and the central noradrenergic pathway

This chapter focuses on the central noradrenergic pathway as an important contributor in the antiepileptic effect of VNS. Preclinical and clinical studies demonstrate a role for vagal afferent A and B fibers at the cervical level running to the brainstem NTS. Data obtained from *in vitro* studies show a frequency-dependent inhibition of the NTS with VNS. Our hypothesis further considers the predominantly inhibitory influence of the NTS on the noradrenergic neurons of the LC. VNS as a result causes a disinhibition of the LC. The activity of the LC and its widespread noradrenergic modulation are now increased. This noradrenergic modulation is believed to be responsible, at least in part, for the antiepileptic effect of VNS.

Vagal fibers involved in the antiepileptic effect of VNS

The role of afferent A and B fibers at the cervical level

In 1992, Zabara reported for the first time that the antiepileptic actions of VNS are not mediated by vagal efferent fibers at the cervical level. This conclusion was based on the demonstration that the antiepileptic effect of VNS in dogs was preserved after transection of the vagus nerve distally to the site of cervical vagal stimulation (Zabara 1992). In 1993, McLachlan et al. demonstrated that VNS-induced abolishment of penicillin-induced focal interictal spikes in rats disappeared after cooling of the vagus nerve proximal to the point of stimulation, further supporting the hypothesis that VNS exerts its effect on seizures through cervical afferent fiber activation (McLachlan 1993).

It remains incompletely understood which afferent fibers precisely induce the antiepileptic effect of VNS. Further research in this field may support a more rational choice of stimulation parameters and increase knowledge on the central mechanism of action of VNS. Early animal studies on the therapeutic mechanisms of VNS suggested that the antiepileptic potential of VNS was directly related to the fraction of cervical vagal afferent C fibers stimulated. This was based on a maximal evoked response from A, B and C fibers in an isolated vagus nerve in anaesthetized rats induced by parameters required to suppress seizures in awake rats (Woodbury and Woodbury 1990, 1991). The theory supporting C fiber involvement was discarded after Krahl et al. demonstrated seizure suppression in awake rats following selective destruction of C fibers using capsaicin (Krahl et al. 2001). The discrepancy between the former and latter studies is probably due to the fact that

compound action potentials (CAPs) in the former studies were recorded in anaesthetized animals and may therefore not be relevant to VNS in awake rats. Stimulation in a chronically implanted, awake animal produces electrical shunting through the body, which is not modeled accurately by an acute isolated nerve preparation. The clinical relevance of this finding is that A and B fibers have much lower activation thresholds and that output current intensities required for C fiber activation would unnecessarily induce side effects and shorten battery life. Data from clinical trials support the idea of Krahl et al. (2001) that efficient antiepileptic stimulation levels are subthreshold for the recruitment of C fibers (Binks et al. 2001). In order to determine the vagal A fiber threshold, patients were asked to speak as monotonically and steadily as possible during each output current intensity increment. Increases in stimulus strength were continued until A fiber stimulation was achieved as judged by an audible change in the subject's voice quality, caused by contraction of the vocal cords. In order to determine the vagal C fiber threshold, cardiopulmonary parameters were measured. None of the patients however showed cardiopulmonary changes or sensations expected to be associated with vagal C fiber stimulation even at the highest possible stimulus current the VNS device could deliver. For all patients, the stimulus strength at which seizures were reduced was as such subthreshold for C fiber activation (Binks et al. 2001). Recently, Helmers et al. developed a mathematical model to explore how full activation of the myelinated A and B fibers at the cervical level of the vagus nerve can be achieved through changes in output current intensity and pulse width (Helmers et al. 2012). The model incorporated acute and chronic stimulation conditions in order to study the effect of tissue encapsulation at the site of electrode placement. In the future, the model can be refined to include additional factors that may influence the effectiveness of the VNS therapy, such as duty cycle and frequency of stimulation. Information retained from such a mathematical model could guide clinicians on how to optimize the VNS therapy.

Fiber type selective stimulation and directional selectivity

VNS for epilepsy requires the selective generation and propagation of action potentials in cervical vagal afferent A and/or B fibers. Efferent fibers of larger diameter innervating the laryngeal muscles are unnecessarily stimulated and this results in stimulation-related coughing, throat pain and/or hoarseness. Avoiding stimulation of these larger diameter motor fibers would reduce these side effects. Two techniques that could be used for this purpose are fiber type selective stimulation and directional selectivity.

Fiber type selective stimulation

The potential of fiber type selective stimulation mainly relies on the knowledge that fiber activation and fiber blocking thresholds are inversely proportional to the square of the fiber diameter (Rozman et al. 1993). Activating or blocking the largest fibers requires the least energy or electrical current density. When an axon is electrically activated, action potentials propagate in both directions away from the point of activation, in both the afferent and efferent fibers. Fiber type selective stimulation involves stimulation of all the nerve fibers and then adjusting a conduction block at both sides of the stimulation point in such a way that the action potentials would not propagate further in the fibers with the largest diameter. The theoretical result is a selective activation of smaller diameter fibers (Rozman et al. 1993; Bugbee et al. 2001).

Directional selectivity

A variation on fiber type selective stimulation is directional selectivity. A conduction block placed on the distal side of the stimulation point can ensure that propagation in the large diameter motor fibers takes place in one direction only (Ungar et al. 1986). Application of this technique would be sufficient to avoid VNS-related side effects (coughing, throat pain and/or hoarseness).

Blocking techniques

To obtain fiber type selective stimulation or directional selectivity, a number of conduction block techniques has been developed. One strategy is '*anodal hyperpolarization*', which consists of electrically hyperpolarizing the nerve membrane at such a level that the depolarizing cathodic currents from an action potential are no longer strong enough to depolarize the corresponding region to the excitation threshold (Rijkhoff et al. 1994). A conduction block based on anodal hyperpolarization can theoretically be obtained by application of a triangular or quasi-trapezoidal stimulus pulse. Such a pulse consists of a square leading edge and a plateau phase, followed by an exponential decay (Ungar et al. 1986). Another physiological mechanism used to block a nerve is '*accommodation*'. The depolarization phase of an action potential is the result of sodium ions flowing into the cell through voltage-gated sodium channels. The inwards sodium flow subsequently opens even more sodium channels, resulting in a further depolarization. This process proceeds explosively until all the available ion channels are open. The rapid influx of sodium ions causes the polarity of the plasma membrane to reverse, and the ion channels then rapidly inactivate. Together with the activation of voltage-gated potassium channels, through which potassium ions are transported out of the cell, the inactivation of sodium channels brings the membrane potential back to the resting state. Accommodation refers to the inactivation of sodium channels, while keeping the channel opening

rate low enough, not to trigger an action potential. Accommodation can be achieved by means of exponentially rising waveforms and depolarizing prepulses (Joseph and Butera 2011). When a nerve fiber is stimulated with a subthreshold current, close to the excitation threshold, the membrane accommodates in such a way that the excitation threshold increases, which is attributed to the inactivation of the voltage-gated sodium channels.

Limitations of the blocking techniques

Both fiber type selective stimulation and directional selectivity are theoretical concepts with limitations for practical implementation. Blocking techniques require relatively high power (Vuckovic et al. 2008). Long pulse durations are required as the blocking pulse must comprise the latency of the action potential arrival and the duration of the action potential itself. This often leads to large power consumptions and current densities that are incompatible with chronic use in clinical implants. In addition, an accurate timing is essential and dependent on anatomical or geometric factors as well as the fiber conduction velocity itself. Apart from the power consumption and timing issues, the application of anodal current typically creates virtual cathodes for example at the electrode extremities. These virtual cathodes are able to activate nerve fibers beyond the blocked region. The pseudo-trapezoidal pulses mentioned above are supposed to reduce this secondary stimulation effect (Ungar et al. 1986). The practical result of the timing and virtual cathode problems is that many fibers escape the expected blocking effect and conduct action potentials anyway, so that neither fiber type selective stimulation nor directional selectivity works very efficiently in clinical applications. In current VNS therapy, co-activation of large diameter Aα motor fibers innervating the laryngeal muscles is not prevented, resulting in stimulation-related coughing, throat pain and/or hoarseness.

The nucleus tractus solitarius

Vagal afferent A and B fibers arrive in the medial, ventral and lateral parts of the brainstem NTS (Kalia and Sullivan 1982). They convey information on the status of visceral organs by releasing the excitatory neurotransmitter glutamate, which acts on postsynaptic AMPA receptors to excite the NTS neurons (Fig.8) (Andresen et al. 2013). At baseline physiological firing rates ranging from 0.1 to 16 Hz depending on the species and the vagal afferent fiber type involved (Mathis et al. 1998; Brundson and Grundy 1999; Lynn and Blackshaw 1999; Niijima 2000; Horn and Friedman 2003; Sengupta et al. 2004; Peiris et al. 2011) most of the released glutamate binds to postsynaptic AMPA receptors on NTS neurons. At higher firing rates of vagal afferent fibers, glutamate diffuses outside the synaptic cleft and binds to presynaptic glutamatergic autoreceptors (i.e. mGluRs type II and III), which reduces

glutamate release and further activation of postsynaptic NTS receptors (Fig.8). The result is a frequency-dependent depression in the NTS with increasing vagal afferent input. Andresen and Yang studied the dynamics of sensory transmission on the NTS neurons in a horizontal brainstem slice preparation of the rat (Andresen and Yang 1995). The amplitude of the excitatory post-synaptic potential (EPSP) in NTS neurons was maximal at < 0.5 Hz of vagal afferent stimulation for 2 minutes. EPSP amplitude declined to an average of 57.5% at 10 Hz of stimulation. Within 1 minute of 100 Hz stimulation, EPSP amplitude declined to nearly zero. The role of the mGluRs type II and III is evidenced by the observation that the frequency-dependent depression in the NTS with increasing vagal afferent input is abolished by mGluR blockade (Liu et al. 1998). Furthermore, administration of a selective agonist of the mGluR type II and III in brainstem slices has been shown to reduce the EPSP amplitude in NTS neurons evoked by low-frequency stimulation (0.1-0.2 Hz) of vagal afferent fibers, and this reduction in EPSP amplitude was attenuated by co-administration of a selective antagonist of the mGluR type II and III (Chen et al. 2002). The antagonist data further showed that the inhibitory effect of mGluRs type II and III on synaptic transmission in the NTS in response to electrical stimulation of vagal afferent fibers was negligible at stimulation frequencies < 9 Hz and was increasingly prominent with higher stimulation frequencies (up to 48 Hz). The NTS findings correspond to those in other central nervous system regions regarding the contribution of presynaptic mGluRs type II and III to frequency-dependent synaptic depression (Dubé and Marshall 2000).

In addition, mGluRs are also expressed presynaptically by GABAergic terminals that are interspersed throughout the NTS (Fig.8) (Fernandes et al. 2011). Some of these GABAergic terminals originate from local interneurons, whereas others may well arise from outside the NTS (Jordan et al. 1988; Fong et al. 2005). Binding of released glutamate on these presynaptic mGluRs can either activate or inhibit GABA release, depending on the receptor subtype (Fig.8) (Jin et al. 2004; Fong et al. 2005; Bailey et al. 2008). mGluR type II and III is a Gi-protein coupled receptor and inhibits GABA release, mGluR type I is a Gq-protein coupled receptor and promotes GABA release. GABA on its turn heterosynaptically modulates the release of glutamate by acting on $GABA_A$ or $GABA_B$ receptors on vagal glutamatergic terminals in the NTS (Fig.8) (Bailey et al. 2008; Kang et al. 2012). The $GABA_A$ receptor on vagal terminals in the NTS is a $Na^+K^+Cl^{2-}$-cotransporter that strongly promotes glutamate release. The binding of GABA on $GABA_A$ receptors normally results in an influx of chloride ions, thereby hyperpolarizing the cell membrane and inhibiting the firing of new action potentials. Upon binding of GABA on the $Na^+K^+Cl^2$-cotransporter on vagal glutamatergic terminals in the NTS, one sodium ion and one potassium ion is transported into the cell, while two chloride ions are

transported out of the cell. The net result is a postsynaptic depolarization and increased release of glutamate (Kang et al. 2012). Such $GABA_A$ receptors are expressed by numerous glutamatergic terminals in the brainstem. These glutamatergic terminals are characterized by a developmental high intracellular chloride concentration, which is up to five times higher then in their soma (Price and Trussel 2006). The $GABA_B$ receptor is a Gi-protein coupled receptor and strongly inhibits glutamate release. The precise mechanism remains poorly understood but *in vitro* studies suggest a possible involvement of GABA in signal transmission in the NTS (Glaum and Brook 1995; Glaum and Brooks 1996; Kang et al. 2012). The sustained synaptic depression in the NTS induced by brief periods of high-frequency (50 Hz) vagal afferent stimulation in a slice preparation of the rat medulla was blocked by $GABA_B$ receptor antagonism (Glaum and Brook 1995; Glaum and Brooks 1996). In a more recent study, $GABA_A$ and $GABA_B$ receptor agonists facilitated and reduced the EPSP frequency in isolated mechanically dispersed NTS neurons, respectively. Local application of $GABA_A$ and $GABA_B$ receptor antagonists had opposite effects (Kang et al. 2012). Other mechanisms that could contribute to the synaptic depression in the NTS with increasing vagal afferent input include desensitization of the postsynaptic AMPA receptors and depletion of synaptic vesicles in the vagal glutamatergic terminals (Liu et al. 1998).

It should be noted that the described frequency-dependent inhibition of the NTS with VNS is only based on *in vitro* studies and this should be evidenced by *in vivo* studies in future. Only a few *in vivo* studies evaluated the effect of VNS on the NTS. These studies demonstrated an increased nuclear c-fos expression in the NTS in response to VNS in rats (Naritoku et al. 1995; Osharina et al. 2006; Cunningham et al. 2008). C-fos is the product of the immediate early gene c-fos and it is commonly acknowledged that the expression of c-fos is a valuable marker of increased neuronal activity. These data indicate that the NTS is indeed addressed by VNS, but do not reveal which neuronal population is triggered by VNS. The heterosynaptic crosstalk between glutamatergic and GABAergic terminals in the NTS implies that both terminals are in very close proximity to each other and to the NTS (Bailey et al. 2008). C-fos may therefore stain the nucleus of both the glutamatergic and GABAergic fibers. Osharina et al. demonstrated a frequency-dependent c-fos expression in the NTS with VNS (Osharina et al. 2006). VNS at 10 Hz stained the NTS more intense compared to VNS at 1 Hz. We hypothesize that at 10 Hz, more GABAergic nuclei are stained.

In summary, *in vitro* studies in which the vagal afferent fibers are electrically stimulated have shown that (i) the NTS is maximally active at stimulation frequencies < 0.5 Hz, and (ii) the overall inhibitory effect on glutamatergic synaptic signaling in the NTS is negligible at stimulation frequencies below 9 Hz while becoming prominent at higher stimulation frequencies. The most common VNS frequency applied in clinical practice is 30 Hz. We therefore hypothesize that clinical VNS reduces the activity of

the NTS neurons. The demonstration in rats that local injection of GABA agonists or glutamate antagonists in the NTS reduces susceptibility to limbic motor seizures provides direct evidence that an inhibition of the NTS can have antiepileptic effects (Walker et al. 1999).

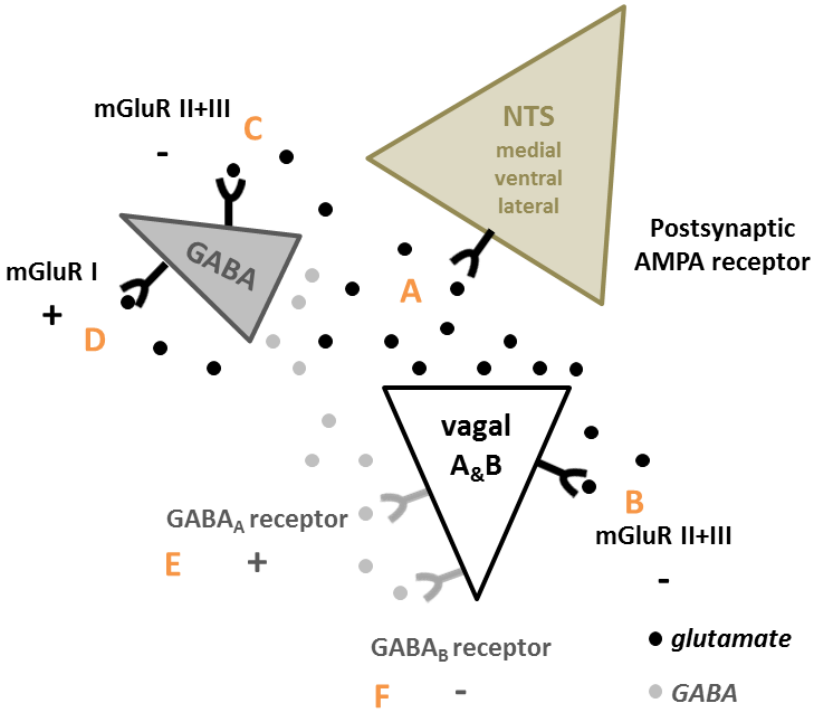


Figure 8| Schematic representation of the heterosynaptic crosstalk between presynaptically released glutamate and GABA to influence glutamatergic signaling onto neurons in the medial, ventral and lateral NTS. The neurotransmitters glutamate and GABA are shown as black and grey dots, respectively. Afferent activity in vagal afferent A and B fibers activates the release of glutamate, which binds to postsynaptic AMPA receptors on NTS neurons **(A)**. In addition, released glutamate can activate mGluRs type II and III on the vagal terminals to reduce subsequent glutamate release **(B)**. Glutamate may also bind to mGluRs on local GABAergic terminals. mGluR type II and III is negatively coupled and inhibits GABA release **(C)**, mGluR type I is positively coupled and stimulates GABA release **(D)**. Released GABA reaches $GABA_A$ and/or $GABA_B$ receptors on the vagal terminals. $GABA_A$ receptors strongly promote glutamate release **(E)**, in contrast to $GABA_B$ receptors which strongly inhibit glutamate release **(F)**. As a result of this complex heterosynaptic interaction, afferent glutamate weighs on both sites of the excitatory and inhibitory balance and this balance determines the outcome of afferent information within the medial, ventral and lateral NTS. (mGluR = metabotropic glutamate receptor)

The locus coeruleus

As a relay station for vagal afferent fibers, the NTS probably plays a key role in connecting the electrical stimulation of cervical vagal afferent A and B fibers to central antiepileptic brain circuits. Identifying structures upstream of the NTS that are engaged by VNS is a further essential step in understanding the antiepileptic effect of VNS. The LC is a strong candidate. The LC is a nucleus of the pontine tegmentum containing a homogeneous compact noradrenergic cell group (Maeda 2000). There are anatomical connections between the NTS and the LC (Fig.9a) (Aston-Jones et al. 1991; Van Bockstaele et al. 1999; Halliday 2004). The LC provides innervation of the entire cortex and is the major source of brain noradrenaline, a neuromodulator with strong antiepileptic effects (Foote et al. 1983). Electrophysiological studies demonstrated that the LC is activated by acute and chronic VNS (Groves et al. 2005; Dorr and Debonnel 2006; Manta et al. 2009).

The noradrenergic LC receives both monosynaptic and disynaptic projections from the NTS (Fig.9a) (Aston-Jones et al. 1991; Van Bockstaele et al. 1999; Halliday 2004). Cell bodies located in the LC have an extensive dendritic network in the pericoerulear region and the monosynaptic projections originating in the NTS influence LC activity through both excitatory and inhibitory synapses with the LC dendrites (Van Bockstaele et al. 1999). Tract-tracing and electrophysiological studies have revealed that the LC proper receives afferents from remarkably few brain loci (Aston-Jones et al. 1991). Major inputs to the LC proper are found in the PGi and the PrH only, which are located in the ventrolateral and dorsomedial rostral medulla, respectively (Aston-Jones et al. 1991). Both the PGi and PrH receive glutamatergic projections from the NTS (Kihara and Kubo 1991; Aston-Jones et al. 1991; Van Bockstaele et al. 1999; Halliday 2004). LC-projecting neurons of the PGi stain positive on markers for adrenalin, enkephalin, corticotropin-releasing factor and glutamate (Aston-Jones et al. 1991). Neuropharmacological and electrophysiological experiments have revealed that the PGi provides a potent excitatory glutamatergic input to the LC, acting primarily on non-NMDA receptors (Ennis and Aston-Jones 1988). Adrenergic as well as non-adrenergic inhibition of the LC could be detected in a minority of PGi cells tested (Aston-Jones et al. 1986). The PrH potently and consistently inhibits LC neurons via GABAergic projections, acting on $GABA_A$ receptors (Aston-Jones et al. 1991). Large proportions of the PrH neurons also stain positive on markers for met-enkephalin. A third disynaptic pathway involves the inhibitory action of GABAergic interneurons surrounding the LC (Aston-Jones et al. 2004). As the NTS efferents to upstream brain structures which among other project to the LC are mainly glutamatergic (Kihara and Kubo 1991; Aston-Jones et al. 1991; Van Bockstaele et al. 1999; Halliday 2004) and ultra-structural analysis has revealed that ± 77% of LC afferents are GABAergic, we hypothesize that the NTS effect on the LC is predominantly inhibitory. By

reducing the activity of the NTS, VNS results in a disinhibition of the LC and a subsequent increased release of noradrenaline (Fig.9b).

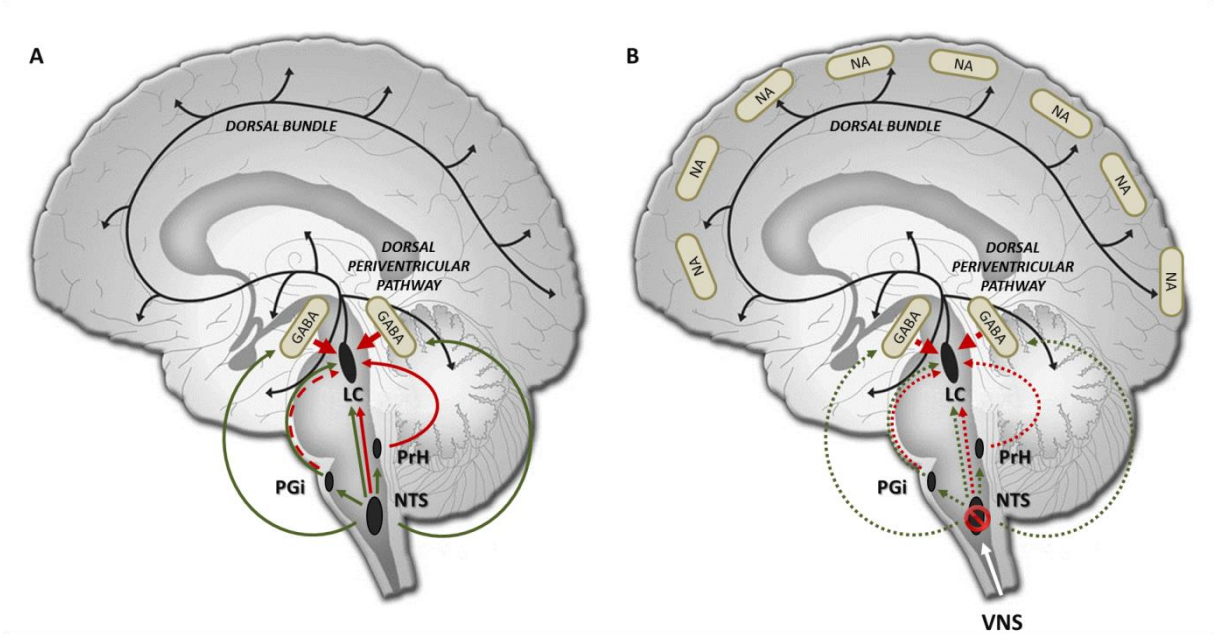


Figure 9| A. Anatomical projections from the NTS to the LC. The green and red arrows represent excitatory and inhibitory projections, respectively. Note the smaller number of inhibitory projections (dotted red arrow) from the PGi to the LC compared to its excitatory projections. The majority of LC afferents are coming from GABAergic interneurons (bold red arrow). **B.** We hypothesize that, by reducing the NTS output, VNS results in a disinhibition of the LC and a subsequent increased release of noradrenaline **(adapted from (Nelson 2007))**. (NTS = nucleus tractus solitarius, LC = locus coeruleus, PGi = nucleus paragigantocellularis, PrH = nucleus prepositus hypoglossi, NA = noradrenaline)

Role of the locus coeruleus and noradrenaline in the antiepileptic effect of VNS

LC neurons have an enormous network of noradrenergic projections throughout the brain (Fig.9) (Ungerstedt 1971; Jones and Moore 1977). LC fibers run within two main ascending fiber systems: the dorsal bundle, which ascends through the central tegmental tract in the pons and midbrain, and the much smaller dorsal periventricular pathway, which is a component of the dorsal longitudinal fasciculus, a white matter fiber tract located within the brainstem (Giorgi et al. 2004). The dorsal bundle provides noradrenergic innervation of the telencephalon and diencephalon. The dorsal periventricular pathway projects to medial and midline thalamic, pretectal and hypothalamic regions (Krahl et al. 2012). The LC axons are characterized by profuse terminal branching and varicosities

(Fornai et al. 2011). The varicosities do not represent a classic synaptic site with a pre- and postsynaptic component, but noradrenaline directly diffuses into the extracellular space to modulate the activity of neurons, glial cells and blood vessels. This paracrine noradrenaline diffusion is quite generalized and can affect the entire cortical activity. Noradrenaline interacts with three families of G-protein coupled adrenergic receptors (α_1, α_2 and β receptors) which are further divided into different subtypes (α_{1A-B}, α_{1D}, α_{2A-C} and β_{1-3} receptors) (Ramos and Arnsten 2007). It has highest affinity for the α_2 adrenergic receptors, which are generally coupled to G_i proteins. The α_2 adrenergic receptor subtype is considered to be the most prominent antiepileptic adrenoreceptor (Weinshenker and Szot 2002). Noradrenaline exerts a variety of central functions and is involved in modulating EEG activity, regulating the sleep-waking cycle by anticipating fluctuations of EEG activity, promoting a state of vigilance, monitoring environmental stimuli with a specific emphasis on alerting stimuli and orienting to novelty (Giorgi et al. 2004).

The noradrenergic system has been implicated in the control of seizure activity (Weinshenker and Szot 2002; Giorgi et al. 2004). When the LC is damaged or the noradrenergic system is genetically impaired, animals demonstrate a higher susceptibility to experimentally evoked seizures. Amygdala and hippocampal kindling were accelerated after noradrenaline depletion with 6-hydroxydopamine in rats (McIntyre and Edson 1981; Bortolotto and Cavalheiro 1986; Corcoran 1988). Genetically engineered mice that lack noradrenaline are shown to have higher susceptibility to different convulsant stimuli (Szot et al. 1999). Other studies demonstrated that seizure susceptibility in these animals can be reduced by restoring the noradrenergic activity. Audiogenic seizures in genetically epilepsy-prone rats were suppressed by intracerebral injections of noradrenaline (Mishra et al. 1993; Yan et al. 1998) and grafted noradrenergic neurons suppressed seizure development in the kindling model (Barry et al. 1987; Bengzon et al. 1990). Also, electrical stimulation of the LC inhibited seizure activity induced by administration of chemoconvulsants (Ferraro et al. 1994). Recently, it was shown that mutations in the α_{2B} adrenoreceptor are involved in autosomal dominant cortical myoclonus and epilepsy (De Fusco et al. 2014). Further evidence for the antiepileptic properties of noradrenaline is related to the ability of several antiepileptic drugs to increase the noradrenergic activity. Valproate and phenytoin increase noradrenaline levels in various brain regions (Baf et al. 1994) and carbamazepine produces a dose-dependent increase in the firing rate of LC neurons (Olpe and Jones 1983), concomitant with higher noradrenaline levels in various brain regions (Baf et al. 1994).

The first evidence for a prominent role of the LC in the antiepileptic activity induced by VNS dates back to 1998, when Krahl et al. showed that the permanent loss or functional inactivation of LC neurons abolished the antiepileptic effects of VNS (Krahl et al. 1998). Results from electrophysiological studies in the rat brain showed that the activity of LC neurons is increased upon

acute and chronic stimulation of the vagus nerve (Groves et al. 2005; Dorr and Debonnel 2006; Manta et al. 2009). Confirming the recruitment of LC neurons during VNS, immediate-early gene mRNAs or their protein transcripts increase within LC neurons, in both rats (Naritoku et al. 1995) and rabbits (Gieroba and Blessing 1994; Cunningham et al. 2008). Increases in extracellular noradrenaline concentrations have been measured by microdialysis in projection areas of the LC, such as the prefrontal cortex (Roosevelt et al. 2006; Follesa et al. 2007; Manta et al. 2013), hippocampus (Roosevelt et al. 2006; Manta et al. 2013) and amygdala (Hassert et al. 2004) in VNS-treated rats.

In addition to noradrenaline, axons pertaining to the LC synthesize a rich spectrum of neuromodulators known to be co-released. The LC neurons store and release a variety of neuropeptides, such as enkephalin, neurotensin, vasopressin, somatostatin, neuropeptide Y and galanin (Olpe and Steinmann 1991). Among these, neuropeptide Y and galanin have shown strong antiepileptic properties when applied locally in the rat hippocampus (Gundlach et al. 1990; Xu et al. 1998; Mazarati and Wasterlain 2002; Meurs et al. 2007). In addition, administration of a galanin receptor antagonist accelerates the development of status epilepticus (Mazarati et al. 1998) and galanin knock-out mice are more susceptible to seizures (Mazarati et al. 2000). It remains to be established to what extent the activity of these noradrenaline co-transmitters contributes to the antiepileptic effect of VNS.

References

Amaral DG. The anatomical organization of the central nervous system. In: Principles of neural science (Kandel ER, Schwartz JH, Jessel TM, eds) 1999. New York: McGraw-Hill.

Andresen MC, Yang M. Dynamics of sensory afferent synaptic transmission in aortic baroreceptor regions of nucleus tractus solitarius. J Neurophysiol 1995;74(4):1518-1528.

Andresen MC, Fawley JA, Hofmann ME. Peptide and lipid modulation of glutamatergic afferent synaptic transmission in the solitary tract. Front Neurosci 2013;10(6) (In Press).

Aston-Jones G, Ennis M, Pieribone VA, Nickell WT, Shipley MT. The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. Science 1986;234(4777):734-737.

Aston-Jones G, Shipley M, Chouvet G, Ennis M, Van Bockstaele E, Pieribone V, Shiekhattar R, Akaoka H, Drolet G, Astier B. Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog Brain Res 1991;88:47-75.

Aston-Jones G, Zhu Y, Card JP. Numerous GABAergic afferents to locus ceruleus in the pericerulear dendritic zone: possible interneuronal pool. J Neuroscience 2004;24(9):2313-2321.

Baf MH, Subhash MN, Lakshmana KM, Rao BS. Sodium valproate induced alterations in monoamine levels in different regions of the rat brain. Neurochem Int 1994;24(1):67-72.

Bailey TW, Appleyard SM, Jin YH, Andresen MC. Organization and properties of GABAergic neurons in solitary tract nucleus (NTS). J Neurophysiol 2008;99(4):1712-1722.

Barry DI, Kikvadze I, Brundin P, Bolwig TG, Björklund A, Lindvall O. Grafted noradrenergic neurons suppress seizure development in kindling-induced epilepsy. Proc Natl Acad Sci USA 1987;84(23):8712-8715.

Bengzon J, Kokaia M, Brundin P, Lindvall O. Seizure suppression in kindling epilepsy by intrahippocampal locus coeruleus grafts: evidence for an alpha-2-adrenoreceptor mediated mechanism. Exp Brain Res 1990;81(2):433-437.

Binks A, Paydarfar D, Schachter S, Guz A, Banzett R. High strength stimulation of the vagus nerve in awake humans: a lack of cardiorespiratory effects. Respir Physiol 2001;127(2-3):125-133.

Bortolotto ZA, Cavalheiro EA. Effect of DSP4 on hippocampal kindling in rats. Pharmacol Biochem Behav 1986;24(3):777-779.

Brooks PA, Glaum SR. GABAb receptors modulate a tetanus-induced sustained potentiation of monosynaptic inhibitory transmission in the rat nucleus tractus solitarii in vitro. J Auton Nerv Syst 1995;54(1):16-26.

Brundson AM, Grundy D. Sensitization of visceral afferents to bradykinin in rat jejunum in vitro. J Physiol 1999;521(Pt2):517-527.

Bugbee M, Donaldson NN, Lickel A, Rijkhoff NJ, Taylor J. An implant for chronic selective stimulation of nerves. Med Eng Phys 2001;23(1):29-36.

Chen CY, Ling EH, Horowitz JM, Bonham AC. Synaptic transmission in nucleus tractus solitarius is depressed by group II and III but not group I presynaptic metabotropic glutamate receptors in rats. J Physiol 2002;538(Pt3):773-786.

Corcoran ME. Characteristics of accelerated kindling after depletion of noradrenaline in adult rats. Neuropharmacology 1988;27(10):1081-1084.

Cunningham J, Mifflin S, Gould G, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation. Neuropsychopharmacol 2008;33(8):1884-1895.

De Fusco M, Vago R, Striano P, Di Bonaventura C, Zara F, Mei D, Kim MS, Muallem S, Chen Y, Wang Q, Guerrini R, Casari G. The α2B-adrenergic receptor is mutant in cortical myoclonus and epilepsy. Ann Neurol 2014;75(1):77-87.

Dorr A, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 2006;318(2):890-898.

Dubé GR, Marshall KC. Activity-dependent activation of presynaptic metabotropic glutamate receptors in locus coeruleus. J Neurophysiol 2000;83(3):1141-1149.

Ennis M, Aston-Jones G. Activation of locus coeruleus from nucleus paragigantocellularis: a new excitatory amino acid pathway in brain. J Neuroscience 1988;8(10):3644-3657.

Fernandes LG, Jin YH, Andresen MC. Heterosynaptic crosstalk: GABA-glutamate metabotropic receptors interactively control glutamate release in solitary tract nucleus. Neuroscience 2011;174:1-9.

Ferraro G, Sardo P, Sabatino M, La Grutta V. Locus coeruleus noradrenaline system and focal penicillin hippocampal epilepsy: neurophysiological study. Epilepsy Res 1994;19(3):215-220.

Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, Puligheddu M, Marrosu F, Biggio G. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res 2007;1179:28-34.

Fong AY, Stornetta RL, Foley CM, Potts JT. Immunohistochemical localization of GAD67-expressing neurons and processes in the rat brainstem: subregional distribution in the nucleus tractus solitarius. J Comp Neurol 2005;493(2):274-290.

Foote S, Bloom L, Aston-Jones G. Nucleus locus coeruleus: new evidence of anatomical and physiological specificity. Physiol Rev 1983;63(3):844-914.

Fornai F, Ruffoli R, Girogi F, Paparelli A. The role of locus coeruleus in the antiepileptic activity induced by vagus nerve stimulation. Eur J Neuroscience 2011;33(12):2169-2178.

Gieroba ZJ, Blessing WW. Fos-containing neurons in medulla and pons after unilateral stimulation of the afferent abdominal vagus in conscious rabbits. Neuroscience 1994;59(4):851-858.

Giorgi FS, Pizzanelli C, Biagioni F, Murri L, Fornai F. The role of norepinephrine in epilepsy: from the bench to the bedside. Neuroscience Biobehav Rev 2004;28(5):507-524.

Glaum SR, Brooks PA. Tetanus-induced sustained potentiation of monosynaptic inhibitory transmission in the rat medulla: evidence for a presynaptic locus. J Neurophysiol 1996;76(1):30-38.

