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INVESTIGATION OF NEUROPHYSIOLOGICAL MECHANISMS OF VAGUS NERVE STIMULATION

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Preface

It is now four years ago since I first arrived in Ghent. At that point I had no idea about the challenges ahead of me. The past four years have by far been the greatest challenge I have ever sustained. My tendency to deeply involve myself in research problems and questions, not resting before they are solved, has meant that I have had to make several sacrifices. I am thankful to my friends, my family, to Kim for the patience that you have shown me. This work is dedicated to you all. I am grateful to several people who are further acknowledged at the end of the thesis.

When I started my work in the Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology (LCEN3), I had presented a project plan, which centered around Vagus Nerve Stimulation (VNS). It did not, however, center around neurophysiological mechanisms of VNS, which ended up becoming the main focus of my works. The original idea was rather to study the effects of VNS on specific cognitive domains, because I had a theoretical reason to believe that VNS could be used to enhance cognitive performance, particularly in the executive domain. We spent countless hours preparing and designing these studies. In fact, I spent the majority of my first year optimizing an attentional set shifting task for rats [1] and we were almost ready to examine our main question: "can VNS be used to enhance attentional set shifting in rats?". We just had this one "little" problem, which I predicted would cause problems for the interpretation of our studies. Previous experience with VNS in rats told us that effective VNS delivery is only observed in a proportion of rats, for reasons that were still under study at the time. This phenomenon would likely have greatly increased the variability, requiring larger numbers of rats and leading to an underestimation of a potential effect.

In order to address this problem, we decided ourselves to initially do a study on effects of VNS on different neurophysiological parameters, in order to identify potential parameters which could be used as a biomarker for effective VNS delivery. This work, however, yielded some very interesting findings. At that point, we were facing a familiar scientific dilemma, which was well described in a recent paper by prof. Barry Komisaruk: "Follow my hypothesis or my findings?" [2]. We decided for the latter, which meant that we put the attentional set shifting work on hold in order to fully dedicate ourselves to explore the meaning of our initial findings.

The thesis itself is divided in three parts, with additional subchapters:

- **Part I** is an introduction to the background material necessary to understand the methodology used and the problems studied
- **Part II** includes the experimental works conducted in relation to the problems formulated, which includes original research articles
- **Part III** summarizes and discusses the impact of the work and ends with a general conclusion and brainstorm on future directions

Any questions concerning the work may be addressed to me at larsemil88@gmail.com

Lars Emil Larsen, Ghent, October 12, 2016

References

[1] J. M. Birrell and V. J. Brown. Medial frontal cortex mediates perceptual attentional set shifting in the rat. *J Neurosci*, 20(11):4320–4, 2000.

[2] B. R. Komisaruk. A scientist's dilemma: follow my hypothesis or my findings? *Behav Brain Res*, 231(2):262–5, 2012.

LIST OF ABBREVIATIONS

ADC: Analog-to-Digital-Converter
ANOVA: Analysis of Variance
CNS: Central Nervous System
dB: Decibel
DC: Direct Current
EEG: Electroencephalography
ECG: Electrocardiography
EMG: Electromyography
EP: Evoked Potential
EPSP: Excitatory Post Synaptic Potential
fEPSP: Field Excitatory Post Synaptic Potential
fMRI: Functional Magnetic Resonance Imaging
HPA: Hypothalamic Pituitary Adrenal
IPSP: Inhibitory Post Synaptic Potential
LC: Locus Coeruleus
LC-NA: Locus Coeruleus Noradrenergic
LFP: Local Field Potential
LMEP: Laryngeal Muscle Evoked Potential
MI: Modulatory Index
NTS: Nucleus of the Solitary Tract
PAC: Phase Amplitude Coupling
PET: Positron Emission Tomography
PFC: Prefrontal Cortex
PS: Population Spike
PSP: Post Synaptic Potential
SD: Standard Deviation
SPECT: Single-Photon-Emission Computed Tomography
t-VNS: Transcutaneous Vagus Nerve Stimulation
VNS: Vagus Nerve Stimulation

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Part I

Background and Presentation of Research Questions

Chapter 1

General Principles

This thesis concerns investigation of neurophysiological mechanisms of Vagus Nerve Stimulation (VNS), which is a treatment alternative in drug resistant epilepsy and depression [4, 24]. Despite major progress in the later years, therapeutic mechanisms of VNS remain elusive. In order to understand the experimental works which form the foundation of the present thesis (presented in **Part II**), it is necessary to have a grasp on principles of neurophysiology, electrophysiology and the use of electricity to stimulate nervous tissue. This chapter is intended to inform readers without a background in neuroscience and give them a basis to understand the problems discussed in the following chapters.

1.1 Neuroanatomy and Neurophysiology

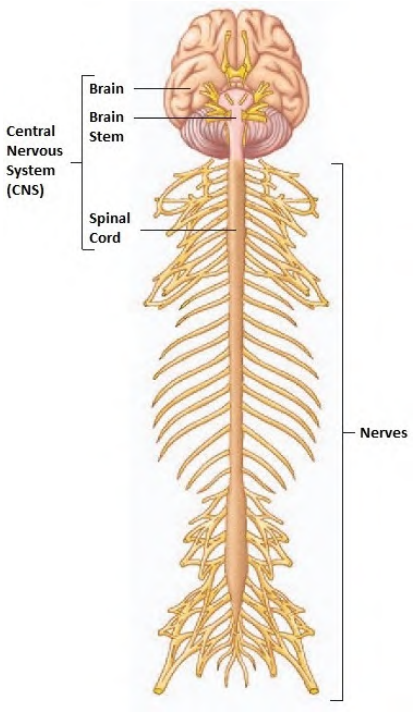

Figure 1.1: The nervous system comprises the central and peripheral nervous systems. The central nervous system is defined as the spinal cord, the brain stem and the brain, while nerves make up the peripheral nervous system. Adapted with permission from Seeley *et al.* 2008 [26].

The nervous system (**Fig. 1.1**) is anatomically divided in a peripheral and a central nervous system (CNS). The peripheral nervous system comprises all peripheral nerves and ganglia, which are collections of neurons situated outside the CNS. Peripheral nerves comprise efferent nerve fibers, which transport information from the CNS towards the periphery and exerts control over organs and muscles, and afferent fibers, which pass sensory information from the periphery towards the CNS. The CNS comprises the spinal cord, the brain stem and the brain. The axons of ventral spinal cord motor neurons leave the ventral spinal cord to innervate muscles of the periphery. Spinal motor neurons are controlled by higher order neurons of of the cortex, which descend through tracts of the spinal cord allowing consciously controlled movement. Somatic sensory information arises from modality specific receptors, such as mechanoreceptors of the skin, and located at the endings of first order sensory neuron. Spinal first order sensory neurons convey the information onto spinal second order sensory neurons, which transmit the information onwards to the brain, where the sensory information reaches conscious awareness. While the brainstem does supply sensory and motor innervation to the face and to some extent to internal organs, it is mainly involved in control of important autonomic functions as blood pressure, heart rate and respiration. The brain is responsible in several higher cognitive functions and executive control [14, 30].

1.1.1 Cells of the Nervous System

The principal functional cell types of the nervous system is the neuron (**Fig. 1.2**). Neurons vary in size and shape, though they generally can be described with regard to three components:

- The cell body containing the cell nucleus and other major cellular organs
- The axons, which are long extensions from the cell body transmitting the neural information, also referred to as the nerve fibers
- The dendrites, which are mostly short outgrowths of the cell body that receive information from other neurons

A neuron mainly receives information from other neurons via the dendrites and transmit information onwards to other neurons (or target organs[1]). When inspecting neural tissue of the CNS, collections of neuron cell bodies, often referred to as nuclei, have a gray appearance (called gray matter), whereas nerve fibers have a white appearance (called white matter). Bundles of nerve fibers in the periphery are called nerves, whereas fiber bundles in the CNS are called tracts. The nervous system has several additional support cells referred to as neuroglia, which generally outnumber neurons by factor 10 to 1, and include astrocytes, oligodendrites and Schwann cells. The astrocytes only reside in the CNS and are mostly involved in metabolic processes and form the blood brain barrier with cellular extensions wrapping around the small vessels of the brain. The oligodendrites are cells which wrap several times around axons of neurons in the CNS to provide myelination (the function of which is explained in explained in the **Section 1.1.2**). Correspondingly, Schwann cells similarly wrap around and thus provide myelination to peripheral nerve fibers. A peripheral nerve is made up by small subunits called fascicles, which contain several nerve fibers bound together by connective tissues. The bundle of fascicles is further surrounded by a sheath of fibrous tissue called the epineurium [14, 30].

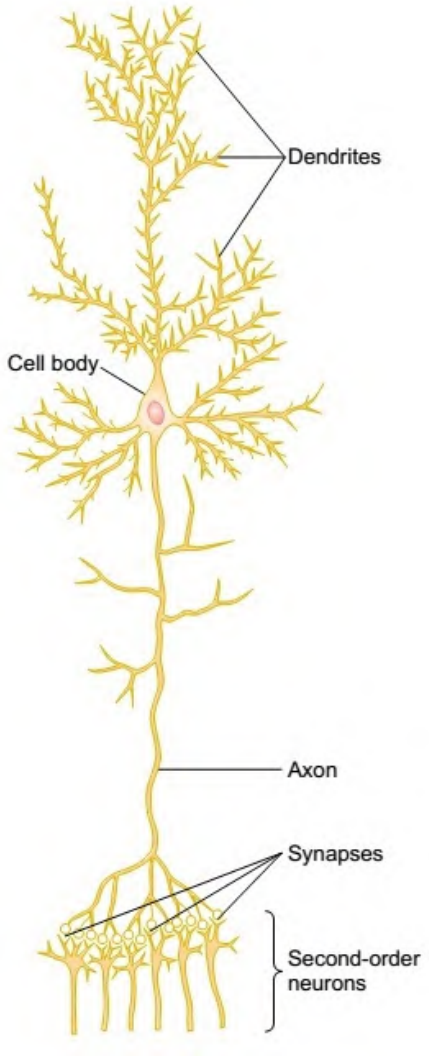

Figure 1.2: The typical structure of a neuron. Neurons receive input from other neurons via their dendrites and transmit information to other neurons or peripheral organs via their axons. The cell body contain the cell nucleus. Adapted with permission from Guyton and Hall 2006 [14].

[1] Spinal motor neurons receive information from higher centers of the brain, but transmit information onwards to muscles, resulting in contraction. Autonomic motorneurons exert control over other organs as the heart, lungs and gastrointestinal tract.

1.1.2 Neural Transmission

The neuronal cell membrane, which is composed of phospholipids, works as an insulator and with exception of water filled ion-specific channels, it is impermeable to molecules possessing electrical charge. The most important ions separated by the membrane are Na^+, K^+, $Cl^-$and Ca^{2+}, with concentrations differing between the intracellular and extracellular fluids. For example, the concentration of $K^+$is approximately 30 times higher on the interior than the exterior side of the membrane, while concentrations of $Na^+$are around 10 times higher on the exterior side. This concentration difference is maintained by membrane pumps, one of them being the sodium-pump, which actively pumps Na^+-ions out of the cell in exchange for K^+-ions. Further, at a resting state, the membrane displays some permeability to K^+-ions that thus will have a tendency to leak to the outside. This in combination with negatively charged intracellular proteins creates a potential difference across the membrane of approximately -70 mV, referred to as the resting membrane potential[2] [11, 14, 30].

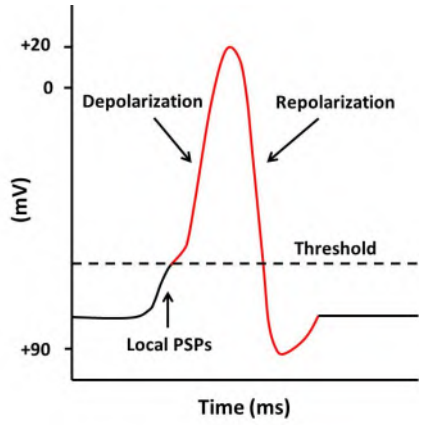

Figure 1.3: A schematic illustration of how the neuronal membrane potential changes in the course of an action potential. At rest the membrane potential situates around -70 mV. If a local depolarizing potential reaches a threshold level, voltage gated sodium-channels open, leading to an influx of Na^+, which depolarizes the membrane to a point where the interior is positive relative to the exterior. As potassium-channels open, resulting in an efflux of K^+, while sodium channels simultaneously close, the membrane potential is repolarized and the resting membrane is restored.

The neuronal action potential (**Fig. 1.3**) is a mechanism which results in rapid changes in the resting membrane potential. The propagation of action potentials along the axons of neurons is the mechanism by which neurons transmit information. The action potential is mediated by ion-specific channels, which open or close upon certain stimuli, such as changing voltage or binding of a ligand to a receptor. If an alteration in the resting membrane potential, which reduces the voltage difference between the interior and exterior (depolarization), reaches a certain threshold level, voltage-sensitive sodium channels situated in the membrane will open, leading to a rapid influx of Na^+-ions. This changes the membrane potential to a point where the interior even becomes positive relative to the exterior. As the membrane depolarizes, voltage sensitive potassium-channels slowly open leading to an efflux of K^+-ions. The simultaneous closing of sodium channels leads to a normalization and reestablishment of the resting membrane potential. Once generated at the axon hillock[3] of the neuron, action potentials depolarize neighboring axonal regions, which generates additional action potentials along the fiber. The speed at which an action potential is propagated along the axon depends on fiber diameter and the extent of myelination, provided by the oligodentrites or Schwann cells. Large diameter and thick myelinated fibers conduct action potentials faster than unmyelinated fibers [11, 14, 30].

Neurons converge to communicate through small knobs called synapses, which are structures formed by the axonal endings of the transmitting neuron, called the presynaptic neuron, and dendritic extensions of the recipient neuron, called the postsynaptic neuron. Though the conduction of information along nerve fibers is electrical, the communication between neurons

[2] The resting membrane can vary significantly throughout the CNS.
[3] The axon hillock is located just at the point where the axon exits the cell body.

in the mammalian brain is by far mostly chemical. As an action potential arrives at the presynaptic terminal this will result in the release of neurotransmitter into the space between the presynaptic and postsynaptic neuron, referred to as the synaptic cleft. Neurotransmitters bind to receptors of postsynaptic neurons and cause alterations in the resting membrane potential referred to as post synaptic potentials (PSPs). An excitatory post synaptic potential (EPSP) is generated by binding of excitatory neurotransmitters as for example glutamate to ligand-gated sodium channels. The opening of sodium channels results in an influx of sodium and as a result depolarizes the membrane. Contrarily, and inhibitory post synaptic potential may be generated by inhibitory neurotransmitters as GABA binding to receptors leading to an opening of chloride-channels. As the extracellular Cl^--concentration is higher than intracellular concentrations, a Cl^--influx results in a hyperpolarization making the generation of an action potential less likely. The nervous system utilizes several neurotransmitters, which all have several receptor systems. A single EPSP is unlikely to result in successful generation of an action potential. This rather requires the simultaneous generation of several EPSPs along the neuronal dendrites that sum up to sufficiently depolarize the cell. On the other hand simultaneous EPSPs and IPSPs may cancel each other out. While neural transmission can be explained with relatively simple models, it is mostly a result of a complex interplay between several factors [11, 14, 30].

1.2 Electrophysiology - Acquisition of Neurophysiological Signals

Electrophysiology is an approach to study the flow of ion currents in biological tissues. As already discussed, the electric nature of the nervous system makes electrophysiology an ideal approach to study the physiology of the nervous system and has in fact been the main contributor to understand the processes described in the previous section. In this section, the aim is to briefly introduce general concepts of electrophysiology, which has been a central part of the experimental work described in **Part II** of the thesis.

1.2.1 General Principles

The field of electrophysiology dates back to Luigi Galvani (1737–1798), an Italian physician, who at the time conducted several anatomic dissections of frogs. During his experiments, he discovered that transfer of static electricity from his scalpel to metal hooks used to suspend the leg of a frog resulted in a muscle twitch. He further observed that similar responses were evoked when the "electricity" was applied to the sciatic nerve, providing motor innervation to the muscle, or spinal cord. At the time, Galvani, described his observation as "animal electricity". The parallel work of other founding fathers in the field of electrotechnology, including Henry Cavendish (1731–1810), Alessandro Volta (1745–1827), André-Marie Ampère (1775–1836) and Georg Ohm (1787–1854), paved the way for the innovative technology used in the field of electrophysiology as we know it today [11].

To measure potentials in biological tissue, electrodes are typically placed in the proximity to the tissue of interest. The signals detected up by these electrodes are additionally often amplified and filtered before they are digitized and stored for analysis (elaborated in **Section 1.2.2**). An important principle is that only differences of potentials are measured by means of electrophysiology, as there is no universal zero potential against which the potentials of the nervous system can be measured. This implies that at least two electrodes are required to record any given potential [11].

Though the following section primarily discusses the application of electrophysiology in the study of neurophysiology, other important clinical applications include electrocardiography (ECG) [20] and electromyography (EMG) [6]. In the study of neurophysiology, electrophysiology

is applied at various levels, which depends on the type of electrodes used and where they are placed. Intracellular recordings may be obtained by inserting a very fine and sharp electrode (tip $<1\mu$m) into the intracellular space and by referencing the recorded potential to an electrode located in the extracellular space. Intracellular recordings were among other applications used to elucidate mechanisms of the action potential and may reach a voltage amplitude of as much as -90 mV. Single-unit activity can also be recorded from the extracellular space using similar very small electrodes (tip ranging from 1-15 μm), though the voltage amplitude of such activity usually is limited to a few mV. Larger electrodes will pick up activity from a larger "field". At first activity from multiple separable units may be obtained, but as the electrode size increases further the activity will increasingly be dominated by current sinks and sources which are the result of an aggregate of synaptic potentials from larger groups of neurons [11]. Such potentials are referred to as "field potentials", which make up the electroencephalogram (EEG) [11], discussed later in **Section 1.2.3**.

1.2.2 Instrumentation

The following section discusses the instruments used in a typical electrophysiology setup. Signals are detected from sources using various designs of electrodes. In a modern setup, signals are usually preamplified, filtered, amplified, digitized and finally stored on a computer for offline analysis (**Fig. 1.4**). These procedures are elaborated in the following subsections.

Filters

It was the French mathematician Jean Baptiste Joseph Fourier (1768-1830) who showed that any periodic signal, such as field potentials, can be described as the sum of several harmonically related sine waves of varying frequencies (described in cycles per second or Hz) [31]. This is the basis of frequency analysis, which is discussed in **Section 1.2.3**. Filters are applied to

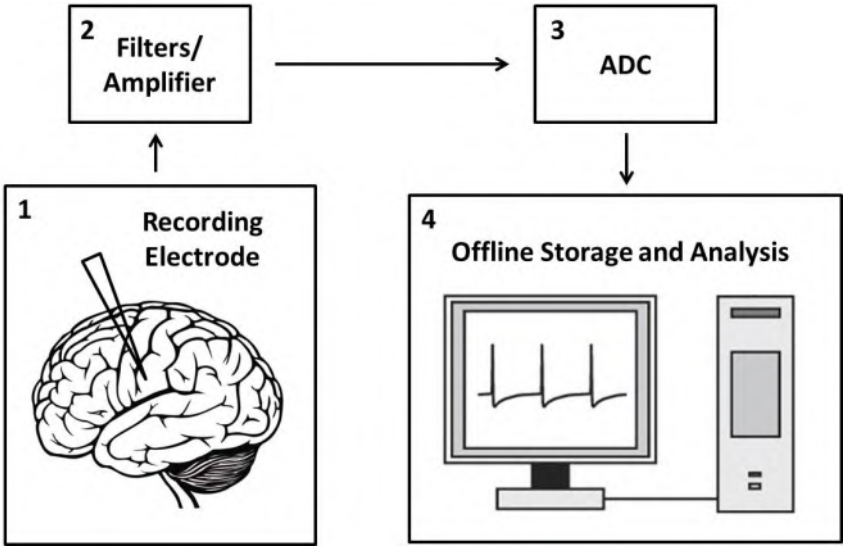

Figure 1.4: A very simplified overview of a basic setup used in electrophysiology. 1. Signals are recorded by electrodes placed in the vicinity of the tissue of interest. 2. The analog signal is subjected to appropriate filtering and amplification. 3. The analog signal is sampled and digitized by and analog-to-digital converter (ADC). 4. The signal is displayed or stored at a computer for offline inspection or analysis.

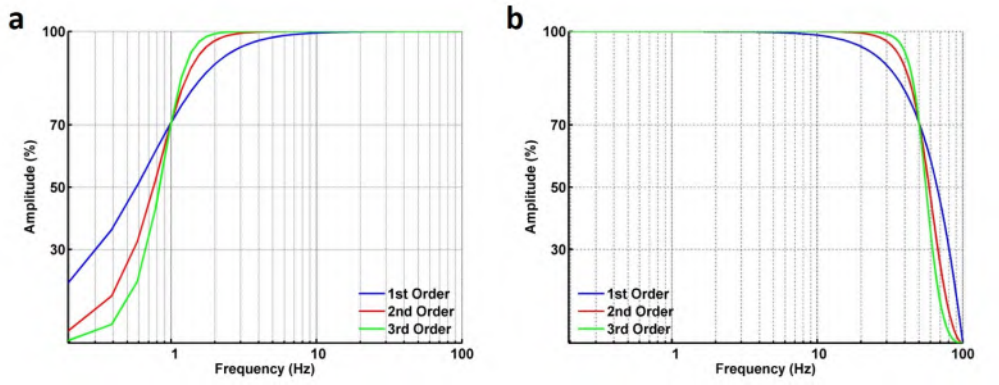

Figure 1.5: The frequency response of a filtered signal can be plotted in the frequency domain in what is called a Bode plot. The plot reveals characteristics of the filter, such as the corner frequency, defined as the frequency at which -3 decibel (dB) (= 70.7% amplitude) attenuation is observed. Further it describes the slope of the filter, which is the speed at which the attenuation occurs around the corner frequency. The higher order of the filter, the steeper is the slope of the filter. In **(a)**, the frequency response of three high-pass filters with a corner frequency of 1 Hz and varying filter orders are shown. Similarly, in **(b)**, three low-pass filters with a corner frequency of 70 Hz and varying slopes are shown.

signals to specify which frequency components of a signal that eventually will be fully amplified and which frequency components that will be attenuated. Filters may be described as low-pass filters when they allow low frequency content to pass but attenuate high frequency activity, while high-pass filters attenuate low frequency activity. Alternatively, band-stop or band-pass filters are a combination of low- and high-pass filters that attenuate or allow amplification of specific frequency ranges, respectively. A filter is characterized by its frequency response curve, as demonstrated in **Fig. 1.5**, and can be described with regard to corner frequencies and filter order, or slope. Filters do not abruptly cut off at specified frequencies, but imposes a more gradual attenuation of frequency components outside the filtered band. The extent to which a filter attenuates a signal at a specified frequency is expressed in decibels (dB) defined by the following formula

$$\text{number of decibels (dB)} = 20 \log_{10} \frac{\text{signal output amplitude}}{\text{signal input amplitude}}$$

This implies, for example, that a 30 % attenuation of a given signal (i.e 70% signal output amplitude) at the specified frequency translates to -3 dB

$$20 \log_{10} \frac{70}{100} \approx \text{-3 dB}$$

A filter is defined by its corner frequency, which is the point where a -3 dB attenuation of the signal occurs. This means, for example that filtering a signal with a low-pass filter defined with a corner frequency of 100 Hz, will result in an attenuation of the amplitude of 100 Hz activity to 70% and further means that attenuation starts already below the specified corner frequency. The slope of a filter, on the other hand, determines how steep the attenuation of the signal is around a specified corner frequency and is expressed in dB per octave, where an octave refers to the doubling or halving of the frequency. This then means that a low-pass filter with a -6 dB (50% amplitude)/octave slope, also known as a first order filter, reduces the amplitude of frequency content by 50% for each time the frequency is doubled. A larger slope value will result

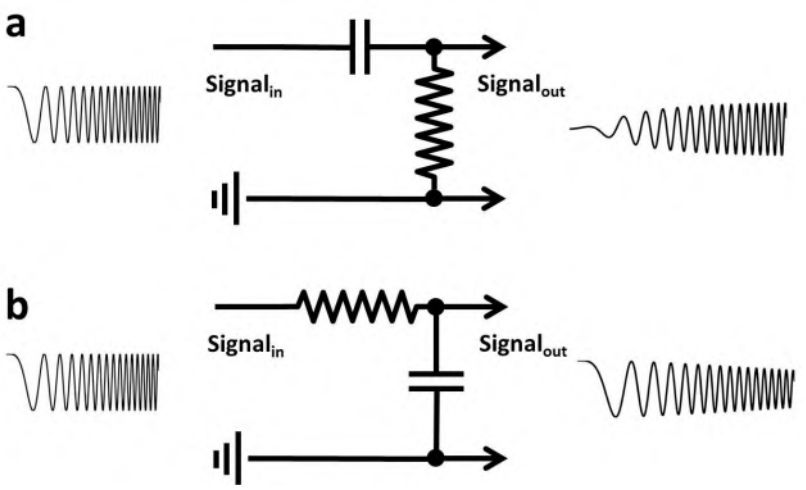

Figure 1.6: In **(a)**, an example of a high-pass filter is seen and it shows how the capacitor blocks low-frequency currents attenuating low-frequency content of an input signal. On the other hand, **(b)** shows a schematic of a simple low-pass filter circuit, which attenuates high-frequency content of an input signal.

in a steeper cut off around the specified cut off frequency, but can produce phase distortion and introduction of unwarranted artificial resonance following large and sharp artifacts in the signal [11].

Analog high-pass and low-pass filters are constructed by relatively simple electronic circuits consisting of a resistor and a capacitor (**Fig. 1.6**). The circuit is a type of voltage divider, which takes an input voltage potential, or as in this case a time varying signal and puts out another voltage potential or time varying signal. A capacitor has the important property that the impedance (Z) depends on the frequency of the signal (F), which can be described with the formula $Z = \frac{1}{2\pi FC}$. C denotes the capacitance of the capacitor in farad. The formula implies that the impedance of a capacitor is high when the frequency of the alternating current is low, and low when the frequency of the alternating current is high. When the circuit is arranged as in **Fig. 1.6a**, most of the signal will cross the capacitor, which blocks or attenuates slowly changing currents, i.e. low frequency components, and thus represents a simple high-pass filter. Contrarily, if the circuit is arranged as in **Fig. 1.6b**, the signal will mainly pass the resistor as the frequency increases, due to decreasing impedance over the capacitor, which attenuates high frequency content [11].

Amplifiers

Amplification of neurophysiological signals is necessary as these typically are in the range of μV to several mVs. Amplifiers additionally serve the task of reducing the sensitivity to interference of electrical noise arising from power sources (50 Hz noise in Europe), radio frequency sources or other static potentials, which may contaminate the signal. The simplest amplifier (marked with a triangle in an electronic circuit schematic) amplifies the signal across two inputs. One input is often specified as the recording site and the other as the reference, which is further connected to the ground (also called earth). While this setup, often referred to as a single ended recording, works for many purposes, it is vulnerable to interferences caused by changing ground potentials, which are not picked up by the active recording electrode, arising from the

aforementioned electronic sources. Alternatively, differential amplifiers are used, which in many ways are superior to single-ended setups, though it requires symmetrical electrode pairs instead of referencing all recording sites to a common reference point. In differential amplifiers, both input signals are recorded with respect to a third reference point (often ground/earth) and subsequently differentiated. This means that ground interference is introduced to both signals but the differentiation cancels it out, a phenomenon referred to as common mode rejection. The common mode rejection ratio of an amplifier refers to how many times the differential signal is amplified relative to the common mode noise, which ideally is as high as possible. A common mode rejection ratio of 10,000:1 therefore means that a common mode noise level of 10,000 μV is displayed at the same level as a 1 μV differential signal. A circuit schematic of a simple differential amplifier, with a feedback system, is shown in **Fig. 1.7**. The output (V_{out}) is proportional to the difference between input signal one (V_1) and input signal two (V_2). In a very simplified explanation the gain of the amplifier will depend on the ratio between the resistors R_1 and R_f, as well as the resistors R_2 and R_g [11].

An important consideration for amplifiers is the input impedance. The impedance of the source of the input signal (i.e. the electrode), together with the input impedance forms a voltage divider. This means that if the electrode impedance is 5 kΩ, and the input impedance 50 kΩ, 10% of the amplitude of the input signal is lost. This means that the input impedance ideally is as high as possible in order to spare as much of the signal as possible. A high input impedance will additionally reduce the influence of impedance differences between pairs of electrodes used for differential recordings. A low electrode impedance is generally favorable, though fine electrodes used for single unit recordings display high impedances in the $M\Omega$-range. What favors the use of recording electrodes with low impedances is the phenomenon of thermal noise, which is attributed to random movement of electrons in the input circuit of the amplifier and will be recordable even in the absence of any other signal. The magnitude of thermal noise can be described according to the following formula

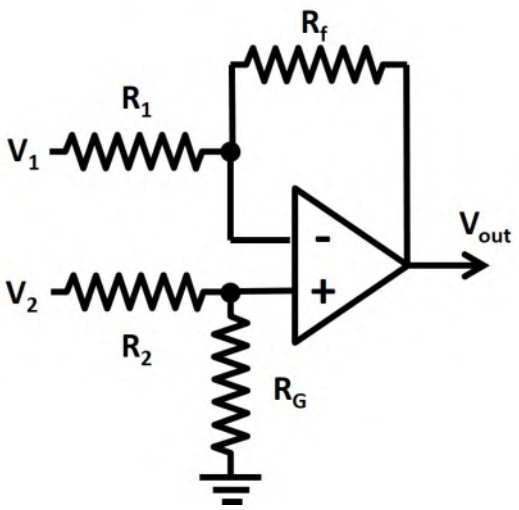

Figure 1.7: A schematic of a typical differential amplifier, where output (V_{out}) is proportional to the difference between two input signals ($V_1 - V_2$). The gain of the amplification depends on ratios between pair of the resistors in the circuit (R_1 vs. R_f as well as R_2 vs. R_g).

$$\mathrm{V}_{tn} = \sqrt{4 \cdot k \cdot T \cdot Z \cdot \triangle f}$$

Here V_{tn} refers to the voltage amplitude of the thermal noise, k is a constant, T the temperature in Kelvin degrees, Z the impedance of the source and $\triangle f$ the bandwidth of the amplifier. Thus, reducing impedance and the bandwidth of the amplifier can all effectively reduce the impact of thermal noise and potentially improve the recording. Changing temperature is often impractical. It should be mentioned in relation to this that a preamplifier, which mostly does not amplify the signal, but simply reduces the impedance from several kΩ to a few ohms may be used to equalize

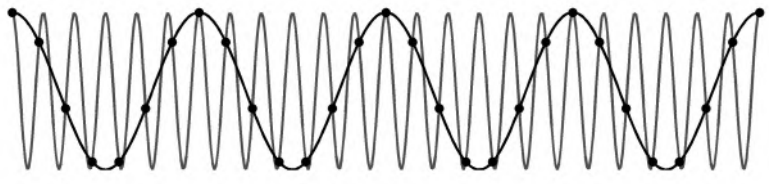

Figure 1.8: A schematic illustration of how aliasing can occur as a result of inadequate sampling of a high frequency source. This will generate a harmonic but artificial frequency in the discrete signal, which can be misinterpreted.

the impedance of the inputs to the amplifier and thus increase the common mode reduction ratio. The preamplification is often done as close to the source as possible, which reduces the possible interference introduced beyond that point towards the amplifier [11].

Digitization

In most setups today, the analog output signal of the amplifier is connected to an analog-to-digital converter (ADC), which samples the signals at a certain rate (denoted the sampling rate) and allows storing a time varying signal as a time series on a hard drive for further analysis. The resolution of an ADC depends on the number of binary digits (commonly coded as 0 or 1's) that the system has to describe each numeric value. A common 16 bit system has $2^{16} = 65536$ binary values. For a voltage range of ± 10 mV, this results in an amplitude resolution of around 0.3 μV.

The sampling rate is an important consideration, depending on which phenomena that are being investigated. An important concept is the Nyquist frequency, which refers to the fact that the maximally detectable frequency content of a sampled time series equals half of the sampling rate. As a result, any signal component above the Nyquist frequency will appear as a lower frequency component. This is called "aliasing" (**Fig. 1.8**). Because filters do not completely eliminate components above their corner frequency, the sampling frequency should be largely above the Nyquist frequency and special 'steep (high order)' filters, called "anti–aliasing" filters should be used to eliminate the high frequency components [11].

1.2.3 Electroencephalography

Electroencephalography (EEG) classically refers to the registration of electrical activity from electrodes pasted to the scalp. The first recordings were performed by Hans Berger in 1920ies, using a galvanometer[4] to record electrical potentials from the scalp [5]. The first regular activity identified was a 10 Hz oscillation in the 50–100 μV-range, which was called alpha-waves (**Fig. 1.9**). Other characteristic rhythms have been named beta (14–30 Hz), delta (<4 Hz), theta (4–7 Hz) and gamma waves (>30 Hz) in the order which they were discovered [11]. This classification, however, only applies to humans and may differ across species [8, 13, 33]. Over the years EEG technology has been gradually improved and today EEG is the most valuable diagnostic tool in the field of clinical neurophysiology [11].

Scalp EEG is the result of field potentials, which arise from current sources and sinks in the superficial cortical layers (**Fig. 1.10**), which do usually not exceed 100 μV under physiological

[4] Named after Luigi Galvani (1737–1798), a galvanometer is a device which detects electric current with a coil. The galvanometer was classically connected with a rotating magnetic needle in order to visualize the flow of currents.

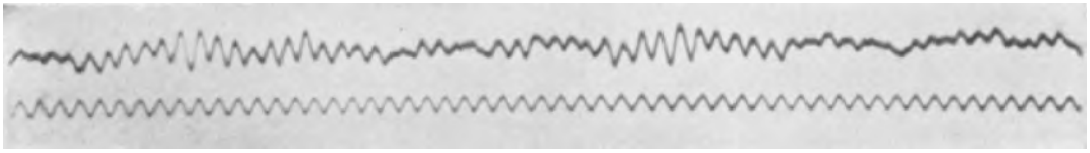

Figure 1.9: One of the initial traces by Hans Berger released in a publication from 1929 [5]. The upper channel shows clear and rhythmic activity, with a frequency around 10 Hz, acquired from the scalp of his son. The activity was plotted against a 10 Hz sinusoid for comparison (lower channel). This frequency pattern was the first discovered in man and named the alpha rhythm. Adapted with permission from Berger 1929 [5].

conditions. Evidence suggests that field potentials mainly are made up by summated post synaptic potentials and that the contribution of action potentials is rather minor. The recording yields maximal amplitude as the flow of ionic current is perpendicular to the surface of the scalp. The electrodes used to record clinical scalp EEG are typically disc–shaped with a diameter of 1-10 mm made of silver/silver-chloride or similar metals. In order to reduce electrode impedance a conducting gel is applied to the electrodes before pasting them to the scalp of the patient. Ideal electrode impedances of such disc electrodes are in the region of 5 kΩ. Conventionally clinically accepted systems have 27 or 32 electrodes placed on the scalp in a standardized manner [17], though modern systems expand the spatial resolution going as far as 128 and 256 electrodes.

EEG, however, may also refer to other field potential recordings, such as those obtained from invasive subdural[5] electrodes or depth electrodes inserted through the brain parenchyma into specific brain regions of interest. Implantation of depth electrodes is usually done by means

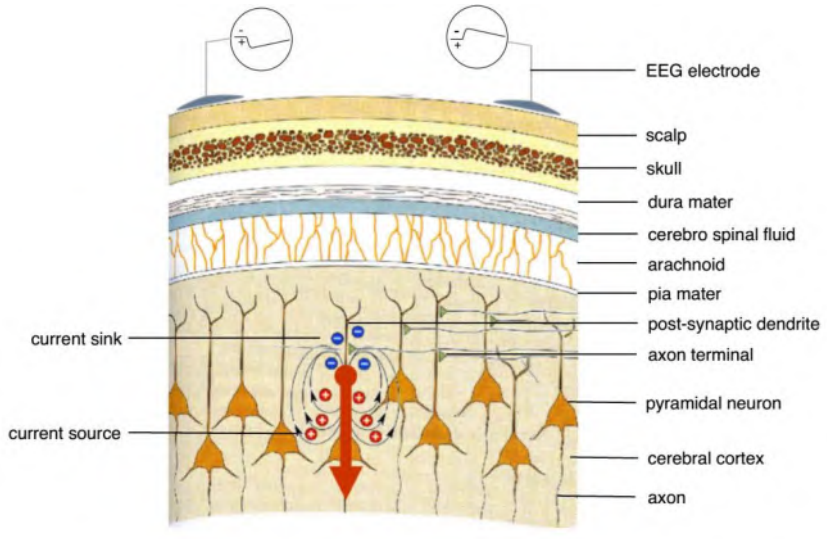

Figure 1.10: A schematic illustration of how extracellular currents flows, generated by current sources and sinks arising from post synaptic potentials in pyramidal neurons in the superficial cortex. The field potential recorded at the site of the electrode is a sum of thousands of synchronous post synaptic potentials. Adapted with permission from Strobbe 2015 [28].

[5] Below the scalp on the surface of the brain.

of stereotaxy[6]. Depth electrodes may be simple wires of platinum or stainless steel, which are insulated with the exception of the tip, which forms the electrode-tissue interface. Subdural electrodes on the contrary are mostly disc-shaped as those used for conventional EEG recordings, with a diameter of 1–3 mm. The advantage of invasive recordings is that the signal amplitude is substantially higher (typically hundreds of μV) than those recorded from the scalp, resulting in a better signal to noise ratio. Invasive EEG is widely used as a part of a presurgical evaluation in for example epilepsy surgery, where the ultimate goal is removal of the zone generating abnormal electrical activity resulting in seizures. Higher quality signals allows more precise diagnosis with regard to the type of epilepsy and gives a better spatial resolution, which allows more precise localization of the region from which the abnormal epileptic electrical activity originates [11, 29].

Depth EEG in order to record local field potentials (LFPs) is also frequently used in preclinical experimental setups and is also central part of the preclinical experimental work presented in **Part II**. In the experimental work presented in **Part II**, such depth electrodes are implanted at the level of the hilus of the dentate gyrus in rats, which is a part of the hippocampal formation. The unique cytoarchitechture of this region where granule cells of the dentate gyrus all are oriented in the same direction creates a uniform flow of ionic current[7], which allows acquisition of high amplitude LFPs. The rhythms of the hippocampal EEG are well characterized as the result of several studies in several species [8]. The perhaps most thoroughly studied hippocampal phenomenon is the theta rhythm (4–12 Hz in rats, see **Fig. 1.11a**), which has been related to several important functions as memory and spatial navigation [27].

Analysis of EEG signals among others include simple measures of amplitude, isolation of frequency bands with digital filters, source localization analyses and various connectivity analyses. One of the most commonly applied analysis for digitized EEG is frequency analysis, which also is a central part of the experimental work presented in **Part II**. The most commonly used algorithm is the Fourier transform, which dates back to Jean Baptiste Joseph Fourier (1768-1830), who showed that any periodic time series can be decomposed into a set of frequency components, formed from harmonically related sine waves. The Fourier transform thus transfers a time varying discrete signal to the frequency domain, focusing on frequency specificity at the expense of time specificity. It is important to acknowledge that though the Fourier transform may yield information on the extent to which a signal occupies specific frequency bands, it is simply a mathematical algorithm. The derived frequency components may not necessarily have a direct physiological meaning [11].

The most commonly used method of Fourier transform for discrete signals is the Discrete Fourier Transform (DFT) calculated by means of the computationally more convenient Fast Fourier Transform (FFT), which is optimized for application of time series containing a number of samples to the power of 2, such as 256, 512 or 1024 samples [10]. The FFT of a time series with a duration T and a number of samples N, will yield N frequency components, often denoted frequency bins, spaced by $1/T$, with the lowest frequency component being a direct current (DC) or 0 Hz component and highest frequency component having a frequency of $N/2T$ Hz[8]. Each frequency bin is a complex number containing both amplitude and phase information. The 2 in $N/2T$ represents the fact that the Fourier transform for each frequency yields both sine and cosine components, which preserves phase information and makes it possible to reconstruct

[6] Stereotaxy refers to the use of a stereotactic frame, in which patients are fixed during an intracranial implantation procedure. The frame has a three dimensional axis system and the use of well characterized reference points allows standardized and precise implantation of depth electrodes.

[7] The dentate gyrus is actually C-shaped, meaning that the most uniform flow of current is obtained if the electrode is inserted between the upper and lower blade with some distance to the bend.

[8] $N/2T$ also equals half of the sampling frequency, also known as the Nyquist frequency.

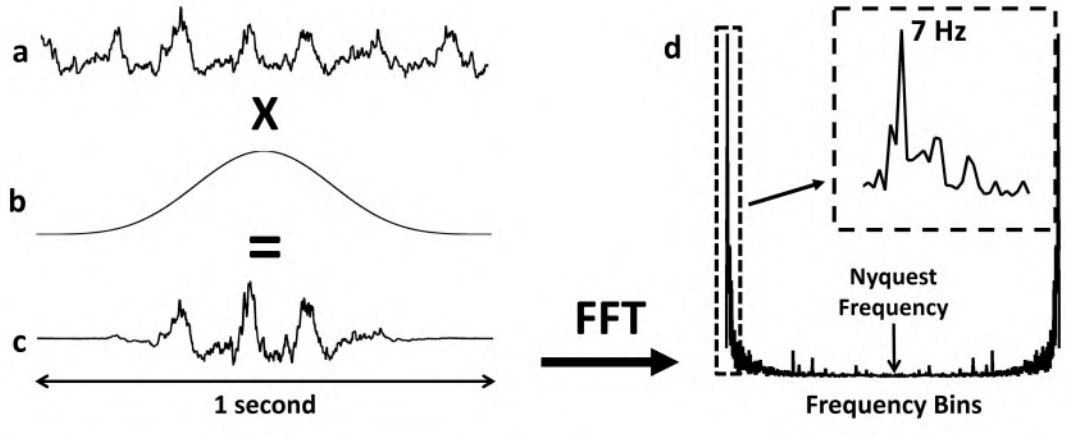

Figure 1.11: Hippocampal EEG, is in specific behavioral states often associated with theta rhythm (4-12 Hz), such as in **(a)**. Before calculating application of the Fast Fourier Transform (FFT), the time domain signal **(a)** is mostly multiplied with a cosine bell **(b, c)** in order to zero out the edges of the signal. Subsequently the power spectrum can be calculated as the modulus of the FFT **(d)**. The power spectrum indicates that most power is distributed at 7 Hz, which also is apparent from the original signal.

the original time series from these components using an inverse transform. Zeroing specific frequency bins and subsequently performing the inverse transform makes it possible to remove specific frequency components, which is a commonly used digital filtering method [11].

What often is of interest is to estimate the power of each frequency bin, which can be done by calculating the modulus[9] of the complex FFT outcome. This makes it possible to assess how the power of a specific signal is distributed in the frequency domain (**Fig. 1.11d**). Though the Fourier transform is applied to a finite signal, it theoretically assumes that the signal is repeated infinitely before and after the end of a signal, which gives rises to potentially sharp edges at the ends of a signal. Such sharp edges will result in "spectral leakage" around prominent frequency components in addition to broadening of potentially relevant peaks in the frequency spectrum. This is dealt with by multiplying the time domain signal with a cosine bell window prior to the Fourier Transform (**Fig. 1.11a**, **b and c**). In addition, it should be mentioned that the DC component of the time series frequently is removed by subtracting the mean or linear trend of the time series from the original time series. An exemplar outcome of the FFT on a hippocampal LFP characterized by 7 Hz theta rhythm is shown in **Fig. 1.11**.

1.2.4 Evoked Potentials

Evoked potentials (EPs) mainly refers to a technique where electrophysiological responses may be recorded in regions of interest in response to a specific stimulus. For example, auditory stimuli will evoke specific responses in regions involved in auditory processing and somatosensory[10] stimuli will evoke responses in somatosensory areas. Alternatively, electrical stimulation may be used to stimulate bundles of nerve fibers projecting to specific regions, in order to record a response from that region. EPs are typically assessed by locking the recording in time relative to the stimulus delivery, allowing averaging of EPs, which sometimes is necessary to analyze

[9] The modulus of a complex number $a + bi$ is $\sqrt{a^2 + b^2}$

[10] Somato means body and somatosensation thus refers to sensations arising from the periphery, such as the skin.

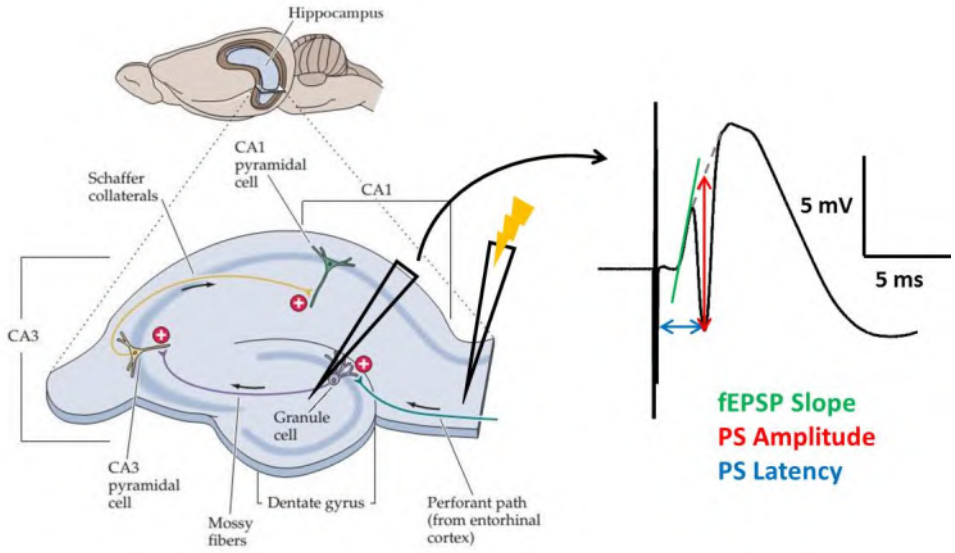

Figure 1.12: An example of how EPs can be recorded in the hilus of the dentate gyus upon stimulation of the perforant path. An exemplar EP with typically assessed parameters is shown on the right side of the figure. Adapted with permission from Purves *et al.* 2004 [21].

low amplitude EPs masked by ongoing background activity. EPs are used both clinically and experimentally. Clinically, EPs may be used to test the integrity of specific systems such as the somatosensory system [15]. Experimentally, evoked potentials have been used to unravel events related to cognitive processing of stimuli as visual impressions or sounds [12]. In preclinical studies, such as those presented in **Chapters 4 and 5**, electrically evoked hippocampal EPs have been used commonly to assess the responsiveness of neuronal circuits, which is altered in some neuronal disorders. In epilepsy, for example, which often is characterized by hyperexcitable neuronal circuits, EPs have been described to be faster and have larger amplitudes [22]. On the contrary, drugs used in the treatment of epilepsy have been shown to decrease this hyperexcitability [1, 2]. Dentate field EPs, as those recorded in **Chapters 4 and 5**, are evoked by electrical stimulation of the perforant path, a hippocampal afferent fiber bundle originating from the entorhinal cortex, forming its first synapse on dendrites of granule cells of the dentate gyrus [19]. The characteristic cytoarchitechture of the dentate gyrus, with the dendrites projecting away from the hilus and the axons leaving towards the hilus gives rise to a characteristic EP recorded from the hilus (**Fig. 1.12**). Stimulating the perforant path will send a volley of action potentials towards the dentate gyrus, which generates EPSPs in dentate granule cells, resulting in a positive potential in the hilus of the dentate gyrus, called the field EPSP(fEPSP). As the depolarization reaches a certain threshold, it will result in a synchronous generation of action potentials in dentate granule cells. Because of the axons directed towards the hilus, these action potentials are propagated towards the hilus. This results in a negative potential superimposed on the fEPSP, called the population spike (PS). These two parameters thus reflect excitatory synaptic transmission and the neuronal response arising as a result of this excitatory neurotransmission, respectively [19].

1.3 Principles of Neurostimulation

With the present thesis centering around vagus nerve stimulation, it is worth-while summarizing some of the principles underlying electrical stimulation of neural tissue. In a historical perspective, electrical stimulation has been used as a therapeutic intervention for neurological disorders even prior to the discovery of basic neuroanatomic or neurophysiological principles [25]. The discovery of basic neurophysiological principles and how electronic principles play a major role in the information processing in the nervous system has paved the way for a wide array of potentially beneficial neurostimulation modalities for various neurological disorders. Some of the major successes include cochlear implants, where electrical pulses are delivered to the auditory nerve [16]; spinal cord stimulation in chronic pain conditions, where electrical stimulation is delivered to the dorsal spinal cord [9]; deep brain stimulation for Parkinson's Disease, where electrodes are implanted in the brain to stimulate the subthalamic nucleus [3]; and vagus nerve stimulation, where the vagus nerve is electrically stimulated in the treatment of epilepsy [4]. Several additional modalities are currently under investigation for a wide spectrum of disorders, extending beyond neurological disorders, and it is very likely that electrical stimulation modalities, facilitated by the continuous improvement of electrotechnology, will provide major therapeutic advances in the following years. The following section will briefly consider general principles of neurostimulation, which apply both to stimulation of the brain with depth electrodes as well as stimulation of peripheral nerves, such as vagus nerve stimulation.

1.3.1 Delivery of Current

The delivery of an electrical current is done by creating a voltage difference between two or more metallic contact points of an implanted electrode, which causes the flow of current through the tissue in the vicinity of the electrodes. The voltage difference between the electrode contacts is typically created in short pulses by a battery driven pulse generator connected to the electrode contacts with leads. In patients, such a pulse generator in addition to the leads are typically implanted under the skin. The system as a whole creates a closed electronic circuit, where the main impedance of the circuit constitutes the interface between the electrode contacts and the tissue. Under optimal conditions, other factors, as lead wire resistance are rather minor contributors. The overall impedance of the circuit is important because it determines how much current that will pass through the tissue for a given voltage, as given by Ohm's law: $I = \frac{U}{R}$, which describes how the flow of current in a circuit (I) is equal to the voltage (U) divided by the resistance (R). Impedance is well related to resistance and describes the resistance in a circuit with alternating currents, whereby it in addition to magnitude value also possesses phase, whereas resistance only has magnitude and only is applicable to constant current circuits.