Groves D, Bowman E, Brown V. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neuroscience Lett 2005;379(3):174-179.

Gundlach A, Rutherfurd S, Louis W. Increase in galanin and neuropeptide Y mRNA in locus coeruleus following acute reserpine treatment. Eur J Pharmacol 1990;184(1):163-167.

Halliday CB. Substantia nigra and locus coeruleus. In: The human nervous system (Paxinos G, Mai JK, eds) 2004. San Diego, USA: Elsevier Academic Press.

Hassert D, Miyashita A, Williams C. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behav Neuroscience 2004;118(1):79-88.

Helmers SL, Begnaud J, Cowley A, Corwin HM, Edwards JC, Holder DL, Kostov H, Larsson PG, Levisohn PM,De Menezes MS, Stefan H, Labiner DM. Application of a computational model of vagus nerve stimulation. Acta Neurol Scand 2012;126(5):336-343.

Horn CC, Friedman MI. Detection of single unit activity from the rat vagus using cluster analysis of principal components. J Neurosci Methods 2003;122(2):141-147.

Jin YH, Bailey TW, Andresen MC. Cranial afferent glutamate heterosynaptically modulates GABA release onto second-order neurons via distinctly segregated metabotropic glutamate receptors. J Neuroscience 2004;24(42):9332-9340.

Jones MT, Moore RY. Ascending projections of the locus coeruleus in the rat. II Autoradiographic study. Brain Res 1977;127(1):25-53.

Jordan D, Mifflin SW, Spyer KM. Hypothalamic inhibition of neurons in the nucleus tractus solitarius of the cat is GABA mediated. J Physiol 1988;399:389-404.

Joseph L, Butera RJ. High-frequency stimulation selectively blocks different types of fibers in frog sciatic nerve. IEEE Trans.Neural Syst Rehabil Eng 2011;19(5):550-557.

Kalia M, Sullivan JM. Brainstem projections of sensory and motor components of the vagus nerve in the rat. J Comp Neurol 1982;211(3):248-265.

Kang YH, Sun B, Park YS, Park CS, Jin YH. GABAA and GABAB receptors have opposite effects on synaptic glutamate release on the nucleus tratus solitarii neurons. Neuroscience 2012;209:39-46.

Kihara M, Kubo T. Immunocytochemical localization of glutamate containing neurons in the ventrolateral medulla oblongata and the nucleus tractus solitarius of the rat. J Hirnforsch 1991;32(1):113-118.

Krahl S, Clark K, Smith D, Browning R. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. Epilepsia 1998;39(7):709-714.

Krahl S, Senanayake S, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 2001;42(5):586-9.

Krahl S, Clark K. Vagus nerve stimulation for epilepsy: a review of central mechanisms. Surg Neurol Int 2012;3(S4):S255-259.

Liu Z, Chen CY, Bonham AC. Metabotropic glutamate receptors depress vagal and aortic baroreceptor signal transmission in the NTS. Am J Physiol 1998;275(5):1682-1694.

Lynn PA, Blackshaw LA. In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. J Physiol 1999;518(Pt1):271-282.

Maeda T. The locus coeruleus: history. J Chem Neuroanat 2000;18(1-2):57-64.

Manta S, Dong J, Debonnel G, Blier P. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. J Psychiatry Neurosci 2009;34(4):272-280.

Manta S, El Mansari M, Debonnel G, Blier P. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. Int J Neuropsychopharmacol 2013;16(2):459-470.

Mathis C, Moran TH, Schwartz GJ. Load-sensitive rat gastric vagal afferents encode volume but not gastric nutrients. Am J Physiol 1998;274(2Pt2):R280-286.

Mazarati AM, Liu H, Soomets U, Sankar R, Shin D, Katsumori H, Langel U, Wasterlain CG. Galanin modulation of seizures and seizure modulation of hippocampalgalanin in animal models of status epilepticus. J Neuroscience 1998;18(23):10070-10077.

Mazarati AM, Hohmann JG, Bacon A, Liu H, Sankar R, Steiner RA, Wynick D, Wasterlain CG. Modulation of hippocampal excitability and seizures by galanin. J Neuroscience 2000;20(16):6276-6281.

Mazarati A, Wasterlain C. Anticonvulsant effects of four neuropeptides in the rat hippocampus during self-sustaining status epilepticus. Neuroscience Lett 2002;331(2):123-127.

McIntyre DC, Edson N. Facilitation of amygdala kindling after norepinephrine depletion with 6-hydroxydopamine in rats. Exp Neurol 1981;74(3):748-757.

McLachlan R. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. Epilepsia 1993;34(5):918-923.

Meurs A, Clinckers R, Ebinger G, Michotte Y, Smolders I. Sigma 1 receptor-mediated increase in hippocampal extracellular dopamine contributes to the mechanism of the anticonvulsant action of neuropeptide Y. Eur J Neuroscience 2007;26(11):3079-3092.

Mishra PK, Kahle EH, Bettendorf AF, Dailey JW, Jobe PC. Anticonvulsant effects of intracerebroventricularly administered norepinephrine are potentiated in the presence of monoamine oxidase inhibition in severe seizure genetically epilepsy-prone rats (GEPR-9s). Life Sci 1993;52(17):1435-1441.

Naritoku D, Terry W, Helfert R. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res 1995;22(1):53-62.

Nelson KR, Mattingly M, Schmitt FA. Out-of-body experience and arousal. Neurology 2007;68:794-795.

Niijima A. Reflex effects of oral, gastrointestinal and hepatoportal glutamate sensors on vagal nerve activity. J Nutr 2000;130(4S):971S-3S.

Olpe HR, Jones RS. The action of anticonvulsant drugs on the firing of locus coeruleus neurons: selective, activating effect of carbamazepine. Eur J Pharmacol 1983;91(1):107-110.

Olpe HR, Steinmann M. Responses of locus coeruleus neurons to neuropeptides. Prog Brain Res 1991;88:241-248.

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

Peiris M, Bulmer DC, Baker MD, Boundouki G, Sinha S, Hobson A, Lee K, Aziz Q, Knowles CH. Human visceral afferent recordings: preliminary report. Gut 2011;60(2):204-208.

Price GD, Trussell LO. Estimate of the chloride concentration in a central glutamatergic terminal: a gramicidin perforated-patch study on the calynx of held. J Neurosci 2006;26(44):11432-11436.

Ramos BP, Arnsten AF. Adrenergic pharmacology and cognition: focus on the prefrontal cortex. Pharmacol Ther 2007;113(3):523-536.

Rijkhoff NJ, Holsheimer J, Koldewijn EL, Struijk JJ, van Kerrebroeck PE, Debruyne FM, Wijkstra H. Selective stimulation of sacral nerve roots for bladder control: a study by computer modeling. IEEE Trans Biomed Eng 1994;41(5):413-424.

Roosevelt R, Smith D, Clough R, Jensen R, Browning R. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res 2006;1119(1):124-132.

Rozman J, Sovinec B, Trlep M, Zorko B. Multielectrode spiral cuff for ordered and reversed activation of nerve fibres. J Biomed Eng 1993;15(2):113-120.

Sengupta JN, Petersen J, Peles S, Shaker R. Response properties of antral mechanosensitive afferent fibers and effects of ionotropic glutamate receptor antagonists. Neuroscience 2004;125(3):711-723.

Szot P, Weinshenker D, White SS, Robins CA, Rust NC, Schwartzkroin PA, Palmiter RD. Norepinephrine-deficient mice have increased susceptibility to seizure-inducing stimuli. J Neuroscience 1999;19(24):10985-10992.

Ungar IJ, Mortimer JT, Sweeney JD. Generation of unidirectionally propagating action potentials using a monopolar electrode cuff. Ann Biomed Eng 1986;14(5):437-450.

Ungerstedt U. Stereotactix mapping of the monoamine pathways in the rat brain. Acta Physiol Scand Suppl. 1971;367:1-48.

Van Bockstaele EJ, Peoples J, Telegan P. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. J Comp Neurol 1999;412(3):410-428.

Vuckovic A, Tosato M, Struijk JJ. A comparative study of three techniques for diameter selective fiber activation in the vagal nerve: anodal block, depolarizing prepulses and slowly rising pulses. J Neural Eng 2008;5(3):275-286.

Walker BR, Easton A, Gale K. Regulation of limbic motor seizures by GABA and glutamate transmission in nucleus tractus solitarius. Epilepsia 1999;40(8):1051–1057.

Weinshenker D, Szot P. The role of catecholamines in seizure susceptibility: new results using genetically engineered mice. Pharmacol Ther 2002;94(3):213-233.

Woodbury D, Woodbury J. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 1990;31(Suppl 2):S7-19.

Woodbury J, Woodbury D. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff electrode for stimulating and recording. Pacing Clin Electrophysiol 1991;14(1):94-107.

Xu Z, Shi T, Hökfelt T. Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and –R2 receptors. J Comp Neurol 1998;392(2):227-251.

Yan QS, Dailey JW, Steenbergen JL, Jobe PC. Anticonvulsant effect of enhancement of noradrenergic transmission in the superior colliculus in genetically epilepsy-prone rats (GEPRs): a microinjection study. Brain Res 1998;780(2):199-209.

Zabara J. Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia 1992;33(6):1005-1012.

Chapter 4

Rationale and research aims

Rationale

Every year, more than 80 new cases of epilepsy occur per 100,000 persons, making it the second most common neurological disorder following neurovascular diseases (Sander and Shorvon 1996; Banerjee and Hauser 2008; Hirose 2013). More than 30% of all epilepsy patients suffer from uncontrolled seizures or experience unacceptable medication-related side effects despite adequate pharmacological treatment (Blume 2008). The inability to adequately treat all patients with refractory epilepsy provides a continuous impetus to investigate novel forms of treatment. One novel treatment option is neurostimulation. This thesis focuses on VNS.

Despite the fact that VNS is an efficacious, safe and worldwide accepted treatment option for patients who are considered unsuitable for resective surgery or in whom surgery failed, specific issues remain to counteract its full therapeutic application.

Mechanism of action

The precise mechanism of action of VNS for refractory epilepsy remains to be elucidated, but the central noradrenergic pathway has been shown to play a key role in the circuitry required for the antiepileptic effects of VNS. The first evidence for a prominent role of the noradrenergic LC in the antiepileptic activity induced by VNS dates back to 1998, when Krahl et al. showed that the permanent loss or functional inactivation of LC neurons abolished the antiepileptic effects of VNS in the MES model (Krahl et al. 1998). Results from electrophysiological studies in the rat brain showed that the activity of LC neurons is increased upon acute and chronic stimulation of the vagus nerve (Groves et al. 2005; Dorr and Debonnel 2006; Manta et al. 2009). Confirming the recruitment of LC neurons during VNS, immediate-early gene mRNAs or their protein transcripts increase within LC neurons, in both rats (Naritoku et al. 1995) and rabbits (Gieroba and Blessing 1994; Cunningham et al. 2008). Increases in extracellular noradrenaline concentrations have been measured by microdialysis in projection areas of the LC, such as the prefrontal cortex (Roosevelt et al. 2006; Follesa et al. 2007; Manta et al. 2013), hippocampus (Roosevelt et al. 2006; Manta et al. 2013) and amygdala (Hassert et al. 2004) in VNS-treated rats. Although these studies show VNS-induced noradrenaline increases, no correlations were made with antiepileptic effects of VNS. One aim of this thesis was therefore to evaluate whether VNS-induced changes in extracellular hippocampal noradrenaline levels are involved in the antiepileptic mechanism of action of VNS.

Stimulation parameters

The stimulation parameters currently used in clinical practice are not evidence based and vary considerably. It is routine clinical practice to uptitrate the output current intensity in order to reach seizure control over several weeks/months. Several preclinical and clinical studies however do suggest that lower current density might be sufficient to achieve clinical efficacy (Woodbury and Woodbury 1990; Zagon and Kemeny 2000; Van Laere et al. 2000; Vonck et al. 2008; Cunningham et al. 2008). A second aim of this thesis was therefore to test the hypothesis that output current intensities in the lower range are sufficient to obtain seizure-suppressing effects.

Predictive factors for response

Clinical response to VNS is variable and unpredictable (Boon et al. 2007). Treatment with VNS reduces seizures with ≥ 50% in 50% of patients. These patients are defined as responders. In about 30% of patients, there is little or no effect. These patients are defined as non-responders. In the other 20% of patients, seizure frequency reduction ranges between 30-50%. These patients are defined as partial responders. So far, no predictive criteria for success have been identified - it is still unclear why some patients do and others do not respond to the VNS therapy. In current clinical practice, physicians are unable to assess true VNS-induced vagal nerve fiber activation. Apart from unravelling and understanding the effect of VNS in the brain, research on activation of the vagus nerve itself remains a key point, as adequate activation of the vagus nerve is ultimately necessary to achieve any positive effects. A third aim of this thesis was therefore to identify a biomarker reflecting true VNS-induced activation of the vagus nerve. Recording CAPs would be a good biomarker.

Research aims

The aims of this thesis are

(i) to investigate whether VNS-induced changes in extracellular hippocampal noradrenaline levels are involved in the antiepileptic mechanism of action of VNS in the intrahippocampal pilocarpine rat model of acute limbic seizures,

(ii) to investigate the effect of various VNS output current intensities on cortical excitability in the motor cortex stimulation rat model, and

(iii) to identify and characterize a neurophysiological parameter reflecting true VNS-induced activation of the vagus nerve.

References

Banerjee PN, Hauser WA. Incidence and prevalence. In Epilepsy: a comprehensive textbook, 2nd Edition (Engel J, Pedley TA, eds.) 2008. Philadelphia, USA: Lippincot Williams & Wilkins.

Blume W. Drug-resistant epilepsy. In: Epilepsy: a comprehensive textbook, 2nd Edition (Engel J, Pedley TA, eds) 2008. Philadelphia, USA: Lippincot Williams & Wilkins.

Boon P, De Herdt V, Vonck K, Van Roost D. Clinical experience with vagus nerve stimulation and deep brain stimulation in epilepsy. Acta Neurochir Suppl 2007;97(Pt2):273-280.

Cunningham JT, Mifflin SW, Gould GG, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation. Neuropharmacology 2008;33(8):1884-1895.

Dorr AE, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 2006 318(2):890-898.

Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, Puligheddu M, Marrosu F, Biggio G. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res 2007;1179:28-34.

Gieroba ZJ, Blessing WW. Fos-containing neurons in medulla and pons after unilateral stimulation of the afferent abdominal vagus in conscious rabbits. Neuroscience 1994;59(4):851-858.

Groves DA, Bowman EM, Brown VJ. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neurosci Lett 2005;379(3):174-179.

Hassert D, Miyashita A, Williams C. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behav Neuroscience 2004;118(1):79-88.

Hirose G. An overview of epilepsy: its history, classification, pathophysiology and management. Brain Nerve 2013;65(5):509-520.

Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure attenuating effects of vagus nerve stimulation. Epilepsia 1998;39(7):709-714.

Manta S, Dong J, Debonnel G, Blier P. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. J Psychiatry Neurosci 2009;34(4):272-280.

Manta S, El Mansari M, Debonnel G, Blier P. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. Int J Neuropsychopharmacol 2013;16(2):459-470.

Naritoku D, Terry W, Helfert R. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res 1995;22(1):53-62.

Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res 2006;1119(1):124-132.

Sander JW, Shorvon SD. Epidemiology of the epilepsies. J Neurol Neurosurg Psychiatry 1996;61(5):433-443.

Van Laere K, Vonck K, Boon P, Brans B, VandenKerckhove T, Dierckx R. Vagus nerve stimulation in refractory epilepsy: SPECT activation study. J Nucl Med 2000;41(7):1145-1154.

Vonck K, De Herdt V, Bosman T, Dedeurwaerdere S,Van Laere K, Boon P. Thalamic and limbic involvement in the mechanism of action of vagus nerve stimulation, a SPECT study. Seizure 2008;17(8):699-706.

Woodbury DM, Woodbury JW. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 1990;31(S2):S7-S19.

Zagon A, Kemeny AA. Slow hyperpolarization in cortical neurons: a possible mechanism behind vagus nerve simulation therapy for refractory epilepsy? Epilepsia 2000;41(11):1382-1389.

Chapter 5

Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model

Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model

Raedt R.[a,1], Clinckers R.[b,1], Mollet L.[a], Vonck K.[a], El Tahry R.[a], Wyckhuys T.[a], De Herdt V.[a], Carrette E.[a], Wadman W.[c], Michotte Y.[b], Smolders I.[b], Boon P.[a] and Meurs A.[a]

[a] Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Ghent, Belgium
[b] Department of Pharmaceutical Chemistry, Drug Analysis & Drug Information, Center for Neuroscience, Vrije Universiteit Brussel, Belgium
[c] Swammerdam Institute of Life Sciences, Department of Neurobiology, University of Amsterdam, Amsterdam, The Netherlands

[1] *These authors contributed equally to this work*

Journal of Neurochemistry 2011;117(3):461-469

Abstract

Introduction: Vagus nerve stimulation (VNS) is an effective adjunctive treatment for medically refractory epilepsy. In this study, we measured VNS-induced changes in hippocampal neurotransmitter levels and determined their potential involvement in the anticonvulsive action of VNS, in order to elucidate the mechanism of action responsible for the seizure suppressing effect of VNS in an animal model for limbic seizures.

Methods: We used in vivo intracerebral microdialysis to measure VNS-induced changes in hippocampal extracellular concentrations of noradrenaline, dopamine, serotonin and GABA in freely moving, male Wistar rats. During the same experiment, the effect of VNS on pilocarpine-induced limbic seizures was assessed using video-EEG monitoring. The involvement of VNS-induced increases in hippocampal noradrenaline in the mechanims of action of VNS was evaluated by blocking hippocampal α_2 receptors.

Results: VNS produced a significant increase in hippocampal noradrenaline concentration (69 ± 16% above baseline levels). VNS also increased the latency between pilocarpine infusion and the onset of epileptiform discharges, and reduced the duration and severity of pilocarpine-induced limbic seizures. A strong positive correlation was found between the noradrenergic and anticonvulsive effects of VNS. Blockade of hippocampal α_2 receptors reversed the seizure-suppressing effect of VNS.

Conclusion: VNS induces increases in extracellular hippocampal noradrenaline, which are at least partly responsible for its seizure-suppressing effect in a model for limbic seizures, and constitute a potential biomarker for the efficacy of VNS in temporal lobe epilepsy.

Introduction

Vagus nerve stimulation (VNS) is an adjunctive treatment for refractory epilepsy in patients who are unsuitable candidates for epilepsy surgery (Ben-Menachem 2002). Worldwide, more than 50,000 epilepsy patients have been treated with VNS. Several studies, including two large double-blind randomized clinical trials (Ben-Menachem et al. 1994; DeGiorgio et al. 2000), have confirmed the efficacy of VNS in different types of epilepsy. Seizure reduction as a result of VNS ranges from 25% to 55%, and varies considerably from patient to patient. In responders, VNS causes either a rapid or a delayed reduction in seizure frequency. However, a significant fraction (approximately one third) of patients does not respond to VNS. Because the mechanism of action of VNS in epilepsy is currently unknown, it is not clear which factors determine the patient's response to the treatment, nor what the most optimal stimulation parameters are.

The vagus nerve is a mixed nerve consisting of 20% efferent (motor) and 80% afferent (sensory) fibers. The nucleus of the solitary tract (NTS) receives the largest number of vagal afferents. The NTS in turn projects to pontine nuclei, the cerebellum and the mesencephalon, but also to regions which are frequently involved in the generation of seizures such as the cortex, thalamus and amygdala. The vagus nerve also projects directly to the raphe nucleus and indirectly to the locus coeruleus (LC). These nuclei are the major sources of serotonergic and noradrenergic neurons in the brain, respectively (Henry 2002). Both send direct projections to the hippocampus, a brain structure that is frequently involved in the generation of epileptic seizures in temporal lobe epilepsy (TLE) (Castle et al. 2005).

Interestingly, bilateral destruction of the LC has been found to reverse the seizure-suppressing effect of VNS in the maximal electroshock model (Krahl et al. 1998). Single-unit recording experiments have shown that the activity of noradrenergic neurons in the LC is increased upon stimulation of the vagus nerve (Dorr and Debonnel 2006a; Groves et al. 2005). Presumably as a result of enhancement of the activity of LC neurons, increases in extracellular noradrenaline concentration have been measured in projection areas of the LC such as the hippocampus and cortex in VNS-treated rats (Roosevelt et al. 2006).

The noradrenergic system has been convincingly implicated in the control of seizure activity, especially for seizures that spread along the limbic system (Giorgi et al. 2004; Weinshenker and Szot

2002a). Animals in which the noradrenergic system has been damaged (McIntyre and Edson 1981; Corcoran 1988; Bortolotto and Cavalheiro 1986) or is genetically impaired (Mishra et al. 1993; Yan et al. 1998; Szot et al. 1999) generally have a higher susceptibility to experimentally evoked seizures and seizure susceptibility in these models can be reduced by restoring noradrenergic activity (Barry et al. 1987; Bengzon et al. 1990; Kokaia et al. 1994; Mishra et al. 1993; Yan et al. 1998; Kokaia et al. 1989; Gross and Ferrendelli 1982). Loss of noradrenaline also attenuates the efficacy of a number of anticonvulsant therapies including the ketogenic diet and valproic acid (Weinshenker 2008; Schank et al. 2005). Conversely, activation of the noradrenergic system can inhibit seizure activity induced by electrical stimulation (McIntyre et al. 1982) or administration of chemoconvulsants (Ferraro et al. 1994). Noradrenaline interacts with three families of G-protein coupled adrenergic receptors (α_1, α_2, β receptors) which are further divided into different subtypes (α_{1A-B}, α_{1D}, α_{2A-C}, β_{1-3} receptors) (Ramos and Arnsten 2007). It has highest affinity for α_2 adrenergic receptors, which are generally coupled to Gi proteins. The α_2 adrenergic receptor subtype is considered to be the most promiscuous anticonvulsant adrenoreceptor (Weinshenker and Szot 2002).

Recent work by our group provides further insight into the relative contribution of different adrenergic receptor subtypes to the anticonvulsive action of noradrenaline. Administation of maprotiline, a selective noradrenaline reuptake inhibitor, in the intrahippocampal pilocarpine model for limbic seizures resulted in increased levels of brain noradrenaline and potent suppression of pilocarpine-induced limbic seizures. Administration of a threshold anticonvulsive dose of maprotiline resulted in a 70% increase of hippocampal noradrenaline levels. Using selective agonists and antagonists for the different adrenergic receptor subtypes, we then showed that seizure suppression was mediated by combined activation of α_2 and β_2 adrenergic receptors. Application of an antagonist of either α_2 (SKF-86466) or β_2 adrenergic receptors (ICI-118551) reversed the anticonvulsive effect of increased hippocampal noradrenaline. On the other hand, combined application of an α_2 (medetomidine) and β_2 adrenoreceptor agonist (salmeterol) was necessary to obtain seizures suppression (Clinckers et al. 2010).

Given the established role of increased noradrenergic transmission in the control of limbic seizures, it is conceivable that VNS produces its anticonvulsive effect by increasing noradrenaline levels in structures that are critically involved in the generation of limbic seizures, such as the hippocampus. To test this hypothesis would require that the noradrenergic and anticonvulsive effects of VNS are measured concomitantly in the same group of animals. This experiment has not been performed to date.

The aim of this study was to evaluate whether VNS-induced changes in hippocampal neurotransmitter levels, particularily those of noradrenaline, are involved in the mechanism of action of VNS in TLE. In a first series of experiments, we determined the effect of VNS on hippocampal noradrenaline, dopamine, serotonin and GABA levels and on pilocarpine-induced limbic seizures in the same group of animals. Subsequently, we tested the hypothesis that increases in hippocampal noradrenaline mediate the anticonvulsive effect of VNS. To this end, we studied the effect of blockade of hippocampal adrenoreceptors on the seizure suppressing action of VNS. We chose to use a selective α_2 adrenoreceptor antagonist in these experiments, given that hippocampal α_2 adrenoreceptor blockade was previously found to be sufficient to completely reverse the anticonvulsive effect of a noradrenaline reuptake inhibitor in the same animal model (Clinckers et al. 2010).

Methods

Chemicals and Reagents

Pilocarpine HCL and SKF-86466 HCl were purchased from Sigma (St. Louis, MO, USA). All other chemicals were analytical reagent grade or better and were obtained from Merck (Darmstadt, Germany). Aqueous solutions were made with purified water (Seralpur pro 90 CN, Belgolabo, Overijse, Belgium) and filtered through a 0.2 µm membrane filter. The aqueous perfusion solution for the microdialysis experiments, subsequently referred to as modified Ringer's solution, consisted of 147 mM NaCl, 2.3mM $CaCl_2$ and 4mM KCl. An antioxidant solution containing 3.3 mM L-cystein, 0.27 mM Na_2EDTA, 12.5 µM ascorbic acid and 100 mM glacial acetic acid was used to stabilize collected monoamines in the dialysates. All compounds were dissolved in modified Ringer's solution and administered via the microdialysis probe.

Animals

Thirty-seven male Wistar rats (Charles River Laboratories, Belgium) weighing 250-275g were used. Animals were treated according to 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%) and implanted with two epidural recording electrodes in the right and left os frontale, a ground (reference) electrode close to the sutura lambdoidea and four anchor screws bilaterally in the os frontale and parietale. A bipolar depth electrode was fixed to a microdialysis guide cannula (CMA/Microdialysis; Solna; Sweden), and both were stereotactically implanted in the left hippocampus (coordinates relative to bregma: rostrocaudal -5.6 mm; medio-lateral -4.6 mm; dorso-ventral -4.6 mm, i.e. 3 mm above final microdialysis probe membrane position). A custom-made silicone spiral cuff-electrode with platinum contacts was implanted around the left vagus nerve. To minimize post-operative pain, buprenorphine (1 mg/kg) was intraperitonally administrated 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 microdialysis probe in the left hippocampus and the cuff electrode around the left vagus nerve was verified post-mortem. Animals with aberrant probe or cuff electrode localization were excluded from the study.

Video-EEG monitoring and intracerebral microdialysis

One week after surgery, rats were placed in specialized neuromonitoring cages equipped for simultaneous performance of VNS, video-EEG monitoring and microdialysis sampling in freely moving conditions. Rats were connected to (i) a custom-built digital video-EEG monitoring system, and (ii) an external current stimulator (NCP, model 100; Cyberonics Inc., Houston, TX, USA) via an electrical swivel (Plastics One, Roanoke, USA). The microdialysis cannula obturator was replaced by a microdialysis probe (CMA12; 3 mm membrane length; theoretical cut-off 20 kDa; CMA/Microdialysis, Solna, Sweden). The microdialysis probe was continuously perfused with modified Ringer's solution at a flow rate of 2 µl/min. A 15 - 20 h interval between probe implantation and the beginning of the experiment was respected to ensure integrity of the blood-brain barrier (Benveniste 1989), absence of excessive reactive gliosis in the tissue surrounding the microdialysis probe (Georgieva et al. 1993) and stable basal neurotransmitter dialysate concentrations (Clapp-Lilly et al. 1999).

Experimental design

In all animals, limbic seizures were evoked by intrahippocampal perfusion of the muscarinic agonist pilocarpine (10 mM) via the microdialysis probe. This rodent model for acute limbic seizures (Millan et al. 1993) has been extensively used in our laboratory (Smolders et al. 1997; Meurs et al. 2008), and

is characterized by the sequential development of typical behavioural patterns, electrographic activity and neurochemical alterations (Meurs et al. 2008).

In a first series of experiments, we studied the effects of VNS on hippocampal neurotransmitter levels and pilocarpine-induced limbic seizure activity. Based on the results of these experiments, we conducted a second series of experiments in which we tested whether the effects of VNS on pilocarpine-induced seizure activity could be reversed by intrahippocampal application of a selective α_2 adrenoreceptor antagonist.

During each experiment, the microdialysis probe was continuously perfused at a flow-rate of 2 µl / min and hippocampal dialysate samples (40 µl) were collected every 20 minutes. Protocols for each experimental group are shown in figure 1 and detailed below:

Effect of VNS on limbic seizure activity and hippocampal neurotransmitter levels

- **SHAM group (n=7)**: the probe was perfused with modified Ringer's solution (R) during the first 15 collection periods. During collection periods 16 and 17, 10 mM pilocarpine was added to the perfusion fluid. Subsequently, perfusion fluid was switched back to modified Ringer's solution and samples were collected for another five collection periods (collection periods 18-22).

- **VNS group (n=12)**: protocol as for the SHAM rats except but VNS was performed from the beginning of collection period 10 until the end of the experiment (i.e. collection period 22). VNS was delivered with the following stimulation parameters: frequency = 30 Hz; intensity = 1 mA; pulse width = 250 µsec; duty cycle = 7 sec on - 18 sec off.

Effect of α_2 adrenoreceptors antagonism on the seizure-suppressing effect of VNS

- **SHAM-SKF group (n=10)**: from collection period 7 until the end of the experiment, 1 nM SKF-86466 was added to the perfusion fluid. During collection period 16 and 17, perfusion was switched to a mixture of 10 mM pilocarpine and 1 nM SKF-86466. Subsequently, perfusion fluid was switched back to the 1 nM SKF-86466 solution and samples were collected for another five collection periods (collection periods 18-22). The SKF-86166 perfusate concentration was selected based on the published affinity constant (Ki= 6nM) (Heal et al. 1995). This concentration was compensated for expected probe recovery (approximately 10%) and multiplied by 2 to obtain full receptor antagonism.

- **VNS-SKF group (n=8)**: protocol as for the SHAM-SKF group, but VNS (frequency = 30 Hz; intensity = 1 mA; pulse width = 250 µsec; duty cycle = 7 sec on - 18 sec off) was delivered from collection period 10 onwards.

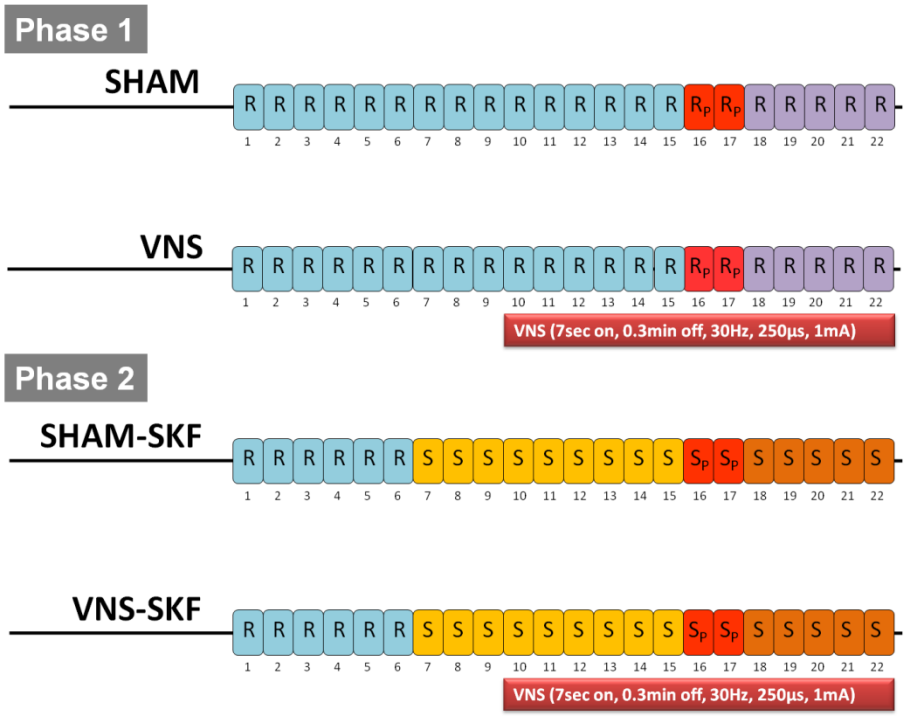


Figure 1| Schematic representation of experimental protocols: Every square represents a 20-min perfusion of the microdialysis probe with: R = modified Ringer's solution; RP = 10 mM pilocarpine dissolved in modified Ringer's solution; S = 1 nM SKF-86466 (α_2 receptor antagonist) dissolved in modified Ringer's solution and SP = 10 mM pilocarpine and 1 nM SKF-86466 dissolved in modified Ringer's solution.

Video-EEG recording

All rats were monitored with continuous video-EEG recording throughout the experiment. Behavioural changes indicative of seizure activity were scored by reviewing video recordings. For each collection period, behavioural changes were rated on a seizure severity scale based on Racine's scale, which was adapted to include all behavioural changes observed in focal limbic seizure models: (0) normal, non-epileptic activity; (1) mouth and facial movements, hyperactivity, grooming, sniffing, scratching, wet dog shakes; (2) head nodding, staring, tremor; (3) forelimb clonus; forelimb extension; (4) rearing, salivating, tonic-clonic activity; (5) falling, status epilepticus. For each of the seven collection periods following the start of the pilocarpine administration (i.e. collection periods 16 to 22) the highest seizure severity score (SSS) was retained. Total seizure severity score (TSSS) for each animal was calculated as the sum of the seizure severity scores (SSS), and used as a measure for seizure severity throughout the experiment. Hippocampal and cortical EEG recordings were reviewed to determine the latency to occurrence of the first epileptiform activity (spikes) and the total duration of the epileptiform activity after the start of the pilocarpine infusion (collection periods 16 to 22).

Microdialysate analysis

Dialysate samples were split for analysis of monoamines (dopamine, serotonin, noradrenaline) (25µL) and GABA (15µL). For monoamine analysis, we performed an off-line microbore liquid chromatography assay (C8, 5 µm; 100 x 1 mm) based on ion-pair reversed phase chromatography, coupled to single-channel electrochemical detection with a low oxidation potential (+450 mV vs. Ag/AgCl) (Decade, Antec, Leiden, The Netherlands), as has previously been described in detail (Smolders et al. 2008). For the analysis of GABA, we performed pre-column derivatisation with ophtalaldehyde/ 2-methyl-2-propanethiol and iodoacetamide followed by reversed-phase isocratic microbore liquid chromatography (C8, 5 µm; 100x1 mm; Unijet, Bioanalytical Systems) and amperometric detection, as has previously been described in detail (Smolders et al. 1995).

Data analysis

All statistical analyses were performed using SPSS 15 for Windows. Data are expressed as mean ± standard error of the mean. The significance level for demonstrating differences between groups was set at α=0.05. To evaluate VNS-induced changes in hippocampal neurotransmitter levels, a Student's T-test for paired comparison was used. In the VNS group, mean neurotransmitter levels during baseline collections 4 to 9 were compared to the mean neurotransmitter levels during the six collection periods after the start of VNS and prior to pilocarpine perfusion (collection periods 10-15). In the VNS-SKF group, mean neurotransmitter levels in the three collections during SKF infusion (collection periods 7-9) were compared to the mean neurotransmitter levels during the six collection periods in which VNS and SKF were co-administered prior to pilocarpine infusion (collection periods 10-15). The effect of VNS on pilocarpine-induced seizure activity was determined by comparing SSS and TSSS (Mann-Whitney U test), and latency to epileptiform discharges and duration of epileptiform discharges on the hippocampal EEG (Student's T test for comparison of independent samples) between the SHAM and VNS group. Pearson correlation tests were performed to analyze correlation between VNS-induced increase of hippocampal NAD levels and seizure parameters (severity, duration and latency).

The effect of hippocampal α_2 adrenoreceptors antagonism on pilocarpine-induced seizures and on the seizure-suppresing effect of VNS were assessed by comparing TSSS (Kruskal-Wallis ANOVA followed by Mann-Whitney U post-hoc tests using adjusted P-levels after Bonferroni correction), and latency and duration of epileptiform discharges on hippocampal EEG (one-way ANOVA followed by Bonferroni-Dunn post-hoc tests for pairwise comparison) between the relevant groups.