It is particularly important to control for stimulation impedance if a constant voltage stimulator is used, as this will determine how much current flows through the tissue. Alternatively, constant current stimulators possess a feedback system, where the voltage automatically is adjusted to the circuit impedance in order to deliver a constant output current. In this case, electrode impedance remains an important issue however, as high impedance results in higher voltage demands in order to deliver a specified current stimulus, which may exceed the maximal voltage range of the stimulator. Additionally high voltages may be disadvantageous from a biosafety point of view and put additional load on stimulation batteries, which is an issue for implanted devices. Electrical currents are typically delivered in short intermittent pulses, which are defined by width and shape, of which the most commonly used are biphasic square wave pulses (**Fig. 1.14a**). A biphasic pulse refers to the alternating polarity of the pulse, i.e. a pulse containing an initial negative phase followed by a positive phase. Biphasic counter-balanced

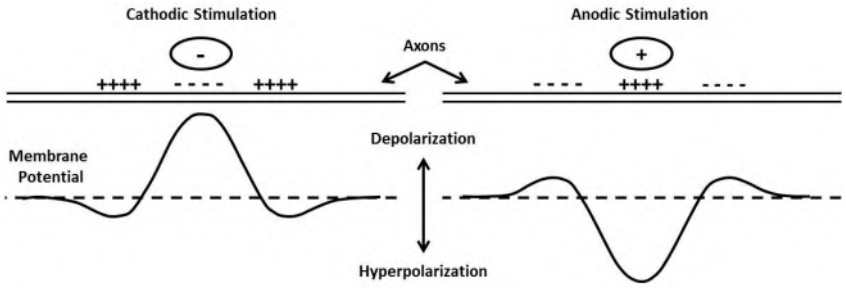

Figure 1.13: Delivery of an cathodic (negative) electrical stimulus will create a negative potential in the extracellular fluid around the electrode. Because the intracellular space usually holds a negative potential relative to the exterior, an extracellular negative potential will decrease (depolarize) the electrical potential across the cell membrane, which usually results in the generation of action potentials in axons. An anodic stimulus has the opposite effect.

pulses are always used for chronic stimulation, because unbalanced currents are associated with irreversible electrode degradation and tissue damage. Electrode degradation happens if sufficient potential builds at the electrode tissue interface and is sustained for sufficient time, whereby electrochemical reactions as reduction and oxidation occur. It is important to note that the potential difference between the contacts of the electrodes does not result in a transfer of electrons from the electrode surface from or to the tissue to a significant degree. Rather, electrons are drawn or transferred to molecules in the extracellular fluid in reduction and oxidation processes. If the reactions are not reversed by counter balanced pulses, irreversible tissue and electrode damage occurs as a result [7].

1.3.2 Impact of Electrical Stimulation on Neural Tissues

As described in the previous section, negative (cathodic) or positive (anodic) electrode potentials result in the flow of ionic currents in the extracellular space. Evidence has suggested that mainly cathodic currents result in depolarization, which is necessary to generate action potentials. This is explained by the effect of a negative potential spreading in the extracellular space on the membrane potential, which was described in **Section 1.1.2**. At rest, the intracellular space is negative relative to the extracellular space. A negative extracellular potential will thus depolarize the membrane. In contrast an anodic potential will have the opposite effect, resulting in adjacent hyperpolarization (**Fig. 1.13**) [7].

In order for electrical stimulation to have effect it typically needs to be delivered at a certain magnitude and it needs to be delivered at the right location. This is particularly the case for stimulation of nerves, where the electrical pulses will need to be delivered with a certain strength, denoted the threshold, in order to activate the neuronal axons. If the magnitude of the electrical pulse does not meet this threshold, nothing happens. The threshold for activation of neuronal axons were described in early experiments by Weiss [32] and reworked by Lapicque [18], who introduced an equation, which is very useful to consider even today

$$I_{th}(PW) = I_{rh}(1 + \frac{T_{ch}}{PW})$$

In the function, the threshold current (I_{th}) for activation of a neuronal fiber is described as a function of the pulse width (PW). The function introduces the "rheobase" current constant (I_{rh}), which refers to the minimal output current necessary to activate a given neuronal axon

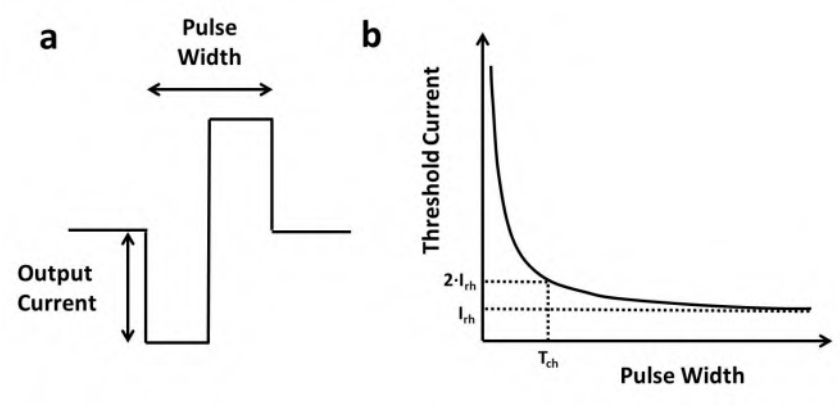

Figure 1.14: An electrical pulse is defined by its shape, amplitude (output current) and width. **(a)** shows an example of a biphasic square wave, which is commonly used in neurostimulation therapy. In **(b)**, it is seen how the threshold for electrical activation of fibers depends on the output current (I_{th}) and the pulse width (PW) of the stimulus. The graph reveals longer pulse widths and higher output currents are more likely to successfully activate neuronal fibers, but that the relationship between these and the threshold for action of a specific fiber is far from linear. I_{rh} is the "rheobase" current, which refers to the minimal output current necessary to activate a given neuronal axon irrespective of pulse width. T_{ch} is the "chronaxie", which is threshold pulse width at an output current of $2I_{rh}$.

for a theoretically infinitely long pulse. It further introduces the "chronaxie" constant (T_{ch}), which is the threshold pulse width at an output current of two times the rheobase current. As expected, the function implies that higher output currents and longer pulses are more likely to result in activation of a fiber, though the relationship between the two is far from linear (**Fig. 1.14b**).

Another relevant principle to consider is the orderly fashion in which fibers are recruited as the output current or pulse width is increased, with thick myelinated fibers recruited at relatively low levels of charge and thin unmyelinated fibers requiring the highest amount of charge for activation [23]. Unmyelinated fibers may display thresholds 100-fold higher than thick myelinated fibers [34]. Another factor worth considering is other stimulation parameters as stimulation frequency (i.e. pulses per unit of time). The stimulation frequency has little influence on whether nerve fibers are activated, but higher frequencies will generally result in a higher frequency of action potentials, which can result in temporal summation of EPSPs at the post synaptic membrane, which can increase the effect of a given outcome. Very high frequencies (>50 Hz), however, have been associated with irreversible tissue damage [7].

References

[1] W. C. Abraham and S. E. Mason. Effects of the nmda receptor/channel antagonists cpp and mk801 on hippocampal field potentials and long-term potentiation in anesthetized rats. *Brain Res*, 462(1):40–6, 1988.

[2] T. E. Albertson and R. M. Joy. Modification of evoked hippocampal dentate inhibition by diazepam and three antagonists in urethane-anesthetized rats. *Exp Neurol*, 106(2):142–9, 1989.

[3] R. J. Andrews. Neuroprotection trek–the next generation: neuromodulation i. techniques–deep brain stimulation, vagus nerve stimulation, and transcranial magnetic stimulation. *Ann N Y Acad Sci*, 993:1–13; discussion 48–53, 2003.

[4] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[5] Hans Berger. Über das elektrenkephalogramm des menschen. *European Archives of Psychiatry and Clinical Neuroscience*, 87(1):527–570, 1929.

[6] Francis J Bonner and Allen B Devleschoward. Aaem minimonograph 45: the early development of electromyography. *Muscle & nerve*, 18(8):825–833, 1995.

[7] Joseph D Bronzino. *Biomedical engineering handbook*, volume 2. CRC press, 1999.

[8] Gyorgy Buzsaki. *Rhythms of the Brain*. Oxford University Press, 2006.

[9] A. K. Compton, B. Shah, and S. M. Hayek. Spinal cord stimulation: a review. *Curr Pain Headache Rep*, 16(1):35–42, 2012.

[10] James W Cooley and John W Tukey. An algorithm for the machine calculation of complex fourier series. *Mathematics of computation*, 19(90):297–301, 1965.

[11] Ray Cooper, Colin D Binnie, and Richard Billings. *Techniques in clinical neurophysiology: A practical manual*. Elsevier Health Sciences, 2005.

[12] Michael W Eysenck and Mark T Keane. *Cognitive psychology: A student's handbook*. Taylor & Francis, 2000.

[13] G. Fang, Q. Chen, J. Cui, and Y. Tang. Electroencephalogram bands modulated by vigilance states in an anuran species: a factor analytic approach. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*, 198(2):119–27, 2012.

[14] A. C. Guyton and J.E. Hall. *Thextbook of Medical Physiology*. Elsevier Sauders, Philadelphia, 11th edition edition, 2006.

[15] R. N. Holdefer, D. B. MacDonald, and S. A. Skinner. Somatosensory and motor evoked potentials as biomarkers for post-operative neurological status. *Clin Neurophysiol*, 126(5):857–65, 2015.

[16] S. Irving, L. Gillespie, R. Richardson, D. Rowe, J. B. Fallon, and A. K. Wise. Electroacoustic stimulation: now and into the future. *Biomed Res Int*, 2014:350504, 2014.

[17] Herbert Henri Jasper. The ten twenty electrode system of the international federation. *Electroencephalography and Clinical Neurophysiology*, 10:371–375, 1958.

[18] Louis Lapicque. *Definition experimentale de l'excitabilite*. 1909.

[19] B. L. McNaughton and C. A. Barnes. Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. *J Comp Neurol*, 175(4):439–54, 1977.

[20] R. J. Noble, J. S. Hillis, and D. A. Rothbaum. Electrocardiography. 1990.

[21] D. Purves, G. J. Augustine, D. Fitzpatrick, W. C. Hall, A. S. LaMantia, J. O. McNamara, and S. M. Williams. *Neuroscience: Third Edition*. Sinauer Associates, Sunderland, USA, 2004.

[22] C. M. Queiroz, J. A. Gorter, F. H. Lopes da Silva, and W. J. Wadman. Dynamics of evoked local field potentials in the hippocampus of epileptic rats with spontaneous seizures. *J Neurophysiol*, 101(3):1588–97, 2009.

[23] F. Rattay. Analysis of models for external stimulation of axons. *IEEE Trans Biomed Eng*, 33(10):974–7, 1986.

[24] T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, I. Heuser, M. Bajbouj, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. Van den Abbeele, Z. Stanga, and M. Hasdemir. Vagus nerve stimulation for depression: efficacy and safety in a european study. *Psychol Med*, 38(5):651–61, 2008.

[25] J. M. Schwalb and C. Hamani. The history and future of deep brain stimulation. *Neurotherapeutics*, ,(1):3–13, 2008.

[26] R. R. Seeley, T. D. Stephens, and P. Tate. *Anatomy and Physiology*. McGraw-Hill, New York, 2008.

[27] W. E. Skaggs, B. L. McNaughton, M. A. Wilson, and C. A. Barnes. Theta phase precession in hippocampal neuronal populations and the compression of temporal sequences. *Hippocampus*, 6(2):149–72, 1996.

[28] G. Strobbe. *Advanced Forward Models for EEG Source Imaging*. PhD thesis, Ghent University, 2015.

[29] P. Van Loo, E. Carrette, A. Meurs, L. Goossens, D. Van Roost, K. Vonck, and P. Boon. Surgical successes and failures of invasive video-eeg monitoring in the presurgical evaluation of epilepsy. *Panminerva Med*, 53(4):227–40, 2011.

[30] Cinnamon VanPutte, Jennifer Regan, Andrew Russo, Philip Tate, Trent Stephens, and Rod Seeley. *Seeley's anatomy & physiology*. McGraw-Hill Higher Education, 2013.

[31] H Joseph Weaver. *Applications of discrete and continuous Fourier analysis*. Wiley-Interscience, 1983.

[32] Georges Weiss. Sur la possibilite de rendre comparables entre eux les appareils servant a l'excitation electrique. *Archives Italiennes de Biologie*, 35(1):413–445, 1990.

[33] I. Q. Whishaw and C. H. Vanderwolf. Hippocampal eeg and behavior: effects of variation in body temperature and relation of eeg to vibrissae movement, swimming and shivering. *Physiol Behav*, 6(4):391–7, 1971.

[34] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

Chapter 2

Vagus Nerve Stimulation

2.1 Vagus Nerve Anatomy and Physiology

In order to facilitate the understanding of vagus nerve stimulation (VNS), it is useful to initially introduce the anatomy and physiology of the vagus nerve, which is more complex than most other nerves. The vagus nerve is commonly referred to as the 10th cranial nerve, consisting of a group of afferent[1] and efferent[2] fibers exiting the brain stem bilaterally at the level of the medulla oblongata [11]. From there, the nerve fibers pass through the foramen magnum and runs in parallel with the carotid artery at the cervical level [11]. Rostally[3], the cervical vagus gives rise to the auricular branch of the vagus nerve, which provides sensory innervation to the external ear [114] (relevant to transcutaneous vagus nerve stimulation, see **Section 2.8**). As the cervical vagus passes caudally[4] and passes the aortic arch, it gives off another branch, the recurrent laryngeal nerve, which innervates the laryngeal and pharyngeal muscles. In the neck, the nerves give off several other branches to the airways, the esophagus and the heart. The left and right vagus nerves pass through the diaphragm along with the esophagus and form the anterior and posterior abdominal or subdiaphragmatic vagal nerve trunks, respectively. The subdiaphragmatic vagal branches further divide to innervate most abdominal organs, including the remaining parts of the gastrointestinal tract, the liver, the pancreas and the adrenal glands [11].

The vagus nerves are mixed nerves composed of approximately 80 % afferent fibers and 20 % efferent fibers [5, 42]. The cell bodies of the afferent fibers harbor mainly in two pairs of ganglia, the nodose and jugular ganglia, located just outside the skull [11]. The afferent fibers project predominantly to the nucleus of the solitary tract [109] and convey sensory information from thoracic and abdominal viscera [67]. This includes changes in blood pH and blood pressure, traced by chemoreceptors and baroreceptors located in the walls of major arteries, but it also includes information on contents passing through the gastrointestinal tract [67]. The efferent proportion of fibers are mainly parasympathetic motor fibers, which arise mainly from the dorsal vagal motor nucleus and nucleus ambiguus, and are involved in regulation of the typical parasympathetic "rest and digest response" [55]. This includes slowing of the heart rate and facilitation of gastrointestinal and glandular activity.

The composition of fibers of the vagus nerve is important to consider with regard to choosing appropriate stimulation parameters for VNS (elaborated in **Section 2.6**). The majority (80-90%) of vagal afferent fibers are thin unmyelinated C-fibers[5] [5], which convey sensory information arising from multimodal chemoreceptors in the lungs, heart and gastrointestinal tract.

[1] Afferent refers to carrying information towards the brain. An afferent root therefore contains fibers which convey sensory information from the periphery towards the brain.

[2] Efferent refers to carrying information from the brain to the body/periphery. An efferent root therefore contains fibers which convey motor information from the brain towards the effector organs.

[3] Rostral means towards the oral or nasal cavity. In relation to the brain this means towards the front (anterior) and upwards in relation to the spinal chord.

[4] Towards the tail. Caudal is the opposite direction of rostral.

[5] Nerve fibers are classified according to their myelination thickness and conduction velocity. Thick myelinated A-fibers conduct signals faster than thin myelinated B-fibers and unmyelinated C-fibers.

The remaining proportion of myelinated B- and A-fibers convey mainly mechanical information. Apart from a small proportion of thickly myelinated motor fibers innervating the laryngeal and pharyngeal muscles, the majority of efferent fibers are either thinly myelinated or unmyelinated fibers [5, 40].

2.2 Historic Perspectives of Vagus Nerve Stimulation

VNS has a long history, with the oldest published work to be found in the pubmed database dating back to 1886[6] [50]. Today, VNS mainly refers to electrical stimulation of the left cervical trunk of the vagus nerve as an established clinical approach, though historically both the left and right vagus nerves in addition to several subbranches of the vagus nerve have been targeted in different contexts, using different stimulation modalities. Research on VNS can be divided into two branches with regard to the aim of the research. In many cases, particularly since the approval of VNS for clinical use, the goal has been to obtain specific clinical effects, which could be beneficial in a therapeutic context (**Section 2.4**). In many other cases, the goal has simply been to identify functions of the vagus nerve.

VNS, as the only neurostimulation device in epilepsy [76], received regulatory approval for drug resistant epilepsy by European and American regulatory agencies in 1994 and 1997, respectively. Inspired by the early findings that mechanical stimulation of the vagus nerve with carotid massage could inhibit seizures [112] and the later finding that electrical stimulation of the vagus nerve could desynchronize the cortical rhythms [19, 154], several investigators initiated preclinical studies to assess anticonvulsant effects of VNS in multiple species [90, 150, 152]. Positive results further motivated full clinical development, with the first epilepsy patient receiving a VNS implant in 1988 [113, 144]. Systematic observations of mood improvement in epilepsy patients, irrespective of anticonvulsant efficacy [36], was further sufficient evidence to support regulatory clearance of VNS for drug resistant depression, which was given in 2005. Today it is estimated that more than 70,000 patients have been treated with VNS [23].

2.3 Surgical Implantation of a Vagus Nerve Stimulator

The commercially available vagus nerve stimulator consists of two silicone helical coils connected via leads to a pulse generator (**Fig. 2.1**). In the clinic, implantation of a vagus nerve stimulator is done under general anesthesia and usually requires two incisions [12, 33]. One incision is made from the left sternocleidomastoid towards the midline, for exposure of the vagus nerve and implantation of the electrode. The cervical branch of the vagus nerve is embedded in a sheath of connective tissue, which also contains both the carotid artery and the internal jugular vein [11]. In order to wrap the electrode around the vagus nerve it is thus required to initially dissect the vagus nerve from the carotid and internal jugular vein [12, 33]. Another incision is made under the left clavicle for implantation of the pulse generator [12, 33]. The leads of the electrode are then tunneled subcutaneously to the pulse generator [12, 33].

Though commercially available systems have been used in dogs or other larger animals[95], the VNS electrode is typically custom made for preclinical studies [35] depending on species. The surgical procedure further varies from laboratory to laboratory. In rats, for example, some investigators isolate the vagus nerve, as in humans, before implanting the electrode around the vagus nerve [35, 116], while other groups implant the electrode around both the vagus nerve and the carotid artery [34, 58]. In addition some investigators suture the electrode to underlying

[6] Using the search words "Vagus Nerve AND Stimulation", www.pubmed.com, done 22/2-2016

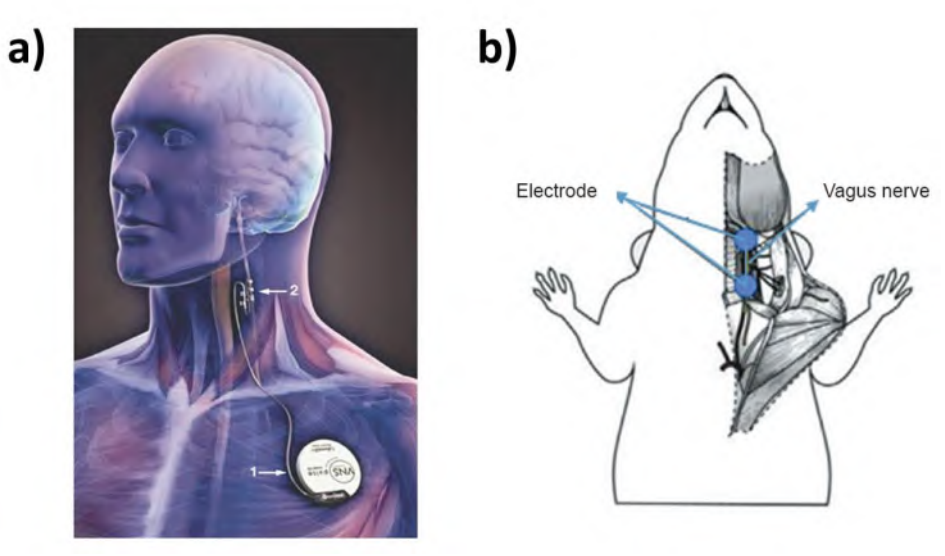

Figure 2.1: A schematic anatomical overview of the VNS electrode implantation in patients **(a)** and rats **(b)**. In **(a)**, 1. illustrates the pulse generator implanted below the clavicle and 2. the helical electrode wrapped around the vagus nerve.

muscles in order to fixate the electrode [34, 58], while others do not [35, 116]. Further, preclinical studies mostly use other stimulators than those used for clinical application of VNS.

2.4 Indications of Vagus Nerve Stimulation

The following section elaborates on evidence supporting the use of VNS in various indication areas. Despite only being approved for the treatment of drug-resistant epilepsy and depression, evidence suggests application of VNS in several other areas, which will also be described in the following.

2.4.1 Epilepsy

Epilepsy is the main indication area of VNS, with VNS being applied in cases of epilepsy that do not respond sufficiently to conventional drug treatment and where epilepsy surgery is not an option[7] [8]. Epilepsy is a neurological disorder, which is characterized by the unexpected and continuous recurrence of seizures [10]. In Europe, the estimated prevalence is 0.5% [44], of which approximately 30% are drug resistant[8] [83] and thus considered for treatment alternatives as VNS. Results of clinical trials with VNS in epilepsy vary, with response rates[9] being reported between 30 and 60% [30, 37, 84, 111]. It must be mentioned that most studies conducted are

[7] Epilepsy surgery most often refers to the resection of the zone that based on presurgical evaluation is estimated to generate the seizures (the epileptogenic zone). Epilepsy surgery is associated with favorable outcomes, with seizure freedom achieved in up to 85% of patients. If resection of the epileptogenic zone is associated with high risk of subsequent neurological disabilities, alternative treatment options as for example VNS are used.

[8] Drug resistant epilepsy can be defined as "failure to adequate trials of two tolerated, appropriately chosen antiepileptic drug schedules (either as monotherapy or in combination)", citation from Kwan *et al.* 2010 [82]

[9] Responders are typically defined as patients that experience an at least 50% reduction in seizure frequency.

open-label trials[10]. A meta-analysis of evidence published prior to 2011 [38], revealed that children, patients with generalized epilepsy[11] and patients with cortical epilepsies arising from malformations are more likely to respond positively to VNS. Further, the outcome is generally found to improve over time [38].

2.4.2 Depression

VNS was approved for depression secondarily to the treatment of drug resistant epilepsy as mood improvements were observed in epilepsy patients [36]. Depressive disorders are highly prevalent with an estimated life-time prevalence of 14% and a one year prevalence of 4-5% in the Western European population [4]. It is estimated that more than 50% of patients are resistant to conventional drug treatment [41], suggesting that a large population could be potential candidates for VNS therapy. Open label trials have generally reported a variable improvement in depressive symptoms, with around 25%-50% of the patients treated experiencing an at least 50% reduction in depressive symptoms following one year of VNS [1, 6, 21, 127, 131]. As for epilepsy, treatment response is generally observed to improve over time [6, 21].

2.4.3 Other Indications

Though VNS is only approved for the treatment of drug resistant epilepsy and depression, it is currently being investigated for several additional applications. Among these indications are pain disorders. The inhibitory influence of vagal afferent activity on nociceptive processing[12] is well documented through a series of rigidly conducted preclinical studies published even before there was concrete evidence of anticonvulsant effects of VNS [118]. Clinically, VNS was found to reduce experimentally induced pain in epilepsy patients [72, 73, 105]. VNS has further been found to reduce pain in fibromyalgia patients [86] and there have been several case reports of therapeutically beneficial effects of VNS on migraines and cluster headaches [17, 46, 65, 87, 96, 128]. VNS has further been found to attenuate inflammation [15], which is clinically beneficial in cases of chronic and painful pathological inflammatory disorders, such as rheumatoid arthritis [75] or inflammatory bowel disorders [14, 94]. VNS has additionally been found to enhance recognition memory [20], which inspired the application of VNS in Alzheimer's disease [99]. The outcome, however, was rather inconclusive, necessitating further study. Finally, several recent studies reported favorable outcomes of application of right sided VNS in cases of heart failure [26, 32, 132].

2.5 Side Effects

The side effects associated with VNS either relate to the risk of surgical intervention or directly to the stimulation applied in the course of the treatment. In relation to the surgical intervention, rare cases of local infections at the site of incision, weakness of the lower facial muscles, vocal cord paresis and bradycardia have been reported [7, 102]. Stimulation related side effects are mainly observed at high output currents and include hoarseness related to the stimulation of motor fibers controlling the vocal cords and pain directly related to the stimulation [7, 102].

[10] In an open-label study, there is often no control condition and both the patient/subject and the investigator are aware of the treatment condition

[11] Generalized seizures result in complete loss of consciousness and typically involve larger regions of the brain [103].

[12] Nociception refers to the transmission of potential harmful stimuli, which typically evoke the perception of pain.

Cardiac side effects have been a theoretical concern owing to the known vagal influence on the heart [11]. This was the reason to apply VNS to the left vagus nerve, considering that the left vagus nerve exerts less cardiac influence [11]. Indeed very few cardiac side effects have been reported [7, 102]. In fact, despite the theoretical concern of cardiac side effects associated with right-sided VNS, application of right-sided VNS has been reported as a safe alternative [97, 104, 135], and as mentioned (section 2.4.3) has even been used to treat heart failure.

Generally, compared to conventional drug treatment, VNS possesses certain advantages. First of all, the device is controlled by an educated physician, which removes the compliance problem associated with self-ingested drugs [64]. Secondly, the tolerability along with the therapeutic efficacy of VNS tends to increase over time [7, 131].

2.6 Stimulation Parameters

Electrical stimulation is delivered to the vagus nerve by creating a voltage difference between the two contacts placed on the nerve resulting in flow of current (principles discussed in **Section 1.3**). If the current sufficiently depolarizes the fibers, it evokes action potentials, which are conducted both orthodromically[13] and antidromically[14]. The threshold for electrical activation of the fibers depends on the amplitude of the charge difference created between the two contacts, but also the duration over which the charge difference is sustained (**Fig. 1.14b**).