Results

Effect of VNS on limbic seizure activity and hippocampal neurotransmitter levels

In the SHAM-treated group, perfusion of a 10 mM pilocarpine solution through the hippocampal microdialysis probe during 40 min elicited a host of behavioural changes including increased exploratory behaviour, wet dog shakes and excessive grooming, beginning 15-20 min after the start of pilocarpine administration. Approximately 30 to 60 min following pilocarpine, these changes progressively evolved into recurring bouts of staring, mouth and facial movements, forelimb clonus, rearing, salivation and rarely falling. Individual behavioural seizures typically lasted between 5 s and 1 min. EEG showed continuous epileptic spikes and intermittent rhythmic epileptiform discharges in the hippocampus, which occasionally generalized to the cortex (fig. 2A). None of the animals developed a status epilepticus or died.

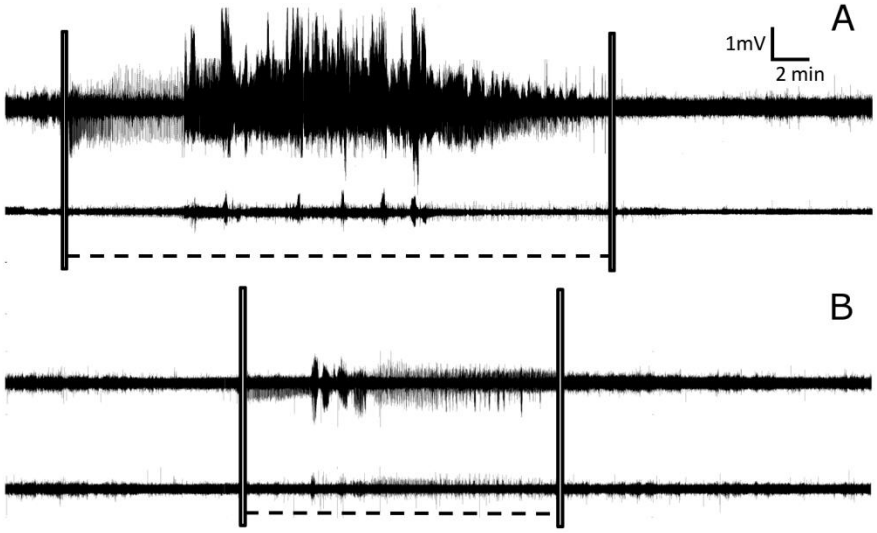


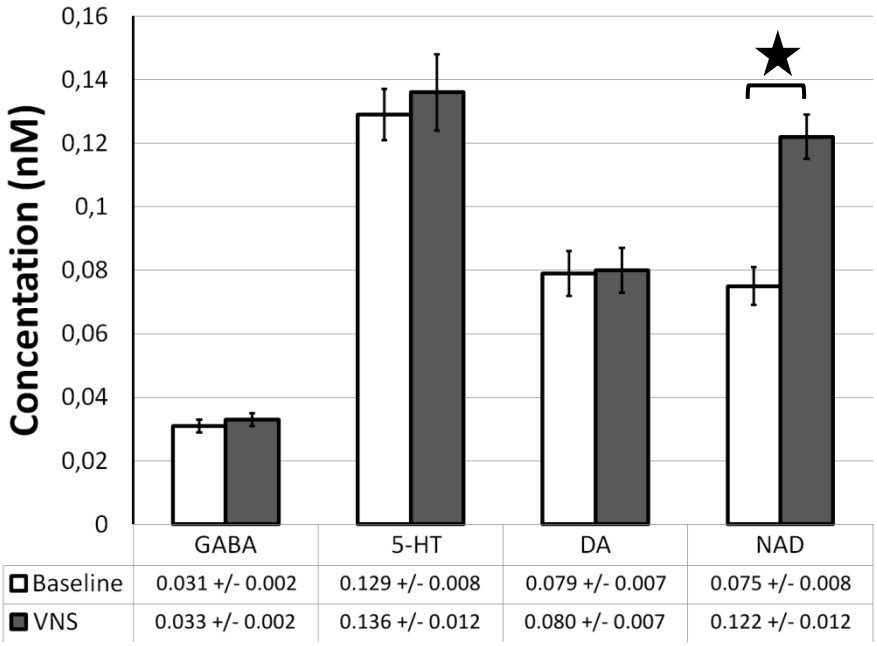
Figure 2| Representative sample of EEG recorded from SHAM- **(A)** and VNS-treated rats **(B)**. The upper traces represent EEG recorded from the left hippocampal depth electrode. The lower traces represent EEG recorded from an epidural electrode positioned over the right frontal cortex. EEG recordings are shown from the start of intrahippocampal pilocarpine infusion until the end of the experiment (i.e. collection periods 16–22, 140 min). The first vertical line represents the onset of epileptiform activity on hippocampal EEG. The second vertical line represents the end of epileptiform activity on hippocampal EEG. Both in SHAM- and VNS-treated rats, intrahippocampal infusion of pilocarpine-induced epileptic spikes and intermittent rhythmic epileptiform discharges, visible on hippocampal EEG and occasionally also on cortical EEG. In VNS-treated animals **(B)**, the latency to first epileptiform activity was significantly increased and the total duration of epileptiform activity was significantly decreased compared with SHAM-treated animals **(A)**.

In the VNS-treated group, pilocarpine-induced behavioural changes indicative of seizure activity were significantly less severe than in the SHAM group (table 1). Overall seizure severity was significantly lower in the VNS-treated group (TSSS = 5 +/- 1) compared to the SHAM group (TSSS = 14 +/- 2) ($p < 0.01$). The latency between the start of pilocarpine administration and the occurrence of epileptiform activity on the hippocampal EEG was significantly prolonged in VNS-treated rats (26 +/- 4 min) compared to SHAM-treated rats (12 +/- 4 min) ($p < 0.05$, table 1). Moreover, the total duration of epileptiform activity on the hippocampal EEG was significantly shorter in VNS-treated rats (67 +/- 12 min) compared to SHAM-treated rats (111 +/- 8 min) ($p < 0.05$, table 1). A representative sample of the EEG recorded from hippocampus and cortex in SHAM- and VNS-treated rats is shown in fig.2.

Table 1| Overview of seizure parameters (severity, latency and duration) in all experimental groups. In VNS and VNS-SKF group, a subdivision was made between non-responders and responders based on VNS-induced noradrenaline increase. In the VNS-SKF group, noradrenaline levels prior to VNS were already increased compared with baseline because of intrahippocampal infusion of 1 nM SKF-86466.

Treatment group	Seizure severity (TSSS)	Seizure latency (min)	Seizure duration (min)	SKF-induced NAD increase (%)	VNS-induced NAD increase (%)
SHAM	14 ± 2	12 ± 4	111 ± 8	N.A.	N.A.
VNS	5 ± 1	26 ± 4	67 ± 12	N.A.	69 ± 16
Non-Resp.	10 ± 1	10 ± 1	106 ± 10	N.A.	11 ± 9
Resp.	2 ± 1	38 ± 3	40 ± 10	N.A.	110 ± 9
VNS-SKF	11 ± 1	20 ± 5	100 ± 8	97 ± 14	57 ± 12
Non-Resp.	11 ± 3	9 ± 3	103 ± 27	101 ± 18	31 ± 11
Resp.	12 ± 1	24 ± 6	99 ± 7	84 ± 14	83 ± 7
SHAM-SKF	13 ± 2	20 ± 5	95 ± 2	84 ± 28	N.A.

During VNS, the mean dialysate concentration of noradrenaline increased by 69% +/- 16% (0.122 +/- 0.012 nM) compared to baseline (0.075 +/- 0.008 nM) ($p < 0.01$). VNS had no significant effect on the dialysate concentration of serotonin, dopamine and GABA (fig 3). VNS-induced increases in the dialysate concentration of noradrenaline were positively correlated with the latency to epileptiform activity on hippocampal EEG ($R2=0.82$; $p < 0.01$), and negatively correlated with the total duration of epileptiform activity on hippocampal EEG ($R2=0.62$; $p < 0.01$) and TSSS ($R2=0.81$; $p < 0.01$) (fig. 4 A-C). Animals could be divided into two distinct groups on the basis of the noradrenergic and anticonvulsive effects of VNS. Rats with a VNS-induced increase in hippocampal noradrenaline concentration of at least 70% (7 out of 12 rats, subsequently referred to as “responders”) had a pronounced reduction in seizure severity. Conversely, animals with a VNS-induced increase in hippocampal noradrenaline concentration of less than 70% (5 out of 12 rats, subsequently referred to as “non-responders”) were not protected from pilocarpine induced seizures. No significant correlation was found between baseline hippocampal NAD levels and seizure parameters (severity, latency and duration).



	GABA	5-HT	DA	NAD
Baseline	0.031 +/- 0.002	0.129 +/- 0.008	0.079 +/- 0.007	0.075 +/- 0.008
VNS	0.033 +/- 0.002	0.136 +/- 0.012	0.080 +/- 0.007	0.122 +/- 0.012

Figure 3| Effect of VNS on hippocampal levels of GABA, serotonin (5HT), dopamine (DA) and noradrenaline (NAD). In the VNS group, mean neurotransmitter levels during baseline collections 4–9 were compared with the mean neurotransmitter levels during the six collection periods after the start of VNS and prior to pilocarpine perfusion (collection periods 10–15). Average dialysate concentrations are presented in the table under the graph (in nM). No significant changes could be demonstrated for GABA, 5HT and DA. VNS did induce a significant increase of hippcampal NAD levels. *Statistical significant difference ($p < 0.05$).

Effect of α_2 adrenoreceptor antagonism on the seizure suppressing effect of VNS

Intrahippocampal infusion of 1 nM SKF-86466 alone induced an increase in mean noradrenaline dialysate concentration of 97 ± 14% in the VNS-SKF group and 84 +/- 28% in the SKF group (table 1). VNS-induced increases in noradrenaline in the VNS-SKF group were determined by comparing mean noradrenaline concentration in the three collections during SKF infusion (collection periods 7- 9) to the mean noradrenaline concentration during the six dialysate collections in which VNS and SKF were co-administered prior to pilocarpine infusion (collection periods 10-15). VNS produced an additional increase in mean noradrenaline dialysate concentration of 57 +/- 12% compared noradrenaline contrations during administration of SKF alone. Out of a total of 8 rats, 4 had a VNSinduced increase in hippocampal noradrenaline of ≥ 70% (further defined as responders).

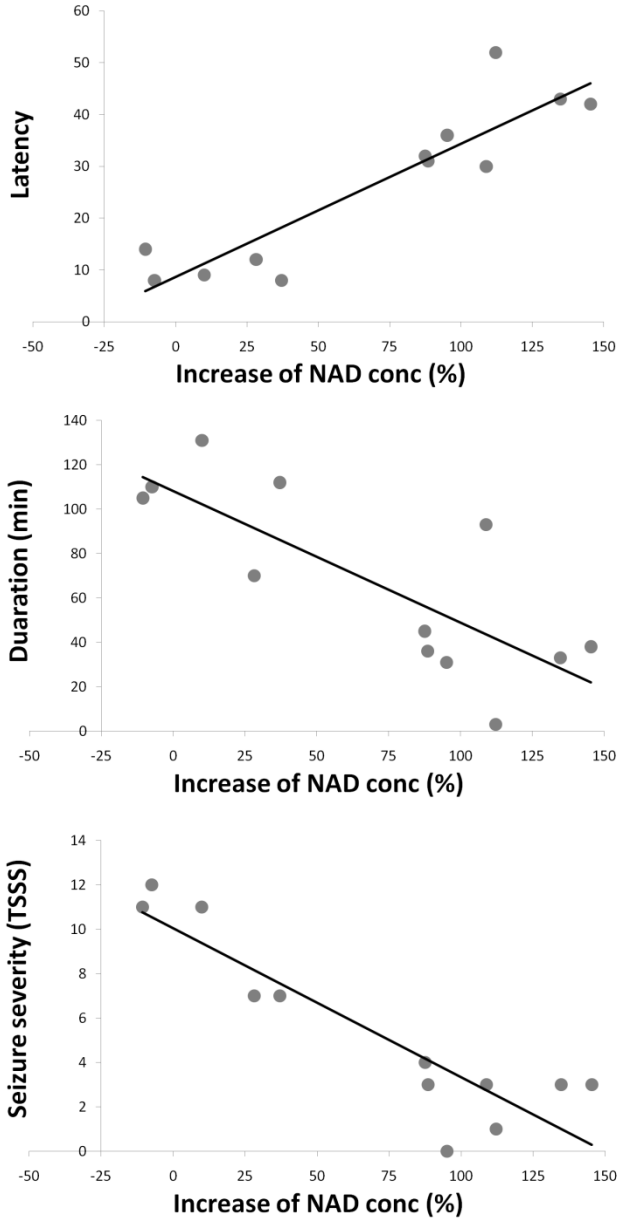


Figure 4| Correlation between VNS-induced changes in hippocampal noradrenaline levels and seizure-suppressing effects. **(A)** VNS-induced increases in hippocampal noradrenaline levels were positively correlated with the latency to epileptiform activity on hippocampal EEG after starting the pilocarpine infusion. VNS-induced increases in hippocampal noradrenaline levels were negatively correlated with **(B)** the total duration of epileptiform activity on hippocampal EEG and **(C)** clinical seizure severity represented by TSSS.

Because the aim of these experiments was to determine the involvement of increased hippocampal noradrenaline in the seizure-suppressing effect of VNS, only responders were included in the statistical analysis of seizure activity.

In responders, intrahippocampal administration of the selective α_2 adrenoreceptor antagonist SKF-86466 completely reversed the seizure-suppressing effect of VNS. Latency to epileptiform discharges,

total duration of epileptiform activity and TSSS were similar for VNS-SKF- and SHAM-SKF-treated rats (fig. 5). Seizure duration and severity in the VNS-SKF group were significantly increased compared to VNS-treated rats, and were comparable to what was observed in SHAM-treated rats (fig.5B-C).

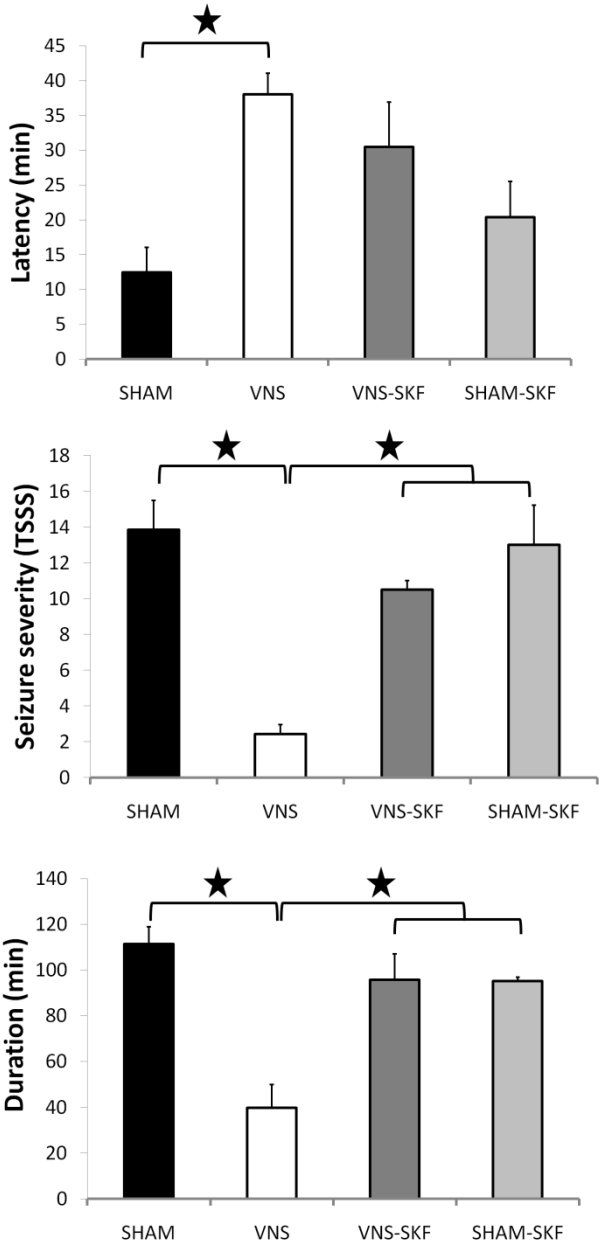


Figure 5| Effect of α_2 adrenoreceptor antagonism on the seizuresuppressive effects of VNS. Intahippocampal infusion of the selective α_2 adrenoreceptor antagonist SKF-86466 completely blocked VNSinduced effects on latency to epileptiform discharges **(A)**, total duration of epileptiform activity **(B)** and clinical seizure severity represented by TSSS **(C)** because these outcome parameters were similar for VNSSKF and SHAM-SKF rats. *Statistical significant difference ($p < 0.05$).

Discussion

The main findings of this study are (i) that VNS induces an increase in the extracellular hippocampal concentration of noradrenaline, but not of dopamine, serotonin and GABA; (ii) that VNS prevents the development of pilocarpine-induced limbic seizures only in those rats in which hippocampal noradrenaline increased by at least 70%; and (iii) that selective α_2 adrenoreceptor antagonism in proximity of the seizure focus abolishes the seizure-suppressing effect of VNS. Taken together, these findings provide convincing evidence for the existence of a strong causal link between the seizuresuppressing effect of VNS and increased hippocampal noradrenergic signaling.

Roosevelt and co-workers have previously reported a bilateral increase in noradrenaline levels in the cortex (39%) and the hippocampus (28%) in response to one hour of VNS (20 Hz, 1 mA, 500 µsec, 30 sec ON - 10 min OFF) (Roosevelt et al. 2006). In our hands, VNS produced a more than two-fold higher increase (69%) in extracellular hippocampal noradrenaline. This may be due to the fact that we used a more intensive stimulation protocol (30 Hz, 1 mA, 250 µsec, 7 sec ON - 18 sec OFF), resulting in the delivery of a total of 30,240 electrical pulses to the vagus nerve compared to 3,600 pulses in the study by Roosevelt and colleagues. The duty cycle used in our experiments (7 sec ON - 18 sec OFF) is referred to as 'rapid cycling', and is used to treat patients in whom VNS with a duty cycle of 30 sec ON - 10 min OFF did not sufficiently improve seizure control (Labar 2004; Liporace et al. 2001). In our hands, VNS suppressed pilocarpine-induced limbic seizures most strongly in those rats with the largest increase in hippocampal noradrenaline. Conversely, rats in which VNS did not increase hippocampal noradrenaline exhibited the most severe seizures. A noradrenaline increase of at least 70% seems to be associated with suppression of limbic seizures. Interestingly, in previous work, we found that hippocampal extracellular noradrenaline increased by 70% in rats that were treated with the selective noradrenaline reuptake inhibitor, maprotiline, at the minimal dose required to suppress pilocarpine-induced seizures (Clinkers et al. 2010).

Based on these observations, we hypothesized that VNS prevents the development of limbic seizures by increasing hippocampal noradrenaline. To test this hypothesis, we verified whether blockade of adrenoreceptors in proximity of the seizure focus would reverse the anticonvulsive effect of VNS. We chose to use a selective α_2 adrenoreceptor antagonist in these experiments, based on previous work in which we showed that application of this antagonist was sufficient to completely reverse the anticonvulsive effect of increased hippocampal noradrenaline (Clinckers et al. 2010).

We found that in rats with a VNS-induced increase in hippocampal noradrenaline of ≥ 70%, concomitant intrahippocampal administration of the selective α_2 adrenoreceptor antagonist SKF-

86466 abolished the anticonvulsive action of VNS. This finding strongly supports the hypothesis that the seizure suppressing effect of VNS is at least partly mediated by increased hippocampal noradrenaline concentration and increased hippocampal α_2 adrenoreceptor activation.

In our hands, only a subset of animals (7 out of 12) exhibited a VNS-induced increase in hippocampal noradrenaline that was sufficient to produce an anticonvulsant effect. This "responderrate" is remarkably similar to what has been observed in clinical trials with VNS.

One small open-label study in patients with TLE and seizures originating independently from the leftand right temporal lobes showed that VNS reduced seizure frequency by at least 50% in 6 out of 10 patients (Alsaadi et al. 2001). Larger clinical trials that have evaluated the efficacy of VNS in various types of refractory epilepsy have consistently shown that VNS is ineffective at reducing seizures in approximately one third of patients (Ben-Menachem et al. 1994; De Herdt et al. 2007; Handforth et al. 1998; Vonck et al. 2004).

Several, though not necessarily similar, factors could account for the inter-subject variablility of the response to VNS in our experiments and in patients suffering from TLE. First, there could be a difference in vagus nerve activation in response to VNS. Although an electrode lead break and stimulator defect was excluded by measuring impedance, it is possible that VNS does not activate vagus nerve fibers due to injury of the nerve induced by induced electrode implantation. Temporary nerve damage could explain the delayed effect of VNS in some patients with epilepsy implanted with a VNS system. Second, genetic variability and differences in external and/or internal environment could lead to differences in the number of noradrenergic neurons, their afferent and/or efferent projections and synaptic strengths within these noradrenergic neuronal networks. This intrinsic variation in the noradrenergic neural network could underlie the variable release of noradrenaline in response to VNS. One argument against this second hypothesis is that our study shows that differences in baseline noradrenaline levels do not explain the variation in clinical response to VNS. indicating that baseline hippocampal NAD level is no critical indicator of VNS mediated anticonvulsant effect.

Given the causal relationship between increased hippocampal noradrenaline and suppression of limbic seizures in response to VNS in rats, VNS-induced increases in hippocampal noradrenaline may be a useful biomarker for the efficacy of VNS in human TLE. If used in combination with a non-invasive technique to deliver VNS (f.i. transcutaneous activation of the vagus nerve (Dietrich et al. 2008)), a biomarker for the efficacy of VNS could help clinicians to reliably identify responders prior to surgical implantation of a VNS device, and to determine optimal stimulation parameters in a rational way.

Conclusion

The main findings of this study are (i) that VNS induces an increase in the extracellular hippocampal concentration of noradrenaline, but not of dopamine, serotonin and GABA; (ii) that VNS prevents the development of pilocarpine-induced limbic seizures only in those rats with VNS-induced increases in hippocampal noradrenaline of at least 70%; and (iii) that selective α_2 adrenoreceptor antagonism in proximity of the seizure focus abolishes the seizure-suppressing effect of VNS. Taken together, these findings provide convincing evidence for the existence of a strong causal link between increased noradrenergic signaling and the anticonvulsant effect of VNS.

Acknowledgements

We acknowledge the excellent technical assistance of Ria Berckmans and Gino De Smet, laboratory technicians at the VUB. Robrecht Raedt is a post-doctoral fellow supported by the Special Research Fund of Ghent University. Ralph Clinckers is a post-doctoral fellow of the Fund for Scientific Research-Flanders (FWO Vlaanderen). Kristl Vonck is supported by a grant from the BOF fund of Ghent University Hospital. Paul Boon is supported by grants from the Fund for Scientific Research- Flanders and Ghent University Research Fund and by the Clinical Epilepsy Grant from Ghent University Hospital.

References

Alsaadi TM, Laxer KD, Barbaro NM, Marks WJ, Jr., Garcia PA. Vagus nerve stimulation for the treatment of bilateral independent temporal lobe epilepsy. Epilepsia 2001;42:954-956.

Aston-Jones G, Shipley MT, Chouvet G et al. Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog Brain Res 1991;88:47-75.

Barry DI, Kikvadze I, Brundin P, Bolwig TG, Bjorklund A, Lindvall O. Grafted noradrenergic neurons suppress seizure development in kindling-induced epilepsy. Proc Natl Acad Sci U S A 1987;84:8712-8715.

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

Ben-Menachem E, Manon-Espaillat R, Ristanovic R et al. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 1994;35:616-626.

Bengzon J, Kokaia M, Brundin P, Lindvall O. Seizure suppression in kindling epilepsy by intrahippocampal locus coeruleus grafts: evidence for an alpha-2-adrenoreceptor mediated mechanism. Exp Brain Res 1990;81:433-437.

Benveniste H. Brain microdialysis. J Neurochem 1989;52:1667-1679.

Bohning DE, Lomarev MP, Denslow S, Nahas Z, Shastri A, George MS. Feasibility of vagus nerve stimulation-synchronized blood oxygenation level-dependent functional MRI. Investigative Radiology 2001;36:470-479.

Bonham AC, Chen CY. Synaptic transmission in nucleus tractus solitarius (NTS). In: Undem BJ, Weinreich D, editors. Advances in vagal afferent neurobiology. Boca Raton: CRC Press; 2005. p. 193-208.

Bortolotto ZA, Cavalheiro EA. Effect of DSP4 on hippocampal kindling in rats. Pharmacol Biochem Behav 1986;24:777-779.

Castle M, Comoli E, Loewy AD. Autonomic brainstem nuclei are linked to the hippocampus. Neuroscience 2005;134:657-669.

Clapp-Lilly KL, Roberts RC, Duffy LK, Irons KP, Hu Y, Drew KL. An ultrastructural analysis of tissue surrounding a microdialysis probe. J Neurosci Methods 1999;90:129-142.

Clinckers R, Zgavc T, Vermoesen K, Meurs A, Michotte Y, Smolders I. Pharmacological and neurochemical characterization of the involvement of hippocampal adrenoreceptor subtypes in the modulation of acute limbic seizures. J Neurochem 2010;115:1595-607.

Contreras RJ, Beckstead RM, Norgren R. The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. J Auton Nerv Syst 1982;6:303-322.

Corcoran ME. Characteristics of accelerated kindling after depletion of noradrenaline in adult rats. Neuropharmacology 1988;27:1081-1084.

Cunningham JT, Mifflin SW, Gould GG, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by Vagal nerve stimulation. Neuropsychopharmacology 2008;33:1884-1895.

De Herdt V, Boon P, Ceulemans B et al. Vagus nerve stimulation for refractory epilepsy: a Belgian multicenter study. Eur J Paediatr Neurol 2007;11:261-269.

DeGiorgio CM, Schachter SC, Handforth A et al. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia 2000;41:1195-1200.

Dietrich S, Smith J, Scherzinger C et al. A novel transcutaneous vagus nerve stimulation leads to brainstem and cerebral activations measured by functional MRI. Biomedizinische Technik 2008;53:104-111.

Dorr AE, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 2006b;318:890-898.

Dorr AE, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 2006a;318:890-898.

Ferraro G, Sardo P, Sabatino M, La G, V. Locus coeruleus noradrenaline system and focal penicillin hippocampal epilepsy: neurophysiological study. Epilepsy Res 1994;19:215-220.

Georgieva J, Luthman J, Mohringe B, Magnusson O. Tissue and microdialysate changes after repeated and permanent probe implantation in the striatum of freely moving rats. Brain Res Bull 1993;31:463-470.

Giorgi FS, Pizzanelli C, Biagioni F, Murri L, Fornai F. The role of norepinephrine in epilepsy: from the bench to the bedside. Neurosci Biobehav Rev 2004;28:507-524.

Gross RA, Ferrendelli JA. Relationships between norepinephrine and cyclic nucleotides in brain and seizure activity. Neuropharmacology 1982;21:655-661.

Groves DA, Bowman EM, Brown VJ. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neurosci Lett 2005;379:174-179.

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

Heal DJ, Cheetham SC, Butler SA, Gosden J, Prow MR, Buckett WR. Receptor binding and functional evidence suggest that postsynaptic alpha 2-adrenoceptors in rat brain are of the alpha 2D subtype. Eur J Pharmacol 1995;277:215-221.

Henry TR. Therapeutic mechanisms of vagus nerve stimulation. Neurology 2002;59:S3-14.

Henry TR, Bakay RA, Votaw JR et al. Brain blood flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: I. Acute effects at high and low levels of stimulation. Epilepsia 1998;39:983-990.

Kokaia M, Bengzon J, Kalen P, Lindvall O. Noradrenergic mechanisms in hippocampal kindling with rapidly recurring seizures. Brain Res 1989;491:398-402.

Kokaia M, Cenci MA, Elmer E et al. Seizure development and noradrenaline release in kindling epilepsy after noradrenergic reinnervation of the subcortically deafferented hippocampus by superior cervical ganglion or fetal locus coeruleus grafts. Exp Neurol 1994;130:351-361.

Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizureattenuating effects of vagus nerve stimulation. Epilepsia 1998;39:709-714.

Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 2001;42:586-9.

Labar D. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. Seizure 2004;13:392-398.

Levitt P, Moore RY. Noradrenaline neuron innervation of the neocortex in the rat. Brain Res 1978;139:219-231.

Liporace J, Hucko D, Morrow R et al. Vagal nerve stimulation: adjustments to reduce painful side effects. Neurology 2001;57:885-886.

McIntyre DC, Edson N. Facilitation of amygdala kindling after norepinephrine depletion with 6-hydroxydopamine in rats. Exp Neurol 1981;74:748-757.

McIntyre DC, Edson N, Chao G, Knowles V. Differential effect of acute vs chronic desmethylimipramine on the rate of amygdala kindling in rats. Exp Neurol 1982;78:158-166.

Meurs A, Clinckers R, Ebinger G, Michotte Y, Smolders I. Seizure activity and changes in hippocampal extracellular glutamate, GABA, dopamine and serotonin. Epilepsy Res 2008;78:50-59.

Millan MH, Chapman AG, Meldrum BS. Extracellular amino acid levels in hippocampus during pilocarpine-induced seizures. Epilepsy Res 1993;14:139-148.

Mishra PK, Kahle EH, Bettendorf AF, Dailey JW, Jobe PC. Anticonvulsant effects of intracerebroventricularly administered norepinephrine are potentiated in the presence of monoamine oxidase inhibition in severe seizure genetically epilepsy-prone rats (GEPR-9s). Life Sci 1993;52:1435-1441.

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

Nieuwenhuis S, Aston-Jones G, Cohen JD. Decision making, the P3, and the locus coeruleusnorepinephrine system. Psychol Bull 2005;131:510-532.

Olejniczak PW, Fisch BJ, Carey M, Butterbaugh G, Happel L, Tardo C. The effect of vagus nerve stimulation on epileptiform activity recorded from hippocampal depth electrodes. Epilepsia 2001;42:423-429.

Ramos BP, Arnsten AF. Adrenergic pharmacology and cognition: focus on the prefrontal cortex. Pharmacol Ther 2007;113:523-536.

Rhoton AL, Jr., O'Leary JL, Ferguson JP. The trigeminal, facial, vagal, and glossopharyngeal nerves in the monkey. Afferent connections. Arch Neurol 1966;14:530-540.

Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res 2006;1119:124-132.

Schank JR, Liles LC, Weinshenker D. Reduced anticonvulsant efficacy of valproic acid in dopamine beta-hydroxylase knockout mice. Epilepsy Res 2005;65:23-31.

Shaw GY, Sechtem P, Searl J, Dowdy ES. Predictors of laryngeal complications in patients implanted with the Cyberonics vagal nerve stimulator. Ann Otol Rhinol Laryngol 2006;115:260-267.

Smolders I, Clinckers R, Meurs A et al. Direct enhancement of hippocampal dopamine or serotonin levels as a pharmacodynamic measure of combined antidepressant-anticonvulsant action. Neuropharmacology 2008;54:1017-1028.

Smolders I, Sarre S, Michotte Y, Ebinger G. The analysis of excitatory, inhibitory and other amino acids in rat brain microdialysates using microbore liquid chromatography. J Neurosci Methods 1995;57:47-53.

Smolders I, Van BK, Ebinger G, Michotte Y. Hippocampal and cerebellar extracellular amino acids during pilocarpine-induced seizures in freely moving rats. Eur J Pharmacol 1997;319:21-29.

Szot P, Weinshenker D, White SS et al. Norepinephrine-deficient mice have increased susceptibility to seizure-inducing stimuli. J Neurosci 1999;19:10985-10992.

Van Bockstaele EJ, Peoples J, Telegan P. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. J Comp Neurol 1999;412:410-428.

Vonck K, De H, V, Bosman T, Dedeurwaerdere S, Van LK, Boon P. Thalamic and limbic involvement in the mechanism of action of vagus nerve stimulation, a SPECT study. Seizure 2008;17:699-706.

Vonck K, Thadani VY, Gilbert K et al. Vagus nerve stimulation for refractory epilepsy: A transatlantic experience. Journal of Clinical Neurophysiology 2004;21:283-289.

Weinshenker D. The contribution of norepinephrine and orexigenic neuropeptides to the anticonvulsant effect of the ketogenic diet. Epilepsia 2008;49 Suppl 8:104-107.

Weinshenker D, Szot P. The role of catecholamines in seizure susceptibility: new results using genetically engineered mice. Pharmacol Ther 2002;94:213-233.

Yan QS, Dailey JW, Steenbergen JL, Jobe PC. Anticonvulsant effect of enhancement of noradrenergic transmission in the superior colliculus in genetically epilepsy-prone rats (GEPRs): a microinjection study. Brain Res 1998;780:199-209.

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

Zalvan C, Sulica L, Wolf S, Cohen J, Gonzalez-Yanes O, Blitzer A. Laryngopharyngeal dysfunction from the implant vagal nerve stimulator. Laryngoscope 2003;113:221-225.

Chapter 6

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

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

Mollet L.[a], Grimonprez A.[a], Raedt R.[a], Delbeke J.[b], El Tahry R.[a], De Herdt V.[a], Meurs A.[a], Wadman W.[c], Boon P.[a], Vonck K.[a]

[a] Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology (LCEN3), Department of Neurology, Institute for Neuroscience, Ghent University Hospital, Belgium
[b] Institute of Neuroscience, Université catholique de Louvain, Medical School, Brussels, Belgium
[c] Swammerdam Institute of Life Sciences, Department of Neurobiology, University of Amsterdam, Amsterdam, The Netherlands

Acta Neurologica Scandinavica 2013;128(6):391-396.

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 mA, 0.25 mA, 0.5 mA and 1 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. $\Delta MST_{baseline}$, ΔMST_{VNS1}, $\Delta MST_{wash\text{-}out1}$, ΔMST_{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 ΔMST_{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 ΔMST_{VNS} at 0 mA, 0.25 mA, 0.5 mA and 1 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 ΔMST_{VNS} at 0.25 mA, 0.5 mA and 1 mA were significantly larger compared to the mean ΔMST_{VNS} at 0 mA ($p=0.002$ for 0.25 mA; $p=0.001$ for 0.5 mA; $p=0.011$ for 1 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 (Boon et al. 2001; Ben-Menachem 2002). 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: 0.25-3.5 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 (Heck et al. 2002). 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 (Ben-Menachem 2002; Henry 2002; Elliott et al. 2011).

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 (Woodbury and Woodbury 1990, 1991; Krahl et al. 2001). 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 (Naritoku et al. 1992; Henry et al. 1999; De Herdt et al. 2010; Raedt et al. 2011).

One clinical drawback of current VNS therapy is the variable therapeutic outcome (Uthman et al. 1993; Ben-Menachem et al. 1994; Handforth et al. 1998; Koo et al. 2001). Currently, VNS is succesfull in only one third of the treated patients (Boon et al. 2007). 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 (Van Laere et al. 2000; Vonck et al. 2008; Cunningham et al. 2008).

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 (De Herdt et al. 2010). In this rat model, the threshold for evoking focal, motor seizures is determined by electrical stimulation of the motor cortex in

unanaesthetized rats (Voskuyl et al. 1989; Liebetanz et al. 2006). 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.

Material and methods

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 (0.0 mA, 0.25 mA, 0.5 mA and 1.0 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 $MST_{baseline}$), $\Delta MS_{Twash\text{-}out1}$ (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 ΔMST_{VNS1} (i.e. MST_{VNS1} minus $MST_{baseline}$) and ΔMST_{VNS2} (i.e. MST_{VNS2} minus $MST_{baseline}$) were calculated. Finally, the mean ΔMST_{VNS} per VNS output current intensity was calculated as the mean of all $\Delta MST_{VNS}1$ and ΔMST_{VNS2} values.

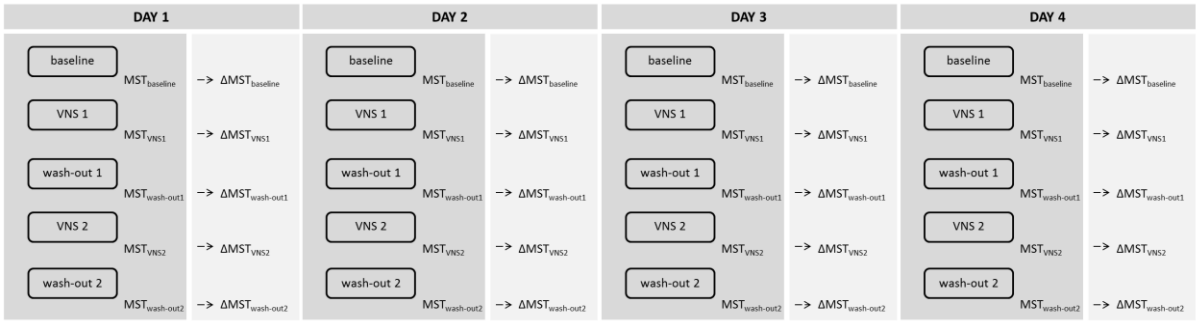


Figure 1| Schematic representation of the experimental design. Vagus nerve stimulation (VNS) at four output current intensities was administered randomly in each rat on four consecutive days. Per output current intensity, five-one-hour periods were conducted and the motor seizure threshold (MST) was 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 ΔMST_{VNS} at 0.25 mA, 0.5 mA and 1.0 mA with the mean ΔMST_{VNS} at 0.0 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 α=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 ΔMSTbaseline, the mean ΔMSTwash-out1 and the mean ΔMSTwash-out2, showing that VNS-induced changes in MST were transient and returned to baseline in the inter-stimulus periods.

The mean ΔMST_{VNS} as a function of VNS output current intensity is plotted in figure 2. The mean ΔMST_{VNS} at 0.0 mA, 0.25 mA, 0.5 mA and 1.0 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 ΔMST_{VNS} at 0.25 mA, 0.5 mA and 1.0 mA were significantly larger compared to the mean ΔMST_{VNS} at 0.0 mA (p = 0.002 for 0.25 mA; p = 0.001 for 0.5 mA and p = 0.011 for 1.0 mA).

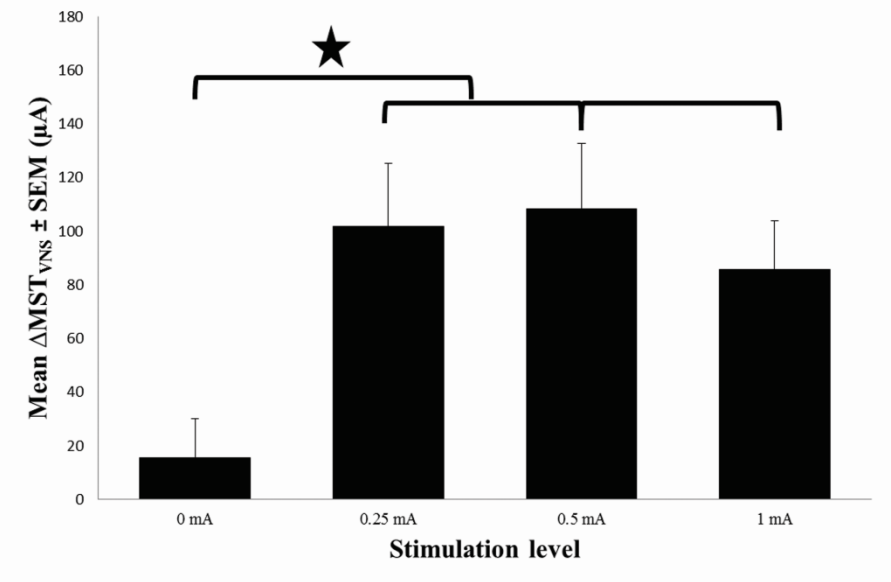


Figure 2| Effect of various vagus nerve stimulation (VNS) output current intensities on the motor seizure threshold (MST). The mean ΔMSTVNS (SEM) is plotted as a function of the VNS output current intensity.

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 VNS at 0.25 mA, 0.5 mA and 1.0 mA significantly increases the threshold for evoking focal, motor seizures compared to stimulation at 0.0 mA and 2) 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 (De Herdt et al. 2010). 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 (Woodbury and Woodbury 1991; Naritoku et al. 1992; Santiago-Rodriguez et al. 2006).

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 (Cunningham et al. 2008). 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 (Van Laere et al. 2000). These findings were confirmed in a human imaging study by Vonck et al. (Vonck et al. 2008).

Our observation, together with the observation of De Herdt et al. (De Herdt et al. 2010), that VNS at 0.25 mA, 0.5 mA, 0.75 mA and 1.0 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 (0.02-0.2 mA) are the large, myelinated A-fibers. At thresholds of 0.04-0.6 mA, smaller, myelinated B-fibers are recruited. C-fibers are small, unmyelinated fibers with the highest stimulation threshold of above 2 mA (Groves et al. 2005; Bunch et al. 2007). Initially, it was thought that the antiepileptic effect of VNS was directly related to the extent of C-fiber activation (Woodbury and Woodbury 1990). This theory was discarded after Krahl et al. demonstrated seizure suppression in rats even following selective destruction of C-fibers using capsaicin (Krahl et al. 2001). Furthermore, the group of Bunch concluded that therapeutically effective stimulation levels are below the threshold for C-fiber activation (Bunch et al. 2007). In an electrophysiological study by Evans et al., a C-fiber response was identified in 4 out of 8 patients using therapeutic VNS parameters (Evans et al. 2004). However, because the C-fiber response (i) was apparent in 2 of the 4 patients only with 2 or 3 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 0.2-0.5 mA already reduced chemically-induced seizures in rats (Woodbury and Woodbury 1990). 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 (Zagon et al. 2000). 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. (Takaya et al. 1996), 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 (Manta et al. 2009), in the effect of VNS on recognition memory in rats and humans (Clark et al. 1998, 1999) and in the effect of VNS on human tolerance for pain (Ness et al. 2000).

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 (Helmers et al. 2012). 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 helicoïdal); 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 (El Tahry et al. 2010).

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.

Acknowledgements, conflict of interest and sources of funding

There are no conflicts of interest to be reported. Lies Mollet is supported by a junior researcher (‘Aspirant’) grant from the “Fonds voor Wetenschappelijk Onderzoek (FWO)”. Prof. Dr. R. Raedt and

Prof. Dr. K. Vonck are supported by a grant from the “Bijzonder Onderzoeksfond (BOF)” from Ghent University Hospital. Prof. Dr. P. Boon is supported by grants from the FWO, grants from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital.

References

Ben-Menachem E, Manon-Espaillat R, Ristanovic R et al. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Group. Epilepsia 1994;35:616-626.

Ben-Menachem E. Vagus-nerve stimulation for the treatment of epilepsy. Lancet Neurol 2002;1:477-482.

Boon P, Vonck K, De Reuck J, Caemaert J. Vagus nerve stimulation for refractory epilepsy. Seizure 2001;10:448–455.

Boon P, De Herdt V, Vonck K, Van Roost D. Clinical experience with vagus nerve stimulation and deep brain stimulation in epilepsy. Acta Neurochir Suppl 2007;97:273-280.

Bunch S, DeGiorgio C, Krahl S et al. Vagus nerve stimulation for epilepsy: is output current correlated with acute response? Acta Neurol Scand 2007;116:217–220.

Clark K, Smith D, Hassert D, Browning R, Naritoku D, Jensen R. Posttraining electrical stimulation of vagal afferents with concomitant vagal efferent inactivation enhances memory storage processes in the rat. Neurobiol Learn Mem 1998;70:364-373.

Clark K, Naritoku D, Smith D, Browning R, Jensen R. Enhanced recognition memory following vagus nerve stimulation in human subjects. Nat Neurosci 1999;2:94–98.

Cunningham J, Mifflin S, Gould G, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation. Neuropharmacol 2008;33:1884-1895.

De Herdt V, De Waele J, Raedt R et al. Modulation of seizure threshold by vagus nerve stimulation in an animal model for motor seizures. Act Neurol Scand 2010;121:271-276.

Elliott R, Morsi A, Kalhorn S et al. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: long-term outcomes and predictors of response. Epilepsy Behav 2011;20:57-63.

El Tahry R, Raedt R, Mollet L et al. A novel implantable vagus nerve stimulation system (ADNS-300) for combined stimulation and recording of the vagus nerve: Pilot trial at Ghent University Hospital. Epilepsy Res 2010;92:231-239.

Evans M, Verma-Ahuja S, Naritoku D, Espinosa J. Intraoperative human vagus nerve compound action potentials. Acta Neurol Scand 2004;110:232-238.

Groves D, Bowman E, Brown V. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neurosci Lett 2005;379:174–179.

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

Heck C, Helmers S, DeGiorgio M. Vagus nerve stimulation therapy, epilepsy, and device parameters, scientific basis and recommendations to use. Neurology 2002;59:31-37.

Helmers S, Begnaud J, Cowley A et al. Application of a computational model of vagus nerve stimulation. Acta Neurol Scand 2012 (in press).

Henry T, Votaw J, Pennell P et al. Acute blood flow changes and efficacy of vagus nerve stimulation in partial epilepsy. Neurology 1999;52:1166-1173.

Henry T. Therapeutic mechanisms of vagus nerve stimulation. Neurology 2002;59:3-14.

Koo B, Ham S, Sood S, Tarver B. Human vagus nerve electrophysiology – a guide to vagus nerve stimulation parameters. J Clin Neurophysiol 2001;18:429-433.

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

Liebetanz D, Klinker F, Hering D et al. Anticonvulsant effects of transcardial direct-current stimulation (tDCS) in the rat cortical ramp model of focal epilepsy. Epilepsia 2006;47:1216-1224.

Manta S, Dong J, Debonnel G, Blier P. Optimization of vagus nerve stimulation parameters using the firing activity of serotonin neurons in the rat dorsal raphe. Eur Neuropsychopharmacol 2009;19:250-255.

Naritoku D, Morales A, Pencer T, Winkler D. Chronic vagus nerve stimulation increases the latency of the thalamocortical somatosensory evoked potential. Pacing Clin Electrophysiol 1992;15:1572-1578.

Ness T, Fillingim R, Randich A, Backensto E, Faught E. Low intensity vagal nerve stimulation lowers human thermal pain thresholds. Pain 2000;86:81–85.

Raedt R, Clinckers R, Mollet L et al. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. J Neurochem 2011;117:461-469.

Santiago-Rodriguez E, Alonso-Vanegas M, Cardenas-Morales L, Harmony T, Bernardino M, Fernandez-Bouzas A. Effects of two different cycles of vagus nerve stimulation on interictal epileptiform discharges. Seizure 2006;15:615-620.

Takaya M, Terry W, Naritoku D. Vagus nerve stimulation induced a sustained anticonvulsant effect. Epilepsia 1996;37:1111-1116.

Uthman B, Wilder B, Penry J et al. Treatment of epilepsy by stimulation of the vagus nerve. Neurology 1993;43:1338-13345.

Van Laere K, Vonck K, Boon P, Brans B, Vandekerckhove T, Dierckx R. Vagus nerve stimulation in refractory epilepsy: SPECT activation study. J Nucl Med 2000;41:1145-1154.

Vonck K, De Herdt V, Bosman T, Dedeurwaerdere S, Van Laere K, Boon P. Thalamic and limbic involvement in the mechanism of action of vagus nerve stimulation, a SPECT study. Seizure 2008;17:699-706.

Voskuyl R, Dingemanse J, Danhof M. Determination of the threshold for convulsions by direct cortical stimulation. Epilepsy Res 1989;3:120-129.

Woodbury D, Woodbury J. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 1990;31:7-19.

Woodbury J, Woodbury D. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff-electrode for stimulating and recording. Pacing Clin Electrophysiol 1991;14:94-107.

Zagon A, Kemeny A. Slow hyperpolarization in cortical neurons: a possible mechanism behind vagus nerve simulation therapy for refractory epilepsy? Epilepsia 2000;41:1382–1389.

Chapter 7

Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats

Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats

El Tahry R.[a,1], Mollet L.[a,1], Raedt R.[a,c], Delbeke J.[b], De Herdt V.[a,c], Wyckhuys T.[a,c], Hemelsoet D.[a,c], Meurs A.[a,c], Vonck K.[a,c], Wadman W.[a], Boon P.[a,c]

[a] Ghent University Hospital, Laboratory for Experimental and Clinical Neurophysiology, De Pintelaan 185, 9000 Gent, Belgium
[b] Institute of Neuroscience (IoNS), Université catholique de Louvain, Medical School, Brussels, Belgium
[c] Reference Center for Refractory Epilepsy, Department of Neurology, Ghent University Hospital, Gent, Belgium

[1] *These authors contributed equally to this work*

Journal of Neuroscience Methods 2011;198(2):287-293

Abstract

Rationale: Vagus nerve stimulation (VNS) is an adjunctive treatment for patients with refractory epilepsy. In more than 30% of the patients VNS has no therapeutic effect. The goal of this study was to find an objective parameter that can be used as an indicator of effective stimulation of the vagus nerve.

Methods: The electrophysiological response to VNS was recorded from the vagus nerve, recurrent laryngeal nerve and larynx muscles. Nerve lesions and muscle relaxing agent were used to find the source of the electrophysiological response. A cuff-electrode for chronic stimulation and recording was implanted for chronic recording of the VNS-induced electrophysiological response after implantation. Dose‑response curves were determined daily during a follow-up period of 2 months.

Results: VNS induced an electrophysiological response around 3 ms after start of the stimulation. This response was identified as a larynx compound action potential (LCMAP) LCMAP could be recorded immediately after surgery in 11/21 rats, while in the other 10/21 rats, a recovery period with an average of 25 days was required. Once the LCAMP could be recorded, the latency and overall characteristics of the doses response curves of the LCMAP remained stable during the entire follow-up period.

Conclusions: In this study, we provide an objective electrophysiological parameter for vagus nerve activation. LCAMP may indicate recovery of the vagus nerve after implantation, which may help to determine when uptitration of VNS therapy can be initiated. LCAMP could be of value in future experiments for objectification of VNS in animal models for epilepsy.

Introduction

Epilepsy is a neurological disorder characterized by recurrent aberrant electrical activity in the central nervous system that typically manifests itself as seizures. It is estimated that 1-2% of the population is affected worldwide. About 30% of the patients with epilepsy do not respond to antiepileptic drugs and are considered medically refractory (Hauser et al. 1996; Fisher et al. 2005). For these patients, alternative treatment modalities such as epilepsy surgery or neurostimulation, such as deep brain stimulation or vagus nerve stimulation, may be useful.

The vagus nerve is a mixed cranial nerve that consists of 80% afferent fibers innervating the heart, aorta, lungs and gastro intestinal tract and 20% efferent fibers that provide parasympathetic innervations of these structures and innervate the voluntary striated muscles of the larynx and pharynx through the recurrent laryngeal nerve, which is similar in rats (Dahlqvist et al. 1982; Paxinos 2004) and humans (Berthoud and Neuhuber 2000; Jotz et al. 2011). Moreover, the proportion of myelinated axons in the cervical left vagus nerve of rats is comparable to humans (Hofmann and Schitzlein 1961; Soltanpour and Santer 1996). At last, the left superior laryngeal nerve splits from the vagus nerve identically in rats and humans, as it runs back superiorly behind the aortic arch in a groove between oesophagus and trachea to finally enter into the larynx (Berthoud and Neuhuber 2000; Paxinos 2004).

Electrical stimulation of the left vagus nerve is used as an adjunctive treatment for patients with refractory seizures (Holder et al. 1992; BenMenachem et al. 1994; Handforth et al. 1998a,b; DeGiorgio et al. 2000; Uthman et al. 2003). The mechanism of action of vagus nerve stimulation (VNS) remains incompletely understood. There is little or no information available about the electrophysiology of the vagus nerve, although activation of the nerve is essential to its antiepileptic effect (Holder et al. 1992; BenMenachem et al. 1994; Handforth et al. 1998a,b). Many questions in VNS therapy remain unresolved, for example why some patients experience beneficial effects and others do not respond to the treatment. In current clinical epilepsy practice, no investigation is available to assess whether the vagus nerve is successfully activated by VNS or not. Defining a parameter reflecting stimulation-induced activation of the vagus nerve activation might provide a better understanding of the electrophysiological properties of the nerve. This in turn, could lead to further optimization of VNS treatment.

The aim of this study was to identify a marker reflecting effective stimulation of the vagus nerve. In the first part of the study an electrophysiological response to VNS was measured using thin-point recording electrodes placed near the stimulation cuff electrode. By inducing lesions at various levels

along the vagus and recurrent laryngeal nerves, performing simultaneous EMG recording and applying a muscle paralyzing agent the electrophysiological response was identified to be a far field potential of a VNS-induced larynx compound muscle action potential (LCAMP). During the second part of this study a new self-sizing cuff electrode for combined stimulation and recording was designed and used to record VNS-induced LCAMP on a daily basis and for several weeks after implantation of the stimulation electrode. In humans, intra-operative VNS-induced LCAMP was described (Ardesch et al. 2010), but no studies, animal nor human, report chronic LCAMP recordings.

Materials and methods

Design of the cuff-electrode

The self-sizing spiral cuff electrode is composed of two 80 μm thick silicone rubber sheets (Statice Santé, France) glued together with an adhesive which polymerizes at room temperature (Parts A and B MED 4-4210, Nusil). The internal sheet is stretched during curling (stretch factor of 0.5) in order to obtain a self-curling spiral cuff. The cuff has an internal diameter of 1 mm and a total length of 9 mm.

For the acute experiments two pieces of platinum (Alfa Aesar, 99.9% metal basis, 0.25 mm thick) are inserted between the silicone sheets to form the stimulation contacts (Fig. 1b). The inter-electrode distance between stimulation of the anode and cathode (each 3 mm $\times$ 1 mm) was 1 mm. Windows of 500 μm diameter are cut out in the internal silicone sheet in order to give the platinum contacts to the nerve.

For the chronic experiments a cuff electrode was manufactured with an extra contact for recording (Fig. 1a and c). Therefore a third piece of platinum (1 mm $\times$ 1 mm) was inserted between the silicone sheet at 2 mm from the cathode, near the cuff edge directed towards the head.

Teflon coated stainless steel wires (FWM 1 $\times$ 7 $\times$ 0.02/316LVM/EFTE, Fort Wayne metals) of 20 cm were welded to each platinum contact before their insertion between the electrode silicone sheets. Connector pins were soldered at the other extremity of the leads, allowing connection to an external stimulator or a recording device.

Animals

Adult male Wistar rats (Harlan, The Netherlands), 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 08/37). All animals were

kept under environmentally controlled conditions (12 h light/dark cycles, 20–23 ° C and 50% relative humidity) with food and water *ad libitum*.

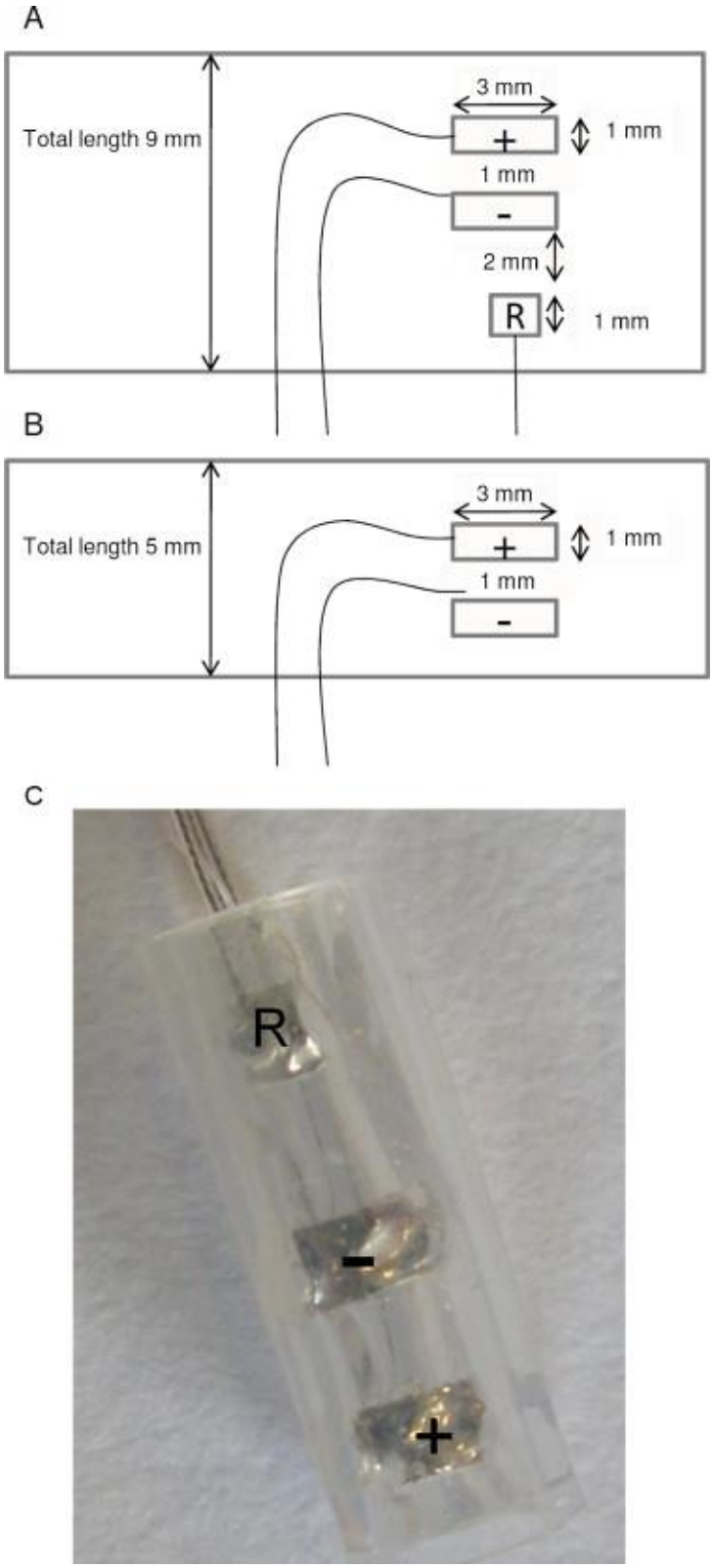


Figure 1| Schematic representation of a self-sizing cuff-electrode for combined stimulation and recording **(A,C)** and for stimulation only **(B)**.

Surgery

- **Acute experiments**

Wistar rats (250‑350 g) (n = 4) were implanted with a spiral cuff stimulation electrode around the left vagus nerve. Rats were anesthetized with gas isoflurane anesthesia (5% isoflurane for induction, 2% isoflurane for maintenance). An incision was made over the left anterior cervical region. The left vagus nerve was carefully dissected from the aortic sheet and the cuff electrode was wound around the nerve with the anode placed caudally. To record the electrophysiological response to VNS thin-point, stainless steel electrodes (125 µm diameter) were placed on different anatomical structures, including the vagus nerve, as well as the recurrent laryngeal nerve and the muscles surrounding the larynx. An epidural electrode, placed over posterior occipital cortex, was used as reference/ground electrode. In order to induce lesions along the nerves a nylon wire was strapped around the nerves. Vecuronium (Norcuron, 1 ml of 2 mg/ml solution), a muscle relaxing agent, was applied to paralyze the larynx muscles. At the end of each acute experiment, animals were sacrificed with an overdose of pentobarbital (180 mg/kg i.p).

- **Chronic experiments**

Wistar rats (250‑350 g) (*n* = 21) were implanted with a spiral cuff electrode for stimulation and recording of the left vagus nerve. The rats were anesthetized with a ketamine/xylazine (respectively, 80 mg/kg and 7.5 mg/kg, i.p.) mixture. For chronic use, the electrode leads were tunnelled to an incision made in the skin above the skull. The connector pins were fixed to a skull head stage of acrylic cement. Four epidural stainless steel anchor screws were screwed bilaterally into the skull above parietal and occipital cortex. The posterior right screw, placed over occipital cortex, served as ground/reference for chronic electrophysiological recording.

Recording of the larynx compound action muscle potential

The vagus nerve was stimulated with biphasic square wave pulses of 100 µs duration in the acute experiments in order to keep stimulation artifact as low as possible and allow recording of possible early physiological signals in response to stimulation. For chronic experiments 500 µs block-pulses were used because this pulse width is mostly used in chronic studies on efficacy of VNS in humans and animals. Stimuli were delivered by a constant current stimulator. Dose‑response curves were determined using a stimulus intensity ranging between 40 µA and 800 µA. Signals were recorded from stainless steel wire point electrodes placed on the vagus nerve, recurrent laryngeal nerve and larynx muscle or from the monopolar contact inside the cuff electrode. Signals were amplified 500

times before high pass filtering at 0.15 Hz in order to remove DC components. Thereafter, the data were digitized using a National Instruments acquisition board (NI DAQ PAD 6259) and finally stored on a personal computer. Recording and analysis of signals were done using Matlab (2007a, the MathWorks, Natick, Massachusetts

Data analysis

Both in the acute and chronic experiments, the latency of the LCAMP was determined. The latency is defined as the delay between the onset of the stimulus artifact and the occurrence of the major negative peak. In addition, acute and chronic dose response curves of the LCAMP were determined. In the chronic experiments LCAMP dose response curves were determined on a daily basis for five days per week during a follow-up period of 8 weeks after surgery. At each session, the rats were anesthetized using isoflurane (induction 5%, maintenance 2%) in order to reduce movement artefacts. The time span, during which vagus nerve was stimulated but no response could be recorded, was considered to represent a recovery period after surgery.

A Boltzmann function: ($M = M_{max}/1 + e(I_{50}–x)/k$) was fitted to the measured dose‑response curves. M_{max} is defined as the maximal muscle potential amplitude. I_{50} is the intensity needed to obtain a response with half the maximal amplitude and slope factor k describes the recruitment homogeneity of the vagus nerve fibers (Fig. 2). For each stimulus intensity, 20 sweeps were averaged to improve the signal to noise ratio. Besides the latency, I_{50} and k, the impedance of the stimulation contacts was recorded over time. The contact impedance of the stimulation electrodes was expressed in kOhms and defined as the voltage to current ratio measured at the end of a stimulation pulse of 100 µA amplitude and 500 µs duration. At the end of the 8 week follow-up period animals were sacrificed with an overdose of pentobarbital (180 mg/kg i.p).

Statistical analysis

From the chronic LCAMP recordings, parameters of the dose‑response curves (latency, I_{50} and k) and electrode impedances were averaged for each rat and for each of the eight follow-up weeks. Sensitivity analysis showed that there was no bias effect of pooling the results of the animals with a recovery period to the results of the rats in which the LCAMP could be recorded from the start of the chronic recordings. Therefore, the means and standard errors of the mean (SEM), presented in figures, were calculated from all rats in which an LCAMP could be recorded during a specific week. Statistical analysis was performed using mixed model linear regression analysis, including random intercepts in order to account for dependent observations. A Bonferonni correction was used to

correct for type I error in multiple comparisons. Calculated residuals were normally distributed, which supports the validity of the used statistical model.

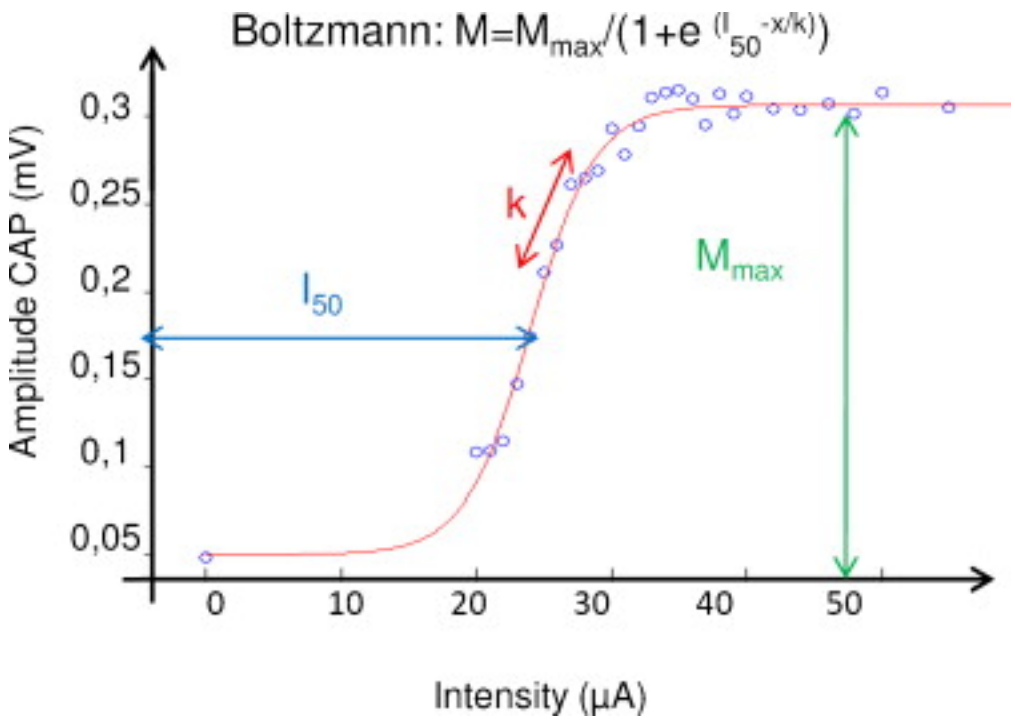


Figure 2| A representative example of a dose response curve of the larynx compound action muscle potential (LCAMP) in response to VNS. A Boltzmann function ($M = M_{max}/1 + e(I_{50}-x)/k$) was fitted to the dose response curve. I_{max} is defined as the intensity needed to achieve a response with maximal amplitude. I_{50} is the intensity needed to obtain a response with half of the maximal amplitude and k is the slope factor representing recruitment homogeneity of the vagus nerve fibers.

Results

Acute experiments: identification of the larynx compound muscle action potential

VNS reproducibly induced a large negative peak at 2.6 ms ± 0.2 ms after onset of the stimulation artifact (N_1) (Fig. 3a). Based on its long latency and large amplitude we hypothesized that this response is a far field potential corresponding to larynx muscle activation induced by co-activating of the recurrent laryngeal nerve with VNS. The following observations support this hypothesis: (1) A lesion of the vagus nerve distal to the stimulation electrode but proximal to the aortic arch, abolished the recorded signals (Fig. 3b); (2) A proximal lesion of the vagus nerve did not abolish the signal (Fig. 3a); (3) An electromyography (EMG) recording of the laryngeal muscles shows a very large response at latency of 2.6 ms (Fig. 3c), and (4) Finally, all the signals recorded at the level of the vagus nerve,

recurrent laryngeal nerve and larynx muscle disappeared immediately when applying the muscle blocking agent Vecuronium (1 ml of Norcuron, 4 mg/2 ml ampoule) to the larynx muscles.

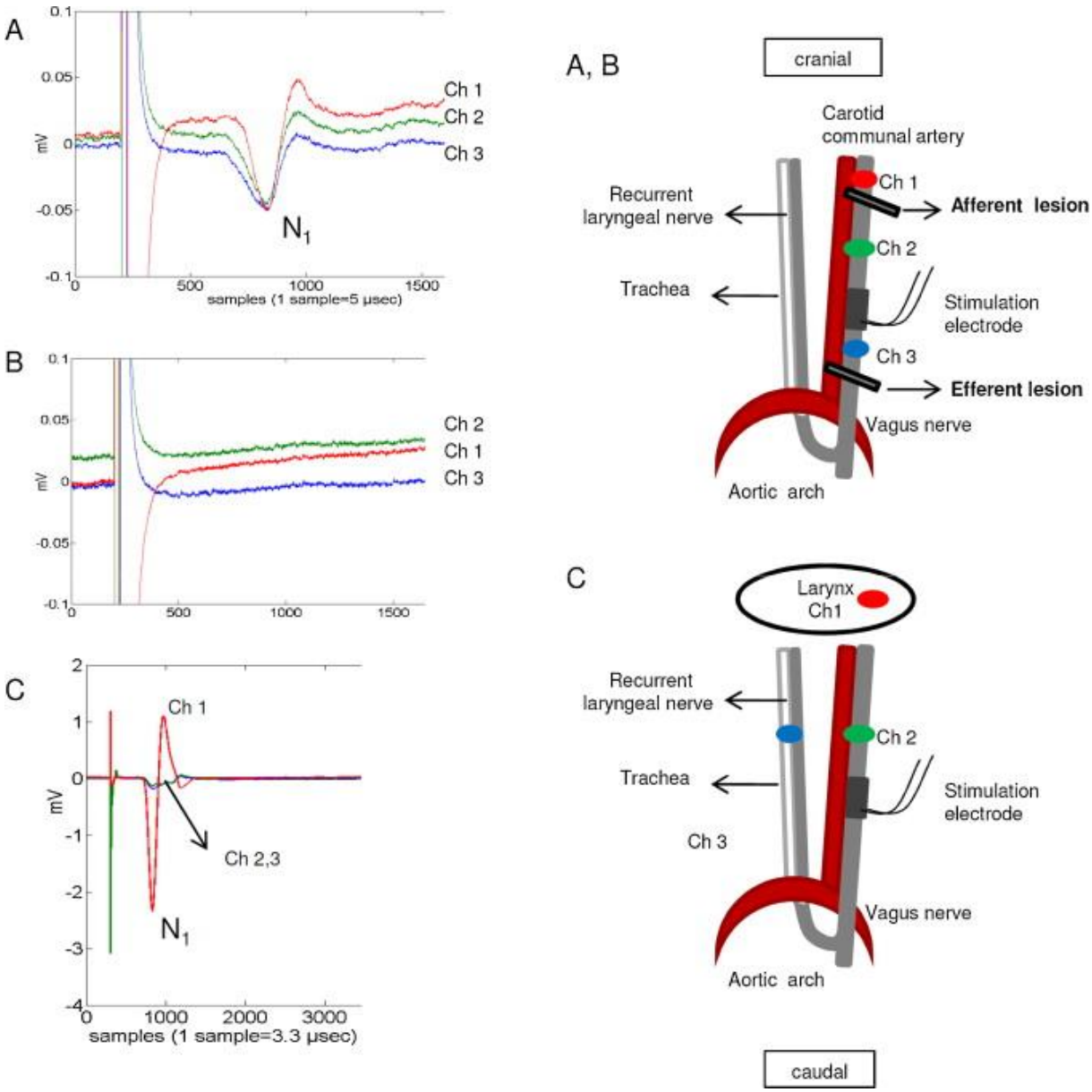


Figure 3| (A) VNS-induced neurophysiological response measured at different locations on the vagus nerve (Ch 1-3), characterized by a major negative peak (N_1). The response remains preserved after inducing a vagus nerve lesion rostral to the stimulation electrode. **(B)** Lesion of the vagus nerve distal to the stimulation electrode but proximal to the aortic arch, abolished all signals recorded at the level the vagus nerve (Ch 1-3). **(C)** Signals recorded from larynx muscles (Ch 1), the vagus nerve (Ch 2), the recurrent laryngeal nerve (Ch 3) showed no difference in latency. Moreover, the larynx EMG channel (Ch 1) exhibited much larger amplitude than signals recorded from other channels. All signals were abolished when applying Vecuronium into the larynx muscles.