Commercially available vagus nerve stimulators allow adjustment of VNS parameters with regard to four different parameters (**Fig. 2.2**)[85]:

- Output current, expressed in mA
- Pulse width, expressed in μs
- Frequency, referring to pulses per second (Hz)
- Duty cycle, referring to the duration of VNS ON and OFF periods

Stimulation parameters on commercially available implantable stimulators can be programmed and adjusted via a wireless connection to a computer. The stimulator is typically only switched on two weeks after surgery, which allows the wounds to heal. The output current is typically

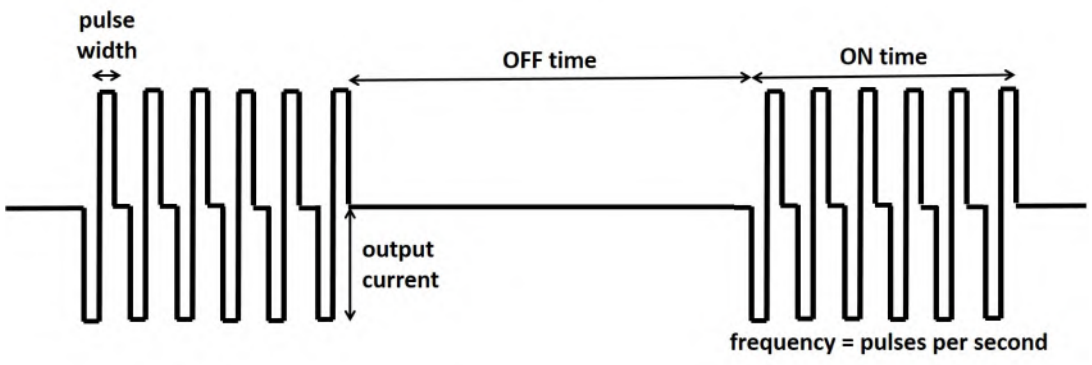

Figure 2.2: A schematic overview of different stimulation parameters. Pulse width refers the the width of the pulses, output current refers to the amplitude of the pulses, the frequency refers to the frequency of pulses per second and the ON and OFF times refer to the durations where VNS is ON and OFF, respectively.

[13] Along with the natural direction of the nerve.
[14] The opposite direction of the natural direction of the nerve.

adjusted to the lowest possible at 0.25 mA, but is gradually increased during monthly consultations to the maximally tolerable level[15]. The pulse width is typically set at either 250 or 500 μs and the frequency of the stimulation is set between 20 and 30 Hz. In preclinical settings, higher frequencies have been associated with irreversible nerve injury [2] and lower frequencies were reported as less effective [150]. VNS is further applied with a duty cycle as the effects of VNS have been found to outlast the active stimulation period [137, 152].

Recommendations for adjustment of VNS parameters in the clinic are mainly based on empirical clinical experience [24], as there is very little systematically acquired clinical evidence comparing the efficacy of stimulation parameters [60]. To clinically evaluate the efficacy of a specific stimulation parameter setting, such as the influence of different duty cycles, long stimulation periods are often required. This is particularly the case for epilepsies with a very low seizure rate. The only option has thus been to retrospectively assess the experience collected, which has been done in a few cases [31, 84]. However, retrospective studies tend to be subject to several confounding factors. For example, patients are typically initially treated with a "standard" duty cycle of 30 seconds ON and 300 or 600 seconds OFF. If the patients shows little response, the duty cycle is intensified (i.e, the %-ON time is increased). This means that as the standard duty cycle is compared to more intense duty cycles, the population treated with intenser duty cycles will tend to be the most treatment resistant population, constituting an important confounding factor to these studies.

2.7 Mechanism of Action

The mechanisms triggered by VNS are complex and still under intense study and are also the main focus of this thesis. Following the overview of some of the more prominent areas indicated for treatment with VNS (**Section 2.4**), it is clear that VNS is associated with a wide array of clinical effects. The extensive projections and consequently physiological implications of the vagus nerve (summarized in **Section 2.1**) provide a basis to understand how a single intervention simultaneously can produce so many different effects. The summary of working mechanisms of VNS given in this section is divided into subsections discussing the following aspects:

- Electrical stimulation of the vagus nerve - what is being stimulated?
- Effects of VNS on the central nervous system (central effects)
- Effects of VNS on other organs (peripheral effects)

The information is graphically summarized in **Fig. 2.3**. While this section mainly focuses on anticonvulsant mechanisms of VNS, parallels are drawn to observations studying other effects as these can assist the general understanding of mechanisms associated with VNS.

2.7.1 Activation of the Vagus Nerve

As discussed previously (**Section 2.1**), the vagus nerve is a mixed nerve with both afferent and efferent fibers. Effective stimulation of the vagus nerve will under all circumstances result in a bidirectional activation of both afferent and efferent fibers, though a unidirectional activation often is desirable. Anticonvulsant effects of VNS, for example have been attributed mainly to stimulation of vagal afferents, as efficacy is retained despite transection of the vagus nerve distal to the electrode [152]. Meanwhile, lesioning the vagus nerve proximal to the stimulation site eliminates anticonvulsant effects of VNS [98]. Thus, historically, the anode of the VNS electrode

[15] Referring to a level where patients do not experience excessive pain in relation to the stimulation or an excess of other side effects as voice disturbances.

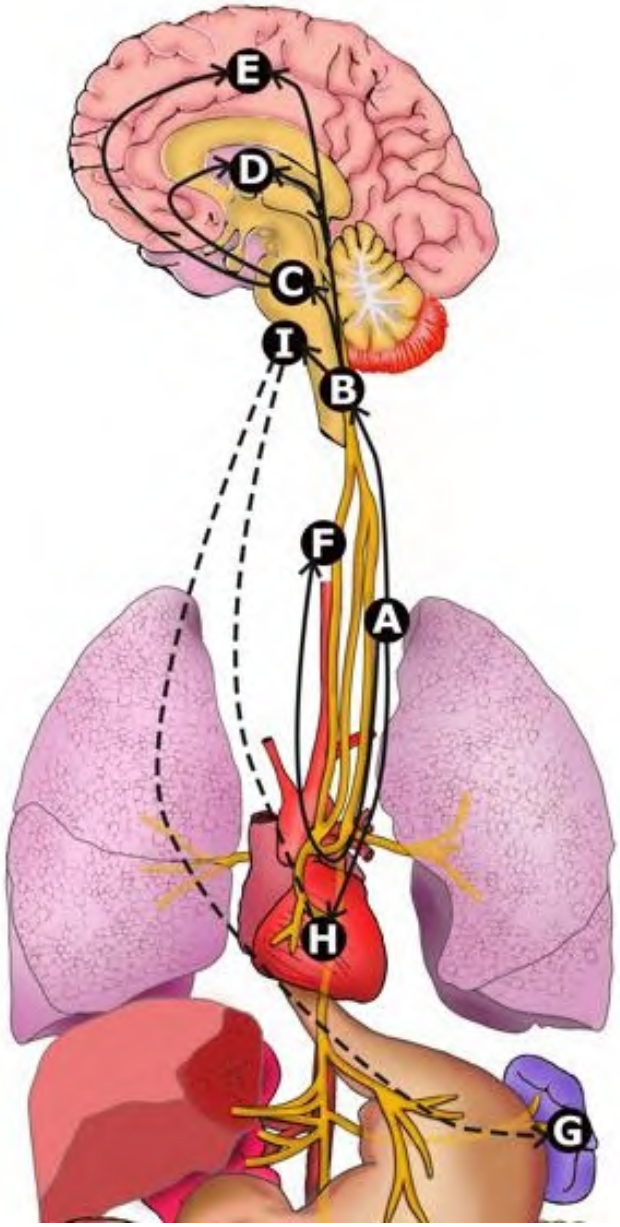

Figure 2.3: A schematic summary of mechanisms observed in various studies with VNS. VNS is typically applied to the cervical trunk of the left vagus nerve **(A)**. Vagal afferent activity relays predominantly in the nucleus of the solitary tract (NTS) **(B)**. As a result, modulation of activity has been observed in connected nuclei as the locus coeruleus (LC) and the raphe nuclei **(C)**, which results in a release of norepinephrine and serotonin in various brain regions. Modulation of several limbic **(D)** and cortical **(E)** structures has been observed, either as a result of altered signaling of neuromodulatory substances or as another upstream consequence from the NTS. Activation of efferent vagal fibers may result in activation of laryngeal and pharyngeal muscles **(F)**, which can be recorded as muscle potentials. Activation of vagal efferents has further been found to reduce inflammatory responses, via an action on the spleen **(G)** and in slowing of the heart **(H)**, though this mainly applies to right sided VNS. The later two effects may theoretically (thus marked with dashed lines) also arise as a result of reflex activation of vagal motor nuclei of the brainstem **(I)**, which receive input from the NTS.

has been placed distally, in order to induce a theoretical anodal block[16] of efferently conducted action potentials during VNS [142], and thus block some of the side effects associated with stimulation of efferents, such as voice alterations and coughing [102]. However, there is evidence that the anodal block is merely a theoretical phenomenon, as switching the polarity of the two VNS contacts results in the same activation of efferent fibers responsible for the generation of the laryngeal muscle evoked potential (LMEP), which can be measured during VNS [52].

When electrically stimulating peripheral nerves, nerve fibers are recruited in an orderly fashion, with thick myelinated and fast conducting type A fibers recruited at the lowest intensities, thin myelinated fibers type B fibers recruited at intermediate intensities and unmyelinated type C fibers recruited at the highest intensities [101, 151]. Initially, relatively high stimulation intensities (5-15 mA, 500 μs pulse width) were used to induce anticonvulsant effects in dogs [152], and anticonvulsant effects of VNS were thus attributed to the activation of vagal C-fibers [150]. However, anticonvulsant effects of VNS were conserved in an acute seizure model in rats, when C-fibers were selectively lesioned with capsaicin [78]. Antinociceptive effects of VNS, in contrast, were eliminated by application of capsaicin and thus attributed to stimulation of C-fibers [119]. Comparative studies have not been conducted for other indications. The only way to have an idea about which fibers that are responsible for the respective effects observed is to examine the VNS intensities used (i.e. output current and pulse width). The electrical recruitment properties of the vagus nerve were studied in anesthetized dogs [151], which along with pigs are the best comparison to the human vagus nerve owing to the relative diameter of the nerve as well as the number of fibers it contains [139, 148, 151]. This study showed that with a pulse width of 300 μs, afferent A-fibers had a mean recruitment threshold of 0.37 mA $\pm$ 0.18 mA (mean $\pm$ standard error of the mean), fast B-fibers a threshold of 1.6 mA $\pm$ 0.36 mA, slow B-fibers a threshold of 3.8 mA $\pm$ 0.84 mA and C-fibers a threshold of 17 mA $\pm$ 7.6 mA [151]. Apart from confirming the fact that thick myelinated fibers are recruited at the lowest intensities, the study also revealed less variable and more homogenous recruitment threshold for type-A and type-B afferents [151], which suggest that full recruitment could occur within a narrow window of VNS intensities. Finally, the study revealed that the threshold for recruitment of thick myelinated motor fibers, responsible for the production of an LMEP, was practically identical to the threshold for recruitment of type-A afferents (0.36 mA $\pm$ 0.17 mA vs. 0.37 mA $\pm$ 0.18 mA) [151]. A very similar finding was obtained in anesthetized rats [101]. Remarkably, the threshold for an LMEP in rats was estimated to be around 0.3 mA, using a pulse width of 250 μs [52], suggesting that the data from dogs is comparable to the data obtained from rats. Anticonvulsant effects of VNS have been reported in several rat studies [3, 77–79, 116, 121, 137]. VNS was further found to decrease cortical excitability [27, 100]. Considering that the intensities used in these studies is below the threshold for recruitment of even fast B-fibers but above the threshold for recruitment of A-fibers [151], the current general idea is that anticonvulsant effects of VNS are mediated by thick myelinated A-fibers. This idea is further a part of the rationale underlying the experimental work presented in **Chapter 5**.

As mentioned previously, past evidence suggests that while anticonvulsant effects of VNS are mediated by A-fibers, antinociceptive effects indeed are mediated by C-fibers recruited at relatively high intensities [118]. Interestingly, a recent study examining the application of VNS in heart failure in order to reduce heart rate, showed that slowing of heart rat was only seen from around 2 mA, using a pulse width of 500 μs [71]. Compared to the previously discussed data, this clearly indicates the necessity of recruiting B-fibers. This implies that while it appears clear that thick

[16] Anodal block refers to the phenomenon that the anode initially holds a positive polarity compared to the cathode. This may in some conditions result in a hyperpolarization of the axons, which theoretically could hinder the propagation of action potentials past the point of the anode.

myelinated type A afferents mediate anticonvulsant effects of VNS, other fibers may mediate other potentially beneficial therapeutic effects necessitating other stimulation parameters.

2.7.2 Central Effects

Though several pieces of the puzzle with regard to how VNS affects brain physiology remain to be identified, significant progress has been made. Vagal afferents enter via the vagal rootlets at the level of the medulla of the brainstem and terminate predominantly in the ipsilateral[17] nucleus of the solitary tract (NTS) [70, 109]. Few vagal afferents project to the contralateral[18] NTS and to other nuclei, including the dorsal vagal motor nucleus, the nucleus ambiguous, the reticular formation of the medulla as well as the trigeminal nucleus [70, 109]. Application of a local anesthetic block to the NTS was indeed described to eliminate all antinociceptive effects of VNS [117]. The NTS is described as a sensory relay center, and conveys sensory information to several other brain stem nuclei, including trigeminal, facial and hypoglossal nuclei, as well as vagal motor nucleus and the nucleus ambiguus [108]. The NTS also connects to autonomic control centers involved in regulation of the cardiovascular system and respiration and connects to the hypothalamus via the parabrachial nucleus [16]. Finally, the NTS connects to brainstem monoaminergic systems as the locus coeruleus (LC) and the raphe nuclei, which provides noradrenergic and serotonergic innervations to large parts of the remaining central nervous system [126]. The NTS, however, also sends direct bilateral projections to hypothalamic, thalamic and cortical regions [120]. The complexity and multitude of projections from the NTS, the predominant recipient of vagal afferent information, perfectly illustrates the complexity of VNS and explains how one intervention can be associated with so many effects as VNS has been. Further, it implies that the mechanisms underlying certain therapeutic effects are likely to be multifactorial.

Anticonvulsant and antidepressive mechanisms of VNS have been attributed to the facilitatory effect of VNS on the release of norepinephrine from the LC and release of serotonin from the raphe nucleus [43, 51]. Thus, administration of the neurotoxin DSP-4, which lesions noradrenergic neurons, including the LC, was found to suppress acute anticonvulsant and antidepressive effects of VNS in rats [53, 77]. VNS was indeed found to increase the tonic activity of LC and serotonergic dorsal raphe neurons in urethane anesthetized rats [34]. Following one hour of VNS, the tonic activity of LC neurons had increased only slightly ($\sim 20\%$), while two weeks of VNS almost doubled the tonic LC activity [34]. Increased tonic activity of serotonergic raphe neurons was only observed after two weeks of VNS [34]. In support of these findings, investigators have found increased c-fos[19] and DeltaFosB[20] staining in the LC and dorsal raphe nucleus following chronic VNS in rats [22]. VNS is associated with increased extracellular concentrations of norepinephrine in both the prefrontal cortex [91, 125] and hippocampus [91, 116, 125]. In a limbic seizure model in rats, anticonvulsant effects of VNS were found to be correlated to the effect of VNS on intrahippocampal norepinephrine concentrations [116]. Anticonvulsant effects of VNS were further attenuated by intrahippocampal administration of an α_2-adrenoceptor antagonist, providing evidence for a causal role of norepinephrine in anticonvulsant effects of VNS.

Recent evidence has further implicated a potential role for cholinergic modulation in the mechanisms underlying effects of VNS on cortical excitability and synchrony [106]. The LC is indeed known to project to the nucleus basalis of the forebrain, which supplies hippocampal and cortical structures with cholinergic innervation [39, 153]. VNS has additionally been shown

[17] Same side, i.e. left vagal nerve fibers terminate in the left NTS
[18] Opposite side.
[19] A marker for acute neuronal activation.
[20] A marker for chronic neuronal activation.

to decrease the concentrations of the excitatory neurotransmitters glutamate and aspartate, while simultaneously increasing the concentration of the inhibitory neurotransmitter GABA in cerebrospinal fluid of patients with epilepsy [9]. At a functional level, VNS has been found to reduce motor cortex excitability in awake freely moving rats [27, 101]. As hyperexcitability is thought to be a key aspect of epilepsy [115], this could reflect anticonvulsant efficacy of VNS. Other studies, examining hippocampal evoked potentials during VNS, however, have yielded more complex outcomes and have even been described to enhance synaptic transmission [133, 143], contradicting an anticonvulsant effect. Anesthesia as used in these studies, however, is known to exert great influence on hippocampal EPs [134] and is thus also likely to have influenced the study outcomes. The question of how VNS affects excitability is crucial to the rationale of the experimental work presented in **Chapter 4**.

The evidence discussed this far has mainly focused on preclinical research, which mainly has considered the involvement of particular neurotransmitter systems in therapeutic mechanisms of VNS. Another branch of VNS research has used various imaging techniques as single-photon emission computed tomography (SPECT), positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) to visualize changes in brain activation patterns during VNS in patients (extensively reviewed by Chae *et al.* [18]). While the aim of this branch of research mainly has been to identify factors that predict therapeutic response, it also has potential to provide information on structures and mechanisms which are important to VNS. The research, has however been limited by clinical heterogeneity in the patient populations studied and though many studies find significant associations between clinical efficacy and effects of VNS on specific brain activity patterns, it has been difficult to draw a common consensus across the studies [18]. Most of the studies, however, implicate thalamic involvement, though PET and fMRI studies have reported increased thalamic activity [61–63, 89] and PET studies decreased thalamic activity [122, 145, 147].

Other investigators have attempted to find factors in the electroencephalography (EEG) predicting therapeutic efficacy, but with somewhat variable success. Some investigators have reported no effect of VNS on EEG in any state [57, 129]. Others have however found changes in interictal[21] and sleep EEG [13, 29, 47, 56, 74, 80, 81, 88, 93, 110, 123, 130, 149]. Generally, VNS has been found to reduce the frequency of interictal spiking and spike and wave activity [56, 74, 80, 81, 110, 130, 149], which is consistent with preclinical reports [98, 154]. The effect of VNS on interictal epileptic discharges has generally been found to be correlated to a general effect of VNS on seizures [56, 74, 80, 149].

Early studies only focusing on changes in the EEG power spectrum have reported no changes during VNS [57, 129], though one group has reported a global increase in 20-50 Hz gamma power after one year of VNS in scalp EEG of epilepsy patients [93]. While data has suggested that VNS has little effect on the general power spectrum, VNS instead has been described to desynchronize EEG rhythms [13, 47, 93]. Through early findings in cats [19, 154], desynchronization of highly synchronous epileptic activity has been a frequently postulated hypothesis of the anticonvulsant mechanisms underlying therapeutic VNS [68, 69]. One study reported increased synchrony of gamma rhythms, while low frequency activity was desynchronized both within and between hemispheres [93]. Decreased symmetry of scalp EEG has indeed been found in epilepsy patients responding to VNS relative to non-responding patients [13, 48]. The effect has further been reported to be stronger during the VNS ON phase than during the VNS OFF phase [13]. There are however examples of studies reporting contradictory synchronizing effects [29] of VNS, which probably is explained by differences in acquisition and analysis methods.

[21] Referring to the periods between seizures.

2.7.3 Peripheral Effects

Peripheral effects of VNS occur either as a direct result of activation of vagal efferents or activation of vagal afferents that subsequently result in reflex activation of effector/motor nuclei that mediate peripheral responses [14]. As elaborated in **Section 2.1**, vagal efferents are an important component of the parasympathetic nervous system, exerting influence on several organs [11]. Vagal afferents are known to be sensitive to certain proinflammatory cytokines, which are capable of activating the hypothalamic pituitary adrenal (HPA) axis in response to a peripheral inflammatory insult [25]. This facilitates the release of corticosterones from the adrenal medulla, which has known immunosuppressive properties [140]. Increased serum levels of corticosteroids has indeed been observed following VNS in rats [28].

Another pathway, which has received a lot of attention recently is the cholinergic anti-inflammatory pathway [141]. This pathway is mediated by cholinergic efferents of the vagus nerve, which may be activated directly or indirectly through activation of vagal afferents that subsequently as a reflex activates vagal efferents [141]. The net effect of the pathway is attenuation of the inflammatory response [141], which has been found during VNS [15]. Importantly this may be suggested as a secondary mechanism by which VNS can suppress seizures as inflammation is thought to play an important role in increasing excitability and as a result increase the susceptibility to develop epileptic seizures [92].

2.8 Transcutaneous Vagus Nerve Stimulation

Transcutaneous vagus nerve stimulation (t–VNS) is a non-invasive alternative neurostimulation technique, which initially was introduced as a theoretical concept by Ventureyra in 2000 [146]. t–VNS, as the name implies is applied transcutaneously, with the aim of activating sensory nerve endings of the auricular branch of the vagus nerve innervating the external acoustic meatus [114]. Like vagal afferents targeted by VNS, a significant proportion of afferent fibers in the auricular branch of the vagus nerve project to the NTS, though an additional significant proportions project to the trigeminal nucleus [107]. Further, mechanical stimulation of the auricular branch of the vagus nerve has been associated with typical vagal responses as cough and cardiac inhibition [49, 54, 138], which suggest a functional connection to the vagal afferent system. Using fMRI, t–VNS has been observed to activate networks typically activated by VNS, including the locus coeruleus [45]. Clinical evidence, however, is limited. A recent proof of concept trial with seven epilepsy patients reported limited therapeutic efficacy [136]. On the other hand, a group from China, where auricular acupuncture has a long history [66], has reported remarkable success with t–VNS, with seizure freedom reported in up to 15% of patients and a reduction in seizure frequency in another 25% [59, 124]. These results remain to be reproduced by other investigators.

References

[1] S. T. Aaronson, L. L. Carpenter, C. R. Conway, F. W. Reimherr, S. H. Lisanby, T. L. Schwartz, F. A. Moreno, D. L. Dunner, M. D. Lesem, P. M. Thompson, M. Husain, C. J. Vine, M. D. Banov, L. P. Bernstein, R. B. Lehman, G. E. Brannon, G. A. Keepers, J. P. O'Reardon, R. L. Rudolph, and M. Bunker. Vagus nerve stimulation therapy randomized to different amounts of electrical charge for treatment-resistant depression: acute and chronic effects. *Brain Stimul*, 6(4):631–40, 2013.

[2] W. F. Agnew and D. B. McCreery. Considerations for safety with chronically implanted nerve electrodes. *Epilepsia*, 31 Suppl 2:S27–32, 1990.

[3] G. M. Alexander and J. O. McNamara. Vagus nerve stimulation elevates seizure threshold in the kindling model. *Epilepsia*, 53(11):2043–52, 2012.

[4] J. Alonso, M. C. Angermeyer, S. Bernert, R. Bruffaerts, T. S. Brugha, H. Bryson, G. de Girolamo, R. Graaf, K. Demyttenaere, I. Gasquet, J. M. Haro, S. J. Katz, R. C. Kessler, V. Kovess, J. P. Lepine, J. Ormel, G. Polidori, L. J. Russo, G. Vilagut, J. Almansa, S. Arbabzadeh-Bouchez, J. Autonell, M. Bernal, M. A. Buist-Bouwman, M. Codony, A. Domingo-Salvany, M. Ferrer, S. S. Joo, M. Martinez-Alonso, H. Matschinger, F. Mazzi, Z. Morgan, P. Morosini, C. Palacin, B. Romera, N. Taub, and W. A. Vollebergh. Prevalence of mental disorders in europe: results from the european study of the epidemiology of mental disorders (esemed) project. *Acta Psychiatr Scand Suppl*, 420:21–7, 2004.

[5] S. A. Asala and A. J. Bower. An electron microscope study of vagus nerve composition in the ferret. *Anat Embryol (Berl)*, 175(2):247–53, 1986.

[6] M. Bajbouj, A. Merkl, T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. van den Abbeele, K. Matthews, D. Christmas, S. Eljamel, and I. Heuser. Two-year outcome of vagus nerve stimulation in treatment-resistant depression. *J Clin Psychopharmacol*, 30(3):273–81, 2010.

[7] E. Ben-Menachem. Vagus nerve stimulation, side effects, and long-term safety. *J Clin Neurophysiol*, 18(5):415–8, 2001.

[8] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[9] E. Ben-Menachem, A. Hamberger, T. Hedner, E. J. Hammond, B. M. Uthman, J. Slater, T. Treig, H. Stefan, R. E. Ramsay, J. F. Wernicke, and et al. Effects of vagus nerve stimulation on amino acids and other metabolites in the csf of patients with partial seizures. *Epilepsy Res*, 20(3):221–7, 1995.

[10] A. T. Berg, S. F. Berkovic, M. J. Brodie, J. Buchhalter, J. H. Cross, W. van Emde Boas, J. Engel, J. French, T. A. Glauser, G. W. Mathern, S. L. Moshe, D. Nordli, P. Plouin, and I. E. Scheffer. Revised terminology and concepts for organization of seizures and epilepsies: report of the ilae commission on classification and terminology, 2005-2009. *Epilepsia*, 51(4):676–85, 2010.

[11] H. R. Berthoud and W. L. Neuhuber. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci*, 85(1-3):1–17, 2000.

[12] C. D. Binnie. Vagus nerve stimulation for epilepsy: a review. *Seizure*, 9(3):161–9, 2000.

[13] C. Bodin, S. Aubert, G. Daquin, R. Carron, D. Scavarda, A. McGonigal, and F. Bartolomei. Responders to vagus nerve stimulation (vns) in refractory epilepsy have reduced interictal cortical synchronicity on scalp eeg. *Epilepsy Res*, 113:98–103, 2015.

[14] B. Bonaz, C. Picq, V. Sinniger, J. F. Mayol, and D. Clarencon. Vagus nerve stimulation: from epilepsy to the cholinergic anti-inflammatory pathway. *Neurogastroenterol Motil*, 25(3):208–21, 2013.