Acute and chronic recordings of the of the larynx compound muscle action potential

In the acute experiments, latency of N_1 of the LCAMP recorded at the level of the vagus nerve, was 2.6 ms ± 0.2 ms after onset of the stimulation artifact. The I_{50} and slope factor of acute doses response curves were respectively, 125.8 ± 35.7 µA and 28 ± 30 µA. In the chronic experiments, 21 rats were implanted with a cuff electrode for combined stimulation and recording of the vagus nerve. LCMAP could be recorded immediately after surgery in 11/21 rats, while in the other 10/21 rats a recovery period ranging between 2 and 7 weeks (mean 25 days) was needed (Table 1).

Table 1| Amount of rats per week after surgery in which an LCAMP could be recorded. In 11/21 rats, LCAMP could be measured immediately after implantation. In the remaining rats (10/21), a post-surgical recovery period was required before LCAMP could be recorded adequately.

Time after surgery (weeks)	Amount of rats with recordable LCAMP
0	11
1	11
2	13
3	13
4	13
5	16
6	20
7	21

The latency of N1 was 3.2 ms ± 0.1 ms and did not change significantly over time during the 8 weeks of follow up ($p = 0.88$) (Fig. 4a). The I50 calculated from the doses response curves did not significantly change over time and varied between 56 µA ± 7 µA and 74 µA ± 18 µA ($p = 0.77$) ($p = 0.77$) (Fig. 4b). The slope factor of the doses response curves varied between 4.2 µA ± 0.7 µA and 6.7 µA ± 2.0 µA, indicating that implanted electrodes were able to activate vagus nerve fibers in a stable manner over the entire follow-up period ($p = 0.82$) (Fig. 4c).

The impedance of the stimulation contacts significantly increased over time ($p < 0.001$), values of week 4 to week 8 being significantly higher in comparison to the first week (post-hoc analysis with bonferonni correction) (Fig. 5).

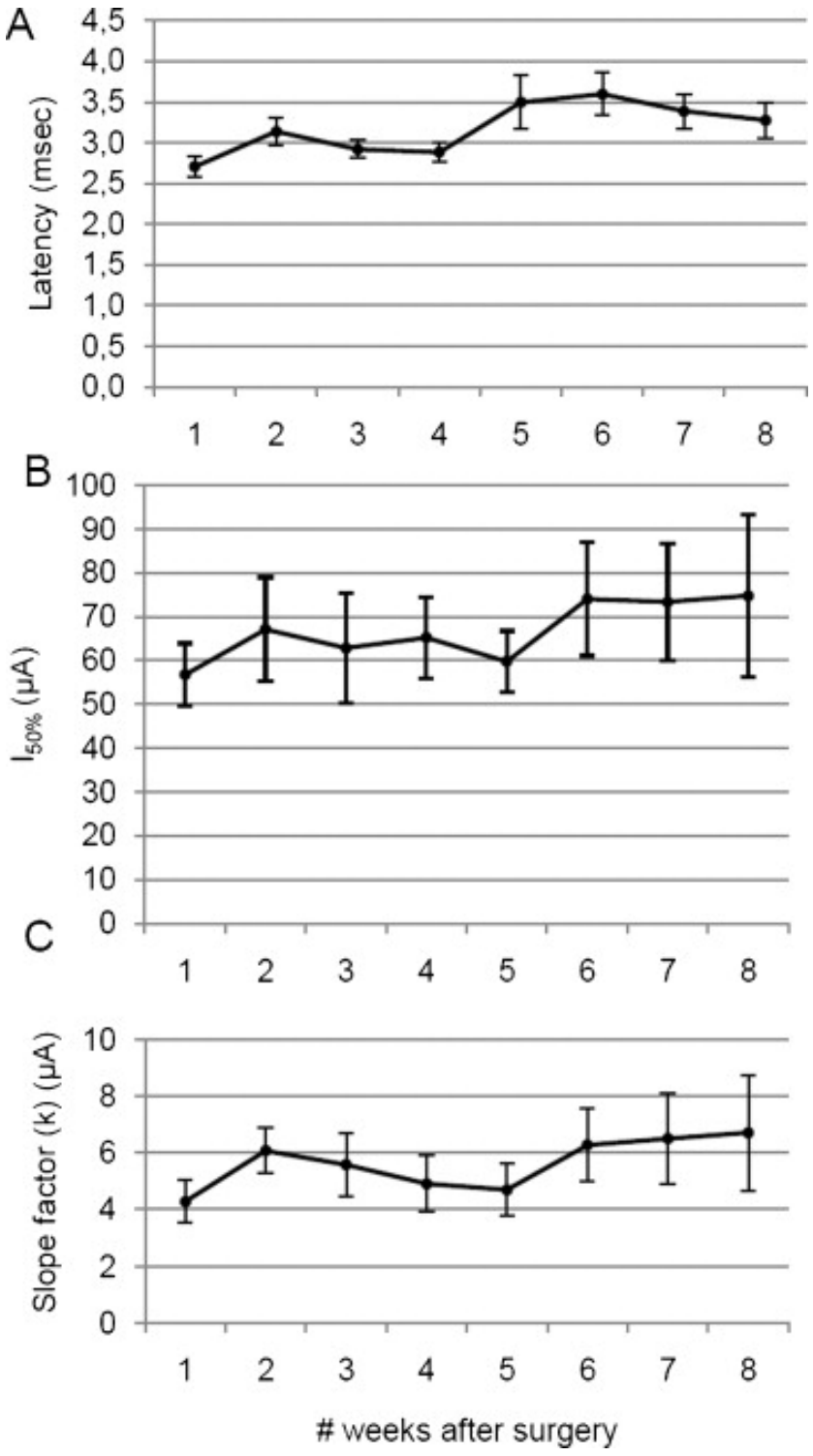


Figure 4| (A) The latency of N_1 was 3.2 ms $\pm$ 0.1 ms and did not change significantly over time during the 8 weeks of follow up. **(B)** I_{50} and **(C)** slope factor (k) deduced from the dose response curves remained stable during 8 weeks of follow-up. Means and standard errors of the mean (SEM) presented in figures, were calculated from all rats in which an LCAMP could be recorded during a specific week.

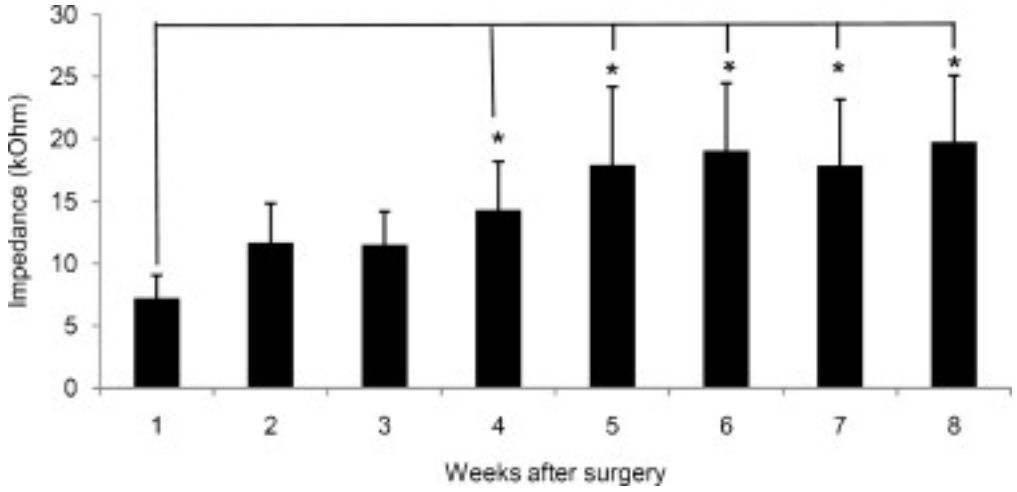


Figure 5| The impedance of the stimulation contacts increased over time. Values of week 4 to week 8 were significantly higher in comparison to the first week. * represents a corrected p-value lower than 0.05.

Discussion

The primary objective of this study was to identify an objective electrophysiological parameter to assess effective vagus nerve stimulation. In the first part of the study, the LCAMP was identified as a candidate marker for effective vagus nerve stimulation. In the second part of this study an implantable electrode system for chronic stimulation and recording of VNS-induced LCMAP in rodents was developed. In half of the rats implanted with this electrode system for chronic stimulation and recording a response to VNS could only be measured after delay of several weeks. From the moment the LCAMP could be measured up to eight weeks after electrode implantation input‑output relationship between stimulus intensity and amplitude of the LCAMP remained stable over time although the impedance of the stimulation electrodes increased slightly during the eight weeks after implantation.

Characteristics of the LCAMP

By introducing lesions distal to the stimulation electrode and application of a muscle relaxing agent we showed that the measured electrophysiological response to VNS corresponds to muscle potentials of the larynx and not to any neural potential originating from the vagus nerve. Most likely the LCAMP is evoked by VNS-induced activation of the Aα efferent motor fibers of the vagus nerve. In rats but also in other mammals these vagal Aα fibers innervate the laryngeal muscles. The LCAMP was recordable in a reproducible manner during a follow-up period of two months, although a variable recovery period after surgery was found. The latency of the LCAMP, I_{50} and slope factor of dose response curves remained relatively stable during the follow-up period after electrode

implantation. However, as our experiments count a small group of animals (N = 21), subtle changes over time cannot fully be excluded. Nevertheless, our results indicate that once the LCAMP could be recorded, the vagus nerve remained excitable, despite the development of a fibrous capsule around the electrode‑nerve interface (Grill and Mortimer 2000; Thil et al. 2006).

The increased impedance values of the stimulation electrodes between 4 and 8 weeks after implantation, support the idea that a fibrous encapsulated electrode-nerve interface is formed. Importantly, values for the I_{50} and the slope factor of *acutely dose response* curves were larger than the same results obtained in *chronic* experiments, but a smaller pulse width was used. In addition, a shunting effect of physiological water that was added to the dissection pouch in order to moisturize the vagus nerve while performing surgery may also explain the obtained results.

LCMAP as a marker for vagus nerve stimulation

Laryngeal activation is the result of efferent stimulation, while VNS in epilepsy is focused on stimulating afferents in order to obtain beneficial effects in the brain (Woodbury and Woodbury 1990, 1991; Krahl et al. 2001). The use of LMCAP as a marker for adequate vagus nerve stimulation thus remains an indirect surrogate parameter, as it does not necessarily reflect activation of the specific fiber population with antiseizure effect (Zagon and Kemeny 2000; Krahl et al. 2001). Nevertheless, Aα fibers, provide motor activation of striated muscles of the larynx and represent a relatively low threshold fiber population. Importantly, these fibers are the most sensitive to anoxia and injury due to surgical manipulation (Agnew and McCreery 1990; Woodbury and Woodbury 1991). Consequently, alteration in Aα function may imply damage to other afferent vagus nerve fibers which are thought to provide anti seizure effect of VNS. On the other hand, a histological study by Evans and Murray (Evans and Murray 1954), in the rabbit vagus nerve showed that myelinated motor fibers of the vagus nerve seem to gather in the deep lateral part of the vagus nerve bundle, which implicates that damage to these fibers would not necessarily imply injury to the medial afferent myelinated fibers. Data about precise configuration of different fibers bundles in the cervical vagus nerve in humans is lacking, therefore possible hypothesises in this field remain purely speculative.

VNS induced vocal cord EMG in humans

A study performed in humans by Ardesh et al, in which intra-operative vocal cord EMG was recorded after VNS implantation showed a very similar VNS induced LCMAP. The shape was identical, but longer latencies and higher amplitudes were reported (Ardesch et al. 2010). Activation of the larynx

in humans was obtained by applying a VNS pulse of 0.5 mA and 130 µs, while in our experiments maximal muscle activation was already reached at approximately 65 µA and 100 µs.

In humans, a temporary paresis of vocal cords after VNS surgery has been described (Zalvan et al. 2003; Shaffer et al. 2005; Shaw et al. 2006) indicating that the surgical procedure and implantation of the electrode often causes a transient vagus nerve failure, hence requiring a recovery period before to become functional again.

Clinical relevance of the VNS induced LCAMP

In clinical practice, the idea that in some patients there is a delayed effect of VNS might in some cases be explained by a temporary failure of VNS-induced activation of the nerve due to nerve damage after electrode implantation. In our study, nearly half of the implanted rats required an average recovery period of 5 weeks (range 2‑7) before VNS could efficiently induce a LCAMP. In clinical practice, ramping up of VNS output generally starts two weeks after surgery (Vonck et al. 2004; De Herdt et al. 2006). Our results and the larynx studies cited above, suggest that an individualized approach might be more beneficial, although comparisons between humans and rats must be made with caution. Currently, there is no specific investigation indicating whether the vagus nerve recovered sufficiently to start up titration of VNS therapy. In this context, recording a VNS-induced LCAMP by EMG of the larynx before and at different time points after surgery, could possibly serve as a new investigative tool in VNS therapy. More research on this topic is needed to confirm this idea.

Future experiments which investigate the relationship between LCAMP occurrence and therapeutic response to VNS treatment could be interesting. The lack of therapeutic effect of VNS may not only be the result of a lack of VNS effects on the brain, but also simply be the consequence of inadequate local recovery of the vagus nerve.

Conclusion

Twenty-one rats were successfully implanted with a custom-made self-sizing stimulation/recording electrode around the left vagus nerve, allowing repeated recording of the LCMAP over time. Our method provides an objective indication of effective vagus nerve activation, which could be of great value in all VNS experiments in animal models for epilepsy.

Acknowledgments

Riëm El Tahry is supported by a Bijzonder Onderzoeks Fonds (BOF) grant from University of Ghent. Prof. P. Boon is supported by grants from the Fund for Scientific Research-Flanders (FWO); grants from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital.

References

AgnewWF,McCreery DB. Considerations for safety with chronically implanted nerve electrodes. Epilepsia 1990:S27–32.

Ardesch JJ, Sikken JR, Veltink PH, Van Der Aa HE, Hageman G, Buschman HP. Vagus nerve stimulation for epilepsy activates the vocal folds maximally at therapeutic levels. Epilepsy Res 2010;89:227–31.

BenMenachem E, Manonespaillat R, Ristanovic R, Wilder BJ, Stefan H, Mirza W, et al.Vagus Nerve-stimulation for treatment of partial seizures. 1. A controlled-study of effect on seizures. Epilepsia 1994;35:616–26.

Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. Auton Neurosci 2000;20:1–17.

Dahlqvist A, Carlsöö B, Hellström S. Fiber components of the recurrent laryngeal nerve of the rat: a study by light and electron microscopy. Anat Rec 1982;204(4):365–70.

De Herdt V, Vonck K, Verschelde H, Legros B, Hauman H, van Rijckevorsel K, et al.Vagus nerve stimulation for refractory epilepsy: pooled results from epilepsy centres in Belgium. Epilepsia 2006;47:205–6.

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

Evans DH, Murray JG. Histological and functional studies on the fibre composition of the vagus nerve of the rabbit. J Anat 1954;88:320–37.

Fisher RS, Van Emde Boas W, Blume W, Elger C, Genton P, Lee P, et al. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 2005;46:470–2.

Grill WM, Mortimer JT. Neural and connective tissue response to long-term implantation of multiple contact nerve cuff electrodes. J Biomed Mater Res 2000;50:215–26.

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

Handforth A, DeSalles AAF, Asatourian AA, Krahl SE, Us Vns SG. Seizure response to long-interval vagus nerve stimulation (VNS) is predictive of response to conventional VNS, suggesting a long-duration antiepileptic mechanism. Neurology 1998b;50:S05006.

Hauser WA, Annegers JF, Rocca WA. Descriptive epidemiology of epilepsy: contributions of population-based studies from Rochester. Minn Mayo Clin Proc 1996;71:576–86.

Hofmann HH, Schitzlein HN. The numbers of nerve fibers in the vagus nerve of man. Anat Rec 1961;139:429–35.

Holder LK, Wernicke JF, Tarver WB. Treatment of refractory partial seizures: preliminary results of a controlled study. Pacing Clin Electrophysiol 1992;15:1557–71.

Jotz GP, de Campos D, Rodrigues MF, Xavier LL. Histological asymmetry of the human recurrent laryngeal nerve. J Voice 2011;25:8–14.

Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 2001;42:586–9.

Paxinos G. The rat nervous system. San Diego: Elsevier Academic Press; 2004. pp. 77–84.

Shaffer MJ, Jackson CE, Szabo CA, Simpson CB. Vagal nerve stimulation: clinical and electrophysiological effects on vocal fold function. Ann Otol Rhinol Laryngol 2005;14:7–14.

Shaw GY, Sechtem P, Searl J, Dowdy ES. Predictors of laryngeal complications in patients implanted with the Cyberonics vagal nerve stimulator. Ann Otol Rhinol Laryngol 2006;115:260–7.

Soltanpour N, Santer RM. J Auton Nerv Syst 1996;60:93–101. Thil MA, Tran Duy D, Colin IM, Delbeke J. Time course of tissue remodelling and electrophysiology in the rat sciatic nerve after spiral cuff electrode implantation. J Neuroimmunol 2006;185:103–14.

Uthman B, Reichl A, Dean J, Reid S, Roper S, Eisenschenck S, et al. Vagus nerve stimulation for epilepsy: a twelve-year experience. Epilepsia 2003;44:123–4.

Vonck K, Thadani V, Gilbert K, Dedeurwaerdere S, De Groote L, De Herdt V, et al. Vagus nerve stimulation for refractory epilepsy: a transatlantic experience. J Clin Neurophysiol 2004;21:283–9.

Woodbury DM, Woodbury JW. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 1990;31:S7–19.

Woodbury JW, Woodbury DM. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff electrode for stimulating and recording. Pacing Clin Electrophysiol 1991;14:94–107.

Zagon A, Kemeny AA. Slow hyperpolarization in cortical pyramidal neurones – a possible mechanism behind vagus nerve stimulation therapy for refractory epilepsy? J Physiol Lond 2000;526:59–60.

Zalvan C, Sulica L, Wolf S, Cohen J, Gonalez-Yanes O, Blitzer A. Laryngopharyngeal dysfunction from the implant vagal nerve stimulator. Laryngoscope 2003;113:221–5.

Chapter 8

Electrophysiological responses from vagus nerve stimulation in rats

Electrophysiological responses from vagus nerve stimulation in rats

Mollet L.[a], Raedt R.[a], Delbeke J.[a], El Tahry R.[a], Grimonprez A.[a], Dauwe I.[a], De Herdt V.[a], Meurs A.[a], Wadman W.[b], Boon P.[a], Vonck K.[a]

[a] Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology (LCEN3), Department of Neurology, Institute for Neuroscience, Ghent University Hospital, Belgium
[b] Swammerdam Institute of Life Sciences, Department of Neurobiology, University of Amsterdam, Amsterdam, The Netherlands

International Journal of Neural Systems 2013;23(6):epub ahead of print

Abstract

The mechanism of action of vagus nerve stimulation (VNS) for pharmacoresistant epilepsy is unknown and the therapeutic outcome is highly variable. We investigated stimulation-induced vagus nerve electrophysiological responses in rats using various stimulation parameters. Conduction velocity, I50, rheobase and chronaxie were calculated. We identified an early and late component corresponding to an afferent compound action potential (CAP) and a remote laryngeal motor-evoked potential (LMEP), respectively. The conduction velocity (CAP: 26.2 ± 1.4 m/s; LMEP: 32.4 ± 2.4 m/s) and I50 (CAP: 2.4 ± 0.3 mA; LMEP: 1.8 ± 0.2 mA) were significantly different for both components, the rheobase (CAP: 140 ± 30 μA; LMEP: 110 ± 26 μA) and chronaxie (CAP: 66 ± 7 μs; LMEP: 73 ± 9 μs) were not. Using a pulse of 10 μs, the CAP saturated between 4–5 mA. Our method can be used to record VNS-induced electrophysiological responses in rats and provides an objective biomarker for electrical stimulation with various parameters in an experimental set-up. Our findings are potentially useful for clinical purposes in the sense that combination of VNS and recording of vagal nerve CAPs may help clinicians to determine the individual optimal intensity required to fully activate fast-conducting afferent fibers.

Keywords: Vagus nerve recording; nerve fiber type; laryngeal muscle potential; vagus nerve stimulation.

Introduction

Vagus nerve stimulation (VNS) is indicated in patients with medically refractory epilepsy who are unsuitable candidates for epilepsy surgery (Boon et al. 2009; Ben-Menachem 2002).

A pulse generator is implanted subcutaneously in the left subclavicular area and delivers intermittent electrical stimuli via an electrode that is wound around the left vagus nerve at the cervical level (Shoeb et al. 2009). Different studies have established the clinical efficacy and safety of VNS both during short- and long-term follow-up in various types of epilepsy (Ben-Menachem 2002; Penry and Dean 1990; Wilder et al. 1991; Handforth et al. 1998; Vonck et al. 1999; DeGiorgio et al. 2001; Schachter 2002).

The vagus nerve is a mixed cranial nerve consisting of 20% efferent (motor) and 80% afferent (sensory) fibers. The efferent fibers of the vagus nerve originate from the dorsal motor nucleus and nucleus ambiguus. A fraction of the efferent fibers of the vagus nerve provides parasympathetic innervation to the abdominal viscera, while another fraction contributes to the recurrent laryngeal nerve. The recurrent laryngeal nerve branches from the vagus nerve at the level of the aortic arch, ascends next to the trachea and carries low-threshold vagal motor neurons to the larynx, pharynx and vocal cords. The afferent fibers of the vagus nerve originate from the jugular and nodosal ganglion and convey visceral information, taste information and somatosensory information to the brain. The afferent fibers primarily project to the nucleus of the solitary tract (NTS), but the vagus nerve also sends ipsilateral projections to the dorsal motor nucleus, nucleus ambiguus and medullary reticular formation (Krahl and Clark 2012). The NTS in turn has widespread projections to numerous areas in the brain, including important areas for epileptogenesis such as the amygdala and the thalamus (Schachter and Saper 1998; Vonck et al. 2008; Fanselow 2012), structures involved in the pathophysiology of epilepsy such as the limbic system (Castle et al. 2005), and monoaminergic structures such as the serotonergic dorsal raphe nucleus and the noradrenergic locus coeruleus (Herbert and Saper 1992; Van Bockstaele 1999). Finally, the NTS has numerous diffuse cortical connections (Jean 1991).

The antiepileptic mechanism of action of VNS remains incompletely understood. There is convincing evidence that VNS acts via activation of afferent vagal fibers (Krahl and Clark 2012; Zabara 1992; McLachlan 1993; Rijkers et al. 2010). These afferent fibers may indirectly activate the thalamus and thalamocortical projection pathways, thereby causing a desynchronization of the EEG (Aalbers et al. 2011). In addition, the monoaminergic system may account, at least in part, for the antiepileptic effect of VNS (Krahl et al. 1998; Groves et al. 2005; Dorr and Debonnel 2006; Raedt et al. 2011). In our group, we demonstrated a positive correlation between the antiepileptic effect of VNS and VNS-induced increases in hippocampal noradrenaline in the focal pilocarpine model (Raedt et al. 2011). Others hypothesize that VNS exerts its antiepileptic effect via nonspecific arousal by activation of the reticular system in the brainstem (Aalbers et al. 2011).

In VNS therapy for medically refractory epilepsy the large variation in therapeutic outcome remains a concern (Handforth et al. 1998; Uthman et al. 1993; Ben-Menachem et al. 1994; Koo et al. 2001). To date, VNS is successful in one third of treated patients (Boon et al. 2007). It is unknown why some patients experience beneficial effects and others do not respond to the treatment. In current clinical practice, physicians are unable to assess effective VNS-induced vagal nerve fiber activation. Individualized vagal nerve recordings reflecting stimulation-induced activation of the vagus nerve and correlation of these recordings with therapeutic efficacy could lead to the optimization of VNS treatment and increase the response rate.

At the cervical level, the majority of vagus nerve fibers are unmyelinated, high-threshold, slow-conducting C-type fibers (65–80%) and a smaller proportion are myelinated, low-threshold, fast-conducting A- and B-type fibers (Erlanger and Gasser 1930; Krahl 2012; Helmers et al. 2012). It is believed that afferent, fast-conducting A- and B-type fibers initiate VNS-induced antiepileptic effects in the brain (Fanselow 2012). Animal experiments demonstrate that selective destruction of C fibers using capsaicin does not abolish VNS-induced seizure suppression (Krahl et al. 2001). Stimulation levels that are therapeutically effective for seizure suppression are below the threshold for C fiber activation (Bunch et al. 2007). As a result of co-activation of efferent, fast-conducting laryngeal motor fibers, side effects such as voice alteration and hoarseness are not uncommon (Ben-Menachem 2002; Banzett et al. 1999; Kersing et al. 2002; Uthman et al. 2004).

The aim of the current study was to investigate the feasibility of recording VNS-induced electrophysiological responses from fast-conducting vagal fibers in rats, and to determine the characteristics of these responses. These recordings can then be applied in an experimental setting as a biological marker reflecting effective vagal fiber activation when electrical neurostimulation is applied to optimize VNS stimulation parameters.

Material and Methods

Animals

Seven male Wistar rats (Harlan, The Netherlands) weighing 250–275 g were used. Animals were treated according to 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 08/37). All animals were kept under environmentally controlled conditions (12 h 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%). Buprenorphine (Temgesic, 0.03mg/kg subcutaneously) was administered to minimize pain. An incision was made over the left, anterior cervical region. The cervical, left vagus nerve was carefully dissected from the arteria carotis communis and a bipolar custom-made silicone cuff electrode (platinum contacts, 3mm^2 area each, 1mm space between them) was implanted around the vagus nerve, with the anode placed caudally and the cathode placed rostrally (Fig. 1).

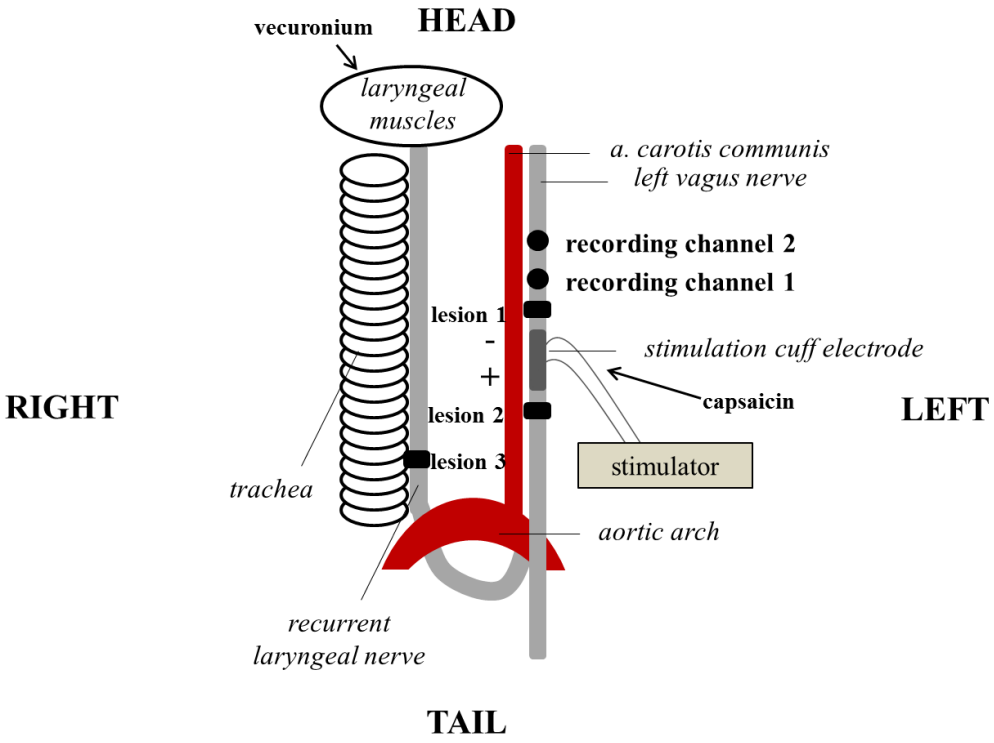


Figure 1| Ventral view of the left, anterior cervical region and experimental set-up.

Animals were sacrificed at the end of each experiment with an overdose of pentobarbital (Nembutal, 180mg/kg intraperitoneally).

Electrophysiological recordings

Electrophysiological recordings were performed under anesthesia using two thin needle electrodes (125 µm diameter) consisting of a stainless steel wire (California Fine Wire, California, USA) placed on the vagus nerve rostral to the stimulating cathode (Fig. 1). The distance between the stimulating cathode and both recording electrodes was determined under microscopic control. A

reference/ground electrode (also consisting of a stainless steel wire) was placed in contact with the surgical field between the stimulation and recording electrodes.

Experimental set-up and protocol

The custom-made silicone cuff electrode was connected to a current stimulator (DS4, Digitimer Ltd., Hertfordshire, England) (Fig. 1). The vagus nerve was stimulated with biphasic, charge-balanced, square-wave pulses. Total pulse duration was 10 µs. Longer stimulus durations, even with sufficient length of nerve exposed, led to broader stimulus artifacts, with the risk of masking very fast electrophysiological responses.

Electrophysiological recordings were amplified 500 times and high-pass filtered at 0.15Hz. Data were digitized at 100 kHz using a National Instruments acquisition board (NI USB 6259) and stored on a personal computer. Matlab (2007b, The Math- Works, Natick, Massachusetts) was used to record and analyze the electrophysiological signals.

In order to investigate the precise origin of different components in the evoked electrophysiological response, three different methodological approaches were investigated (Fig. 1):

(i) The vagus nerve and recurrent laryngeal nerve were ligated using silk wire (lesion 1, 2, 3).

(ii) The muscle-paralyzing agent vecuronium (Norcuron, 2mg/ml solution, 1ml) was applied to the laryngeal muscles.

(iii) The C fiber-specific neurotoxic agent capsaicin (10% Tween 80, 10% ethanol and 80% saline, 0.5mg/ml) was administered locally at the cervical level of the vagus nerve.

Data analysis

The response latency and conduction velocity of different components in the evoked electrophysiological response were determined. The *response latency* was defined as the delay between the onset of the stimulus and the onset of the evoked electrophysiological component measured at the first recording electrode. The *conduction velocity* was calculated from the distance between the two different recording positions divided by the difference in response latency at both recording positions.

Stimulus-response curves were determined by increasing the stimulus intensity from 0 mA to 6 mA in steps of 0.3 mA. The upper limit was set at a high intensity level because of the short pulse duration that was applied (10 µs). For each stimulus intensity, ten response sweeps (duration/sweep: 25 ms)

were averaged. After assessing response onset and response peak of the averaged electrophysiological component, a time interval of 50 µs and 200 µs was defined around both, wherein the local maximum and minimum was determined, respectively. The mean amplitudes within a time interval of 100 µs around the local maximum and minimum were then calculated and the absolute difference between the two latter values was retained as the peak amplitude. A Boltzmann function (Eq. (1)) was fitted to the recorded stimulus-response curves and its goodness of fit was calculated via residual sum of square analysis. Only fittings with a minimum goodness of fit of 0.90 were retained.

$$y(I) = y_{max}/(1 + \exp(I_{50} - I)/k), \quad (1)$$

where y_{max} is the maximal peak amplitude, I_{50} is the stimulus intensity required to evoke an electrophysiological response with half the maximal amplitude and the *slope factor k* describes the steepness of the stimulus-response curve. These parameters were determined based on the fitted Boltzmann function.

Strength-duration curves were determined by defining the minimal current required to evoke an electrophysiological response using various pulse widths. Total pulse duration was increased from 10 µs to 100 µs in steps of 10 µs and from 100 µs to 200 µs in steps of 20 µs. The Lapicque equation (Eq. (2)) was fitted to the measured strength-duration curves.

$$I_{stim} = I_{rh}(1 + T_{ch}/T), \quad (2)$$

where I_{stim} is the minimum stimulation current required to evoke an electrophysiological response, I_{rh} is the rheobase current, T_{ch} is the chronaxie and T is the stimulus duration. *Rheobase* is the minimal electrical current of infinite duration required to activate a single nerve fiber. *Chronaxie* is the stimulus duration required to reach threshold when the current intensity value is twice the rheobase. At threshold level, long duration stimulation pulses correspond to larger power consumption and hazardous electric charges while voltage and current density becomes prohibitive with very short pulses. A commonly accepted optimum for stimulation pulse duration is a value in the range of the chronaxie. Both the rheobase and chronaxie characterize the response to our specific stimulation configuration and were calculated for all components in the evoked electrophysiological response based on the fitted Lapicque function.

Data is expressed as mean ± standard error of the mean (SEM). Comparisons were made with a Student's t-test. $P < 0.05$ was assumed to indicate a significant difference.

Results

In all rats an electrophysiological response to VNS could be recorded from the vagus nerve rostral to the stimulating cathode. This electrophysiological response consisted of an early and a late component (Fig. 2). The early, but not the late component, disappeared by ligating the vagus nerve between the stimulating cathode and the first recording electrode (Fig. 2). A ligation distal to the stimulating anode but proximal to the aortic arch abolished only the late component (Fig. 2). The late component also disappeared by blocking the recurrent laryngeal nerve and by applying vecuronium to the laryngeal muscles (Fig. 2).

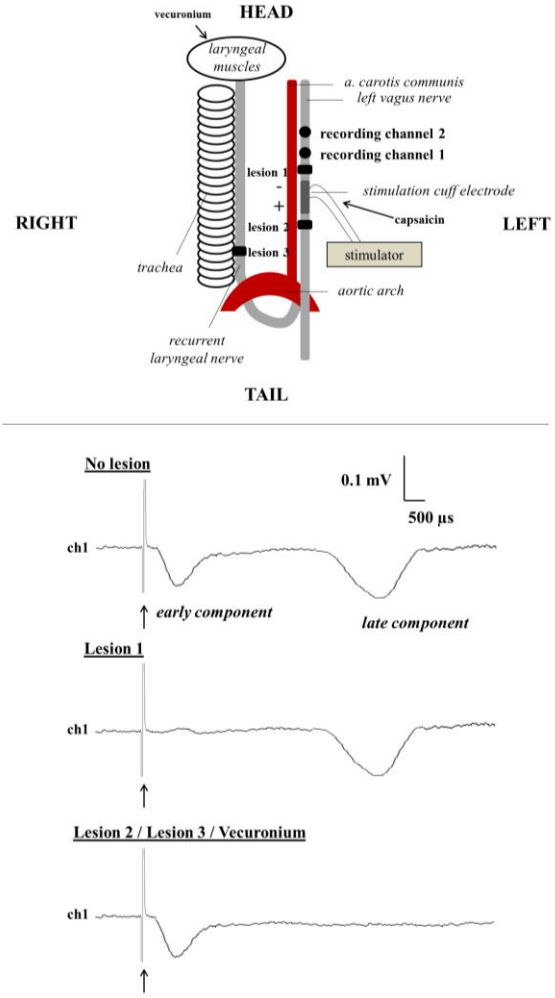


Figure 2| Top: Ventral view of the left, anterior cervical region in rats and experimental set-up. **Bottom:** Recordings on channel 1 are shown. The arrow indicates the stimulation artifact. The evoked electrophysiological response from the vagus nerve consisted of an early and a late component. A lesion of the vagus nerve proximal of the stimulation electrode (lesion 1) abolished only the early component. The late component was abolished by (i) a distal lesion of the vagus nerve (lesion 2), (ii) a lesion of the recurrent laryngeal nerve (lesion 3) and (iii) application of the muscle-paralyzing agent vecuronium to the laryngeal muscles.

The latter provides evidence that the late component reflects laryngeal muscle activity, rather than nerve conduction. Taken together, at this point, the early component represented a rostrally propagated vagus nerve compound action potential (CAP), while the late component was a remote VNS-induced laryngeal motor-evoked potential (LMEP).

A difference in response latency between both recording positions was found for the CAP, but not for the LMEP (Fig. 3(a)).

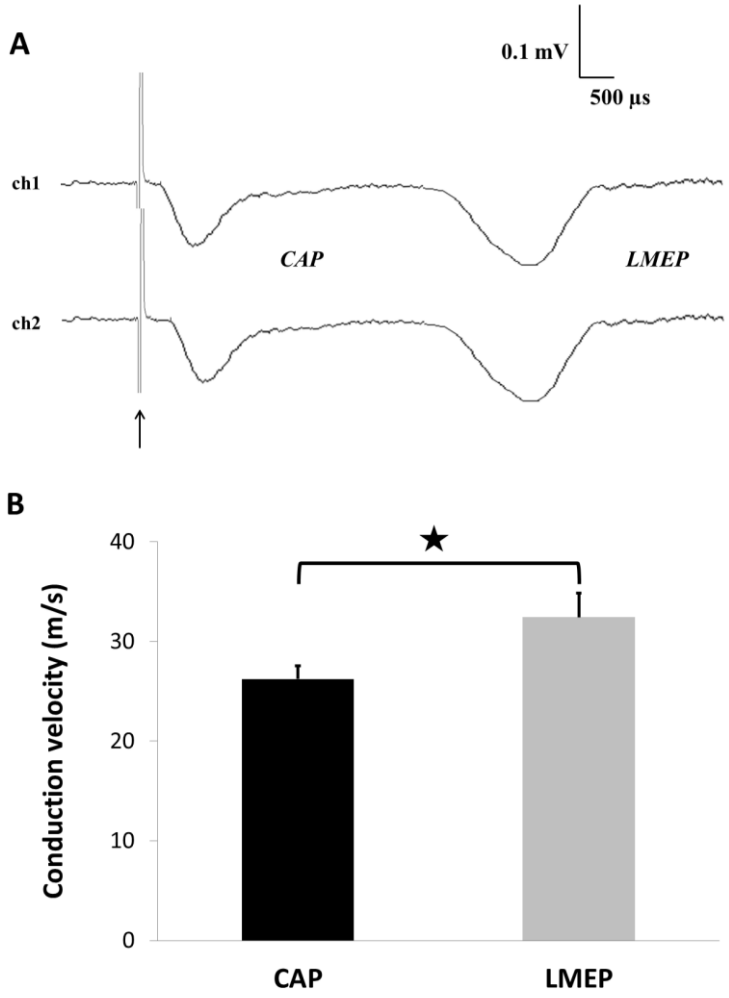


Figure 3| (A) Recordings on channel 1 (upper trace) and channel 2 (lower trace) are shown in one rat. The arrow indicates the stimulation artifact. A difference in response latency between both recording channels was observed only for the CAP. **(B)** A statistically significant difference in conduction velocity between the CAP and LMEP was observed. The conduction velocity for the CAP was calculated based on the distance and latency difference between both recording positions. The conduction velocity for the LMEP was calculated using a single latency measurement, regardless of recording position, and distance measurement (i.e. nerve length between the stimulating cathode and the laryngeal muscles).

The presence of a latency difference for the CAP confirmed our hypothesis that the first wave is a propagated axonal potential, while the absence of a latency difference for the LMEP confirmed our hypothesis that the second wave is a remote VNS induced muscle potential. At 1.5 ± 0.1 mm rostral to the stimulating cathode (recording channel 1), the latencies of the CAP and LMEP were 0.18 ± 0.02 ms and 2.88 ± 0.27 ms, respectively. At 3.4 ± 0.3 mm rostral to the stimulating cathode (recording channel 2), the latencies were 0.25 ± 0.03 ms and 2.88 ± 0.27 ms, respectively. Based on the difference in response latency for the CAP, conduction velocity was calculated to be 26.2 ± 1.4 m/s. Based on the nerve length between the stimulating cathode and the laryngeal muscles (89 ± 2 mm) that was measured under microscopic control, conduction velocity of the efferent action potentials leading to the LMEP was calculated to be 32.4 ± 2.4 m/s. The observed difference between the conduction velocity for the CAP and LMEP was statistically significant (Fig. 3(b)).

In Fig. 4 the stimulus-response curves for both the CAP and LMEP are shown for individual rats. A Boltzmann function was fitted to the curves. The individual and mean (± SEM) I_{50} values, k values and y_{max} values are shown in Table 1. For each rat, the I_{50} for the CAP was higher compared to the I_{50} for the LMEP. The mean I_{50} for the CAP (2.4 ± 0.3 mA) was significantly higher compared to the mean I_{50} for the LMEP (1.8 ± 0.2 mA). No differences were found between the mean slope factor k for the CAP (0.27 ± 0.03) and the mean slope factor k for the LMEP (0.20 ± 0.04). The mean y_{max} for the CAP and LMEP was 0.08 ± 0.03 mV and 0.21 ± 0.10 mV, respectively.

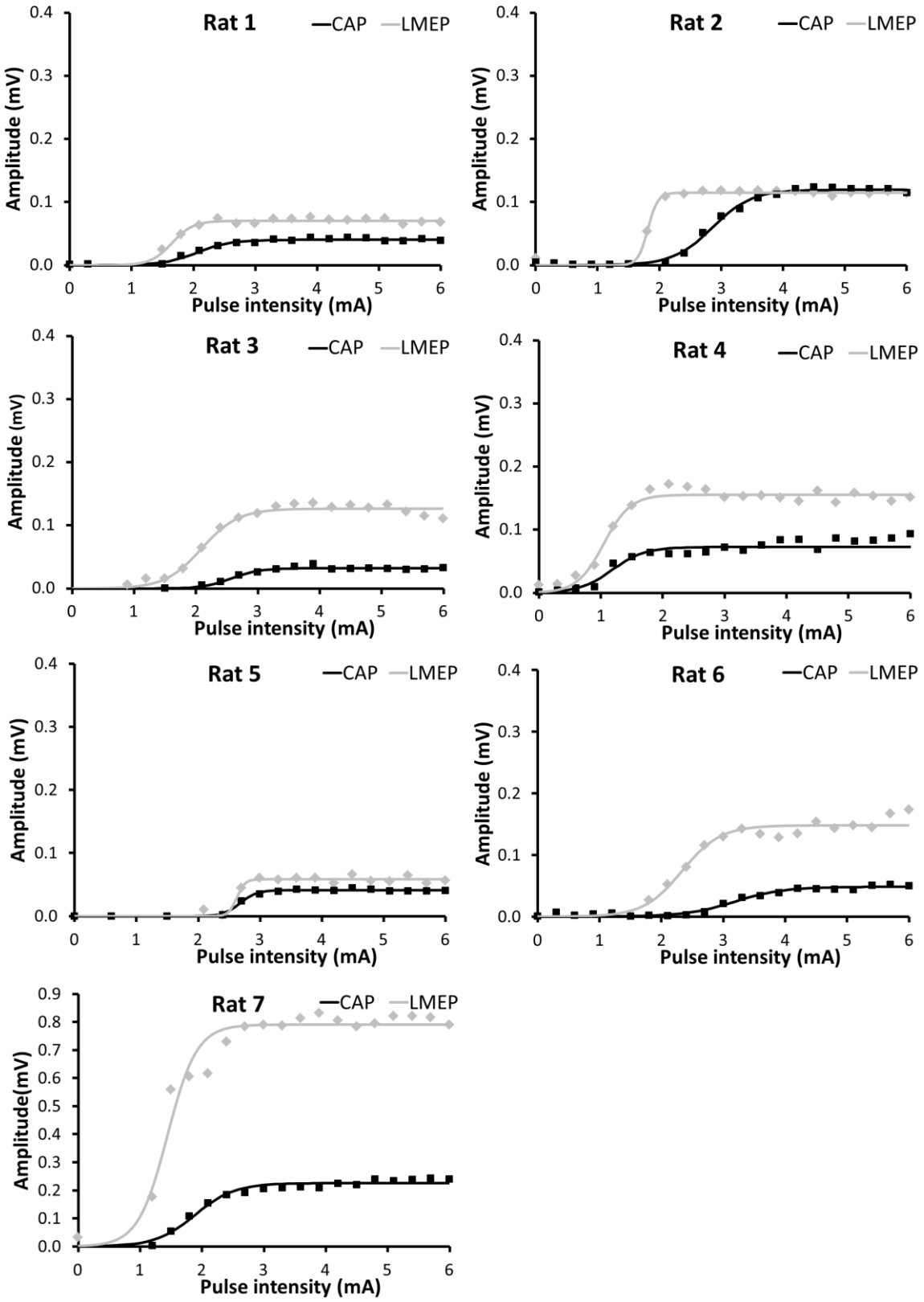


Figure 4| Plots of the amplitude of the CAP (black) and LMEP (grey) as a function of the pulse intensity and fitted Boltzmann curves are shown for individual rats.

Table 1| Individual and mean (±SEM) I_{50} values, k values and y_{max} values. For each rat, the I_{50} for the CAP was higher compared to the I_{50} for the LMEP. The mean I_{50} for the CAP was significantly higher compared to the mean I_{50} for the LMEP.

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Mean ± (SEM)
I_{50} CAP (mA)	2.1	2.9	2.6	1.2	2.7	3.2	1.9	2.4 ± 0.3
I_{50} LMEP (mA)	1.7	1.8	2.1	1.0	2.6	2.3	1.4	1.8 ± 0.2
k CAP	0.27	0.31	0.23	0.26	0.13	0.40	0.32	0.27 ± 0.03
k LMEP	0.17	0.08	0.29	0.21	0.07	0.31	0.24	0.20 ± 0.04
y_{max} CAP (mV)	0.04	0.12	0.03	0.07	0.04	0.05	0.23	0.08 ± 0.03
y_{max} LMEP (mV)	0.07	0.11	0.13	0.16	0.06	0.15	0.79	0.21 ± 0.10

For each component, strength-duration curves were determined and a Lapicque equation was fitted to the curves (Fig. 5). The rheobase for the CAP and LMEP was 140 ± 30 µA and 110 ± 26 µA, respectively. The chronaxie for the CAP and LMEP was 66 ± 7 µs and 73 ± 9 µs, respectively. The differences between the CAP and the LMEP were not significant for either parameter.

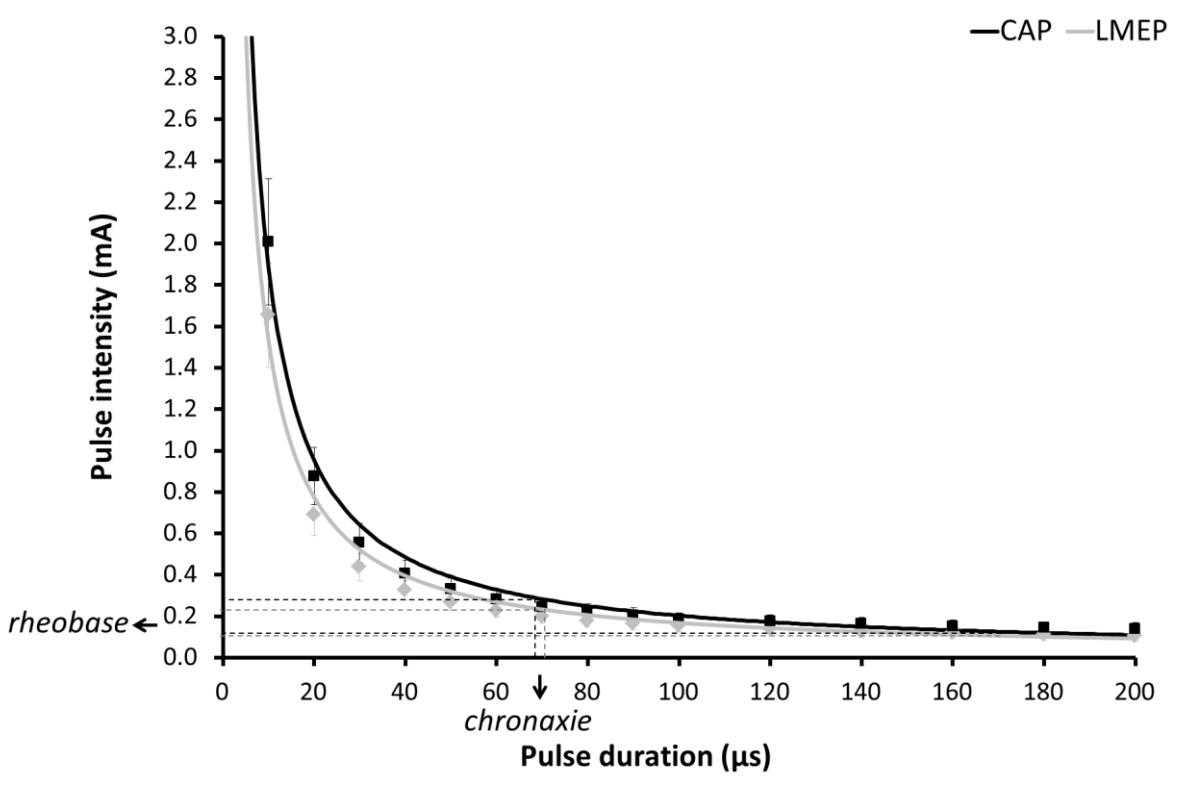


Figure 5| Strength-duration curves for the CAP (black) and LMEP (grey) are shown. The rheobase and chronaxie are indicated. No significant differences for the rheobase and chronaxie were found between the CAP and the LMEP.

When conducting the strength-duration experiments, it was examined in all rats whether additional components could be observed. A third electrophysiological component with a threshold above 1 mA was observed in one out of seven rats, when a pulse width of 100 µs was applied (Fig. 6). The latency of this component was 2.8 ms, and at a distance of 4 mm between the stimulating cathode and the first recording electrode, this latency corresponds to a conduction velocity of 1.4 m/s. Local application of the C fiber-specific neurotoxic agent capsaicin to the cervical level of the vagus nerve (Fig. 1) abolished this additional electrophysiological component (Fig. 6).

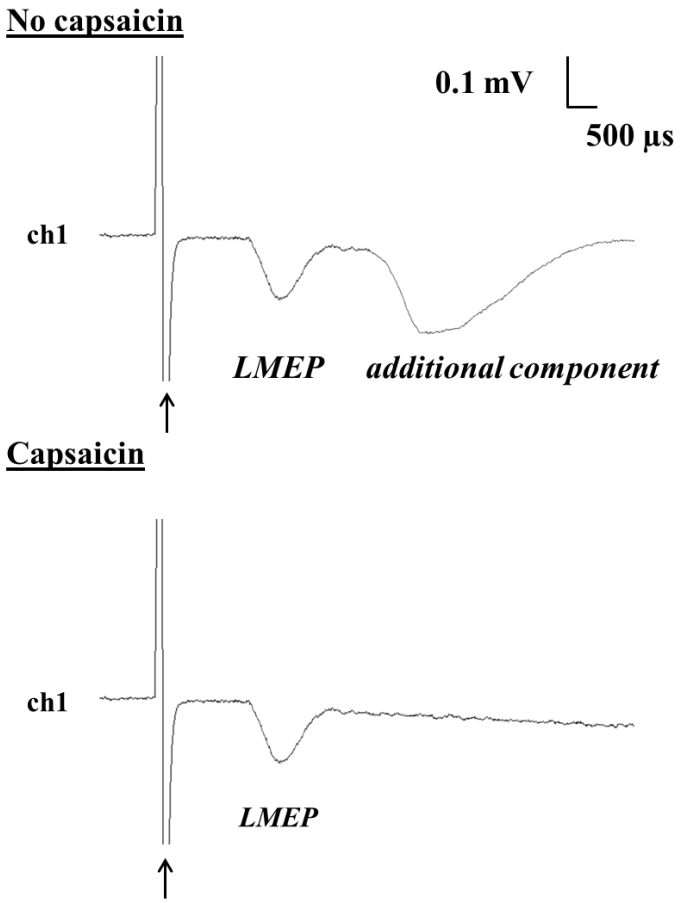


Figure 6| In addition to the CAP and LMEP, a third electrophysiological component was observed in one out of seven rats **(upper trace)**. The arrow indicates the stimulation artifact. This component disappeared by local application of the C fiber-specific neurotoxic agent capsaicin **(lower trace)**. The CAP is hidden within the stimulation artifact.

Discussion

The primary objective of this study was to investigate the feasibility of recording VNS-induced electrophysiological responses from fast-conducting vagal fibers in rats, and to determine the characteristics of these responses. The results indicate that, using our electrode configuration, it is feasible to record VNS-induced electrophysiological responses from the vagus nerve in rats. A short (10 μs), biphasic, charge-balanced, square-wave stimulus to the vagus nerve with an intensity of 1–6 mA induces rostrally and caudally propagating action potentials.

In our study, electrical stimulation of the vagus nerve elicited a bidirectional activation of nerve fibers, with the CAP and LMEP reflecting rostrally propagating nerve action potentials and efferent laryngeal muscle activation, respectively. Based on the lesion experiments, no distinction can be made between retrograde activity in efferent fibers and anterograde activity in afferent fibers with regard to the CAP. As a result, both the CAP and LMEP could possibly, but not necessarily, result from activation of the same motor fibers, the first reflecting a nerve potential and the second a muscle potential. However, we hypothesize that afferent fibers were also recruited, based on a number of observations. *Firstly*, the I_{50} for the CAP was significantly higher compared to the I_{50} for the LMEP. It is therefore likely that the CAP does not (only) represent activation of the same low-threshold laryngeal motor fibers. *Secondly*, the conduction velocity of the fibers leading to the CAP was significantly lower compared to the conduction velocity of the laryngeal motor fibers leading to the LMEP, indicating that different fiber types were recruited. Furthermore, the conduction velocity of the laryngeal motor fibers calculated in this study is probably an underestimation, as we did not take into account an estimated delay for (i) the nerve activation (± 0.32 ms), (ii) the terminal slowing due to narrowing and branching of the laryngeal motor fibers (± 0.8 ms) and (iii) the neuromuscular transmission (± 1 ms).

One puzzling finding is the slower conduction velocity in the proximally recorded CAP compared to the distal muscle LMEP response, especially taking into account the likely underestimation of the last value. A likely hypothesis is that the motor fibers are not numerous enough to be detected as a CAP but produce a clear LMEP due to the muscle “amplification” effect. Another hypothesis is local damage to the nerve resulting in slowing of the conduction velocity near the implantation site. The nerve remains unaffected further away so that the longer path for the LMEP is mostly healthy while almost all of the explored conduction path for the CAP would be subjected to slowing. These are issues for further research, but this finding indicates that in this kind of work, the observed conduction velocities must be interpreted cautiously.

If indeed the CAP and LMEP represent activation of different vagal fiber types, one could expect to see differences in their strength-duration curves. However, the chronaxie only depends on the electrical resistance and capacitance of the axonal membrane and is therefore expected to be similar for all myelinated nerve fibers, for all unmyelinated nerve fibers and to be very different for muscles (Geddes 2004). The absence of a difference in rheobase on the other hand, can be explained by the fact that this is a threshold value, only taking into account the most excitable fibers and not the average fiber population. The variability of the fiber characteristics could further obscure the difference between the two fiber populations sampled at the lowest threshold. In contrast, the I_{50} obtained from the stimulus-response curves concerns fibers with modal characteristics and is therefore a more optimal representation of the average fiber population.

With regard to the afferent CAP, we hypothesize that fast-conducting vagal A, and perhaps B, fibers were activated. In other experiments, conduction velocity estimates for vagal A and B afferent fibers were in the range of 10-30 m/s (Woodbury and Woodbury 1990; Usami et al. 2012), which is in line with the conduction velocity found here. Also in cats and dogs, a similar conduction velocity for type A and B fibers was found (Li et al. 1976, 1977; Castoro et al. 2001). The afferent CAP observed in our study may therefore reflect activation of the specific fiber population within the vagus nerve that addresses the neural pathways involved in the seizure-suppressing effect of VNS. Indeed, vagal afferent fibers with low-to-moderate activation thresholds (i.e. A and B fibers) are responsible for activating the seizure-suppressing mechanisms of VNS in the brain (Krahl et al. 2001; Bunch et al. 2007; Groves and Brown 2005). Due to spatial constraints in rats, recording electrodes were placed at a small distance from the stimulation electrode. As a consequence, the electrophysiological response of A and B afferent fibers were likely superimposed and their contribution to the overall electrophysiological response could not be differentiated. Even in dogs, the electrophysiological component from small A fibers is clearly superimposed with the component from B fibers for conduction distances of 8 cm or less (Castoro et al. 2001).

A third electrophysiological response was recorded in one out of seven animals. Because the conduction velocity is in the range of C fiber conduction velocity and the component disappeared by local application of capsaicin, the component was interpreted as a C fiber response. Higher stimulation intensities are needed to consistently measure C fiber responses. As levels of VNS that are therapeutically effective for seizure suppression are below threshold for C fiber activation (Bunch et al. 2007), it was beyond the scope of this study to look for an electrophysiological response from C fibers.

We suggest that the CAP can be used as an objective biomarker for effective stimulation-induced activation of fast-conducting afferent vagal fibers. In a recent study by our group, the LMEP was suggested as a marker for adequate VNS (El Tahry et al. 2011). As, however, the LMEP is the result of efferent stimulation and VNS in epilepsy is focused on stimulating afferents in order to obtain beneficial effects in the brain (Krahl et al. 2001; Woodbury and Woodbury 1990, 1991), the LMEP remains an indirect surrogate marker (El Tahry et al. 2011). Furthermore, CAPs of the fast-conducting afferent vagal fibers may serve as a new tool to identify more adequate stimulation parameters. In clinical practice, one of the most important issues is whether higher output current intensities can provide better efficacy (DeGiorgio et al. 2001). VNS is gradually uptitrated according to the reported seizure reduction and side effects (Rajdev et al. 2011), due to a lack of electrophysiological information about the vagus nerve itself. In this context, the stimulus-response curve for the CAP obtained in our study illustrates that recruitment of fast-conducting afferent vagal fibers rapidly reaches a saturation level in rats. Using a pulse of 10 µs, saturation was reached between 4 mA and 5 mA. On the basis of equal pulse charge, these intensities are approximately equivalent to 0.16-0.20 mA using a pulse of 250 µs. We therefore hypothesize that output current intensities of this order should be sufficient to reduce seizure frequency and higher output current intensities should not be required in the rat. Our results from rats still have to be translated to humans. Combination of VNS and recording of vagal nerve CAPs could help to decide at what level this change in strategy should be applied in individual patients. The last years, attention is paid to the development of VNS devices that include an electrode for combined stimulation and recording. One feasibility study has shown that is it possible to record CAPs using such a VNS device (El Tahry et al. 2010). Recording CAPs postoperatively may help clinicians to determine the individual optimal intensity required to fully activate fast-conducting afferent vagal nerve fibers, which in turn could be used as a patient-specific optimal VNS output current value. In addition, this may lead to more insights in the subpopulations to be targeted as, hypothetically, non-responders to the VNS therapy could be those patients in whom the vagus nerve does not recover sufficiently from surgery and in whom no CAPs can be recorded. Finally, recording from the vagus nerve might help to develop stimulation methods that have the potential to reduce the LMEP, which reflects VNS-related throat discomfort, while maximizing the CAP.

Acknowledgments, Conflicts of Interest and Sources of Funding

There are no conflicts of interest to be reported. Lies Mollet is supported by a junior researcher (‘Aspirant’) grant from the “Fonds voor Wetenschappelijk Onderzoek (FWO)”. Annelies Grimonprez is supported by a PhD-grant from the Ghent Institute for Neuroscience. Prof. Dr. R. Raedt and Prof. Dr.

K. Vonck are supported by a grant from the “Bijzonder Onderzoeksfond (BOF)” from Ghent University. Prof. Dr. P. Boon is supported by grants from the FWO, grants from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital.

References

P. Boon, R. Raedt, V. De Herdt, T. Wyckhuys and K. Vonck, Electrical stimulation for the treatment of epilepsy, Neurotherapeutics 6 (2009) 218–227.

E. Ben-Menachem, Vagus-nerve stimulation for the treatment of epilepsy, Lancet Neurol. 1 (2002) 477– 482.

A. Shoeb, T. Pang, J. Guttag and S. Schachter, Non-invasive computerized system for automatically initiating vagus nerve stimulation following patient-specific detection of seizures or epileptiform discharges, Int. J. Neural Syst. 19 (2009) 157–172.

J. Penry and J. Dean, Prevention of intractable partial seizures by intermittent vagal stimulation in humans: Preliminary results, Epilepsia 31 (1990) 40–43.

B.Wilder, B. Uthman and E. Hammond, Vagal stimulation for control of complex partial seizures in medically refractory epileptic patients, Pacing Clin. Electrophysiol. 14 (1991) 108–115.

A. Handforth, C. DeGiorgio, S. Schachter, B. Uthman, D. Naritoku and E. Tecoma, Vagus nerve stimulation therapy for partial-onset seizures: A randomized active-control trial, Neurology 51 (1998) 48–55.

K. Vonck, P. Boon, M. D’Have, T. Vandekerckhove, S. O’Connor and J. De Reuck, Long-term results of vagus nerve stimulation in refractory epilepsy, Seizure 8 (1999) 328–334.

C. DeGiorgio, J. Thompson, P. Lewis, S. Arrambide, D. Naritoku and A. Handforth, Vagus nerve stimulation: Analysis of device parameters in 154 patients during the long-term XE5 study, Epilepsia 42 (2001) 1017–1020.

S. Schachter, Vagus nerve stimulation: Where are we?, Curr. Opin. Neurol. 15 (2002) 201–206.

S. Krahl and K. Clark, Vagus nerve stimulation for epilepsy: A review of central mechanisms, Surg. Neurol. Int. 3 (2012) 255–259.

S. Schachter and C. Saper, Vagus nerve stimulation, Epilepsia 39 (1998) 677–686.

K. Vonck, V. De Herdt, T. Bosman, S. Dedeurwaerdere, K. Van Laere and P. Boon, Thalamic and limbic involvement in the mechanism of action of vagus nerve stimulation, a SPECT study, Seizure 17 (2008) 699–706.

E. Fanselow, Central mechanisms of cranial nerve stimulation for epilepsy, Surg. Neurol. Int. 3 (2012) 247–254.

M. Castle, A. Comoli and A. Loewy, Autonomic brainstem nuclei are linked to the hippocampus, Neuroscience 134 (2005) 657–669.

H. Herbert and C. Saper, Organization of medullary adrenergic and noradrenergic projections to the periaqueductal gray matter in the rat, J. Comp. Neurol. 315 (1992) 34–52.

E. Van Bockstaele, J. Peoples and P. Telegan, Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: Evidence for a monosynaptic pathway, J. Comp. Neurol. 412 (1999) 410–428.

A. Jean, The nucleus tractus solitarius: Neuroanatomic, neurochemical and functional aspects, Arch. Int. Physiol. Biochim. Biophys. 99 (1991) 3–52.

J. Zabara, Inhibition of experimental seizures in canines by repetitive vagal stimulation, Epilepsia 33 (1992) 1005–1012.

R. McLachlan, Suppression of interictal spikes and seizures by stimulation of the vagus nerve, Epilepsia 34 (1993) 918–923.

K. Rijkers,M. Aalbers, G. Hoogland, L. vanWinden, J. Vles, H. Steinbusch and M. Majoie, Acute seizuresuppressing effect of vagus nerve stimulation in the amygdala kindled rat, Brain Res. 1319 (2010) 155-163.

M. Aalbers, J. Vles, S. Klinkenberg, G. Hoogland, M. Majoie and K. Rijkers, Animal models for vagus nerve stimulation in epilepsy, Exp. Neurol. 230 (2011) 167–175.

S. Krahl, K. Clark, D. Smith and R. Browning, Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation, Epilepsia 39 (1998) 709–714.

D. Groves, E. Bowman and V. Brown, Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat, Neurosci. Lett. 379 (2005) 174–179.

A. Dorr and G. Debonnel, Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission, J. Pharmacol. Exp. Ther. 318 (2006) 890–898.

R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, De Herdt V, E. Carrette, W. Wadman, Y. Michotte, I. Smolders, P. Boon and A. Meurs, Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model, J. Neurochem. 117 (2011) 461–469.

B. Uthman, B. Wilder, J. Penry, C. Dean, R. Ramsay, S. Reid, E. Hammond, W. Tarver and J. Wernicke, Treatment of epilepsy by stimulation of the vagus nerve, Neurology 43 (1993) 1338–1345.

E. Ben-Menachem, R. Manon-Espaillat, R. Ristanovic, B. Wilder, H. Stefan, W. Mirza, W. Tarve and J. Wernicke, Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Group, Epilepsia 35 (1994) 616–626.

B. Koo, S. Ham, S. Sood and B. Tarver, Human vagus nerve electrophysiology — a guide to vagus nerve stimulation parameters, J. Clin. Neurophysiol. 18 (2001) 429–433.

P. Boon, V. De Herdt, K. Vonck and D. Van Roost, Clinical experience with vagus nerve stimulation and deep Suppl. 97 (2007) 273–280.

J.Erlangerand H. Gasser, The action potential in fibers of slow conduction in spinal roots and somatic nerves, Am. J. Physiol. 92 (1930) 43–82.

S. Krahl, Vagus nerve stimulation for epilepsy: A review of the peripheral mechanisms, Surg. Neurol. Int. 3 (2012) 47–52.

S. Helmers, J. Begnaud, A. Cowley, H. Corwin, J. Edwards, D. Holder, H. Kostov, P. Larsson, P. Levisohn, M. De Menezes, H. Stefan and D. Labiner, Application of a computational model of vagus nerve stimulation, Acta. Neurol. Scand. 126 (2012) 336– 343.

S. Krahl, S. Senanayake and A. Handforth, Destruction of peripheral C fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats, Epilepsia 42 (2001) 586–589.

S. Bunch, C. DeGiorgio, S. Krahl, J. Britton, P. Green, M. Lancman, J. Murphy, P. Olejniczak, J. Shih and C. Heck, Vagus nerve stimulation for epilepsy: Is output current correlated with acute response?, Acta. Neurol. Scand. 116 (2007) 217–220.

R. Banzett, A. Guz, D. Paydarfar, S. Shea, S. Schachter and R. Lansing, Cardiorespiratory variables and sensation during stimulation of the left vagus in patients with epilepsy, Epilepsy Res. 35 (1999) 1–11.

W. Kersing, P. Dejonckere, A. Van der Aa and H. Buschman, Laryngeal and vocal changes during vagus nerve stimulation in epileptic patients, J. Voice. 16 (2002) 251–257.

B. Uthman, A. Reichl, J. Dean, S. Eisenschenk, R. Gilmore, S. Reid, S. Roper and B. Wilder, Effectiveness of vagus nerve stimulation in epilepsy patients: A 12-year observation, Neurology 63 (2004) 1124– 1126.

L. Geddes, Accuracy limitations of chronaxie values, IEEE Trans. Biomed. Eng. 51 (2004) 176–181.

D. Woodbury and J. Woodbury, Effects of vagal stimulation on experimentally induced seizures in rats, Epilepsia 31 (1990) 7–19.

K. Usami, K. Kawai, M. Sonoo and N. Saito, Scalprecorded evoked potentials as a marker for afferent nerve impulse in clinical vagus nerve stimulation, Brain Stimul. (2012) (Epub ahead of print).

C. Li, G. Mathews and A. Bak, Excitability characteristics of the A- and C fibers in a peripheral nerve, Exp. Neurol. 50 (1976) 67–79.

C. Li, G. Mathews and A. Bak, Action potential of somatic and autonomic nerves, Exp. Neurol. 56 (1977) 527–537.

M. Castoro, P. Yoo, J. Hincapie, J. Hamann, S. Ruble, P. Wolf and W. Grill, Excitation properties of the right cervical vagus nerve in adult dogs, Exp. Neurol. 227 (2001) 62–68.

D. Groves and V. Brown, Vagal nerve stimulation: A review of its applications and potential mechanisms that mediate its clinical effects, Neurosci. Biobehav. Rev. 29 (2005) 493–500.

R. El Tahry, L. Mollet, R. Raedt, J. Delbeke, V. De Herdt, T. Wyckhuys, D. Hemelsoet, A. Meurs, K. Vonck, W. Wadman and P. Boon, Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats, J. Neurosci. Methods 198 (2011) 287–293.

J. Woodbury and D. Woodbury, Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: Use of a cuff electrode for stimulating and recording, Pacing Clin. Electrophysiol. 14 (1991) 94–107.

P. Rajdev, M. Ward and P. Irazoqui, Effect of stimulus parameters in the treatment of seizures by electrical stimulation in the kainate animal model, Int. J. Neural Syst. 21 (2011) 151–162.

R. El Tahry, R. Raedt, L. Mollet, V. De Herdt, T. Wyckhuys, A. Van Dycke, A. Meurs, F. Dewaele, D. Van Roost, P. Doguet, J. Delbeke, W. Wadman, K. Vonck and P. Boon, A novel implantable vagus nerve stimulation system (ADNS-300) for combined stimulation and recording of the vagus nerve: Pilot trial at Ghent University Hospital, Epilepsy Res. 92 (2010) 231–239.

Chapter 9

Conclusion, discussion and future perspectives

Conclusion

Successful treatment of patients with medically refractory epilepsy remains a challenge due to the inherent intractable nature of the disorder. Electrical stimulation of the left vagus nerve is one of several treatment options for these patients. Despite the fact that VNS is accepted in epilepsy centers worldwide as a valuable and reliable therapeutic option for patients who are considered unsuitable for resective surgery or in whom surgery failed, specific issues remain to counteract its full therapeutic application. VNS research comprises 3 major topics: (i) identification of the antiepileptic mechanism of action, (ii) identification of the most optimal stimulation parameter settings, and (iii) identification of responder characteristics. In this thesis, research was done on these 3 major topics.

Mechanism of action

From this thesis we can conclude that:

1. Afferent A and B fibers at the cervical level of the vagus nerve may be responsible for the antiepileptic effect of VNS.
2. VNS induces an increase in the extracellular hippocampal concentration of noradrenaline, which is at least partly responsible for the antiepileptic effect of VNS in the intrahippocampal pilocarpine model of acute limbic seizures.
3. VNS is able to modulate cortical excitability in rats.

Stimulation parameters

From this thesis we can conclude that:

1. VNS at intensities of 0.25 mA, 0.5 mA and 1 mA has similar effects on cortical excitability in the motor cortex stimulation rat model. This finding indicates that 0.25 mA is sufficient to modulate cortical excitability and higher output current intensities may not be required.
2. CAP recordings could be used to guide the search for a more appropriate choice of stimulation parameters.

Predictive factors for response

From this thesis we can conclude that:

1. Extracellular hippocampal noradrenaline levels/changes are a potential biomarker for the efficacy of VNS in MTLE.
2. VNS-induced electrophysiological responses recorded from the vagus nerve reflect true vagal fiber activation. These electrophysiological responses may be used to determine recovery of the vagus nerve after implantation, to determine when VNS therapy in a specific experiment should be initiated and to optimize VNS parameters in an experimental set-up.

Discussion

Mechanism of action

The antiepileptic mechanism of action of VNS is not completely understood. Nevertheless, knowledge of the mechanism of action of a treatment can be crucial to increase its efficacy.