[15] L. V. Borovikova, S. Ivanova, M. Zhang, H. Yang, G. I. Botchkina, L. R. Watkins, H. Wang, N. Abumrad, J. W. Eaton, and K. J. Tracey. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*, 405(6785):458–62, 2000.

[16] Eva K Bystrzycka and BS Nail. Brain stem nuclei associated with respiratory, cardiovascular and other autonomic functions. *The rat nervous system*, 2:95–110, 1985.

[17] A. P. Cecchini, E. Mea, V. Tullo, M. Curone, A. Franzini, G. Broggi, M. Savino, G. Bussone, and M. Leone. Vagus nerve stimulation in drug-resistant daily chronic migraine with depression: preliminary data. *Neurol Sci*, 30 Suppl 1:S101–4, 2009.

[18] J. H. Chae, Z. Nahas, M. Lomarev, S. Denslow, J. P. Lorberbaum, D. E. Bohning, and M. S. George. A review of functional neuroimaging studies of vagus nerve stimulation (vns). *J Psychiatr Res*, 37(6):443–55, 2003.

[19] M. H. Chase, Y. Nakamura, C. D. Clemente, and M. B. Sterman. Afferent vagal stimulation: neurographic correlates of induced eeg synchronization and desynchronization. *Brain Res*, 5(2):236–49, 1967.

[20] K. B. Clark, D. K. Naritoku, D. C. Smith, R. A. Browning, and R. A. Jensen. Enhanced recognition memory following vagus nerve stimulation in human subjects. *Nat Neurosci*, 2(1):94–8, 1999.

[21] P. Cristancho, M. A. Cristancho, G. H. Baltuch, M. E. Thase, and J. P. O'Reardon. Effectiveness and safety of vagus nerve stimulation for severe treatment-resistant major depression in clinical practice after fda approval: outcomes at 1 year. *J Clin Psychiatry*, 72(10):1376–82, 2011.

[22] J. T. Cunningham, S. W. Mifflin, G. G. Gould, and A. Frazer. Induction of c-fos and deltafosb immunoreactivity in rat brain by vagal nerve stimulation. *Neuropsychopharmacology*, 33(8):1884–95, 2008.

[23] Cyberonics. Cyberonics images and captions, 2013.

[24] Cyberonics. Vns therapy system physician's manual, 2014.

[25] R. Dantzer, J. P. Konsman, R. M. Bluthe, and K. W. Kelley. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? *Auton Neurosci*, 85(1-3):60–5, 2000.

[26] G. M. De Ferrari and P. J. Schwartz. Vagus nerve stimulation: from pre-clinical to clinical application: challenges and future directions. *Heart Fail Rev*, 16(2):195–203, 2011.

[27] V. De Herdt, J. De Waele, R. Raedt, T. Wyckhuys, R. El Tahry, K. Vonck, W. Wadman, and P. Boon. Modulation of seizure threshold by vagus nerve stimulation in an animal model for motor seizures. *Acta Neurol Scand*, 121(4):271–6, 2010.

[28] V. De Herdt, L. Puimege, J. De Waele, R. Raedt, T. Wyckhuys, R. El Tahry, C. Libert, W. Wadman, P. Boon, and K. Vonck. Increased rat serum corticosterone suggests immunomodulation by stimulation of the vagal nerve. *J Neuroimmunol*, 212(1-2):102–5, 2009.

[29] C. C. de Vos, L. Melching, J. van Schoonhoven, J. J. Ardesch, A. W. de Weerd, H. C. van Lambalgen, and M. J. van Putten. Predicting success of vagus nerve stimulation (vns) from interictal eeg. *Seizure*, 20(7):541–5, 2011.

[30] C. M. DeGiorgio, S. C. Schachter, A. Handforth, M. Salinsky, J. Thompson, B. Uthman, R. Reed, S. Collins, E. Tecoma, G. L. Morris, B. Vaughn, D. K. Naritoku, T. Henry, D. Labar, R. Gilmartin, D. Labiner, I. Osorio, R. Ristanovic, J. Jones, J. Murphy, G. Ney, J. Wheless, P. Lewis, and C. Heck. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. *Epilepsia*, 41(9):1195–200, 2000.

[31] C. M. DeGiorgio, J. Thompson, P. Lewis, S. Arrambide, D. Naritoku, A. Handforth, D. Labar, P. Mullin, and C. Heck. Vagus nerve stimulation: analysis of device parameters in 154 patients during the long-term xe5 study. *Epilepsia*, 42(8):1017–20, 2001.

[32] R. Dennert, H. J. Crijns, J. Smid, H. Klein, A. Gavazzi, S. Raspopovic, G. Milasinovic, M. Borggrefe, M. Zabel, G. De Ferrari, and P. J. Schwartz. Long-term benefits of vagal nerve stimulation therapy in heart failure. *Circulation*, 126(21), 2012.

[33] B. J. Dlouhy, S. V. Viljoen, D. K. Kung, T. W. Vogel, M. A. Granner, 3rd Howard, M. A., and H. Kawasaki. Vagus nerve stimulation after lead revision. *Neurosurg Focus*, 32(3):E11, 2012.

[34] A. E. Dorr and G. Debonnel. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *J Pharmacol Exp Ther*, 318(2):890–8, 2006.

[35] 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(2):287–93, 2011.

[36] G. Elger, C. Hoppe, P. Falkai, A. J. Rush, and C. E. Elger. Vagus nerve stimulation is associated with mood improvements in epilepsy patients. *Epilepsy Res*, 42(2-3):203–10, 2000.

[37] R. E. Elliott, A. Morsi, S. P. Kalhorn, J. Marcus, J. Sellin, M. Kang, A. Silverberg, E. Rivera, E. Geller, C. Carlson, O. Devinsky, and W. K. Doyle. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: long-term outcomes and predictors of response. *Epilepsy Behav*, 20(1):57–63, 2011.

[38] D. J. Englot, E. F. Chang, and K. I. Auguste. Vagus nerve stimulation for epilepsy: a meta-analysis of efficacy and predictors of response. *J Neurosurg*, 115(6):1248–55, 2011.

[39] R. A. Espana and C. W. Berridge. Organization of noradrenergic efferents to arousal-related basal forebrain structures. *J Comp Neurol*, 496(5):668–83, 2006.

[40] D. H. Evans and J. G. Murray. Histological and functional studies on the fibre composition of the vagus nerve of the rabbit. *J Anat*, 88(3):320–37, 1954.

[41] M. Fava. Diagnosis and definition of treatment-resistant depression. *Biol Psychiatry*, 53(8):649–59, 2003.

[42] James O. Foley and Franklin S. DuBois. Quantitative studies of the vagus nerve in the cat. i. the ratio of sensory to motor fibers. *The Journal of Comparative Neurology*, 67(1):49–67, 1937.

[43] F. Fornai, R. Ruffoli, F. S. Giorgi, and A. Paparelli. The role of locus coeruleus in the antiepileptic activity induced by vagus nerve stimulation. *Eur J Neurosci*, 33(12):2169–78, 2011.

[44] L. Forsgren, E. Beghi, A. Oun, and M. Sillanpaa. The epidemiology of epilepsy in europe - a systematic review. *Eur J Neurol*, 12(4):245–53, 2005.

[45] E. Frangos, J. Ellrich, and B. R. Komisaruk. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fmri evidence in humans. *Brain Stimul*, 8(3):624–36, 2015.

[46] A. Franzini, G. Messina, M. Leone, A. P. Cecchini, G. Broggi, and G. Bussone. Feasibility of simultaneous vagal nerve and deep brain stimulation in chronic cluster headache: case report and considerations. *Neurol Sci*, 30 Suppl 1:S137–9, 2009.

[47] M. Fraschini, M. Demuru, M. Puligheddu, S. Floridia, L. Polizzi, A. Maleci, M. Bortolato, A. Hillebrand, and F. Marrosu. The re-organization of functional brain networks in pharmaco-resistant epileptic patients who respond to vns. *Neurosci Lett*, 580:153–7, 2014.

[48] M. Fraschini, M. Puligheddu, M. Demuru, L. Polizzi, A. Maleci, G. Tamburini, S. Congia, M. Bortolato, and F. Marrosu. Vns induced desynchronization in gamma bands correlates with positive clinical outcome in temporal lobe pharmacoresistant epilepsy. *Neurosci Lett*, 536:14–8, 2013.

[49] X. Y. Gao, Y. H. Li, K. Liu, P. J. Rong, H. Ben, L. Li, B. Zhu, and S. P. Zhang. Acupuncture-like stimulation at auricular point heart evokes cardiovascular inhibition via activating the cardiac-related neurons in the nucleus tractus solitarius. *Brain Res*, 1397:19–27, 2011.

[50] W. H. Gaskell. The electrical changes in the quiescent cardiac muscle which accompany stimulation of the vagus nerve. *J Physiol*, 7(5-6):451–2, 1886.

[51] A. Grimonprez, R. Raedt, I. Dauwe, L. Mollet, L. E. Larsen, A. Meurs, V. De Herdt, W. Wadman, J. Delbeke, K. Vonck, and P. Boon. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. *Brain Stimul*, 8(1):13–20, 2015.

[52] A. Grimonprez, R. Raedt, L. De Taeye, L. E. Larsen, J. Delbeke, P. Boon, and K. Vonck. A preclinical study of laryngeal motor-evoked potentials as a marker vagus nerve activation. *Int J Neural Syst*, page 1550034, 2015.

[53] A. Grimonprez, R. Raedt, J. Portelli, I. Dauwe, L. E. Larsen, C. Bouckaert, J. Delbeke, E. Carrette, A. Meurs, V. De Herdt, P. Boon, and K. Vonck. The antidepressant-like

effect of vagus nerve stimulation is mediated through the locus coeruleus. *J Psychiatr Res*, 68:1–7, 2015.

[54] D. Gupta, S. Verma, and S. K. Vishwakarma. Anatomic basis of arnold's ear-cough reflex. *Surg Radiol Anat*, 8(4):217–20, 1986.

[55] A. C. Guyton and J.E. Hall. *Thextbook of Medical Physiology*. Elsevier Sauders, Philadelphia, 11th edition edition, 2006.

[56] T. Hallbook, J. Lundgren, G. Blennow, L. G. Stromblad, and I. Rosen. Long term effects on epileptiform activity with vagus nerve stimulation in children. *Seizure*, 14(8):527–33, 2005.

[57] E. J. Hammond, B. M. Uthman, S. A. Reid, and B. J. Wilder. Electrophysiologic studies of cervical vagus nerve stimulation in humans: Ii. evoked potentials. *Epilepsia*, 33(6):1021–8, 1992.

[58] A. Handforth and S. E. Krahl. Suppression of harmaline-induced tremor in rats by vagus nerve stimulation. *Mov Disord*, 16(1):84–8, 2001.

[59] W. He, X. H. Jing, B. Zhu, X. L. Zhu, L. Li, W. Z. Bai, and H. Ben. The auriculo-vagal afferent pathway and its role in seizure suppression in rats. *BMC Neurosci*, 14:85, 2013.

[60] C. Heck, S. L. Helmers, and C. M. DeGiorgio. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. *Neurology*, 59(6 Suppl 4):S31–7, 2002.

[61] T. R. Henry, R. A. Bakay, P. B. Pennell, C. M. Epstein, and J. R. Votaw. Brain blood-flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: Ii. prolonged effects at high and low levels of stimulation. *Epilepsia*, 45(9):1064–70, 2004.

[62] T. R. Henry, R. A. Bakay, J. R. Votaw, P. B. Pennell, C. M. Epstein, T. L. Faber, S. T. Grafton, and J. M. Hoffman. Brain blood flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: I. acute effects at high and low levels of stimulation. *Epilepsia*, 39(9):983–90, 1998.

[63] T. R. Henry, J. R. Votaw, P. B. Pennell, C. M. Epstein, R. A. Bakay, T. L. Faber, S. T. Grafton, and J. M. Hoffman. Acute blood flow changes and efficacy of vagus nerve stimulation in partial epilepsy. *Neurology*, 52(6):1166–73, 1999.

[64] J. C. Hodges, J. Treadwell, A. D. Malphrus, X. G. Tran, and A. P. Giardino. Identification and prevention of antiepileptic drug noncompliance: the collaborative use of state-supplied pharmaceutical data. *ISRN Pediatr*, 2014:734689, 2014.

[65] E. D. Hord, M. S. Evans, S. Mueed, B. Adamolekun, and D. K. Naritoku. The effect of vagus nerve stimulation on migraines. *J Pain*, 4(9):530–4, 2003.

[66] P. W. Hou, H. C. Hsu, Y. W. Lin, N. Y. Tang, C. Y. Cheng, and C. L. Hsieh. The history, mechanism, and clinical application of auricular therapy in traditional chinese medicine. *Evid Based Complement Alternat Med*, 2015:495684, 2015.

[67] W. Janig. Neurobiology of visceral afferent neurons: neuroanatomy, functions, organ regulations and sensations. *Biol Psychol*, 42(1-2):29–51, 1996.

[68] H. Jaseja. Mechanism of vagal nerve stimulation (vns) anti-convulsant action. *Med Hypotheses*, 66(3):680–1, 2006.

[69] H. Jaseja. Eeg-desynchronization as the major mechanism of anti-epileptic action of vagal nerve stimulation in patients with intractable seizures: clinical neurophysiological evidence. *Med Hypotheses*, 74(5):855–6, 2010.

[70] M. Kalia and J. M. Sullivan. Brainstem projections of sensory and motor components of the vagus nerve in the rat. *J Comp Neurol*, 211(3):248–65, 1982.

[71] G. Kember, J. L. Ardell, J. A. Armour, and M. Zamir. Vagal nerve stimulation therapy: what is being stimulated? *PLoS One*, 9(12):e114498, 2014.

[72] A. Kirchner, F. Birklein, H. Stefan, and H. O. Handwerker. Left vagus nerve stimulation suppresses experimentally induced pain. *Neurology*, 55(8):1167–71, 2000.

[73] A. Kirchner, H. Stefan, K. Bastian, and F. Birklein. Vagus nerve stimulation suppresses pain but has limited effects on neurogenic inflammation in humans. *Eur J Pain*, 10(5):449–55, 2006.

[74] B. Koo. Eeg changes with vagus nerve stimulation. *J Clin Neurophysiol*, 18(5):434–41, 2001.

[75] F. A. Koopman, P. R. Schuurman, M. J. Vervoordeldonk, and P. P. Tak. Vagus nerve stimulation: a new bioelectronics approach to treat rheumatoid arthritis? *Best Pract Res Clin Rheumatol*, 28(4):625–35, 2014.

[76] S. E. Krahl and K. B. Clark. Vagus nerve stimulation for epilepsy: A review of central mechanisms. *Surg Neurol Int*, 3(Suppl 4):S255–9, 2012.

[77] S. E. Krahl, K. B. Clark, D. C. Smith, and R. A. Browning. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia*, 39(7):709–14, 1998.

[78] S. E. Krahl, S. S. Senanayake, and A. Handforth. Destruction of peripheral c-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. *Epilepsia*, 42(5):586–9, 2001.

[79] S. E. Krahl, S. S. Senanayake, and A. Handforth. Right-sided vagus nerve stimulation reduces generalized seizure severity in rats as effectively as left-sided. *Epilepsy Res*, 56(1):1–4, 2003.

[80] R. Kuba, M. Guzaninova, M. Brazdil, Z. Novak, J. Chrastina, and I. Rektor. Effect of vagal nerve stimulation on interictal epileptiform discharges: a scalp eeg study. *Epilepsia*, 43(10):1181–8, 2002.

[81] R. Kuba, D. Nesvadba, M. Brazdil, H. Oslejskova, M. Ryzi, and I. Rektor. Effect of chronic vagal nerve stimulation on interictal epileptiform discharges. *Seizure*, 19(6):352–5, 2010.

[82] P. Kwan, A. Arzimanoglou, A. T. Berg, M. J. Brodie, W. Allen Hauser, G. Mathern, S. L. Moshe, E. Perucca, S. Wiebe, and J. French. Definition of drug resistant epilepsy: consensus proposal by the ad hoc task force of the ilae commission on therapeutic strategies. *Epilepsia*, 51(6):1069–77, 2010.

[83] P. Kwan and M. J. Brodie. Early identification of refractory epilepsy. *N Engl J Med*, 342(5):314–9, 2000.

[84] D. Labar. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. *Seizure*, 13(6):392–8, 2004.

[85] D. M. Labiner and G. L. Ahern. Vagus nerve stimulation therapy in depression and epilepsy: therapeutic parameter settings. *Acta Neurol Scand*, 115(1):23–33, 2007.

[86] G. Lange, M. N. Janal, A. Maniker, J. Fitzgibbons, M. Fobler, D. Cook, and B. H. Natelson. Safety and efficacy of vagus nerve stimulation in fibromyalgia: a phase i/ii proof of concept trial. *Pain Med*, 12(9):1406–13, 2011.

[87] M. E. Lenaerts, K. J. Oommen, J. R. Couch, and V. Skaggs. Can vagus nerve stimulation help migraine? *Cephalalgia*, 28(4):392–5, 2008.

[88] L. C. Lin, C. S. Ouyang, C. T. Chiang, H. C. Wu, and R. C. Yang. Early evaluation of the therapeutic effectiveness in children with epilepsy by quantitative eeg: a model of mozart k.448 listening–a preliminary study. *Epilepsy Res*, 108(8):1417–26, 2014.

[89] W. C. Liu, K. Mosier, A. J. Kalnin, and D. Marks. Bold fmri activation induced by vagus nerve stimulation in seizure patients. *J Neurol Neurosurg Psychiatry*, 74(6):811–3, 2003.

[90] J. S. Lockard, W. C. Congdon, and L. L. DuCharme. Feasibility and safety of vagal stimulation in monkey model. *Epilepsia*, 31 Suppl 2:S20–6, 1990.

[91] S. Manta, M. El Mansari, G. Debonnel, and P. Blier. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. *Int J Neuropsychopharmacol*, pages 1–12, 2012.

[92] M. Maroso, S. Balosso, T. Ravizza, J. Liu, E. Aronica, A. M. Iyer, C. Rossetti, M. Molteni, M. Casalgrandi, A. A. Manfredi, M. E. Bianchi, and A. Vezzani. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. *Nat Med*, 16(4):413–9, 2010.

[93] F. Marrosu, F. Santoni, M. Puligheddu, L. Barberini, A. Maleci, F. Ennas, M. Mascia, G. Zanetti, A. Tuveri, and G. Biggio. Increase in 20-50 hz (gamma frequencies) power spectrum and synchronization after chronic vagal nerve stimulation. *Clin Neurophysiol*, 116(9):2026–36, 2005.

[94] R. Marshall, I. Taylor, C. Lahr, T. L. Abell, I. Espinoza, N. K. Gupta, and C. R. Gomez. Bioelectrical stimulation for the reduction of inflammation in inflammatory bowel disease. *Clin Med Insights Gastroenterol*, 8:55–9, 2015.

[95] V. Martle, L. M. Van Ham, P. Boon, J. Caemaert, M. Tshamala, K. Vonck, R. Raedt, I. Polis, and S. Bhatti. Vagus nerve stimulator placement in dogs: Surgical implantation technique, complications, long-term follow-up, and practical considerations. *Vet Surg*, 45(1):71–8, 2016.

[96] A. Mauskop. Vagus nerve stimulation relieves chronic refractory migraine and cluster headaches. *Cephalalgia*, 25(2):82–6, 2005.

[97] A. McGregor, J. Wheless, J. Baumgartner, and D. Bettis. Right-sided vagus nerve stimulation as a treatment for refractory epilepsy in humans. *Epilepsia*, 46(1):91–6, 2005.

[98] R. S. McLachlan. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. *Epilepsia*, 34(5):918–23, 1993.

[99] C. A. Merrill, M. A. Jonsson, L. Minthon, H. Ejnell, C. son Silander H, K. Blennow, M. Karlsson, A. Nordlund, S. Rolstad, S. Warkentin, E. Ben-Menachem, and M. J. Sjogren. Vagus nerve stimulation in patients with alzheimer's disease: Additional follow-up results of a pilot study through 1 year. *J Clin Psychiatry*, 67(8):1171–8, 2006.

[100] L. Mollet, A. Grimonprez, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. *Acta Neurol Scand*, 128(6):391–6, 2013.

[101] L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, D. E. Herdt V, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Electrophysiological responses from vagus nerve stimulation in rats. *Int J Neural Syst*, 23(6):1350027, 2013.

[102] 3rd Morris, G. L. and W. M. Mueller. Long-term treatment with vagus nerve stimulation in patients with refractory epilepsy. the vagus nerve stimulation study group e01-e05. *Neurology*, 53(8):1731–5, 1999.

[103] George L Morris. A clinical guide to epileptic syndromes and their treatment. *JAMA*, 299(3):346–352, 2008.

[104] M. Navas, E. G. Navarrete, J. M. Pascual, R. Carrasco, J. A. Nunez, S. F. Shakur, J. Pastor, and R. G. Sola. Treatment of refractory epilepsy in adult patients with right-sided vagus nerve stimulation. *Epilepsy Res*, 90(1-2):1–7, 2010.

[105] T. J. Ness, R. B. Fillingim, A. Randich, E. M. Backensto, and E. Faught. Low intensity vagal nerve stimulation lowers human thermal pain thresholds. *Pain*, 86(1-2):81–5, 2000.

[106] J. A. Nichols, A. R. Nichols, S. M. Smirnakis, N. D. Engineer, M. P. Kilgard, and M. Atzori. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience*, 189:207–14, 2011.

[107] S. Nomura and N. Mizuno. Central distribution of primary afferent fibers in the arnold's nerve (the auricular branch of the vagus nerve): a transganglionic hrp study in the cat. *Brain Res*, 292(2):199–205, 1984.

[108] R. Norgren. Projections from the nucleus of the solitary tract in the rat. *Neuroscience*, 3(2):207–18, 1978.

[109] P. Norman and A. J. Bower. An autoradiographic study of the brainstem projections of vagal visceral afferent fibres in the domestic hen. *J Anat*, 134(Pt 3):583–9, 1982.

[110] P. W. Olejniczak, B. J. Fisch, M. Carey, G. Butterbaugh, L. Happel, and C. Tardo. The effect of vagus nerve stimulation on epileptiform activity recorded from hippocampal depth electrodes. *Epilepsia*, 42(3):423–9, 2001.

[111] I. Orosz, D. McCormick, N. Zamponi, S. Varadkar, M. Feucht, D. Parain, R. Griens, L. Vallee, P. Boon, C. Rittey, A. K. Jayewardene, M. Bunker, A. Arzimanoglou, and L. Lagae. Vagus nerve stimulation for drug-resistant epilepsy: a european long-term study up to 24 months in 347 children. *Epilepsia*, 55(10):1576–84, 2014.

[112] CH Parry. On the effects of compression of the arteries in various diseases, and particularly in those of the head; with hints towards a new mode of treating nervous disorders. *Mem Med Soc Lond*, 3:77–113, 1792.

[113] J. K. Penry and J. C. Dean. Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. *Epilepsia*, 31 Suppl 2:S40–3, 1990.

[114] E. T. Peuker and T. J. Filler. The nerve supply of the human auricle. *Clin Anat*, 15(1):35–7, 2002.

[115] D. A. Prince, B. W. Connors, and L. S. Benardo. Mechanisms underlying interictal-ictal transitions. *Adv Neurol*, 34:177–87, 1983.

[116] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[117] A. Randich and S. A. Aicher. Medullary substrates mediating antinociception produced by electrical stimulation of the vagus. *Brain Res*, 445(1):68–76, 1988.

[118] A. Randich, C. L. Thurston, P. S. Ludwig, J. D. Robertson, and C. Rasmussen. Intravenous morphine-induced activation of vagal afferents: peripheral, spinal, and cns substrates mediating inhibition of spinal nociception and cardiovascular responses. *J Neurophysiol*, 68(4):1027–45, 1992.

[119] K. Ren, M. Zhuo, A. Randich, and G. F. Gebhart. Vagal afferent stimulation-produced effects on nociception in capsaicin-treated rats. *J Neurophysiol*, 69(5):1530–40, 1993.

[120] J. A. Ricardo and E. T. Koh. 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*, 153(1):1–26, 1978.

[121] K. Rijkers, M. Aalbers, G. Hoogland, L. van Winden, J. Vles, H. Steinbusch, and M. Majoie. Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat. *Brain Res*, 1319:155–63, 2010.

[122] H. A. Ring, S. White, D. C. Costa, R. Pottinger, J. P. Dick, T. Koeze, and J. Sutcliffe. A spect study of the effect of vagal nerve stimulation on thalamic activity in patients with epilepsy. *Seizure*, 9(6):380–4, 2000.

[123] P. Rizzo, M. Beelke, F. De Carli, P. Canovaro, L. Nobili, A. Robert, P. Tanganelli, G. Regesta, and F. Ferrillo. Chronic vagus nerve stimulation improves alertness and reduces rapid eye movement sleep in patients affected by refractory epilepsy. *Sleep*, 26(5):607–11, 2003.

[124] P. Rong, A. Liu, J. Zhang, Y. Wang, W. He, A. Yang, L. Li, H. Ben, H. Liu, P. Wu, R. Liu, Y. Zhao, F. Huang, X. Li, and B. Zhu. Transcutaneous vagus nerve stimulation for refractory epilepsy: a randomized controlled trial. *Clin Sci (Lond)*, 2014.

[125] R. W. Roosevelt, D. C. Smith, R. W. Clough, R. A. Jensen, and R. A. Browning. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res*, 1119(1):124–32, 2006.

[126] R. Ruffoli, F. S. Giorgi, C. Pizzanelli, L. Murri, A. Paparelli, and F. Fornai. The chemical neuroanatomy of vagus nerve stimulation. *J Chem Neuroanat*, 42(4):288–96, 2011.