A first research line consists of investigating the role of the different fiber types that constitute the vagus nerve at the cervical level. There is convincing evidence that VNS acts via activation of afferent vagal fibers at the cervical level (Zabara 1992; McLachlan 1993). The earliest animal studies suggested that the antiepileptic potential of VNS was directly related to the fraction of vagal afferent C fibers stimulated. This was based on a maximal evoked response from A, B and C fibers in an isolated vagus nerve preparation induced by parameters required to suppress seizures in awake rats (Woodbury and Woodbury 1990, 1991). In rats, A fibers are recruited between 0.02-0.2 mA, B fibers between 0.04-0.6 mA and C fibers above 1 mA (Groves et al. 2005). The theory supporting C fiber involvement was discarded after Krahl et al. demonstrated seizure suppression with VNS in awake rats following selective destruction of C fibers using capsaisin (Krahl et al. 2001). The CAP recordings conducted by Woodbury and Woodbury (1990, 1991) were performed in anaesthetized animals and such a preparation does not take electrical shunting through the body fluid into account. Our observation that VNS at 0.25 mA, 0.5 mA and 1 mA has similar effects on cortical excitability in the motor cortex stimulation rat model supports the theory that vagal afferent fibers with low-to-moderate activation thresholds (i.e. A and B fibers) may be responsible for the central antiepileptic mechanisms of VNS.

A second branch of research consists of identifying the potential role of central nervous system structures that are located on the anatomical pathways from the cervical part of the vagus nerve up to the cortex and their neurotransmitters in the antiepileptic effect of VNS. The vagus nerve indirectly projects to the noradrenergic LC via the NTS. Several studies indicate that the

noradrenergic LC is a critical structure in the antiepileptic effect of VNS. The seizure suppression induced by VNS in the MES model is lost with the bilateral inactivation of the LC (Krahl et al. 1998). Single-unit recording experiments in rats have shown that the activity of LC neurons is increased upon acute and chronic stimulation of the vagus nerve (Groves et al. 2005; Dorr and Debonnel 2006; Manta et al. 2009). Confirming the recruitment of LC neurons during VNS, immediate-early gene mRNA's or their protein transcripts increase within LC neurons in response to VNS, in both rats (Naritoku et al. 1995) and rabbits (Gieroba and Blessing 1994; Cunningham et al. 2008). Increases in extracellular noradrenaline concentration have been measured by microdialysis in projection areas of the LC, such as the prefrontal cortex (Roosevelt et al. 2006; Follesa et al. 2007; Manta et al. 2013), hippocampus (Roosevelt et al. 2006; Manta et al. 2013) and amygdala (Hassert et al. 2004) in VNS-treated rats. Also we found significant increases in the extracellular hippocampal concentration of noradrenaline in response to VNS. Based on *in vitro* studies, we hypothesize that VNS reduces the activity of the NTS in a frequency-dependent manner. This should be evidence by *in vivo* studies in future. Our hypothesis further considers the predominantly inhibitory influence of the NTS on LC neurons. VNS as a result causes a disinhibition of the LC. The activity of the LC and its widespread noradrenergic modulation of various brain regions are now increased.

Although the above mentioned studies all suggest a role for noradrenaline in the antiepileptic effect of VNS, we are the first to show a causal relationship between VNS-induced noradrenaline increases and VNS-induced antiepileptic effects. In our hands, VNS suppressed pilocarpine-induced limbic seizures only in those rats with a hippocampal noradrenaline increase of at least 70%. Conversely, rats in which VNS did not increase hippocampal noradrenaline exhibited the most severe seizures. Furthermore, concomitant intrahippocampal administration of a selective α_2 adrenorecepor antagonist abolished the antiepileptic effects of VNS. These findings strongly support the hypothesis that the antiepileptic effect of VNS in MTLE is at least partly mediated by increased hippocampal noradrenaline and increased hippocampal α_2 adrenorecepor activation.

The brainstem DRN contains the largest number of serotonergic neurons in the brain. These serotonergic neurons represent a diffusely projecting system that innervates virtually all areas of the central nervous system (Krahl and Clark 2012). Pharmacological treatments that increase the concentration of serotonin (5-HT) at its postsynaptic receptors produce antiepileptic effects in a variety of epilepsy models, while treatments that decrease the 5-HT concentration exert proconvulsant effects (Przegalinksi 1985; Giorgi et al. 2004). Although less extensively studied compared to the effect of VNS on the LC and its noradrenaline transmission, VNS might also suppress

seizures through activation of the DRN and its serotonergic transmission. While acute VNS produces an increase in c-fos expression in the LC, the gene product specific to delayed and persistent neuronal activation, i.e. delta fosB, is expressed in the DRN only after prolonged VNS treatment (≥ 14 days) (Cunningham et al. 2008). These data are consistent with electrophysiological studies showing that VNS increases the firing rate of LC neurons after 1 hour of stimulation (Groves et al. 2005) while the firing rate of DRN neurons only increases after 14 days of stimulation (Dorr and Debonnel 2006). Interestingly, this last group also demonstrated that the LC must remain intact in order for VNS to affect DRN activity (Dorr and Debonnel 2006). LC lesions completely prevented the increase in DRN activity in response to chronic VNS. Furthermore, administration of an α_1 adrenoreceptor antagonist and agonist in the DRN reduced and increased its firing rate, respectively (Manta et al. 2009). The levels of 5-HIAA, a metabolite of serotonin, were increased in the cerebrospinal fluid of patients receiving VNS (Ben-Menachem et al. 1995) and the VNS-induced suppression of PTZ-induced seizures in rats was abolished when serotonergic neurons are destroyed with 5,7-dihydroxytryptamin, a selective 5-HT neurotoxin (Browning et al. 1997). Long-term VNS treatment has been shown to increase the tonic activation of postsynaptic 5-HT_{1A} receptors and to suppress neuronal firing in the hippocampus (Manta et al. 2013).

Based on the knowledge that seizures are characterized by highly synchronized EEG and on the finding that VNS alters EEG activity in animal models (Zabara 1985; Woodbury and Woodbury 1990, 1991; Sunderam et al. 2001; Sahin et al. 2009), it was initially hypothesized that the main mechanism of action of VNS consists of desynchronisation of cortical activity. This desynchronization may be the result of the VNS-induced activation of the noradrenergic and serotonergic system, but one report also suggests a possible role for acetylcholine (Nichols et al. 2011). The acitivity in the rat auditory cortex was suppressed by VNS and the muscarinic receptor antagonist scopolamine attenuated these suppressive effects. In addition, desynchronization of cortical activity may also be the result of VNS-induced activation of brain structures that have been shown to play a role in the regulation of seizures such as the amygdala, limbic cortex and thalamus, and that are anatomically connected with the vagus nerve (Hopkins and Holstege 1978; Ito and Craig 2005). VNS inhibited cortical responses evoked by amygdala stimulation (Lyubashina and Panteleev 2009) and evoked slow hyperpolarization in rat cortical neurons (Zagon and Kemeny 2000). In line with these results, VNS has been shown to increase the threshold for evoking focal motor seizures in the motor cortex stimulation rat model (De Herdt et al. 2010). We confirmed this last finding, indicating that VNS is able to modulate cortical excitability.

Stimulation parameters

The currently applied VNS parameters are not evidence based, but supported by a limited number of animal studies on efficacy and safety (Zanchetti et al. 1952; Stoica and Tudor 1967; Woodbury and Woodbury 1990, 1991; Zabara 1992). VNS is intermittently administered in order to reduce stimulation-related nerve damage (Agnew and McCreery 1990) and prolong battery life. In addition efficacy studies have shown that the effect of stimulation outlasts the stimulus duration (Zabara 1992; Takaya et al. 1996; Santiago-Rodriguez et al. 2006; Carrette et al. 2007). Agnew et al. have demonstrated in rats that electrically induced damage to the vagus nerve was greatly reduced when lower stimulation frequencies (20 Hz versus 50-100 Hz) were used (Agnew et al. 1989). They also showed that continuous high-frequency (> 50 Hz) stimulation could cause nerve injury.

The discovery that intermittent VNS was antiepileptic and safe in animals led to the development of intermittent VNS for human use. In many open label studies, the use of different stimulation parameters was investigated. Two randomized trials have shown that “high” settings in terms of *duty cycle, frequency* and *pulse width* (30 s ON, 5 min OFF, 30 Hz, 500 µsec, 0.25 to 3.5 mA) are significantly more effective than “low” settings (30 s ON, 180 min OFF, 1 Hz, 130 µsec, 0.25 to 3.5 mA) (Handforth et al. 1998). When patients originally randomized to “low” settings were crossed over to “high” settings, a robust improvement in efficacy resulted (DeGiorigio et al. 2000). Furthermore, when the duty cycle was increased above 22% or when the off time was decreased to ≤ 1.1 min, a significant improvement in efficacy was observed (DeGiorgio et al. 2001). In an uncontrolled, open label retrospective Belgian multicenter study, analysis of the used stimulation parameters revealed that the efficacy of VNS was comparable between patients with a 10 min OFF period and patients with shorter OFF periods (3 or 5 min) (De Herdt et al. 2007). Regarding the pulse width, reductions from 500 µs to 250 µs have been shown to increase tolerability (Liporace et al. 2001). Few data are available describing the use of pulse durations of less than 250 µsec in humans. Therefore, the use of such low pulse durations is not recommended (Heck et al. 2002).

Regarding the *output current intensity*, several animal and human studies corroborate the possibility that less charge density could be sufficient to obtain VNS efficacy. Acute VNS at 0.25 mA in conscious rats already increased staining for c-fos, an indirect marker of neuronal activity, in the NTS and many regions that receive its projections (Cunningham et al. 2008). In a study of Woodbury and Woodbury, VNS at 0.2-0.5 mA already reduced chemically-induced seizures in dogs (Woodbury and Woodbury 1990). 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 (Van Laere et al. 2000). These findings were confirmed in another human imaging study by our group (Vonck et al. 2008). In our study, we found that VNS at 0.25 mA, 0.5 mA and 1 mA has similar effects on cortical excitability in the motor cortex stimulation rat model. These results indicate that 0.25 mA is sufficient to decrease cortical excitability in rats and higher output current intensities may not be required. A direct translation to clinical practice is hampered by a large number of factors: (i) experimental rats versus humans; (ii) much smaller diameter of rat vagus nerve and (iii) different electrode configurations. Future prospective clinical studies comparing high versus low output current intensities are required to confirm if less charge density is sufficient to exert antiepileptic effects in humans.

The lowest output current intensity tested in the motor cortex stimulation model was 0.25 mA. The question remains if even lower output current intensities are sufficient to exert an antiepileptic effect. This question could be answered by means of CAP recordings. The stimulus-response curve for the afferent CAP described in our study illustrates that recruitment of fast-conducting afferent vagal A and B fibers rapidly reaches a saturation level in rats. Using a single pulse of 10 μs, saturation was already reached between 4 and 5 mA. On the basis of equal pulse charge, these intensities are approximately equivalent to 0.16-0.2 mA using a pulse of 250 μs. We therefore hypothesize that output current intensities of this order might be sufficient to reduce seizure frequency and higher output current intensities might not be required in the rat.

A recently developed mathematical model allows achievement of full activation of myelinated A and B fibers at the cervical level of the vagus nerve through changes in output current intensity and pulse width (Helmers et al. 2012). By adding additional factors that may influence the effectiveness of the VNS therapy, such as virtual cathodes and duty cyle and frequency of stimulation, the model could give clinicians complete information on how to optimize the VNS parameter settings.

Predictive factors for response

Another way to optimize the VNS treatment, apart from defining optimal stimulation paradigms, is the search for predictive factors for response. Clinical response to VNS is variable and unpredictable (Boon et al. 2007). Treatment with VNS (especially long-term treatment) reduces seizures with ≥ 50% in 50% of patients. These patients are defined as responders. In about 30% of patients, there is little or no effect. These patients are defined as non-responders. In the other 20% of patients, seizure frequency reduction ranges between 30-50%. These patients are defined as partial responders. So far, no criteria for success have been identified - despite the growing application of VNS, it is still not

possible to predict which patients will respond to what extent to the VNS therapy. Determining the success of VNS is important in counseling patients and in optimizing the VNS treatment. Moreover, because of the invasive nature of the procedure, the possible hazards of chronic implantation and the relatively high costs of the treatment, factors indicating a poor prognosis could favour other treatment options, e.g. a ketogenic diet or newly developed AEDs.

Most studies that attempt to predict the success of VNS are based upon clinical characteristics, the localization of the seizure focus or the epilepsy syndrome. However, these predictors for success are still elusive. It was found that VNS responsiveness was associated with older age and longer epilepsy duration (Labar 2004) or rather to be independent of epilepsy duration (Tecoma and Iragui 2006) and associated with younger age (Ghaemi et al. 2010; Marras et al. 2013). Bilateral or multifocal epilepsy seems to be associated with a good clinical outcome, while Lennox-Gastaut syndrome typically does not benefit from VNS (Labar 2004; Marras et al. 2013). Few studies evaluated whether success of VNS can be forecasted using the EEG. Janszky et al. showed that absence of bilateral interictal epileptiform discharges in the EEG before VNS implantation was associated with a seizure free outcome (Janszky et al. 2005). Elliott et al. noted that patients with focal epilepsy had optimal responses to VNS therapy (Elliott et al. 2011). De Vos et al. found that EEG symmetric features, based on brain symmetry index, could predict VNS efficacy (De Vos et al. 2011). In their study, the asymmetric spectral characteristics of the interictal EEG proved more typical of non-responder than responder patients, indicating a role of interictal EEG in predicting the success of VNS.

Further identification of VNS responders based on clinical and EEG characteristics require large prospective studies targeted at specific subpopulations of patients based on their unique characteristics. This remains a real challenge in a population as diverse as patients with refractory epilepsy. Identification of other, biological, neurochemical or neurophysiological markers may represent potential candidates (Janszky et al. 2005). When used in combination with a non-invasive technique to deliver VNS (e.g. transcutaneous activation of the vagus nerve (Dietrich et al. 2008)), such a biomarker for efficacy of VNS could help clinicians to reliably identify responders prior to surgical implantation of a VNS device.

We are the first to show that the antiepileptic effect of VNS in MTLE is at least partly mediated by increased hippocampal noradrenaline and increased hippocampal α_2 adrenorecepor activation. Noradrenaline levels/changes are therefore a potential biomarker for the efficacy of VNS in MTLE. Translation to human MTLE patients is hampered by the fact that limbic seizures in our study are acutely evoked. It should therefore be first evaluated if the same type of correlation between the

hippocampal noradrenaline content and antiepileptic effects of VNS also exists in a model of spontaneous and not acutely evoked limbic seizures (e.g. the post-status model). Furthermore, to date no non-invasive techniques are available to directly measure noradrenaline levels/changes in the human brain.

In current clinical practice, physicians are unable to assess true VNS-induced vagal nerve fiber activation. In our studies, we focused on the identification of an electrophysiological parameter that relfects true activation of the vagus nerve by VNS. Electrophysiological responses to VNS were measured using thin-point recording electrodes placed on the vagus nerve near the stimulation electrode. In the first study, a remote Larynx Muscle Evoked Potential (LMEP) was recorded. As, however, the LMEP is the result of efferent stimulation and VNS in epilepsy is focused on stimulating afferents in order to obtain beneficial effects in the brain (Woodbury and Woodbury 1990,1991; Krahl et al. 2001), the LMEP remains an indirect surrogate marker for adequate VNS. In the second study, CAPs of the fast-conducting afferent vagal A and B fibers were recorded. This CAP may provide an objective parameter for adequate VNS. In addition, both the LMEP and the CAP may be used to determine recovery of the vagus nerve after implantation and to determine when VNS therapy should be initiated.

Future perspectives

Mechanism of action

An important finding of this thesis is the causal relationship between VNS-induced hippocampal noradrenaline increases and VNS-induced suppression of limbic seizures in the intrahippocampal pilocarpine model. We hypothesize that the VNS-induced hippocampal noradrenaline increases are the result of a VNS-induced enhancement of the activity of LC neurons. This hypothesis could be evidenced by selective lesioning of the LC noradrenergic neurons in the intrahippocampal pilocarpine model. We anticipate that following selective lesioning of the LC noradrenergic neurons in the intrahippocampal pilocarpine model, the VNS-induced hippocampal noradrenaline increases will be no longer present. If this correlates with a loss of antiepileptic efficacy of VNS, it would be a strong evidence for a crucial role of the LC in the antiepileptic effect of VNS in MTLE.

Different lesioning approaches for selective destruction of LC noradrenergic neurons can be used. The LC can be lesioned following intraperitoneal administration of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). An alternative, more selective approach makes use of intrahippocampal injection with an antiserum against Dopamine-β-Hydroxylase (DbH) conjugated with the neurotoxin

saporin. DbH is the enzyme that converts dopamine to noradrenaline at the level of noradrenergic nerve terminals. The neurotoxin is taken up retrogradely by noradrenergic neurons that project to the hippocampus. Eventually, these neurons will be lesioned. Using intrahippocampal microdialysis and the acute pilocarpine model, the impact of LC lesions on the hippocampal noradrenergic response following VNS and on the antiepileptic effect of VNS in MTLE can be investigated.

Limbic seizures in the intrahippocampal pilocarpine model are acutely evoked. A next step is to study the role of hippocampal noradrenaline in the antiepileptic effect of VNS in a model of spontaneous limbic seizures.

In our laboratory a rat model of spontaneous limbic seizures is available. In this model, a status epilepticus and anatomopathological changes are induced by intraperitoneal injection of kainic acid, an analogue of the excitatory neurotransmitter glutamate. A few days to weeks after the status epilepticus rats develop spontaneous seizures in which the hippocampus is heavily involved. The epileptic rats could be implanted with a VNS electrode, EEG registration electrodes and a microdialysis guide cannula in the hippocampus. During two weeks of baseline monitoring the frequency, duration and severity of epileptic seizures could be determined. Then VNS could be initiated and freely moving animals could be monitored again for two weeks using the video-EEG setup to determine the frequency, duration and severity of spontaneous seizures. While continuing the VNS treatment, hippocampal perfusion of an α_2 adrenorecepor antagonist could now be initiated. The influence of the antagonist treatment on the effects of VNS on seizures in the kainic acid model could be monitored for 2 weeks. We hypothesize that VNS will reduce limbic seizure activity in the kanic acid model and that this antiepileptic effect will be abolished by the antagonist treatment. If this is indeed the case, it would provide evidence for a role of hippocampal noradrenaline and α_2 adrenorecepors in the antiepileptic effect of VNS in a model of spontaneous limbic seizures. If this is not the case, spontaneous limbic seizures and entailed anatomopathological lesions are likely to compromise noradrenergic neurotransmission. We hypothezise that possible antiepileptic effects of VNS are then the result of other, more chronic VNS-induced neuromodulatory effects, such as induction of neuronal plasticity and neurogenesis (Revesz et al. 2008).

We hypothezise that VNS in clinical practice reduces the activity of the NTS neurons. Our hypothesis further considers the predominantly inhibitory effect of the NTS on the LC. As a result of VNS, LC neurons are relieved from this inhibitory influence of the NTS, and their activity is increased. Our hypothesis could be evidenced by determining the effect of NTS antagonism on the LC firing rate. The NTS could be inhibited by local application of an AMPA-receptor antagonist. If NTS antagonism

increases the firing rate of LC neurons, it would provide direct evidence that NTS inhibition might exert antiepileptic effects.

LC neurons not only contain noradrenaline, but the noradrenaline co-transmitters Neuropeptide Y (NP-Y) and galanin are also expressed in LC neurons (Gundlach et al. 1990; Xu et al. 1998). Both neuropeptides have shown strong antiepileptic properties when applied locally into the hippocampus (Mazarati et al. 2002; Meurs et al. 2007). For these reasons, we hypothesize that hippocampal levels of both neuropeptides will increase in response to VNS and that these neuropeptides are also active contributors to the antiepileptic effect of VNS.

The effect of VNS on hippocampal NP-Y and galanin release can be studied in different ways. Post-mortem analysis of hippocampal NP-Y and galanin expression in VNS-treated rats can be performed using immunohistochemistry and ELISA techniques. The results can be compared to data obtained from LC-lesioned or sham-treated animals. It is worthwhile to investigate if the same ELISA technique can be used to analyze hippocampal microdialysates for NP-Y and galanin contents as well. If VNS indeed increases the hippocampal NP-Y and/or galanin concentration, selective antagonists for NP-Y and/or galanin receptors can be locally administered into the hippocampus to unravel the potential contribution of both neuropeptides to the antiepileptic effect of VNS in the intrahippocampal pilocarpine model of acute limbic seizures.

In this thesis we confirmed that VNS is able to increase the threshold for evoking focal motor seizures in the motor cortex stimulation rat model, indicating that VNS is able to modulate cortical excitability. Further research may be performed within the motor cortex stimulation rat model to explore the detailed underlying mechanisms responsible for the VNS-induced modulation of cortical excitability. Using microdialysis, neurochemical effects of VNS could be measured and correlated with the VNS-induced increases in seizure threshold. Based on the knowledge that the generalized paracrine noradrenaline diffusion affects the entire cortical activity, we hypothesize that noradrenaline will be involved in the VNS-induced modulation of cortical excitability. Also SPECT imaging studies could identify a neuronal correlate for the attenuation of cortical excitability with VNS in this animal model. The SPECT tracer ^{99m}Tc-ethyl cysteine dimer is characterized by a high and immediate cerebral uptake after intravenous injection (less than 10% of the administered activity remains in the blood after 5 minutes) (Shishido et al. 1994), making quick imaging at the moment of tracer injection possible. At the end of a VNS trial, the threshold for evoking focal motor seizures could be determined, immediately followed by SPECT imaging. In addition to the LC, we hypothezise that VNS-induced cerebal blood flow changes will be observed in brain structures that have been shown to play a role in the regulation of seizures such as the amygdala, limbic cortex and thalamus,

and that these changes will correlate with clinical efficacy of VNS in the motor cortex stimulation model.

Stimulation parameters

The lowest output current intensity tested in the motor cortex stimulation model, i.e. 0.25 mA, was sufficient to affect cortical excitability. Based on the stimulus-response curve for the vagal afferent A and B fibers described in our other study, we hypothesize that even lower output current intensities may be sufficient to significantly reduce cortical excitability. A combination of CAP recordings and threshold determinations in the motor cortex stimulation rat model could give more insight in this hypothesis.

Among the currently applied VNS parameters, many different combinations are optional and the efficacy of different paradigms should be investigated in a prospective way to evaluate potential superiority of certain paradigms with regards to efficacy as well as battery life. It is however likely to assume that not one ultimate set of stimulation parameters will benefit all different types of epilepsy and all refractory individuals. Individually guided stimulation parameter titration may be a more successful avenue.

Recording CAPs of afferent A and B fibers at the cervical level of the vagus nerve may represent a new tool for guiding individual parameter titration. Over the last years, attention is paid to the development of VNS devices that include an electrode for combined stimulation and recording (El Tahry et al. 2010). Recording CAPs post-operatively may help clinicians for example to determine the individual optimal intensity required to fully activate fast-conducting afferent vagal A and B fibers, which in turn could be used as a patient-specific optimal VNS output current value. In addition, this tool might help to develop stimulation methods that have the potential to reduce the LMEP, which reflects VNS-related throat discomfort, while maximizing the afferent CAP.

Predictive factors for response

Based on our rat study, we hypothesize that noradrenaline may be a useful biomarker for the efficacy of VNS in human MTLE. Even more, based on the knowledge that the generalized paracrine noradrenaline diffusion affects the entire cortical activity, we hypothesize that noradrenaline may be a useful biomarker for VNS efficacy in other types of epilepsy as well. The challenge is to measure noradrenaline in the human brain. Although no non-invasive techniques are currently available to directly measure noradrenaline in the human brain, an increase in noradrenaline may be indirectly

evaluated through parameters which are modulated by central noradrenergic signalization, such as the pupil diameter and the P300 component of event-related potentials. Recently, a retrospective clinical trial, including patients with diverse seizure foci, has been successfully performed at the Center for Neurophysiological Monitoring (CNM) at Ghent University Hospital. Patients with a good therapeutic response to VNS showed an increase in the amplitude of the P300 component of event-related potentials during the VNS ON phase compared to the VNS OFF phase. As the amplitude of the P300 component of event-related potentials is modulated by central noradrenergic signalization, these results indirectly indicate that VNS in these patients increases the noradrenaline levels and that the P300 component of event-related potentials can be used a biomarker for efficacy of VNS. A prospective clinical study is currently being conducted on this topic.

Another non-invasive and indirect technique to assess changes in central noradrenaline release could be the use of radioligands. Recently, it was shown that the PET tracer 11C-yohimbine, an α_2 adrenoreceptor antagonist, can be used to picture pharmacologically-induced changes in noradrenaline in the pig brain (Landau et al. 2012). Based on this study, a pilot trial is currently being performed in our laboratory to evaluate if 11C-yohimbine can be used to picture changes in noradrenaline concentration in the rat brain during VNS. If the results are satisfactory and can be correlated with seizure reduction in the intrahippocampal pilocarpine model, clinical trials could be initiated to evaluate if the PET tracer 11C-yohimbine can be used as a surrogate biomarker for the efficacy of VNS in human epilepsy.

References

Agnew WF, McCreery DB, Yuen TG, Bullara LA. Histologic and physiologic evaluation of electrically stimulated peripheral nerve: considerations for the selection of parameters. Ann Biomed Eng 1989;17(1):39-60.

Agnew WF, McCreery DB. Considerations for safety with chronically implanted nerve electrodes. Epilepsia 1990;31(S2):S27-S32.

Ben-Menachem E, Hamberger A, Hedner T, Hammond EJ, Uthman BM, Slater J, Treig T, Stefan H, Ramsay RE, Wernicke JF, Wilder BJ. Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. Epilepsy Res 1995;20(3):221-227.

Boon P, De Herdt V, Vonck K, Van Roost D. Clinical experience with vagus nerve stimulation and deep brain stimulation in epilepsy. Acta Neurochir Suppl 2007;97(Pt2):273-280.

Browning RA, Clark KB, Naritoku DK, Smith DC, Jensen RA. Loss of anticonvulsant effect of vagus nerve stimulation in the pentylenetetrazol seizure model following treatment with 6-hydroxydopamine or 5,7-dihydroxytryptamine. Soc Neurosci Abstr 1997;23:2424.

Carrette E, De Herdt V, Manders J. Correlation of interictal spikes and long-term efficacy in patients with refractory epilepsy treated with vagus nerve stimulation. Epilepsia 2007;48:195-196.

Cunningham JT, Mifflin SW, Gould GG, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation. Neuropharmacology 2008;33(8):1884-1895.

DeGiorgio CM, Schachter SC, Handforth A, Salinsky M, Thompson J, Uthman B, Reed R, Collins S, Tecoma E, Morris GL, Vaughn B, Naritoku DK, Henry T, Labar D, Gilmartin R, Labiner D, Osorio I, Ristanovic R, Jones J, Murphy J, Ney G, Wheless J, Lewis P, Heck C. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia 2000;41(9):1195-2200.

DeGiorgio CM, Thompson J, Lewis P, Arrambide S, Naritoku D, Handforth A, Labar D, Mullin P, Heck C; VNS U.S. Study Group. Vagus nerve stimulation: analysis of device parameters in 154 patients during the long-term XE5 study. Epilepsia *2001;42(8):1017–1020.*

De Herdt V, Boon P, Ceulemans B, Hauman H, Lagae L, Legros B, Sadzot B, Van Bogaert P, van Rijckevorsel K, Verhelst H,Vonck K. Vagus nerve stimulation for refractory epilepsy: a Belgian multicenter study. Eur J Paediatr Neurol 2007;11(5):261-269.

De Herdt V, De Waele J, Raedt R, Wyckhuys T, El Tahry R, Vonck K, Wadman W, Boon P. Modulation of seizure threshold by vagus nerve stimulation in an animal model for motor seizures. Acta Neurol Scand 2010;121(4):271-276.

De Vos CC, Melching L, van Schoonhoven J, Ardesch JJ, de Weerd AW, van Lambalgen HC, van Putten MJ. Predicting success of vagus nerve stimulation (VNS) from interictal EEG. Seizure 2011;20(7):541-545.

Dietrich S, Smith J, Scherzinger C, Hofmann-Preiss K, Freitag T, Eisenkolb A, Ringler R. A novel transcutaneous vagus nerve stimulation leads to brainstem and cerebral activations measured by functional MRI. Biomedizinische Technik 2008;53(3):104-111.

Dorr AE, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 2006;318(2):890-898.

Elliott RE, Morsi A, Kalhorn SP, Maracus J, Sellin J, Kang M, Silverberg A, Rivera E, Geller E, Carlson C, Devinsky O, Doyle WK. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: long-term outcomes and predictors of response. Epilepsy Behav 2011;20(1):57-63.

El Tahry R, Raedt R, Mollet L, De Herdt V, Wyckhuys T, Van Dycke A, Meurs A, Dewaele F, Van Roost D, Doguet P, Delbeke J, Wadman W, Vonck K, Boon P. A novel implantable vagus nerve stimulation system (ADNS-300) for combined stimulation and recording of the vagus nerve: Pilot trial at Ghent University Hospital. Epilepsy Res 2010;92(2-3):231-239.

Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, Puligheddu M, Marrosu F, Biggio G. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res 2007;1179:28-34.

Ghaemi K, Elsharkawy AE, Schulz R, Hoppe M, Polster T, Pannek H, Ebner A. Vagus nerve stimulation: outcome and predictors of seizure freedom in long-term follow-up. Seizure 2010;19(5):264-268.

Gieroba ZJ and Blessing WW. Fos-containing neurons in medulla and pons after unilateral stimulation of the afferent abdominal vagus in conscious rabbits. Neuroscience 1994;59(4):851-858.

Giorgi FS, Pizzanelli C, Biagioni F, Murri L, Fornai F. The role of norepinephrine in epilepsy: from the bench to the bedside. Neuroscience Biobehav Rev 2004;28(5):507-524.

Groves DA, Bowman EM, Brown VJ. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neurosci Lett 2005;379(3):174-179.

Gundlach AL, Rutherfurd SD, Louis WJ. Increase in galanin and neuropeptide Y mRNA in locus coeruleus following acute reserpine treatment. Eur J Pharmacol 1990;184(1):163-167.

Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, Henry TR, Collins SD, Vaughn BV, Gilmartin RC, Labar DR, Morris GL 3rd, Salinsky MC, Osorio I, Ristanovic RK, Labiner DM, Jones JC, Murphy

JV, Ney GC, Wheless JW. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 1998;51(1):48-55.

Hassert D, Miyashita A, Williams C. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behav Neurosci 2004;118(1):79-88.

Heck C, Helmers SL, DeGiorgio CM. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. Neurology 2002;59(6S4):S31-S37.

Helmers SL, Begnaud J, Cowley A, Corwin HM, Edwards JC, Holder DL, Kostov H, Larsson PG, Levisohn PM,De Menezes MS, Stefan H, Labiner DM. Application of a computational model of vagus nerve stimulation. Acta Neurol Scand 2012;126(5):336-343.

Hopkins DA, Holstege G. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp Brain Res 1978;32(4):529-547.

Ito S, Craig A. Vagal-evoked activity in the parafascicular nucleus of the primate thalamus. J Neurophysiol 2005;94(4):2976–2982.

Janszky J, Hoppe M, Benhe F, Tuxhorn I, Pannek HW, Ebner A. Vagus nerve stimulation: predictors of seizure freedom. J Neurol Neurosurg Psychiatry 2005;76(3):384-389.

Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure attenuating effects of vagus nerve stimulation. Epilepsia 1998;39(7):709-714.

Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 2001;42(5):586-589.

Krahl SE, Clark KB. Vagus nerve stimulation for epilepsy: a review of central mechanisms. Surg Neurol Int 2012;3(Suppl4):S255-259.

Labar D. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. Seizure 2004;13(6):392-398.

Liporace J, Hucko D, Morrow R, Barolat G, Nei M, Schnur J, Sperling M. Vagal nerve stimulation: adjustments to reduce painful side effects. Neurology *2001;57(5):885–886.*

Landau AM, Doudet DJ, Jakobsen S. Amphetamine challenge decreases yohimbine binding to α2 adrenoreceptors in Landrace pig brain. Psychopharmacology 2012;222(1):155-163.

Lyubashina O, Panteleev S. Effects of cervical vagus nerve stimulation on amygdala-evoked responses of the medial prefrontal cortex neurons in rat. Neurosci Res 2009;65(1):122-125.

Manta S, Dong J, Debonnel G, Blier P. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. J Psychiatry Neurosci 2009;34(4):272-280.

Manta S, El Mansari M, Debonnel G, Blier P. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. Int J Neuropsychopharmacol 2013;16(2):459-470.

Marras CE, Chiesa V, De Benedictic A, Franzini A, Rizzi M, Villani F, Ragona F, Tassi L, Vignoli A, Freri E, Specchio N, Broggi G, Casazza M, Canevini MP. Vagus nerve stimulation in refractory epilepsy: new indications and outcome assessment. Epilepsy and Behav 2013;28(3):374-378.

Mazarati A, Wasterlain CG. Anticonvulsant effects of four neuropeptides in the rat hippocampus during self-sustaining status epilepticus. Neurosci Lett 2002;331(2):123-127.

McLachlan RS. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. Epilepsia 1993;34(5):918-923.

Meurs A, Clinckers R, Ebinger G, Michotte Y, Smolders I. Sigma 1 receptor-mediated increase in hippocampal extracellular dopamine contributes to the mechanism of the anticonvulsant action of neuropeptide Y. Eur J Neurosci 2007;26(11):3079-3092.

Naritoku DK, Terry WJ, Helfert RH. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res 1995;22(1):53-62.

Nichols JA, Nichols AR, Smirnakis SM, Engineer ND, Kilgard MP, Atzori M. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. Neuroscience 2011;189:207-214.

Przegalinski E. Monoamines and the pathophysiology of seizure disorders. In: Antiepileptic drugs (Frey HH, Janz D, eds) 1985. Berlin, Germany: Springer-Verlag.

Revesz D, Tjernstrom M, Ben-Menachem E, Thorlin T. Effects of vagus nerve stimulation on rat hippocampal progenitor proliferation. Exp Neurol 2008;214(2):259-265.

Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res 2006;1119(1):124-132.

Sahin D, Ilbay G, Imal M, Bozdogan O, Ates N. Vagus nerve stimulation suppresses generalized seizure activity and seizure-triggered postictal cardiac rhythm changes in rats. Physiol Res 2009;58(3):345 ‑350.

Santiago-Rodriguez E, Alonso-Vanegas M, Cardenas-Morales L, Haarmony T, Bernardino M, Fernandez-Bouzas A. Effects of two different cycles of vagus nerve stimulation on interictal epileptiform discharges. Seizure 2006;15(8):615-620.

Shishido F, Uemura K, Murakami M, Inugami A, Ogawa T, Fujita H, Shimosegawa E, Kanno I, Aizawa Y, Nagata K. Cerebral uptake of Tc-99m-bicisate in patients with cerebrovascular disease in comparison with CBF and CMRO;I2 measured by positron emission tomography. J Cereb Blood Flow Metab 1994;14(Suppl1):S66-S75.

Stoica I, Tudor I. Effects of vagus afferents on strychnine focus on coronal gyrus. Rev Roum Neurol 1967;4:287-295.

Sunderam S, Osorio I, Watkins JF 3rd, Wilkinson SB, Frei MG, Davis RE. Vagal and sciatic nerve stimulation have complex, time-dependent effects on chemically-induced seizures: a controlled study. Brain Res 2001;918(1-2):60 ‑66.

Takaya M, Terry WJ, Naritoku DK. Vagus nerve stimulation induces a sustained anticonvulsant effect. Epilepsia 1996;37(11):1111-1116.

Tecoma ES, Iragui VJ. Vagus nerve stimulation use and effect in epilepsy: what have we learned? Epilepsy Behav 2006;8(1):127-136.