[127] A. J. Rush, L. B. Marangell, H. A. Sackeim, M. S. George, S. K. Brannan, S. M. Davis, R. Howland, M. A. Kling, B. R. Rittberg, W. J. Burke, M. H. Rapaport, J. Zajecka, A. A. Nierenberg, M. M. Husain, D. Ginsberg, and R. G. Cooke. Vagus nerve stimulation for treatment-resistant depression: a randomized, controlled acute phase trial. *Biol Psychiatry*, 58(5):347–54, 2005.

[128] R. M. Sadler, R. A. Purdy, and S. Rahey. Vagal nerve stimulation aborts migraine in patient with intractable epilepsy. *Cephalalgia*, 22(6):482–4, 2002.

[129] M. C. Salinsky and K. J. Burchiel. Vagus nerve stimulation has no effect on awake eeg rhythms in humans. *Epilepsia*, 34(2):299–304, 1993.

[130] E. Santiago-Rodriguez, M. Alonso-Vanegas, L. Cardenas-Morales, T. Harmony, M. Bernardino, and A. Fernandez-Bouzas. Effects of two different cycles of vagus nerve stimulation on interictal epileptiform discharges. *Seizure*, 15(8):615–20, 2006.

[131] T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, I. Heuser, M. Bajbouj, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. Van den Abbeele, Z. Stanga, and M. Hasdemir. Vagus nerve stimulation for depression: efficacy and safety in a european study. *Psychol Med*, 38(5):651–61, 2008.

[132] P. J. Schwartz, G. M. De Ferrari, A. Sanzo, M. Landolina, R. Rordorf, C. Raineri, C. Campana, M. Revera, N. Ajmone-Marsan, L. Tavazzi, and A. Odero. Long term vagal stimulation in patients with advanced heart failure: first experience in man. *Eur J Heart Fail*, 10(9):884–91, 2008.

[133] M. Y. Shen, Y. L. Luo, C. H. Yang, T. Ruan, and C. J. Lai. Hypersensitivity of lung vagal c fibers induced by acute intermittent hypoxia in rats: role of reactive oxygen species and trpa1. *Am J Physiol Regul Integr Comp Physiol*, 303(11):R1175–85, 2012.

[134] Y. Shirasaka and C. G. Wasterlain. The effect of urethane anesthesia on evoked potentials in dentate gyrus. *Eur J Pharmacol*, 282(1-3):11–7, 1995.

[135] S. Spuck, G. Nowak, A. Renneberg, V. Tronnier, and J. Sperner. Right-sided vagus nerve stimulation in humans: an effective therapy? *Epilepsy Res*, 82(2-3):232–4, 2008.

[136] H. Stefan, G. Kreiselmeyer, F. Kerling, K. Kurzbuch, C. Rauch, M. Heers, B. S. Kasper, T. Hammen, M. Rzonsa, E. Pauli, J. Ellrich, W. Graf, and R. Hopfengartner. Transcutaneous vagus nerve stimulation (t-vns) in pharmacoresistant epilepsies: a proof of concept trial. *Epilepsia*, 53(7):e115–8, 2012.

[137] M. Takaya, W. J. Terry, and D. K. Naritoku. Vagus nerve stimulation induces a sustained anticonvulsant effect. *Epilepsia*, 37(11):1111–6, 1996.

[138] A. Thakar, K. K. Deepak, and S. S. Kumar. Auricular syncope. *J Laryngol Otol*, 122(10):1115–7, 2008.

[139] M. Tosato, K. Yoshida, E. Toft, V. Nekrasas, and J. J. Struijk. Closed-loop control of the heart rate by electrical stimulation of the vagus nerve. *Med Biol Eng Comput*, 44(3):161–9, 2006.

[140] K. J. Tracey. The inflammatory reflex. *Nature*, 420(6917):853–9, 2002.

[141] K. J. Tracey. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest*, 117(2):289–96, 2007.

[142] I. J. Ungar, J. T. Mortimer, and J. D. Sweeney. Generation of unidirectionally propagating action potentials using a monopolar electrode cuff. *Ann Biomed Eng*, 14(5):437–50, 1986.

[143] H. Ura, Y. Sugaya, H. Ohata, I. Takumi, K. Sadamoto, T. Shibasaki, and E. Maru. Vagus nerve stimulation induced long-lasting enhancement of synaptic transmission and decreased granule cell discharge in the hippocampal dentate gyrus of urethane-anesthetized rats. *Brain Res*, 1492:63–71, 2013.

[144] B. M. Uthman, B. J. Wilder, E. J. Hammond, and S. A. Reid. Efficacy and safety of vagus nerve stimulation in patients with complex partial seizures. *Epilepsia*, 31 Suppl 2:S44–50, 1990.

[145] K. Van Laere, K. Vonck, P. Boon, J. Versijpt, and R. Dierckx. Perfusion spect changes after acute and chronic vagus nerve stimulation in relation to prestimulus condition and long-term clinical efficacy. *J Nucl Med*, 43(6):733–44, 2002.

[146] E. C. Ventureyra. Transcutaneous vagus nerve stimulation for partial onset seizure therapy. a new concept. *Childs Nerv Syst*, 16(2):101–2, 2000.

[147] K. Vonck, P. Boon, K. Van Laere, M. D'Have, T. Vandekerckhove, S. O'Connor, B. Brans, R. Dierckx, and J. De Reuck. Acute single photon emission computed tomographic study of vagus nerve stimulation in refractory epilepsy. *Epilepsia*, 41(5):601–9, 2000.

[148] A. Vuckovic, M. Tosato, and J. J. Struijk. 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*, 5(3):275–86, 2008.

[149] Y. Wang, J. Cui, R. C. Chan, Y. Deng, H. Shi, X. Hong, Z. Li, X. Yu, Q. Y. Gong, and D. Shum. Meta-analysis of prospective memory in schizophrenia: nature, extent, and correlates. *Schizophr Res*, 114(1-3):64–70, 2009.

[150] D. M. Woodbury and J. W. Woodbury. Effects of vagal stimulation on experimentally induced seizures in rats. *Epilepsia*, 31 Suppl 2:S7–19, 1990.

[151] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

[152] J. Zabara. Inhibition of experimental seizures in canines by repetitive vagal stimulation. *Epilepsia*, 33(6):1005–12, 1992.

[153] L. Zaborszky, W. E. Cullinan, and V. N. Luine. Catecholaminergic-cholinergic interaction in the basal forebrain. *Prog Brain Res*, 98:31–49, 1993.

[154] A. Zanchetti, S. C. Wang, and G. Moruzzi. The effect of vagal afferent stimulation on the eeg pattern of the cat. *Electroencephalogr Clin Neurophysiol*, 4(3):357–61, 1952.

Chapter 3

Research Aims

Vagus Nerve Stimulation (VNS) has become an established therapeutic alternative to medically refractory epilepsy, with more that 70,000 patients treated on a world-wide basis, and is additionally gaining popularity in the treatment of drug resistant depression [1–3]. In Europe, more than 20 years have passed since regulatory approval of VNS. Though significant progress has been made, there is still a long way to understanding the mechanisms underlying therapeutic effects of VNS. A majority of studies have examined the effects of VNS on various transmitter systems in the brain, using a variety of biochemical and histochemical techniques [4–7]. Very few studies have used means of electrophysiology to characterize effects of VNS on neurophysiology and even fewer studies have conducted such experiments in the awake state. This was the rationale for initiation of our initial investigations on neurophysiological mechanisms of VNS, using various electrophysiological techniques, in awake freely moving rats, whereby well documented interfering neurophysiological effects of anesthetics are avoided.

The initial goal of this thesis was to use means of electrophysiology to identify markers of effective VNS delivery (**Chapter 4**). This included electroencephalography (EEG) and evoked potentials. The subsequent aim was to use the same markers to characterize effects of VNS on hippocampal excitability (**Chapter 4**). Due to the limitations of traditional clinical outcomes (e.g. seizure counts in epilepsy), where longer treatment durations are necessary to obtain an estimate of the treatment efficacy, the use of neurophysiological outcomes may constitute a feasible alternative to obtain a more immediate estimate of the effects associated with VNS. Following identification of markers of effective VNS delivery in **Chapter 4**, we thus aimed to use the same markers to assess the effectiveness of various VNS parameters (**Chapter 5**). In the following experimental work (**Chapter 6**), we follow up on the findings made in the initial experimental works (**Chapter 4 and 5**) and explore potential effects of VNS on thermoregulation in rats, which was indicated by the electrophysiological outcomes. Due to the extensive physiological influences of temperature, this constituted a potential pivotal finding in VNS research. Despite decades of VNS research, no previous studies have reported any effects of VNS on temperature. In a final study (**Chapter 7**), the aim was to further assess the translatability of our findings, by comparing effects of VNS on electrophysiological correlates in rats and patients.

References

[1] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[2] T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, I. Heuser, M. Bajbouj, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. Van den Abbeele, Z. Stanga, and M. Hasdemir. Vagus nerve stimulation for depression: efficacy and safety in a european study. *Psychol Med*, 38(5):651–61, 2008.

[3] D. J. Englot, E. F. Chang, and K. I. Auguste. Vagus nerve stimulation for epilepsy: a meta-analysis of efficacy and predictors of response. *J Neurosurg*, 115(6):1248–55, 2011.

[4] D. L. Hassert, T. Miyashita, and C. L. Williams. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. *Behav Neurosci*, 118(1):79–88, 2004.

[5] R. W. Roosevelt, D. C. Smith, R. W. Clough, R. A. Jensen, and R. A. Browning. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res*, 1119(1):124–32, 2006.

[6] A. E. Dorr and G. Debonnel. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *J Pharmacol Exp Ther*, 318(2):890–8, 2006.

[7] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

Part II

Experimental Work

Chapter 4

Modulation of Hippocampal Activity by Vagus Nerve Stimulation in Freely Moving Rats

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Abstract

Background: Vagus Nerve Stimulation (VNS) has seizure-suppressing effects but the underlying mechanism is not fully understood. To further elucidate the mechanisms underlying VNS-induced seizure suppression at a neurophysiological level, the present study examined effects of VNS on hippocampal excitability using dentate gyrus evoked potentials (EPs) and hippocampal electroencephalography (EEG). *Methods:* Male Sprague–Dawley rats were implanted with a VNS electrode around the left vagus nerve. A bipolar stimulation electrode was implanted in the left perforant path and a bipolar recording electrode was implanted in the left dentate gyrus for EEG and dentate field EP recording. Following recovery, VNS was applied in freely moving animals, using a duty cycle of 7 s on/18 s off, 30 Hz frequency, 250 μs pulse width, and an intensity of either 0 (SHAM), 25 μA or 1000 μA, while continuously monitoring EEG and dentate field EPs. *Results:* VNS at 1000 μA modulated dentate field EPs by decreasing the field excitatory post-synaptic potential (fEPSP) slope and increasing the latency and amplitude of the population spike. It additionally influenced hippocampal EEG by slowing theta rhythm from 7 Hz to 5 Hz and reducing theta peak and gamma band power. No effects were observed in the SHAM or 25 μA VNS conditions. *Conclusion:* VNS modulated hippocampal excitability of freely moving rats in a complex way. It decreased synaptic efficacy, reflected by decreased fEPSP slope and EEG power, but it simultaneously facilitated dentate granule cell discharge indicating depolarization of dentate granule cells.

Introduction

Vagus Nerve Stimulation (VNS) is approved for the treatment of pharmacoresistant epilepsies [9] and depression [10]. We recently documented significant acute anticonvulsant effects of a rapid cycle VNS paradigm in a rat model of temporal lobe seizures, which depended on noradrenergic signaling in the hippocampus [32]. This has prompted further investigation of anticonvulsant mechanisms of VNS at a neurophysiological level. Among other neurophysiological parameters, dentate field evoked potentials (EPs) have previously been used extensively to probe excitability

of dentate neurotransmission [22, 23, 31]. Upon electrical stimulation of the perforant path, a field excitatory post-synaptic potential (fEPSP) is evoked as a result of excitatory synaptic transmission, which will result in a synchronous discharge of dentate granule cells as the discharge threshold is reached, reflected by a superimposed population spike. Perforant path evoked dentate field EPs thus constitute an opportunity to investigate different aspects of excitability, which is enhanced and considered a core aspect of epilepsy [30]. Considering the anticonvulsant efficacy of VNS, a general reduction in hippocampal excitability, reflected by a reduction in excitatory neurotransmission, could be expected. However, activation of noradrenergic signaling, which is involved in the anticonvulsant effect of VNS [32], is typically associated with an increased discharge of dentate granule cells and thus a larger population spike [14, 15, 27, 35]. To gain more insight into the effects of a VNS paradigm with anticonvulsant properties on hippocampal excitability, changes in dentate field EPs and spontaneous hippocampal electroencephalography (EEG) during rapid cycle VNS were monitored in freely moving rats.

Material and methods

Thirty male Sprague–Dawley rats (Harlan Laboratories) were used in this study. Upon arrival, animals were housed under environmentally controlled conditions at a 12 h/12 h light/dark cycle with ad libitum access to food and water. Prior to any experimental procedures, animals were handled daily by experimenters for at least 2 weeks. All procedures were conducted in accordance with the local animal experimental committee (Ghent University Hospital, Ghent, Belgium, ECD 12/63) and in accordance with the European directive 2010/63/EU.

Surgery

After the initial handling period, rats (300–380 g) underwent surgery under isoflurane anesthesia (5% induction, 2% maintenance, mixed with medical oxygen). Animals were implanted with a custom-made cuff electrode around the left vagus nerve (details of the procedures have been previously described [32]). In addition, a bipolar electrode for recording, consisting of two twisted 70 μm polyimide coated stainless steel wires (0.9 mm tip separation), was implanted in the hilus (most ventral tip) of the left dentate gyrus (3.8 mm posterior and 1.9 mm lateral relative to bregma, 3 mm ventral relative to brain surface) using electrophysiological feedback. A bipolar electrode for stimulation, consisting of two twisted 125 μm polyimide coated stainless steel wires, was placed in the left dorsomedial perforant path [23] (7–8 mm posterior and 3.9 mm lateral relative to bregma, 2.5 mm ventral to brain surface) at the point where a maximal dentate population spike was evoked. In a subset of eight animals, an additional bipolar recording electrode (0.9 mm tip separation) was implanted in the left prefrontal cortex (3.2 mm anterior and 0.6 mm lateral relative to bregma, 3.8 mm ventral relative to brain surface). All electrode leads were assembled in a connector block anchored to the skull with microscrews and dental acrylic cement. Animals were left for at least 6 weeks to recover and analgesics (buprenorphine 0.03 mg/kg/day, subcutaneously) were administered on the first 2 days after surgery. Handling was resumed from the second week of recovery until the moment of the first recording session.

Recording sessions

After at least 6 weeks of recovery, animals were connected to a video-EEG setup allowing free movement via an electric swivel. Following habituation to the setup for at least a day, animals were subjected to recording sessions, during which effects of VNS on dentate field EPs and hippocampal EEG were assessed. Recording sessions (**Fig. 4.1**) consisted of a 1 hour baseline

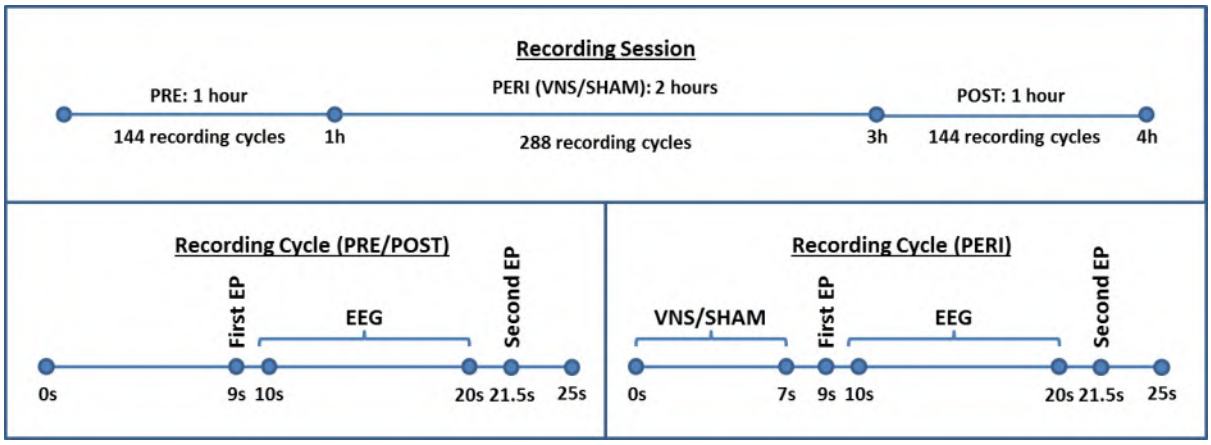


Figure 4.1: Schematic illustration of the recording design. For all recording sessions, a 1 hour baseline period (PRE) preceded a 2 hour Vagus Nerve Stimulation (VNS) or SHAM period (PERI) followed by another hour of recordings after VNS or SHAM (POST). All data were acquired during the off periods of the VNS duty cycle (7 s on, 18 s off), allowing the acquisition of two perforant path evoked dentate field potentials (EPs) and a 10 second EEG sweep during each cycle. Each animal underwent three recording sessions using VNS intensities of 0 (SHAM), 25 μA and 1000 μA.

recording (PRE), followed by a 2 hour recording period during VNS or SHAM treatment (PERI) and concluded by 1 hour of recordings after VNS or SHAM treatment (POST). VNS or SHAM treatment was applied with a rapid cycle (7 s on, 18 s off), which is one of the clinically used regimens to treat patients not responding to the more conventional regimen (30 s on, 300 or 600 s off) [19, 20]. Previous work from our laboratory showed a robust anticonvulsant efficacy along with an increase in hippocampal norepinephrine, using this rapid VNS duty cycle [32]. Animals underwent three recording sessions with different VNS intensities: 0 (SHAM), 25 μA, and 1000 μA conducted in the dark phase, where animals typically are awake. Intensities (25 μA and 1000 μA) were chosen to be respectively below and above the threshold for activation of vagal afferents, based on previous work [7, 25, 40]. VNS with an output current of 800 μA–1000 μA has previously yielded significant anticonvulsant effects [16–18, 32, 34]. The pulse width was 250 μs and stimulation frequency 30 Hz. In the VNS 'off' phase of the duty cycle (i.e. 18 s), two dentate field EPs were acquired, separated by the acquisition of a 10 s spontaneous EEG sweep. Prior to the recording sessions, an input/output relationship was generated between the dentate field EP response and perforant path stimulation intensities. In the following recording session, dentate field EPs were obtained in response to single bipolar pulses of 100 μs at an intensity evoking a population spike with an amplitude around 75% of the maximal population spike. The data acquisition cycles were repeated throughout all recording sessions, resulting in 144 recording cycles before VNS/SHAM (PRE), 288 during VNS/SHAM (PERI) and 144 after VNS/SHAM (POST). Impedances of the VNS electrode and the perforant path stimulation electrode were assessed before each session. In a separate experiment, using animals (n = 8) implanted with recording electrodes in both the hippocampal formation and the prefrontal cortex, effects of rapid cycle VNS on hippocampal and cortical EEG were assessed simultaneously. Following a 1 hour baseline period (PRE), VNS was applied for 2 hours. In each 'off' period of the VNS duty cycle, a 10 s EEG sweep was recorded (as the design illustrated, **Fig. 4.1**, but without recording dentate field EPs). One VNS session was conducted during the dark phase, while animals were awake, and another session was conducted during the day, while animals were sleeping.

Electrophysiology

All signals were acquired referencing each recording electrode to a stainless steel microscrew placed epidurally over the right frontal lobe. The signals were subjected to high pass filtering at 0.1 Hz and amplified 100 times. Dentate field EP sweeps were digitized at 20 kHz and EEG was digitized at 1 kHz with a 16 bit dynamic and a ± 3.05 μV resolution. All data were digitized with a USB-6259 National Instruments data acquisition device (National Instruments, Austin, Texas, USA) and stored locally on a computer for offline analysis.

Data analysis

All data were processed using Matlab (The MathWorks, Inc., Natick, US). For dentate field EP sweeps, fEPSP slope, population spike amplitude and population spike latency to the stimulus onset were calculated. fEPSP slope was calculated by fitting a slope to the initial fEPSP before the onset of the population spike using least squares. Population spike amplitude was calculated by drawing a line between positive peak of the fEPSP before population spike onset and the positive peak after the population spike and optimizing the line to become tangent to the fEPSP waveform. Population spike amplitude was calculated as the vertical distance from this line to the negative peak of the population spike. Population spike latency was calculated as the time between the stimulus onset and the negative peak of the population spike. To focus on local hippocampal or cortical activity, the differential hippocampal or cortical EEG was calculated by subtracting both hippocampal or both cortical EEG signals. EEG sweeps were rejected as artifacts when total power deviated more than 3 standard deviations from the mean of all sweeps. Signals were high pass filtered offline at 2 Hz before performing spectral analysis using the Fast Fourier algorithm. Aiming at a frequency resolution of 1 Hz and a frequency range of 2–100 Hz, each 10 s EEG sweep was split into 1 s windows overlapping by 0.5 s. The resulting 19 power spectra were averaged to provide the power spectrum of each 10 s EEG sweep. Theta peak power and frequency were determined by averaging spectrograms covering 20 minute epochs into a single spectrogram. Total band (2–100 Hz) and gamma band (35–100 Hz) power were determined for each sweep by calculating the sum of power within the respective bands. Before any statistical analysis, hippocampal EEG and dentate field EP parameters were normalized to the PRE period mean. For all calculated EEG and EP parameters, outliers were defined as observations deviating by more than 3 standard deviations from the mean across a full session. Statistical analyses were performed using R 2.12.1 (R Development Core Team), SPSS 22.0 (IBM Corporation, Armonk, New York, US) or SigmaPlot 11.0 (Systat Software Inc., San Jose, US). For statistical analysis, all outcomes were averaged over 20 minute epochs and processed in repeated measures two-way ANOVAs with the factor condition (VNS 1000 μA, VNS 25 μA and SHAM) and time (12 epochs of 20 minutes). To assess differences between conditions within specific 20 minute epochs, Student–Newman–Keuls Method for post hoc testing was applied. Methods specific to the creation of specific plots used for data visualization, such as representation of averaged dentate field EP traces or hippocampal spectrograms, are stated in figure legends. All time plots were made with SigmaPlot. Values are expressed as mean $\pm$ standard error of the mean unless otherwise stated.

Results

Hippocampal EEG recorded in awake rats as a predictor of correct VNS delivery

Previous studies [8, 28] used a reduction in cortical EEG power during slow wave sleep to assess effective delivery of VNS in rats. In a first experiment, we examined whether hippocampal EEG

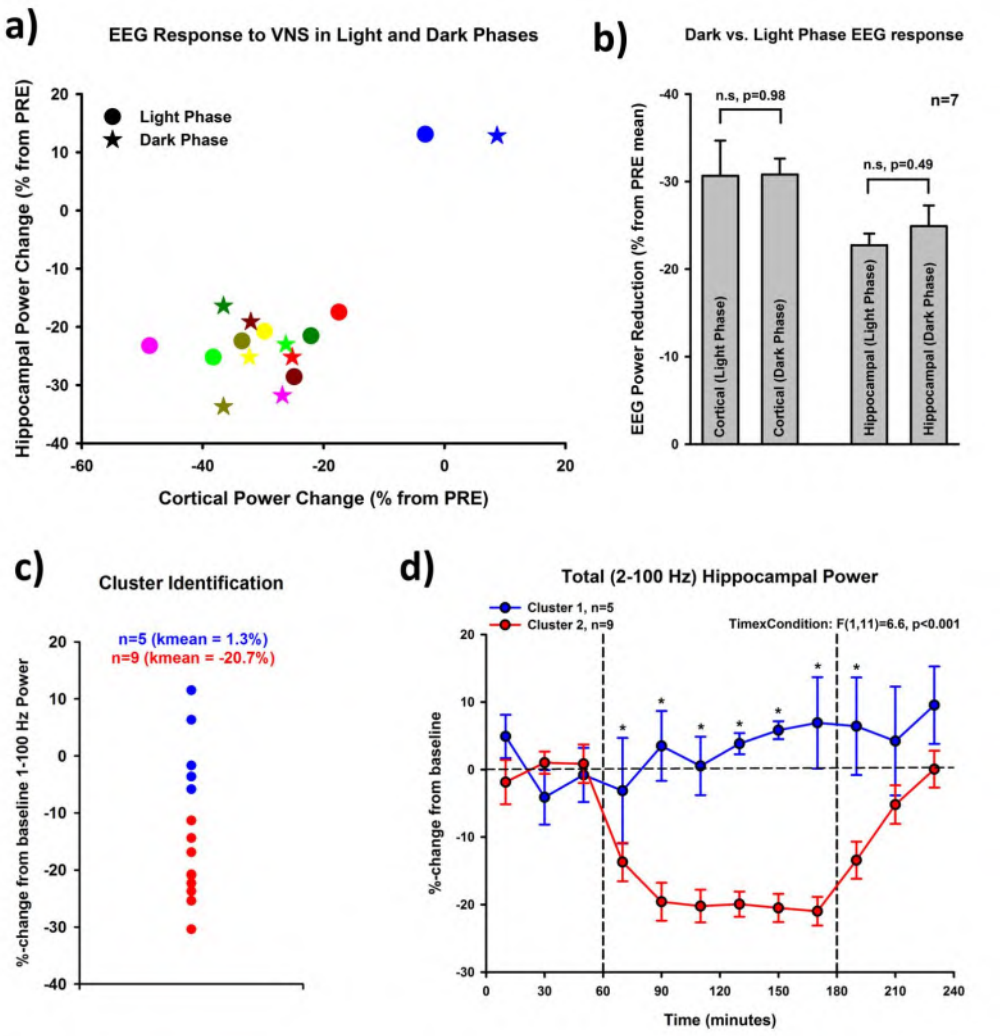


Figure 4.2: EEG power reduction as a predictor of effective Vagus Nerve Stimulation. In a subexperiment, Vagus Nerve Stimulation (VNS) was observed to reduce EEG power both in the prefrontal cortex and in the hippocampal formation. In **(a)**, the percentage change from the PRE period in 2–100 Hz EEG power during the last hour of the PERI is depicted. Circles denote measurements obtained during the light phase, where animals typically sleep, and stars denote measurements obtained during the dark phase, where animals typically are awake. Colors code for individual animals. The data reveal that a reduction in cortical power always was associated with a simultaneous reduction in hippocampal EEG power yielding seven effectively stimulated and one ineffectively stimulated rat. In **(b)**, average ($\pm$ standard error of the mean) cortical and hippocampal EEG responses to VNS obtained during the light and dark phases, from the seven effectively stimulated animals, are depicted. In the subsequent main experiment, hippocampal EEG, recorded during the dark phase where animals typically are awake, was used as a predictor of effective VNS delivery. The average change from the PRE period in EEG power over the last hour of the PERI period, where VNS at 1000 μA was delivered, was used as variable in a k-means clustering analysis. The outcome was two clusters of five and nine rats, with a mean change of 1.3% and -20.7% respectively, identifying an ineffectively and effectively stimulated group of rats **(c)**. In **(d)**, the mean (20 minute epochs $\pm$ standard error of the mean) responses to VNS at 1000 μA of the two clusters have been plotted over time. Vertical dashed lines denote the beginning and end of VNS.

during the awake state can also be used to assess effective VNS delivery. We examined how changes in total (2–100 Hz) cortical EEG power relate to changes in hippocampal EEG power during VNS during the light (mainly sleeping state) and dark (mainly awake state) phases of the day (**Fig. 4.2a**). A k-means cluster analysis revealed that seven out of eight rats displayed a VNS-induced reduction in both cortical and hippocampal EEG power both during the light and dark phases. In the cluster of seven rats responding to VNS with a reduction in cortical and hippocampal EEG power, there was no difference in the response to VNS during light or dark phase (**Fig. 4.2b**; cortical EEG power changes in light vs. dark phases: -30.8% vs. -30.7%, $t(6) = 0.03$, $p = 0.98$; hippocampal EEG power changes in light vs. dark phases: -22.7% vs. -24.9%, $t(6) = 0.73$, $p = 0.49$). In the main experiment, 14/22 rats displayed dentate field EPs of good quality, characterized by prominent population spikes and were used for subsequent recordings. Cluster analysis revealed that application of VNS at 1000 μA reduced hippocampal EEG power, measured during the dark phase, in the 2–100 Hz spectrum in 9/14 rats while no effects were seen in 5/14 rats (**Fig. 4.2c and d**). Subsequent analyses focused on these 9/14 rats.

Baseline relationship between dentate field EP parameters

Input/output relationships, generated prior to recording sessions, revealed a systematic relation between fEPSP, population spike amplitude and population spike latency. As the intensity of the perforant path stimulus increased, fEPSP slope and population spike amplitude increased (**Fig. 4.3a and b**), while population spike latency decreased. This resulted in a positive correlation between fEPSP slope and population spike amplitude (**Fig. 4.3c**), a negative correlation between fEPSP slope and population spike latency and between population spike amplitude and population spike latency (**Fig. 4.3d**) within the perforant path stimulation intensity range evoking a population spike.

Effects of VNS on dentate field EPs

VNS at 1000 μA modulated all aspects of the dentate field EP analyzed (**Fig. 4.4a**). Thus, compared to SHAM, VNS at 1000 μA decreased the fEPSP slope (**Fig. 4.4b**; -15.4 $\pm$ 1.6% at the end of the PERI period), while an increase was observed in the population spike amplitude (**Fig. 4.4c**; 41.3 $\pm$ 23.5% at the end of the PERI period) and in the population spike latency (**Fig. 4.4d**; 24.5 $\pm$ 2.9% at the end of the PERI period). Compared to SHAM, there were no changes in the VNS 25 μA condition. The two-way repeated measures ANOVAs yielded interactions between time and condition for the fEPSP slope ($F(11,2) = 6.3$, $p < 0.001$), the population spike amplitude ($F(11,2) = 3.9$, $p < 0.001$) and for the population spike latency ($F(11,2) = 38.4$, $p < 0.001$).

Effects of VNS on hippocampal EEG

Application of VNS at 1000 μA modulated the entire 2–100 Hz hippocampal spectrum, except from a narrow 20–35 Hz spectral band where no changes were observed (**Fig. 4.5a and b**). Thus, application of VNS at 1000 μA reduced total power within the 2–100 Hz spectrum (**Fig. 4.5c**; –20.9 $\pm$ 2.1% at the end of the PERI period; time $\times$ stimulation condition: $F(11,2) = 3.9$, $p < 0.001$). Similarly, VNS at 1000 μA prominently affected hippocampal theta activity, reducing the theta peak power (**Fig. 4.5d**; -25.8 $\pm$ 4.2% at the end of the PERI period; time $\times$ stimulation condition: $F(11,2) = 2.2$, $p < 0.01$) and shifting the theta peak frequency from 7 Hz at baseline to 5 Hz toward the end of the PERI period (**Fig. 4.5f**; time $\times$ stimulation condition: $F(11,2) = 4.1$, $p < 0.001$). Gamma band power (35–100 Hz) was also reduced by VNS at 1000 μA (**Fig. 4.4e**; -23.9 $\pm$ 1.6% at the end of the PERI period; time $\times$ stimulation

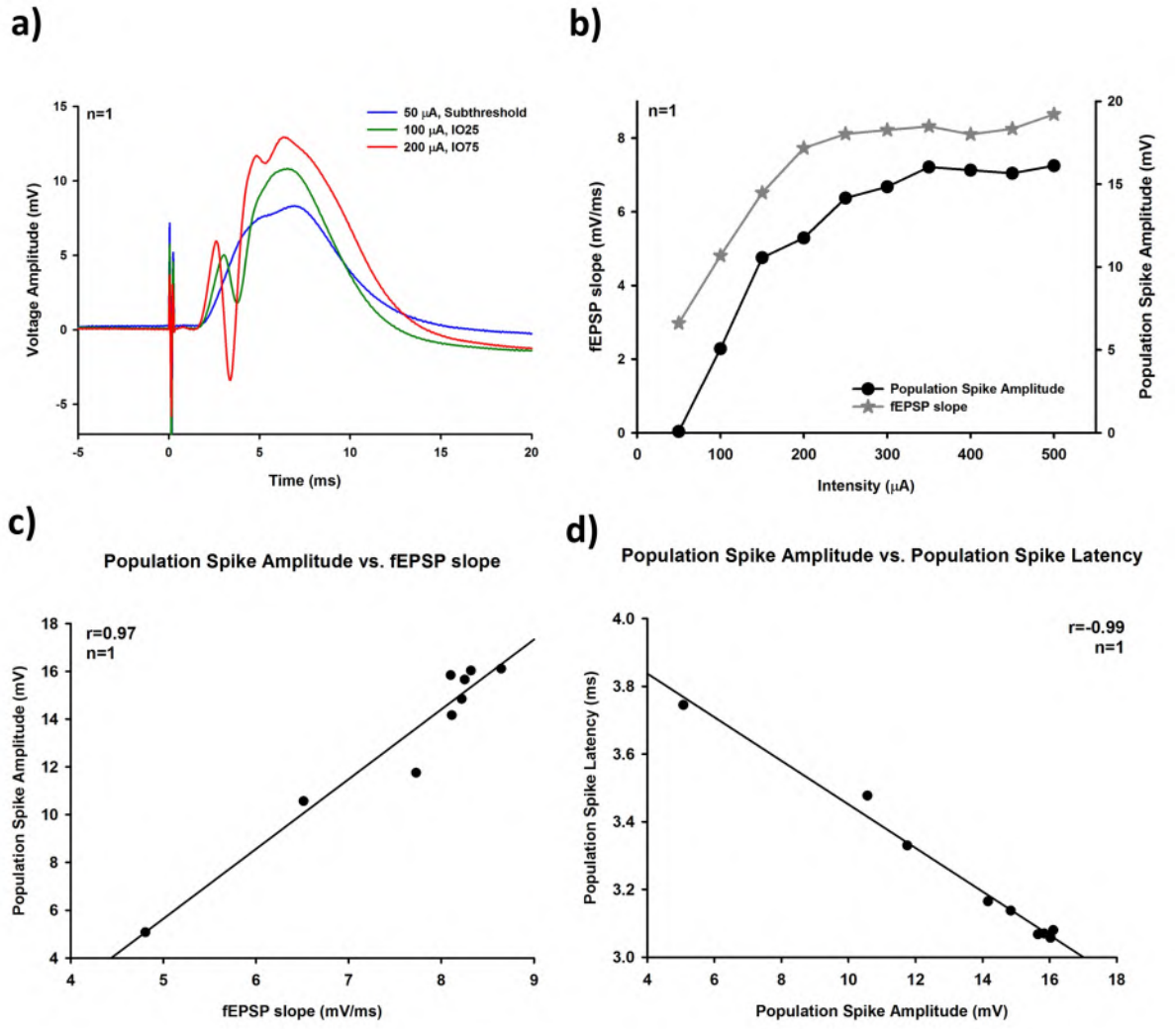


Figure 4.3: Input/output relationships of dentate field evoked potentials. Input/output relationships of dentate field evoked potentials were generated by gradually ramping up the perforant path stimulation intensity. **(a)** Averaged traces (20 traces averaged) from one representative rat for three representative intensities: an intensity below the threshold for a population spike (blue), an intensity evoking a population spike with an amplitude of 25% of the maximal population spike (green), and an intensity evoking a population spike with an amplitude of 75% of the maximal population spike (red). In response to increasing perforant path stimulation intensities, field EPSP (fEPSP) slope and population spike amplitude increased **(b)**, while population spike latency decreased. The relationship between the changes in these three parameters was close to linear in the perforant path stimulation intensity ranges where a population spike was evoked, as exemplified for one rat in **(c)** and **(d)**.

condition: $F(11,2) = 8.7$, $p < 0.001$). Relative to SHAM, there was no effect of VNS at 25 μA on any of the EEG parameters analyzed.

Time course of effects of VNS at 1000 μA on dentate field EPs and hippocampal EEG

Effects of VNS at 1000 μA on dentate field EPs were not immediate and required at least 20 minutes of VNS to commence. Further, effects continued to build up over the course of the

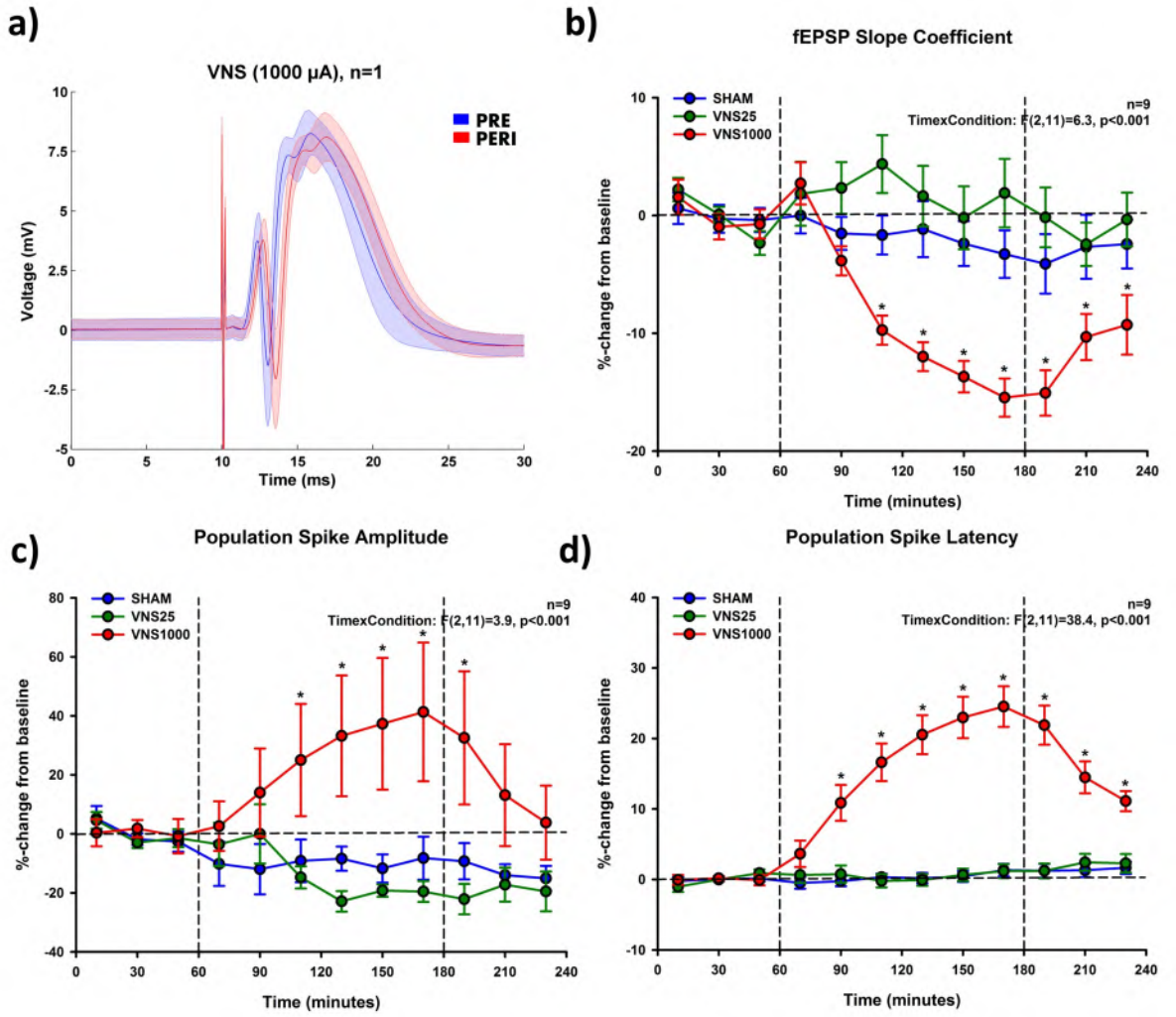


Figure 4.4: Effects of Vagus Nerve Stimulation on perforant path evoked dentate field potentials. Effects of Vagus Nerve Stimulation (VNS) on the perforant path evoked dentate field potential (EP), recorded at a stimulation intensity at baseline evoking a dentate field EP with a population spike of 75% of the maximum. As an example, in **(a)**, dentate field EPs have been averaged for one rat over the PRE period (288 traces; blue) and the last hour of the PERI period (288 traces; red). The shaded bars surrounding the mean trace lines denote the 95% confidence intervals. In **(b)**, **(c)** and **(d)** the parameter field excitatory post-synaptic potential (fEPSP) slope, population spike amplitude and population spike latency have been averaged over 20 minute epochs for plotting and statistical analysis. The main outcome statistic of the two-way repeated measures ANOVA, denoting the interaction between time and condition is stated in the upper right corner. Significant differences between the VNS 1000 μA and the SHAM conditions within single time points are marked with an asterisk. No change was seen after VNS at 25 μA

PERI period and did not saturate. In contrast, effects of VNS at 1000 μA on the hippocampal EEG power had a rapid onset, reaching statistical significance within the first 20 minute epoch, and effects saturated within the three 20 minute epochs of the PERI period. Compared to the effects on hippocampal EEG power, the effects on theta peak frequency had a slower buildup. In addition, the effects induced by VNS at 1000 μA on the fEPSP slope and the population

spike latency of the dentate field EP and the theta peak frequency of the hippocampal EEG did not return to PRE levels within the POST period.

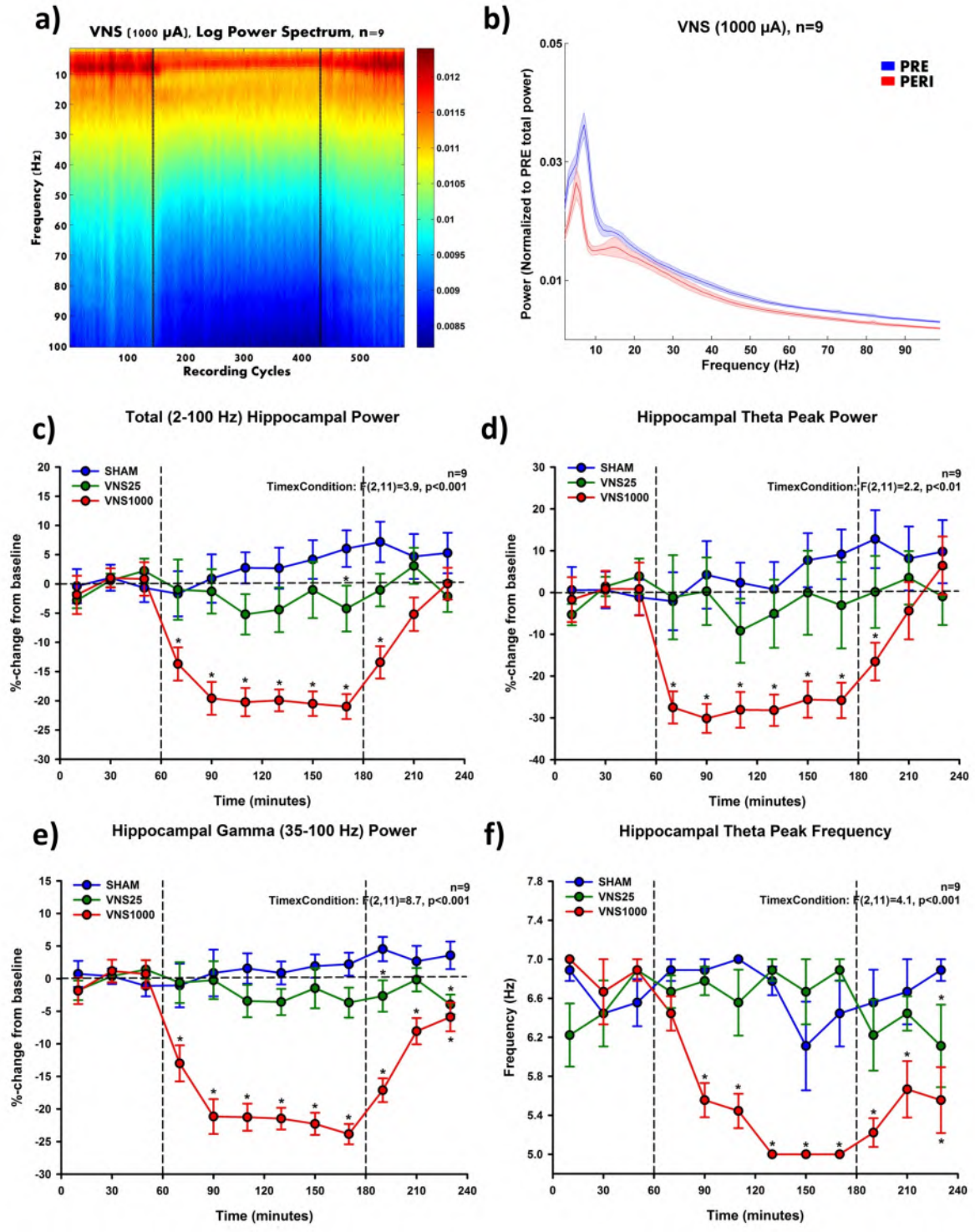
Discussion

The main findings of this study were that application of VNS at 1000 μA in freely moving rats (1) modulated dentate field EPs with a decrease in fEPSP slope, an increase in the population spike latency and an increase in population spike amplitude and (2) modulated hippocampal EEG by slowing the theta rhythm and reduced power both at the theta peak and in the 35–100 Hz gamma band. VNS applied at an intensity below the theoretic threshold for activation of vagal afferents, based on previous studies [7, 25, 40], had no effect.

Zuo et al. (2007) previously reported that VNS had no effects on perforant path dentate field EPs in awake rats but only affected induction of long term potentiation of the dentate field EP, induced with a tetanic burst stimulus applied to the perforant path, in a VNS intensity dependent manner [41]. However, only three 30 s trains of VNS, separated by 60 s, were used, whereas we continuously cycled VNS with a heavier duty cycle over a period of 2 hours. Indeed the effects of VNS on dentate field EPs observed in the present study only appeared after at least 20 minutes of VNS. Another study reported an increase in fEPSP slope and a reduction in population spike amplitude in response to VNS in urethane anesthetized rats [38], which is the opposite of the observation of the present study. The use of urethane, however, is known to exert a major influence on dentate field EPs [36].

Without any external manipulation, input/output relationships demonstrate a positive correlation between fEPSP slope and population spike amplitude, which reflects that more efficacious excitatory neurotransmission leads to increased postsynaptic response [13]. During VNS, however, the efficacy of excitatory neurotransmission is decreased while the activation of granule cells is increased. This suggests a complex modulation of excitability of the perforant path-dentate granule cell synapse. Reduced hippocampal EEG power during VNS likely reflects a suppression of synaptic potentials, the main contributor to EEG [3], and may be coupled with the finding of decreased efficacy of synaptic transmission as suggested by decreased fEPSP slope of dentate field EPs. We previously observed robust anticonvulsant effects in a rat model of temporal lobe seizures, using the exact same VNS paradigm as used in the present study [32], and we can thus assume the presence of an anticonvulsant effect in the present study as well. With that in mind, it is reasonable to believe that a decrease in synaptic efficacy, as observed in

Figure 4.5 ***(following page)*****:** Effects of Vagus Nerve Stimulation on hippocampal EEG. Application of Vagus Nerve Stimulation (VNS) resulted in pronounced changes in the hippocampal EEG spectrum. In **(a)**, the power spectrum has been normalized to the average total (2–100 Hz) power of the PRE period before averaging the resulting power spectra across rats (n = 9). The power spectrum was further logarithmically transformed and a Gaussian smoothing filter (3 × 3 with a sigma of 0.7) was applied to the matrix before creating the plot. Dashed vertical lines indicate the beginning and end of the PERI period. In **(b)**, the absolute 2–100 Hz power spectrum has been averaged over the PRE period (blue) and last hour of the PERI period (red) and subsequently normalized to the PRE period mean total power in the 2–100 Hz spectrum. Shaded areas surrounding the solid mean lines across rats (n = 9) denote the 95% confidence interval. In **(c)**, **(d)**, **(e)** and **(f)** the outcomes total 2–100 Hz hippocampal EEG power, theta peak power, gamma band (35–100 Hz) power and theta peak frequency have been averaged into 20 minute epochs for statistical analysis. The main outcome of the two-way repeated measures ANOVA, the time × stimulation condition interaction, is stated in the upper right corner of figures. Significant differences between the VNS conditions and SHAM condition are indicated with asterisks for given time points.


a)
VNS (1000 µA), Log Power Spectrum, n=9
Frequency (Hz)
Recording Cycles
b)
VNS (1000 µA), n=9
PRE
PERI
Power (Normalized to PRE total power)
Frequency (Hz)
c)
Total (2-100 Hz) Hippocampal Power
SHAM
VNS25
VNS1000
n=9
TimexCondition: F(2,11)=3.9, p<0.001
%-change from baseline
Time (minutes)
d)
Hippocampal Theta Peak Power
SHAM
VNS25
VNS1000
n=9
TimexCondition: F(2,11)=2.2, p<0.01
%-change from baseline
Time (minutes)
e)
Hippocampal Gamma (35-100 Hz) Power
SHAM
VNS25
VNS1000
n=9
TimexCondition: F(2,11)=8.7, p<0.001
%-change from baseline
Time (minutes)
f)
Hippocampal Theta Peak Frequency
SHAM
VNS25
VNS1000
n=9
TimexCondition: F(2,11)=4.1, p<0.001
Frequency (Hz)
Time (minutes)

the present study, is a likely neurophysiological mechanism underlying anticonvulsant effects of VNS. Furthermore, dentate field EPs recorded from epileptic rats in hyperexcitable or seizure frequent phases display a decrease in population spike latency [21, 31]. VNS, on the other hand, increased population spike latency, which further suggests a decrease in excitability during VNS and supports an anticonvulsant effect. The fact that the amplitude of the population spike increases despite an increase in its latency indicates a depolarization of granule cells in response to VNS. This effect is less obvious to relate to a straightforward anticonvulsant of VNS and illustrates the complexity of VNS induced modulation of hippocampal excitability. A depolarization of the resting membrane potential decreases the impact of leaking potassium currents, which has been described to be associated with a decrease in neuronal resonance frequency [26], providing a likely explanation for the slowing of hippocampal theta rhythm. Depolarization should additionally decrease the electromagnetic driving force upon opening of ligand- and voltage gated sodium channels, such as upon arrival of perforant path input, which results in a slower rate of depolarization as indeed indicated by a reduction in the fEPSP slope.

The fact that neurophysiological parameters were monitored in response to continuously cycled VNS produced another novel finding as it allowed us to assess the time course of the neurophysiological outcome. It was thus observed that effects on EEG power had a more immediate onset, while the effects on EEG theta peak frequency and dentate field EPs required at least 20 minutes of VNS to reach statistical significance. Though the reason for these specific latencies can only be speculated, it either indicates a time dependent and staged recruitment of different VNS-induced effects or that the measured parameters refer to a single VNS-induced effect but display differing dynamics. The time course of the effects may however offer insight into the dynamics of the anticonvulsant effect induced with VNS. Seeing as most effects did not reach maximal amplitude within the first 20 minute epoch, this suggests that optimal anticonvulsant efficacy is not achieved immediately within the first or few VNS trains. Further, the effect of VNS on measured parameters outlasted the active VNS period, but gradually reduced after stopping VNS, which supports that continuous cycling of VNS is required to maintain optimal efficacy. The present study, however, only assessed mechanisms recruited acutely within a 2 hour VNS period, which likely differs from the chronic scenario, where anticonvulsant efficacy generally is observed to increase over time [1].