Van Laere K, Vonck K, Boon P, Brans B, VandenKerckhove T, Dierckx R. Vagus nerve stimulation in refractory epilepsy: SPECT activation study. J Nucl Med 2000;41(7):1145-1154.

Vonck K, De Herdt V, Bosman T, Dedeurwaerdere S,Van Laere K, Boon P. Thalamic and limbic involvement in the mechanism of action of vagus nerve stimulation, a SPECT study. Seizure 2008;17(8):699-706.

Woodbury DM, Woodbury JW. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 1990;31(S2):S7-S19.

Woodbury J, Woodbury D. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff electrode for stimulating and recording. Pacing Clin Electrophysiol 1991;14(1):94-107.

Xu Z, Shi T, Hökfelt T. Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and –R2 receptors. J Comp Neurol 1998;392(2):227-251.

Zabara J. Peripheral control of hypersynchronous discharge in epilepsy. Electroencephalogr Clin Neurophysiol 1985;61(3):S162.

Zabara J. Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia 1992;33(6):1005-1012.

Zagon A, Kemeny AA. Slow hyperpolarization in cortical neurons: a possible mechanism behind vagus nerve simulation therapy for refractory epilepsy? Epilepsia 2000;41(11):1382-1389.

Zanchetti A, Wang S, Moruzzi G. The effect of vagal stimulation on the EEG pattern of the cat. Electroencephalogr Clin Neurophysiol 1952;4(3):357-461.

Summary

Epilepsy is a frequently occurring chronic neurological disorder, characterized by the spontaneous and recurrent occurrence of epileptic seizures. Epilepsy affects approximately 0.5-1% of the population. Despite adequate antiepileptic treatment, 30-40% of patients continue to have seizures or experience unacceptable pharmacological side effects. These patients have medically refractory epilepsy and require a thorough diagnostic and therapeutic evaluation in a specialized epilepsy center. Epilepsy surgery is an invasive but often curative treatment option that aims at removing the ictal onset zone believed to be responsible for seizure occurrence. A substantial number of patients is however rejected from surgery due to the fact that a well circumscribed, unifocal ictal onset zone cannot be identified or due to the fact that the ictal onset zone is located in functional brain tissue. Unsuitable candidates for resective surgery have few options left. Administration of newly developed antiepileptic drugs (AEDs) leads to seizure freedom in only a small number of patients. The inability to adequately treat all patients with refractory epilepsy provides a continuous impetus to investigate novel forms of treatment. One novel treatment option is neuromodulation by electrical stimulation of central nervous system structures. One type of neuromodulation is vagus nerve stimulation (VNS).

VNS involves stimulation of the tenth cranial nerve by means of a spiral stimulation electrode, wound around the vagus nerve at the cervical level and connected to a subclavicularly implanted pulse generator. The first human implant was performed in 1990. Now, over 60,000 patients are treated with VNS worldwide. Despite the established efficacy and safety, the large number of treated patients and more than 20 years of experience with VNS, some specific issues about this treatment remain to be established. The currently existing drawbacks of clinical application of VNS are the lack of responder identification prior to implantation and the insufficient knowledge about the most appropriate stimulation parameters. Improved knowledge of the antiepileptic mechanism of action of VNS may support the solution for these drawbacks. This thesis focused on these unresolved VNS-related topics with the aim of discovering new insights in the treatment modality of VNS for refractory epilepsy.

Several studies indicate that the noradrenergic brainstem nucleus locus coeruleus (LC) is a critical structure in the antiepileptic effect of VNS. Presumably as a result of enhancement of the activity of LC neurons, we observed an increase in the extracellular hippocampal noradrenaline concentration in VNS-treated rats. A strong positive correlation was found between the noradrenergic and antiepileptic effects of VNS in the focal pilocarpine limbic seizure model and blockade of hippocampal α_2 receptors reversed the antiepileptic effect of VNS. These findings

strongly suggest that VNS-induced increases in extracellular hippocampal noradrenaline are at least partly responsible for the antiepileptic effect of VNS in a model for limbic seizures, and constitute a potential biomarker for the efficacy of VNS in temporal lobe epilepsy. When used in combination with a non-invasive technique to deliver VNS (e.g. transcutaneous activation of the vagus nerve), noradrenaline as a biomarker for the efficacy of VNS could help clinicians to reliably identify responders prior to surgical implantation of a VNS device, and to determine optimal stimulation parameters in a more rational way.

With regards to stimulation parameters, we found that VNS at 0.25 mA, 0.5 mA and 1 mA increased the threshold for evoking focal, motor seizures compared to sham stimulation in rats. These results indicate 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. Our results are supported by several animal and human studies and support the theory that vagal afferent fibers with low-to-moderate activation thresholds may be responsible for addressing the antiepileptic mechanisms of VNS. Future prospective clinical trials comparing high versus low output current intensities are required to confirm these findings, as low output current intensities significantly increase battery life and improve tolerability.

In current clinical practice, physicians are unable to assess effective VNS-induced vagal nerve fiber activation. In this thesis, we focused on the identification of an electrophysiological parameter that reflects adequate VNS. Therefore, electrophysiological responses to VNS were measured using thin-point recording electrodes placed on the vagus nerve near the stimulation electrode. The electrophysiological response to VNS consisted of an early and a late component, identified as a compound action potential (CAP) of fast-conducing afferent vagal fibers and a remote laryngeal motor-evoked potential (LMEP), respectively. As the LMEP is the result of efferent co-stimulation, and VNS in epilepsy is focused on stimulating fast-conducting vagal afferents in order to obtain beneficial effects in the brain, the CAP may provide the best objective parameter for adequate VNS. Both the LMEP and CAP may be used to determine the recovery of the vagus nerve after implantation and to determine when VNS therapy can be initiated. In addition, recording CAPs of fast-conducting afferent vagal A and B fibers may represent a new tool for guiding individual parameter titration and might help to develop stimulation methods that have the potentials to reduce the efferent LMEP, which reflects VNS-related throat discomfort, while maximizing the afferent CAP.

Résumé

L’épilepsie est une affection neurologique chronique fréquente, caractérisée par l’apparition spontanée et récurrente des crises d’épilepsie. Malgré les traitements antiépileptiques adéquats, 30 à 40% des patients continuent d’avoir des convulsions ou des effets secondaires pharmaceutiques inacceptables. Ces patients souffrent d'épilepsie réfractaire au traitement médical et nécessitent une évaluation diagnostique et thérapeutique approfondie dans un centre spécialisé. La chirurgie d'épilepsie est une option invasive mais souvent curative qui vise à éliminer la zone ictale qui est responsable de la survenance de crises. Un nombre important de patients est cependant rejeté pour la chirurgie en raison du fait qu’une zone ictale unifocale et bien circonscrit ne peut être identifiée ou en raison du fait que la zone ictale se trouve dans le tissu cérébral fonctionnel. Candidats inappropriés pour une chirurgie d'exérèse ont peu d'autres options. Administration nouvellement mis au point des médicaments antiépileptiques (AEDs) conduit à la contrôle que dans un petit nombre de patients. L'incapacité à traiter correctement tous les patients souffrant d'épilepsie réfractaire fournit une impulsion continue à enquêter sur de nouvelles formes de traitement. Une nouvelle option de traitement est la neuromodulation par stimulation électrique des structures du système nerveux central. Un type de neuromodulation est la stimulation du nerf vague (VNS).

VNS implique la stimulation du dixième nerf crânien au moyen d'une électrode de stimulation en spirale, enroulée autour du nerf vague, au niveau du col et reliée à un générateur d'impulsions implanté dans la région sous-claviculaire. Le premier implant humain a été réalisé en 1990. Maintenant, plus de 60000 patients sont traités par la VNS dans le monde entier. Malgré l'efficacité, la sécurité établie, le grand nombre de patients traités et plus de 20 ans d'expérience avec le système de la VNS, certaines questions spécifiques au sujet de ce traitement restent à établir. Les inconvénients existant actuellement à l’application clinique de la VNS sont le manque d'identification répondeur avant l'implantation et l'insuffisance des connaissances sur les paramètres de stimulation les plus appropriés. Une meilleure connaissance du mécanisme antiépileptique de l'action de la VNS peut soutenir la solution pour ces inconvénients. Cette thèse a porté sur ces sujets non résolus de la VNS liées dans le but de découvrir de nouvelles perspectives dans la modalité de traitement de la VNS pour l'épilepsie réfractaire.

Plusieurs études indiquent que le noyau locus coeruleus (LC) est une structure située dans le tronc cérébral noradrénergique essentielle à l'effet antiepileptique de la VNS. Probablement en raison de l'amélioration de l'activité des neurones LC, nous avons observé une augmentation de la concentration de noradrénaline extracellulaire dans l'hippocampe de rats traités par VNS. Une forte

corrélation a été trouvée entre les effets noradrénergiques et antiepileptique de la VNS dans le modèle limbique pilocarpine focal. En plus, le blocus des récepteurs α_2 de l'hippocampe annulait l'effet antiepileptique de la VNS. Ces résultats suggèrent fortement que les augmentations de la noradrénaline hippocampe extracellulaire induites par la VNS sont au moins partiellement responsable de l'effet antiepileptique de la VNS dans un modèle de crises limbiques, et constituent un bio marqueur potentiel pour l'efficacité de la VNS dans l'épilepsie du lobe temporal. S'il est utilisé en combinaison avec une technique non-invasive pour fournir une VNS (par exemple l'activation transcutanée du nerf vague), la noradrénaline comme un bio marqueur de l'efficacité de la VNS pourrait aider les cliniciens à identifier de manière fiable les respondeurs avant l'implantation chirurgicale d'un dispositif VNS, et à déterminer les paramètres de stimulation optimale d'une manière rationnelle.

En ce qui concerne les paramètres de stimulation, nous avons constaté que la VNS à 0,25 mA, 0,5 mA et 1 mA également augmente le seuil pour évoquer des convulsions moteur focales par rapport à sham stimulation des rats. Ces résultats indiquent que, sur les intensités actuellement testés, 0,25 mA est suffisant pour diminuer l'excitabilité corticale et des intensités de courant plus élevés ne peuvent pas être nécessaire. Nos résultats sont supportés par plusieurs études animales et humaines et soutiennent la théorie selon laquelle les fibres afférentes vagales avec des seuils d'activation faible à modéré jouent un rôle important dans le mécanisme antiépileptique de l'action de la VNS. Essais cliniques prospectifs futurs comparant les intensités de courant élevées par rapport à faible rendement sont nécessaires pour confirmer ces résultats, comme intensités faibles augmentent considérablement l'autonomie de la batterie et améliorent la tolérance.

Dans la pratique clinique actuelle, les médecins sont incapables d'évaluer l'activation des fibres du nerf vague induite par la VNS. Dans cette thèse, nous nous sommes concentrés sur l'identification d'un paramètre électrophysiologique qui reflète une VNS adéquate. Les réponses électrophysiologiques à la VNS ont été mesurées à l'aide des électrodes d'enregistrement mince placées sur le nerf vague près de l'électrode de stimulation. La réponse électrophysiologique à la VNS se composait d'une composante tôt et d'une composante tard, identifié comme un potentiel d'action composite (CAP) de fibres vagales afférentes rapide conducteurs et un potentiel laryngeal évoqué moteur à distance (LMEP), respectivement. Comme le LMEP est le résultat de co-stimulation efférente, et la VNS dans l'épilepsie est axée sur la stimulation rapide conducteur afférences vagales afin d'obtenir des effets bénéfiques dans le cerveau, la CAP peut offrir le meilleur paramètre objectif pour une VNS adéquate. Le LMEP et la CAP peuvent être utilisés les deux pour déterminer le temps de récupération du nerf vague après l'implantation et pour déterminer quand une titration de la thérapie de VNS peut être initiée. En outre, l'enregistrement CAP de rapide conducteur afférente

vagale A et fibres B peut représenter un nouvel outil pour guider le titrage des paramètres individuels et pourraient aider à développer des méthodes de stimulation qui ont le potentiel de réduire le LMEP efferent.

Samenvatting

Epilepsie is een frequent voorkomende chronische neurologische aandoening die gekenmerkt wordt door het herhaaldelijk en episodisch optreden van epileptische aanvallen. Epilepsie komt voor bij 0.5-1% van de bevolking. De meeste patiënten reageren goed op een medicamenteuze behandeling. Bij 30-40% van de epilepsiepatiënten komen de epileptische aanvallen niet of slechts gedeeltelijk onder controle ondanks een optimale behandeling met antiepileptica. Deze patiënten hebben refractaire epilepsie en vereisen een meer uitgebreide diagnostische en therapeutische evaluatie in een gespecialiseerd epilepsiecentrum. De behandelingsmogelijkheden bestaan uit epilepsiechirurgie en/of toediening van recent ontwikkelde antiepileptica in het kader van fase-III klinische studies. Het relatief groot aantal refractaire patiënten en de beperkte beschikbaarheid van middelen om refractaire epilepsie te behandelen onderstreept de nood tot het ontwikkelen van nieuwe behandelingsmodaliteiten. Neuromodulatie door elektrische stimulatie van zenuwstructuren (neurostimulatie) met de bedoeling epileptische aanvallen te onderbreken vormt hiervan een voorbeeld. Concreet betreft het stimulatie van de tiende craniale zenuw, nl. “nervus vagus stimulatie” (NVS).

Bij NVS wordt een spiraalvormige stimulatie elektrode rond de linker nervus vagus in de halsregio gewonden en verbonden met een kleine pulsgenerator die subcutaan thv het sleutelbeen geplaatst wordt. De eerste implantatie in een epilepsiepatiënt vond plaats in 1990. Ondertussen worden wereldwijd reeds meer dan 60000 patiënten behandeld met NVS. Ondanks de doeltreffendheid en veiligheid van de NVS behandeling, het groot aantal behandelde patiënten en meer dan 20 jaar ervaring met NVS, dienen nog een aantal aspecten opgehelderd te worden. De factoren die de klinische doeltreffendheid van NVS in een individuele patient bepalen zijn momenteel ongekend. Daarnaast is er nood aan een identificatie van de meest optimale stimulatie parameters en aan een gedetailleerde kennis van het anti-epileptisch werkingsmechanisme van NVS. Dit proefschrift richt zich op deze NVS-gerelateerde onderwerpen met het oog op het aantonen van nieuwe inzichten in de NVS therapie voor refractaire epilepsie.

Het anti-epileptisch effect van NVS wordt toegeschreven aan de activatie van afferente vezels van de nervus vagus. Een zenuwkern van de nervus vagus die een belangrijke rol lijkt te spelen in het aanvalsonderdrukkend effect van NVS is de locus coeruleus (LC). Deze kern, gelegen in de pons, is rijk aan noradrenerge neuronen en is de voornaamste bron van noradrenaline in de hersenen. Vermoedelijk als gevolg van een verhoogde activiteit van LC neuronen, toonde onze eerste studie een toename van de noradrenaline concentratie in de hippocampus in VNS-behandelde dieren. Deze

studie toonde bovendien aanvalsonderdrukkende effecten van NVS in een diermodel voor temporale kwab epilepsie. De mate van aanvalsonderdrukking bleek positief gecorreleerd te zijn met de toename van de hippocampale noradrenaline concentratie en de anticonvulsieve effecten van NVS werden opgeheven door intrahippocampale toediening van een α_2 adrenoreceptor antagonist. Deze bevindingen suggereren dat de NVS-geïnduceerde toename van de hippocampale noradrenaline concentratie minstens gedeeltelijk verantwoordelijk is voor de aanvalsonderdrukkende effecten in een diermodel voor temporale kwab epilepsie. Hippocampaal noradrenaline zou aldus een potentiële biomarker kunnen zijn voor de potentie van NVS om limbische aanvallen te onderdrukken.

In een tweede studie werd het effect van verschillende NVS intensiteiten op de corticale exciteerbaarheid in ratten geëvalueerd. NVS aan 0.25 mA, 0.5 mA en 1 mA verhoogde de drempel voor het uitlokken van focaal, motorische aanvallen in gelijke mate. Deze resultaten wijzen erop dat NVS aan 0.25 mA volstaat om de corticale exciteerbaarheid in ratten te verlagen, en dat hogere stimulatie intensiteiten niet vereist zijn. Onze resultaten worden ondersteund door verschillende dierexperimentele en humane studies, en ondersteunen de theorie dat vagale afferente vezels met een lage tot matige activeringsdrempel verantwoordelijk zijn voor het activeren van het aanvalsonderdrukkend werkingsmechanisme van NVS. Prospectieve klinische studies zijn nodig om deze bevindingen te bevestigen, aangezien lagere NVS intensiteiten de levensduur van de batterij aanzienlijk verhogen en de bijwerkingen van de NVS therapie aanzienlijk reduceren.

In de huidige klinische praktijk zijn neurologen niet in staat om werkelijke NVS-geïnduceerde activatie van de nervus vagus te beoordelen. In dit proefschrift hebben we ons gericht op de identificatie van een elektrofysiologische parameter die effectieve NVS weerspiegelt. Hiertoe werden NVS-geïnduceerde elektrofysiologische responsen gemeten met behulp van puntelektroden die geplaatst werden op de nervus vagus in de buurt van de stimulatie elektrode. De elektrofysiologische responsen bestonden uit een vroege en een late component, geïdentificeerd als respectievelijk een “compound action potential” (CAP) van de snelgeleidende afferente vagale vezels en een op afstand gemeten “larynx motor-evoked potential” (LMEP). Aangezien de LMEP het resultaat is van efferente co-stimulatie en VNS in epilepsie gericht is op het stimuleren van snelgeleidende afferente vagale vezels, kan de CAP beschouwd worden als de beste objectieve biomarker voor adequate NVS. Zowel de LMEP als de CAP kunnen gebruikt worden om het herstel van de nervus vagus na implantatie te bepalen en om te bepalen wanneer uptitratie van de NVS therapie kan gestart worden. Daarnaast zouden CAP registraties kunnen gebruikt worden als een nieuwe methode om op individuele basis te zoeken naar de meest adequate NVS parameters.

Dankwoord

Dit doctoraatswerk bleek een werk van lange adem te zijn, een weg van mooie ups, maar toch ook downs, eigen aan het voltooien van een dergelijke onderneming. Ongetwijfeld clichés die menig doctoraatstudent zich al bedacht heeft. Hierbij wil ik graag even de tijd nemen om een aantal personen in de bloemetjes te zetten die een belangrijke rol hebben gespeeld bij het voltooien van dit proefschrift.

Als eerste had ik graag **Prof. Dr. Paul Boon** bedankt om mij een plaats te bieden binnen zijn laboratorium. Prof. Boon zorgde en zorgt nog steeds voor een uitstekend kader waarin naar hartelust aan onderzoek kan gedaan worden.

Dankzij de kritische en wetenschappelijke bijdrage van mijn promotor **Prof. Dr. Kristl Vonck** heb ik mijn interesse voor de neurowetenschappen verder kunnen uitbouwen tot een passie, die niet meer weg te denken is uit mijn professionele leven. De regelmatige peilingen naar nieuwe onderzoeksresultaten en het aanwenden van nieuwe nuttige experimenten hebben zeker hun nut bewezen bij het tot stand brengen van dit doctoraatswerk.

Ook zonder mijn co-promotor **Prof. Dr. Robrecht Raedt** had ik dit punt waarschijnlijk nooit bereikt. Zijn bereidheid tot het nalezen van manuscripten, projecten, data en zoveel meer kende de afgelopen jaren geen grenzen. Hij leerde me dat wat moeilijk en onmogelijk lijkt niet altijd onhaalbaar is. Bedankt, Prof. Raedt, voor de professionele ondersteuning, intensieve en stimulerende begeleiding en de waardevolle en kritische wetenschappelijke input.

Prof. Dr. Delbeke en **Prof. Wadman**, jullie zijn voor mij speciale leermeesters geweest. Bedankt voor de vele leerrijke woorden over elektroden en stimulatie.

Doctoreren en eenzaamheid zouden hand in hand kunnen gaan. Maar dat is, op zijn zachtst uitgedrukt, buiten ons labo gerekend. De aangename werkomgeving, waarin naast het druk in de weer zijn ook altijd tijd en ruimte was voor een babbeltje/lolletje over niet-neurologische zaken, zorgde voor een unieke groepsgeest, en vele collega's zijn de afgelopen jaren dan ook in de eerste plaats vrienden geworden. Bedankt **Bregt, Annelies, Lars, Ine, Ine, Lisa, Mathieu** en **Leen**! In het bijzonder wens ik mijn jaargenoten **Ine** en **Bregt** veel succes met het weldra voltooien van hun doctoraat. De jongere garde wens ik ook heel veel succes, mooie resultaten en veel doorzettingsvermogen. Ook een dikke merci aan de immer sympathieke **Corine**. Dankzij jou konden we toch steeds een gaatje vinden in die drukke agenda!

Een woordje van dank is er zeker ook voor de dierenarts **Dr. Ingrid Van Overbeke** en de **dierenverzorgers** van het animalarium voor hun geduld bij het verzorgen van de (niet altijd gemakkelijke) proefdieren.

Gelukkig bleef er ook nog wat vrije tijd over. Bedankt fantastische vrienden voor alle toffe momenten tijdens etentjes, spelletjesavonden, weekenden, reisjes of gewoon op café: **Ilse-Mien, Marlies, Iris, Katrijn, Ine, Kirsten, Thierry, Wendy, Bert, Evelyne, Pieter, Sara, Webbey, Annelies, Christophe, Fanie, Dominique, Debbie, Arvid,…**! Ook een dikke dankjewel voor alle begeleiders en leden van Abajaa! Het jaarlijks zomerkamp was steeds fantastisch!

Mijn grootste supporters wil ik hier uiteraard ook bedanken. In de eerste plaats wens ik mijn lieve ouders van harte te bedanken voor hun onvoorwaardelijke steun. Bedankt **mama** en **papa** voor de geweldige jeugd, het geven van alles kansen waarvan ik kon dromen en de blijvende interesse in mijn onderzoek. Wat er ook moge gebeuren, jullie bereidheid om alles te laten vallen voor jullie kinderen kent geen grenzen. Ook een dikke merci aan **Karen, Karel, Pieter, Karolien, Bart** en **An** om mij op te peppen wanneer nodig en voor de vele leuke momenten. Ik kan mij geen betere (schoon)zussen en (schoon)broers voorstellen! Een dikke zoen ook voor de **allerkleinste Molletjes**! Ook mijn schoonmama **Thérèse** en **Mammoe** wil ik graag in de bloemetjes zetten. Dank je wel!

En als laatste, maar absoluut niet in het minste, wil ik mijn allerliefste schat bedanken. **Karel(tje)**, liefje, merci voor je opkikkertalent, luisterend oor, geduld, eindeloze humor en de enorme vreugde die je brengt in mijn leven! Je bent er altijd om mij op te vangen, letterlijk en figuurlijk. Ik hoop dat we lang van elkaar kunnen genieten!

Dit werk kwam tot stand dankzij de financiële steun van het Fonds voor Wetenschappelijk Onderzoek Vlaanderen.

Curriculum vitae

PERSOONLIJKE GEGEVENS

Naam: Mollet
Voornaam: Lies
Adres: Belgiëlaan 41, 9070 Destelbergen, België
Geboortedatum: 13/04/1986
Nationaliteit: Belg
Burgerlijke staat: samenwonend
Telefoon: 0032 (0)477/35.75.65
Email: molletlies@gmail.com

OPLEIDING

Periode: 1998-2004
Instituut: ASO Don Bosco College Zwijnaarde
Diploma: Wiskunde-Wetenschappen (8u wiskunde)

Periode: 2004-2009
Instituut: Universiteit Gent
Diploma: Master Biomedische Wetenschappen – major Neurologie

- 2004-2005: 1ste bachelor- grootste onderscheiding
- 2005-2006: 2de bachelor - grootste onderscheiding
- 2006-2007: 3de bachelor - grote onderscheiding
- 2007-2008: 1ste master - grote onderscheiding
- 2008-2009: 2de master - grote onderscheiding

Periode: 2009-heden
Instituut: Universiteit Gent
Diploma: Doctor in de Biomedische Wetenschappen, FWO beurs
Extra trainingscertificaten:

- Basic ICH GCP Qualification Training Course and Examination, International Survey of Regulatory Requirements concerning Clinical Research
- Course in Laboratory Animal Science (General and Specific Topics)
- Statistische analyse met behulp van SPSS
- Introductory Statistics: basic of statistical inference
- Effective Scientific Communication

WERKERVARING

Periode: 2009-heden
Organisatie: Universiteit Gent, Departement Neurologie
Functie: Doctoraatsstudent
Taken:

- Plannen, organiseren en uitvoeren van dierexperimentele onderzoeksprojecten in neurofysiologische, neurobiologische en neuropsychologische vakgebieden
- Ervaring met diermodellen voor epilepsie

- Uitvoeren van klinisch onderzoek
- Kennis van state-of-the-art elektrofysiologische technieken
- Praktijktaken: uitvoeren van labowerk en opstellen van SOPs
- Systematische literatuurresearch binnen neurologische vakgebieden
- Statistische analyse en kritische interpretatie van data
- Wetenschappelijk rapporteren en publiceren van data en literatuurreviews
- Schrijven van projecten
- Datapresentatie op nationale en internationale congressen (abstract, posterpresentatie en mondelinge voorstelling)
- Begeleiden van thesisstudenten

VAARDIGHEDEN

COMPUTERKENNIS

Office toepassingen - Word, Excel, Powerpoint: zeer goed
Mail, internet: zeer goed
Software pakket voor statische analyse - SPSS: goed
Software pakket voor wetenschappelijke programmatie– matlab: goed
Wetenschappelijke databases - Pubmed, Web of Science,…: goed

TALENKENNIS

	Lezen	Spreken	Schrijven
Nederlands	moedertaal	moedertaal	moedertaal
Engels	goed	goed	goed
Frans	goed	goed	goed

BREVETTEN

Rijbewijs Categorie B
Animator in het jeugdwerk

WETENSCHAPPELIJKE PUBLICATIES

A1 PUBLICATIES

1. El Tahry R, Raedt R, **Mollet L**, De Herdt V, Wyckhuys T, Van Dycke A, Meurs A, Dewaele F, Van Roost D, Doguet P, Delbeke J, Wadman W, Vonck K, Boon P. A novel implantable vagus nerve stimulation system (ADNS-300) for combined stimulation and recording of the vagus nerve: pilot trial at Ghent University Hospital. Epilepsy Research 2010;92(2-3):231-239.
2. Raedt R*, Clinckers R*, **Mollet L**, Vonck K, El Tahry R, Wyckhuys T, De Herdt V, Carrette E, Wadman W, Michotte Y, Smolders I, Boon P, Meurs A. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. Journal of Neurochemistry 2011;117(3):461-469. *(*equally contributed)*
3. El Tahry R*, **Mollet L***, Raedt R, Delbeke J, De Herdt V, Wyckhuys T, Hemelsoet D, Meurs A, Vonck K, Wadman W, Boon P. Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats. Journal of Neurocsience Methods 2011;198(2):287-293. *(*equally contributed)*

4. **Mollet L**, Grimonprez A, Raedt R, Delbeke J, El Tahry R, De Herdt V, Meurs A, Wadman W, Boon P, Vonck K. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. Acta Neurologica Scandinavica 2013;128(6):391-396.
5. **Mollet L**, Raedt R, Delbeke J, El Tahry R, Grimonprez A, Dauwe I, De Herdt V, Meurs A, Wadman W, Boon P, Vonck K. Electrophysiological responses from vagus nerve stimulation in rat. International Journal of Neural Systems 2013;23(6): epub ahead of print.

A3 PUBLICATIES

1. **Mollet L**, Clinckers R, Raedt R, Meurs A, Wyckhuys T, Van Dycke A, El Tahry R, Vonck K, Wadman W, Michotte Y, Smolders I, Boon P. Antiepileptic effects of vagus nerve stimulation in the focal pilocarpine model. Acta Physiologica 2009;195(S670).
2. Meurs A, **Mollet L**, Clinckers R, Raedt R, El Tahry R, De Herdt V, Vonck K, Smolders I, Michotte Y, Boon P. Increased hippocampal extracellular noradrenalin concentration contributes to the anticonvulsant effect of VNS in the focal pilocarpine rat model for limbic seizures. Epilepsia 2009;50(S11):355-355.
3. Raedt R, Clinckers R, **Mollet L**, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Antiepileptic effects of vagus nerve stimulation in the focal pilocarpine model. European Journal of Neurology 2009;16(S3):141-141.
4. **Mollet L**, Raedt R, Delbeke J, El Tahry R, Delbeke J, De Herdt V, Meurs A, Wadman W, Vonck K, Boon P. Electrophysiological responses to vagus nerve stimulation in rats. Epilepsia 2011;52(S6):160-160.
5. Buffel I, Meurs A, Raedt R, De Herdt V, El Tahry R, Van Nieuwenhuyse B, Dauwe I, **Mollet L**, Sioncke L, Vonck K. Pilot trial: high-frequency, poisson distributed cortical stimulation in a screening model for epileptic seizures. Epilepsia 2011;52(S6):159-160.
6. **Mollet L**, Raedt R, Delbeke J, El Tahry R, De Herdt V, Meurs A, Wadman W, Vonck K, Boon P. Electrophysiological responses from vagus nerve stimulation in rats. Epilepsia 2012;53(S5):170-170.
7. Buffel I, Meurs A, Raedt R, De Herdt V, El Tahry R, Van Nieuwenhuyse B, **Mollet L**, Wadman W, Vonck K, Boon P. The effect of high frequency, poisson distributed cortical stimulation on cortical excitability in rats. Epilepsia 2012;53(S5):175-175.

AWARDS

- Prijs voor beste posterpresentatie op "11th Annual International Clinical Symposium Kempenhaeghe", Heeze, Nederland, 27/03/2009 - Raedt R, Clinckers R, Mollet L, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Enhancement of EC noradrenalin concentration and suppression of pilocarpine-induced seizures in the left hippocampus after vagus nerve stimulation.
- Behorende tot de beste 12 abstracts op "Maastricht Medical Students Research Conference", Maastricht, Nederland, 28/04/2010 - L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P.Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.

PARTICIPATIE (INTER)NATIONALE CONGRESSEN

1. Belgian Brain Congress, Oostende, Belgium, 24/10/2008-25/10/2008
   Contribution: poster presentation

Raedt R, Clinckers R, **Mollet L**, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Enhancement of EC noradrenalin concentration and suppression of pilocarpine-induced seizures in the left hippocampus after vagus nerve stimulation.

2. 11th Annual International Clinical Symposium Kempenhaeghe, Heeze, The Netherlands, 27/03/2009
   Contribution: poster presentation
   Raedt R, Clinckers R, **Mollet L**, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Antiepileptic effects of vagus nerve stimulation in the focal pilocarpine model.
3. 13th Congress of the European Federation of Neurological Societies, Florence, Italy, 12/09/2009-15/09/2009
   Contribution: poster presentation
   Raedt R, Clinckers R, **Mollet L**, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Antiepileptic effects of vagus nerve stimulation in the focal pilocarpine model.
4. Belgian Society of Fundamental and Clinical Physiology and Pharmacology, Ghent, Belgium, 24/10/2009
   Contribution: poster presentation
   Raedt R, Clinckers R, **Mollet L**, Meurs A, Wyckhuys T, El Tahry R, Vonck K, Michotte Y, Boon P, Smolders I: Antiepileptic effects of vagus nerve stimulation in the focal pilocarpine model.
5. 63rd Annual Meeting of the American-Epilepsy-Society, Boston, USA, 04/12/2009-08/12/2009
   Contribution: poster presentation
   Meurs A, **Mollet L**, Clinckers R, Raedt R, El Tahry R, Deherdt V, Vonck K, Smolders I, Michotte Y, Boon P: Increased hippocampal extracellular noradrenalin concentration contributes to the anticonvulsant effect of VNS in the focal pilocarpine model for limbic seizures.
6. Wetenschapsdag Ugent, Gent, Belgium, 11/03/2010
   Contribution: poster presentation
   L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.
7. 12th annual International Clinical Symposium Kempenhaeghe, Heeze, The Netherlands, 26/03/2010
   Contribution: poster presentation
   L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.
8. Maastricht Medical Students Research Conference, Maastricht, The Netherlands, 28/04/2010
   Contribution: oral presentation
   L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.
9. "New approaches for epilepsy treatment in Europe: back to the future. An international state-of-the-art symposium, celebrating the 20th anniversary of the Ghent University Epilepsy Programme, Gent, Belgium, 21/05/2010-22/05/2010
   Contribution: oral presentation
   L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.

10. 13th International Conference on In Vivo Methods, Monitoring Molecules in Neuroscience, VUB, Brussel, Belgium, 12/09/2010-16/09/2010
    Contribution: poster presentation
    L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Enhancement of hippocampal extracellular norepinephrine is responsible for the seizure suppressive effect of vagus nerve stimulation in the focal pilocarpine model for limbic seizures.
11. SWO Midwinter meeting, Amsterdam, The Netherlands, 11/02/2011
    Contribution: oral presentation
    L. Mollet, R. Clinckers, R. Raedt, A. Meurs, T. Wyckhuys, A. Van Dycke, R. El Tahry, K. Vonck, W. Wadman, Y. Michotte, I. Smolders, P. Boon: Vagus nerve stimulation-induced increase in hippocampal noradrenaline levels correlates with seizure control**.**
12. 13th Annual International Clinical Symposium Kempenhaeghe, Heeze, The Netherlands, 25/03/2011
    Contribution: poster presentation
    L. Mollet, R. Raedt, R. El Tahry, J. Delbeke, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
13. 10th Dutch Endo-Neuro-Psycho Meeting, Lunteren, The Netherlands, 31/05/2011
    Contribution: poster presentation
    L. Mollet, R. Raedt, R. El Tahry, J. Delbeke, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
14. 29th International Epilepsy Congress Rome, Rome, Italy, 28/08/2011-01/09/2011
    Contribution: poster presentation
    L. Mollet, R. Raedt, R. El Tahry, J. Delbeke, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
15. American Epilepsy Society, Baltimore, USA, 02/12/2011-06/12/2011
    Contribution: poster presentation
    L. Mollet, A. Grimonprez, R. Raedt, R. El Tahry, J. Delbeke, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Modulation of cortical excitability by vagus nerve stimulation in the cortical stimulation model.
16. Wetenschapsdag Ugent, Gent, Belgium, 14/03/2012
    Contribution: poster presentation
    L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
17. 14th Annual International Clinical Symposium Kempenhaeghe, Heeze, The Netherlands, 24/03/2012
    Contribution: poster presentation
    L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
18. 10th European Congress on Epileptology, London, England, 30/09/2012-04/10/2012
    Contribution: poster presentation
    L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
19. Autumn Meeting Belgian Society of Physiology and Pharmacology, Brussel, Belgium, 26/10/2012
    Contribution: poster presentation
    L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.
20. Belgian Brain Congress, Luik, Belgium, 27/10/2012
    Contribution: poster presentation

L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, K. Vonck, P. Boon: Electrophysiological responses to vagus nerve stimulation in rats.

21. SWO Midwinter meeting, Amsterdam, The Netherlands, 01/02/2013
    Contribution: oral presentation
    L. Mollet, A. Grimonprez, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, P. Boon, K. Vonck: Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability**.**
22. 15th Annual International Clinical Symposium Kempenhaeghe, Heeze, The Netherlands, 22/03/2013
    Contribution: poster presentation
    L. Mollet, A. Grimonprez, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, P. Boon, K. Vonck: Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability.
23. Jaarlijkse wetenschappelijke NVS vergadering Kempenhaeghe, Heeze, The Netherlands, 03/10/2013
    Contribution: oral presentation
    L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, V. De Herdt, A. Meurs, W. Wadman, P. Boon, K. Vonck: Electrophysiological responses to vagus nerve stimulation in rats.