In the previous experiment, where anticonvulsant effects were found using the exact same VNS paradigm as in the present study, the anticonvulsant effect was coupled with an increase in intrahippocampal norepinephrine concentrations [32]. Though we did not measure norepinephrine concentrations, we can assume that increased noradrenergic signaling in the hippocampus also plays a role in the present study. With this in mind, many of the observed changes in the dentate field EPs and hippocampal EEG in response to VNS can be explained by increased noradrenergic signaling. Norepinephrine increases the granule cell discharge for a given fEPSP slope both *in vitro* [4, 5, 11, 29], in anesthetized rats [6, 15, 33] and in freely moving rats [35, 39]. Noradrenergic activation of β-adrenoceptors, which are widely distributed in the dentate gyrus [12], is believed to mediate this effect through depolarization of the resting membrane potential [11, 12]. Norepinephrine may additionally act on presynaptic α_{2A}-adrenoceptors, which are densely located presynaptically in the dentate gyrus [24, 37], and which have been found to oppose presynaptic release of excitatory neurotransmitters [2], constituting another mechanism which could contribute to a reduction in the fEPSP slope.

Conclusions

The present study shows robust VNS induced modulation of both dentate field EPs and hippocampal EEG. VNS decreased synaptic efficacy reflected by a decrease in the fEPSP slope

and EEG power, while simultaneously increasing the dentate granule cell discharge for a given input. These observations reveal that VNS induces a complex modulation of excitability in the rat dentate gyrus.

References

[1] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[2] P. E. Bickler and B. M. Hansen. Alpha 2-adrenergic agonists reduce glutamate release and glutamate receptor-mediated calcium changes in hippocampal slices during hypoxia. *Neuropharmacology*, 35(6):679–87, 1996.

[3] G. Buzsaki, C. A. Anastassiou, and C. Koch. The origin of extracellular fields and currents–eeg, ecog, lfp and spikes. *Nat Rev Neurosci*, 13(6):407–20, 2012.

[4] D. Dahl and J. M. Sarvey. Norepinephrine induces pathway-specific long-lasting potentiation and depression in the hippocampal dentate gyrus. *Proc Natl Acad Sci U S A*, 86(12):4776–80, 1989.

[5] D. Dahl and J. M. Sarvey. Beta-adrenergic agonist-induced long-lasting synaptic modifications in hippocampal dentate gyrus require activation of nmda receptors, but not electrical activation of afferents. *Brain Res*, 526(2):347–50, 1990.

[6] H. T. Edison and C. W. Harley. Medial and lateral perforant path evoked potentials are selectively modulated by pairing with glutamatergic activation of locus coeruleus in the dentate gyrus of the anesthetized rat. *Hippocampus*, 22(3):501–9, 2012.

[7] 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(2):287–93, 2011.

[8] N. D. Engineer, J. R. Riley, J. D. Seale, W. A. Vrana, J. A. Shetake, S. P. Sudanagunta, M. S. Borland, and M. P. Kilgard. Reversing pathological neural activity using targeted plasticity. *Nature*, 470(7332):101–4, 2011.

[9] FDA. July 1997 pma approvals, 1997.

[10] FDA. July 2005 pma approvals, 2005.

[11] H. L. Haas and G. M. Rose. Noradrenaline blocks potassium conductance in rat dentate granule cells in vitro. *Neurosci Lett*, 78(2):171–4, 1987.

[12] C. W. Harley. Norepinephrine and the dentate gyrus. *Prog Brain Res*, 163:299–318, 2007.

[13] D. Johnston and S. Miao-Sin Wu. *Foundations of Cellular Neurophysiology*. The MIT Press, Cambridge, 1995.

[14] G. Klukowski and C. W. Harley. Locus coeruleus activation induces perforant path-evoked population spike potentiation in the dentate gyrus of awake rat. *Exp Brain Res*, 102(1):165–70, 1994.

[15] J. Knight and C. W. Harley. Idazoxan increases perforant path-evoked epsp slope paired pulse inhibition and reduces perforant path-evoked population spike paired pulse facilitation in rat dentate gyrus. *Brain Res*, 1072(1):36–45, 2006.

[16] S. E. Krahl, K. B. Clark, D. C. Smith, and R. A. Browning. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia*, 39(7):709–14, 1998.

[17] S. E. Krahl, S. S. Senanayake, and A. Handforth. Destruction of peripheral c-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. *Epilepsia*, 42(5):586–9, 2001.

[18] S. E. Krahl, S. S. Senanayake, and A. Handforth. Right-sided vagus nerve stimulation reduces generalized seizure severity in rats as effectively as left-sided. *Epilepsy Res*, 56(1):1–4, 2003.

[19] D. Labar. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. *Seizure*, 13(6):392–8, 2004.

[20] J. Liporace, D. Hucko, R. Morrow, G. Barolat, M. Nei, J. Schnur, and M. Sperling. Vagal nerve stimulation: adjustments to reduce painful side effects. *Neurology*, 57(5):885–6, 2001.

[21] J. Matzen, K. Buchheim, and M. Holtkamp. Circadian dentate gyrus excitability in a rat model of temporal lobe epilepsy. *Exp Neurol*, 234(1):105–11, 2012.

[22] B. L. McNaughton. Long-term synaptic enhancement and short-term potentiation in rat fascia dentata act through different mechanisms. *J Physiol*, 324:249–62, 1982.

[23] B. L. McNaughton and C. A. Barnes. Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. *J Comp Neurol*, 175(4):439–54, 1977.

[24] T. A. Milner, A. Lee, S. A. Aicher, and D. L. Rosin. Hippocampal alpha2a-adrenergic receptors are located predominantly presynaptically but are also found postsynaptically and in selective astrocytes. *J Comp Neurol*, 395(3):310–27, 1998.

[25] L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, D. E. Herdt V, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Electrophysiological responses from vagus nerve stimulation in rats. *Int J Neural Syst*, 23(6):1350027, 2013.

[26] R. Narayanan and D. Johnston. The h channel mediates location dependence and plasticity of intrinsic phase response in rat hippocampal neurons. *J Neurosci*, 28(22):5846–60, 2008.

[27] R. S. Neuman and C. W. Harley. Long-lasting potentiation of the dentate gyrus population spike by norepinephrine. *Brain Res*, 273(1):162–5, 1983.

[28] J. A. Nichols, A. R. Nichols, S. M. Smirnakis, N. D. Engineer, M. P. Kilgard, and M. Atzori. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience*, 189:207–14, 2011.

[29] M. R. Pelletier, R. D. Kirkby, S. J. Jones, and M. E. Corcoran. Pathway specificity of noradrenergic plasticity in the dentate gyrus. *Hippocampus*, 4(2):181–8, 1994.

[30] D. A. Prince, B. W. Connors, and L. S. Benardo. Mechanisms underlying interictal-ictal transitions. *Adv Neurol*, 34:177–87, 1983.

[31] C. M. Queiroz, J. A. Gorter, F. H. Lopes da Silva, and W. J. Wadman. Dynamics of evoked local field potentials in the hippocampus of epileptic rats with spontaneous seizures. *J Neurophysiol*, 101(3):1588–97, 2009.

[32] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[33] A. T. Reid and C. W. Harley. An associativity requirement for locus coeruleus-induced long-term potentiation in the dentate gyrus of the urethane-anesthetized rat. *Exp Brain Res*, 200(2):151–9, 2010.

[34] K. Rijkers, M. Aalbers, G. Hoogland, L. van Winden, J. Vles, H. Steinbusch, and M. Majoie. Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat. *Brain Res*, 1319:155–63, 2010.

[35] S. J. Sara and M. Segal. Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition. *Prog Brain Res*, 88:571–85, 1991.

[36] Y. Shirasaka and C. G. Wasterlain. The effect of urethane anesthesia on evoked potentials in dentate gyrus. *Eur J Pharmacol*, 282(1-3):11–7, 1995.

[37] E. M. Talley, D. L. Rosin, A. Lee, P. G. Guyenet, and K. R. Lynch. Distribution of alpha 2a-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J Comp Neurol*, 372(1):111–34, 1996.

[38] H. Ura, Y. Sugaya, H. Ohata, I. Takumi, K. Sadamoto, T. Shibasaki, and E. Maru. Vagus nerve stimulation induced long-lasting enhancement of synaptic transmission and decreased granule cell discharge in the hippocampal dentate gyrus of urethane-anesthetized rats. *Brain Res*, 1492:63–71, 2013.

[39] A. Valjakka, K. Lukkarinen, E. Koivisto, Jr. Riekkinen, P., R. Miettinen, M. M. Airaksinen, R. Lammintausta, and P. Riekkinen. Modulation of eeg rhythmicity and spike activity in the rat hippocampus by systemically administered tetrahydroaminoacridine, scopolamine and atipamezole. *Brain Res Bull*, 26(5):739–45, 1991.

[40] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

[41] Y. Zuo, D. C. Smith, and R. A. Jensen. Vagus nerve stimulation potentiates hippocampal ltp in freely-moving rats. *Physiol Behav*, 90(4):583–9, 2007.

Chapter 5

Vagus Nerve Stimulation Applied with a Rapid Cycle Has More Profound Influence on Hippocampal Electrophysiology Than a Standard Cycle

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Abstract

Although vagus nerve stimulation (VNS) is widely used, therapeutic mechanisms and optimal stimulation parameters remain elusive. In the present study, we investigated the effect of VNS on hippocampal field activity and compared the efficiency of different VNS paradigms. Hippocampal electroencephalography (EEG) and perforant path dentate field evoked potentials were acquired before and during VNS in freely moving rats, using 2 VNS duty cycles: a rapid cycle (7 s on, 18 s off) and standard cycle (30 s on, 300 s off) and various output currents. VNS modulated the evoked potentials, reduced total power of the hippocampal EEG, and slowed the theta rhythm. In the hippocampal EEG, theta (4–8 Hz) and high gamma (75–150 Hz) activity displayed strong phase amplitude coupling that was reduced by VNS. Rapid-cycle VNS had a greater effect than standard-cycle VNS on all outcome measures. Using rapid cycle VNS, a maximal effect on EEG parameters was found at 300 μA, beyond which effects saturated. The findings suggest that rapid-cycle VNS produces a more robust outcome than standard cycle VNS and support already existing preclinical evidence that relatively low output currents are sufficient to produce changes in brain physiology and thus likely also therapeutic efficacy.

Introduction

Vagus nerve stimulation (VNS) is an adjunctive treatment for pharmacoresistant epilepsy and depression [1, 2]. Since gaining regulatory approval, VNS has been used to treat>70,000 patients worldwide [3]. Despite numerous preclinical and clinical studies and its widespread application in patients, VNS remains associated with unpredictable and limited therapeutic response rates [1, 4], constituting a drawback for an invasive treatment. Although some attempts have been made to determine whether VNS efficacy depends on stimulation parameters [5, 6], the results

are generally confounded by design inconsistencies, in addition to the relatively few parameter settings investigated. Guidelines on the individual optimization of stimulation parameters to improve patient outcomes systematically are unavailable owing to the lack of studies systematically investigating dose–response relationships. Conducting systematic investigations of VNS parameter efficacy in patients is challenging and almost impossible owing to the huge variability in the treated VNS population, the numerous potential stimulation parameter combinations, and the typical limitations of seizure counts as an outcome parameter. The effect of VNS on different electrophysiological phenomena is now increasingly being investigated in order to understand mechanisms of VNS at a neurophysiological level [7–12]. We previously found in rats that rapid-cycle (7 s on, 18 s off) VNS at 1000 μA modulates dentate gyrus field evoked potentials (EPs) induced by stimulation of the perforant path, an afferent fiber bundle projecting to the hippocampus [12]. Dentate field EPs consist of a field excitatory postsynaptic potential (fEPSP), reflecting depolarization of dentate granule cells in response to afferent stimulation. Upon reaching the dentate granule cell discharge threshold, the fEPSP is accompanied by a superimposed population spike, which is the summation of the granule cell action potentials [13]. Dentate field EPs allow us to study excitability, which is an important element in epilepsy [14]. In addition, we found that VNS decreases hippocampal EEG power in various frequency bands [12], reflecting reduced magnitude and/or synchrony of both local and global hippocampal activity [15]. In the present study, we investigated hippocampal effects of various VNS parameters by studying effects of VNS on theta–gamma phase amplitude coupling (PAC) in the hippocampal EEG. Theta–gamma PAC has been described both in human and rodent hippocampus [16, 17], and is thought to be the mechanism by which the activity of local hippocampal neurons or small neuronal assemblies (represented by gamma frequency power) is controlled by field potential changes of larger areas (represented by theta frequency phase) [15]. PAC is hypothesized to support memory encoding and retrieval [17, 18]. In addition we aimed to investigate the dose–response relationship between various VNS output currents and effects on hippocampal electrophysiology. Based on previous studies showing that output current < 1000 μA is sufficient to recruit vagal fibers effectively [19] and reduce cortical excitability [20], we hypothesized that output currents < 1000 μA would also be sufficient to modulate hippocampal electrophysiology. In addition, we hypothesized that a rapid cycle would be more effective than a less intense but more clinically used duty cycle.

Methods

Twenty-two male Sprague–Dawley rats (Harlan Laboratories, The Netherlands) were used in the present study. Temperature and humidity were controlled, and rats were housed on a 12 h/12 h light/dark cycle. The experiment was conducted after approval by the local animal experimental committee (Ghent University Hospital, Ghent, Belgium, ECD 12/63) and following European Directive 2010/63/EU.

Surgery

Animals (weighing 300–380 g) were operated under isoflurane anesthesia (5% for induction, 2% for maintenance). A cuff electrode was implanted around the left vagus nerve for VNS, a bipolar recording electrode in the hilus of the left dentate gyrus (3.8 mm posterior and 1.9 mm lateral relative to bregma, approximate depth of 3 mm relative to brain surface), and a bipolar stimulation electrode in the left dorsomedial perforant path (7–8 mm posterior and 3.9 mm lateral relative to bregma, approximate depth of 2.5 mm relative to brain surface) [13]. The depth of the electrodes was carefully adjusted to evoke a maximal population spike in the dentate

gyrus. Bipolar recording electrodes were made from 2 twisted, polyimide-coated, stainless steel wires with a diameter of 70 μm (0.9-mm tip separation). Bipolar stimulation electrodes were made from 2 twisted, polyimide-coated, stainless steel wires with a diameter of 125 μm (0.9-mm tip separation). Electrode leads were collected in a custom-made connector, which was fixed to the skull with stainless steel screws and dental acrylic cement. Following surgery, animals recovered for a minimum of 6 weeks and analgesics (buprenorphine 0.03 mg/kg/day, s.c.) were administered on the 2 days following surgery.

Recording Sessions

Following recovery, animals were connected to a video/electroencephalography (EEG) setup with free movement permitted by a commutator. After habituating to the setup for 1 day, a series of recordings sessions was performed in the freely moving animals. Two electrophysiological parameters were assessed: spontaneous hippocampal EEG and field EPs in the dentate gyrus in response to electrical stimulation of the perforant path. All recording sessions consisted of a 1-h baseline and a 2-h VNS/SHAM period. The data acquisition design was dependent on the VNS duty cycle (**Fig. 5.1**). Two types of VNS duty cycles were investigated: a standard VNS duty cycle (30 s on and 300 s off) and a rapid VNS duty cycle (7 s on, 18 s off) [12, 21], which is often used to treat patients not responding to the former scheme [5, 22]. Two standard cycle conditions were included with VNS applied at intensities of 1000 μA (S1000) and 250 μA (S250). Additionally, 5 rapid cycle conditions with varying VNS intensities were included: 0 (RSHAM), 1000 μA (R1000), 250 μA (R250), 25 μA (R25), and 25 μA below behavioral threshold (RSUB). The behavioral VNS threshold was determined by ramping up the output current in 25 μA increments until the point where rats displayed the first detectable behavioral reaction to the stimulation. The pulse width of the VNS pulses was 250 μs and the frequency was 30 Hz in all cases.

In all sessions, data were acquired in the VNS off period. In standard cycle conditions, the 300-s off period was split into 12 subrecording cycles of 25 s, during which 2 EPs and a 10-s sweep of EEG were acquired (**Fig. 5.1**). This resulted in the acquisition of 24 dentate field EPs and 12 sweeps of EEG between each VNS train. The rapid-cycle recording design resembled the subrecording cycle of the standard-cycle condition, allowing the acquisition of 2 dentate field EPs and a 10-s EEG sweep during each off period of the VNS duty cycle (**Fig. 5.1**). In all sessions, the same cycles for data acquisition were used for both baseline and VNS/SHAM periods, though no VNS was applied during baseline. All dentate field EPs were obtained by delivering biphasic pulses to the perforant path with a pulse width of 100 μs at an intensity adjusted to evoke a dentate population spike of 75% of maximal amplitude based on an input/output relationship generated prior to the recording sessions.

To establish a dose–response relationship between VNS intensity and changes in hippocampal EEG parameters, an additional experiment was carried out in a subset of 6 rats. On consecutive days, rapid cycle recording sessions were performed using VNS intensities ramped up in daily 25 μA increments from 0 to 250 μA (day 1–11), in daily increments of 50 μA from 300 μA to 500 μA (day 12–16), and up to 1000 μA in a final recording session (day 17). In this experiment, only hippocampal EEG was acquired. Impedances of stimulation electrodes (both for VNS and perforant path stimulation) were assessed before all sessions. Dentate field EPs and hippocampal EEG were acquired referencing each hippocampal recording electrode to an epidural ground electrode placed above the right frontal cortex. Dentate field EPs were sampled at 20 kHz and hippocampal EEG was sampled at 1 kHz. All signals were high pass filtered at 0.1 Hz and amplified 100 times, sampling with a 16-bit dynamic and a $\pm$3.05-μV input ampli-

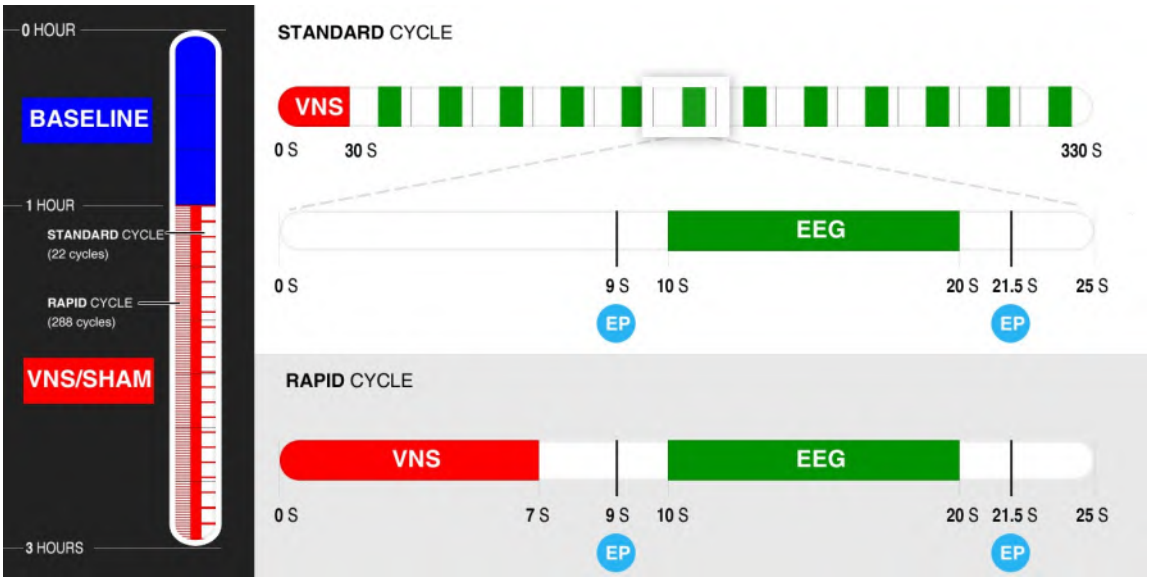

Figure 5.1: A schematic illustration of the recording design in all recording conditions. Two conditions followed a standard vagus nerve stimulation (VNS) duty cycle design (VNS on 30 s, off 300 s) and 5 conditions followed a rapid duty cycle design (c, VNS on 7 s, off 18 s). In all sessions, the 2-h VNS/SHAM period was preceded by a 1-h baseline acquisition period. In the rapid-cycle condition, each duty cycle allowed the acquisition of 2 perforant path-evoked dentate field potentials (EPs) and a 10-s sweep of spontaneous hippocampal field electroencephalography (EEG) during the off period of the cycle. During the standard cycle condition, the off period was split into 12 subrecording cycles of the same length as the rapid cycle, and, correspondingly, 2 EPs and a 10-s sweep of EEG were recorded. The recording cycle during the baseline period was identical to the VNS/SHAM period with exception of no VNS being applied.

tude resolution. Digitization was done with a USB-6259 National Instruments acquisition device (National Instruments, Austin, TX, USA).

Analysis of Dentate Field EPs

EPs were analyzed with Matlab (MathWorks, Inc., Natick, MA, US). Three parameters were extracted from the dentate field EPs: fEPSP slope, population spike amplitude, and population spike latency(**Fig. 5.2a**). fEPSP slope was estimated by fitting a line onto the initial positive deflection of the fEPSP prior to population spike onset, using the least square difference method. Population spike amplitude was estimated by fitting a line between the initial positive peak of the fEPSP, prior population spike onset, and the subsequent positive peak of the fEPSP wave form after the population spike. The line was made tangent to the fEPSP waveform and the population spike amplitude was calculated as the vertical distance from the negative peak of the population spike to this line. Population spike latency was defined as the duration from the stimulus artifact to the negative peak of the population spike.

Spectral Analysis of EEG

Spectral analysis of the hippocampal EEG was done in Matlab. The 2 hippocampal EEG signals were subtracted to focus on local hippocampal activity. EEG sweeps affected by artifacts were detected on the basis of total power deviating $>$ 3 SD from the mean across trials. For power spectral analysis, the EEG traces were subjected to a high pass Butterworth filter at 2 Hz

before splitting the 10-s sweeps into 19 sweeps of 1 s overlapping by 0.5 s. Using the fast Fourier transform, the 1–100 Hz power spectrum was extracted at a frequency resolution of 1 Hz. The outcome was 19 power spectra, which were averaged as a representative for each 10-s EEG sweep. Further analysis focused on parameters within the 2–100 Hz power spectrum. Theta peak frequency was calculated from the mean spectrum of the last hour of the VNS/SHAM period. Total hippocampal power was calculated for each EEG sweep by integrating the power within the 2–100 Hz spectrum and averaged over the last hour of the VNS/SHAM period subsequently.

PAC Analysis

PAC analysis was performed with Matlab. To assess PAC in the hippocampal EEG and the effects of VNS on this coupling, we used a normalized modulatory index (MI), according to the methods of Tort *et al.* [16]. The MI reflects the modulation of the amplitude of faster oscillations by the phase of slower oscillations, and has previously been used to characterize coupling of theta and gamma frequencies in the hippocampal region [16, 23, 24]. To calculate the MI, hippocampal EEG was band pass filtered to isolate the frequencies of interest (see below). The phase and amplitude information was then extracted from the respective signals by calculating the phase angle and modulus of the Hilbert transform, respectively, and were used to construct a composite phase amplitude time series. The amplitude information was binned into phase bins of 20 degrees (18 bins) and the amplitude distribution was calculated by calculating the mean amplitude of each bin. The amplitude distribution was normalized by dividing each bin with the sum of all bins and finally the MI is calculated as the deviation (Kullback–Leibler distance) from a uniform distribution. To initially explore coupled frequencies, we computed a comodulogram (see Fig. 4a) pairing the phase of 2– 20 Hz frequencies to the amplitude of frequencies of 15–200 Hz. Phase frequencies were narrowly band pass filtered (1 Hz width), in steps of 0.5 Hz. The amplitude frequencies were band pass filtered with a filter width of 4 Hz in steps of 2 Hz. Following identification of coupling between the phase of 4–8 Hz theta activity and the amplitude of 75–150-Hz gamma activity, band pass filters in these respective frequency ranges were applied to isolate the activity of interest. Analysis focused on coupling strength in the final hour of the VNS/SHAM period. To estimate the significance of the coupling, the MI representative of the final hour of the VNS/SHAM period in the SHAM condition was compared with coupling calculated from shuffled EEG trials (surrogate SHAM) from the same last hour of the SHAM condition. Finally, the MI of the final hour of the VNS/SHAM period was also calculated from all other VNS conditions, for comparison.

Statistical Analysis

Hippocampal EEG power and dentate field EP parameters were normalized to the mean of the baseline period. For all extracted outcomes, outliers, defined as measurements deviating > 3 SD from the mean of a full session, were removed before statistical analysis. Statistical analysis was conducted with R 2.12.1 (R Development Core Team), SPSS 22.0 (IBM, Armonk, NY, USA), or SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). As reported previously [9, 10, 12, 21], preclinical VNS studies tend to yield an ineffectively and an effectively stimulated group for reasons yet to be elucidated. The subset of ineffectively stimulated rats was identified after performing a k-means cluster analysis on the average total hippocampal EEG power change in response to rapid-cycle VNS at 1000 μA, as described previously [12]. Further analysis only focused on the cluster of rats responding to rapid cycle VNS at 1000 μA with a reduction in hippocampal EEG power. All outcome parameters were averaged over the last hour of the VNS/SHAM period and VNS conditions were compared in a 1-way repeated measures ANOVA.

In cases of normality violation, a Friedman ANOVA was applied. The type of outcome statistics, noted in figures or text, denotes the type of test used. Post-hoc evaluation was performed using the Student Newman–Keuls post-hoc test. Statistical significance was considered at $p < 0.05$. Methods related to the creation of specific graphs are explained in the figure legends. All time graphs were created with SigmaPlot. Values are given as mean ± SEM.

Results

Fourteen of 22 rats displayed dentate field EPs of adequate and stable quality and were used for the subsequent recordings. K-means cluster analysis revealed that VNS in the R1000 session reduced hippocampal EEG power in 9/14 rats, while no effect was seen in the remaining 5/14 rats. Further analysis focused on the 9/14 rats.

The Effect of Various VNS Parameters on Dentate Field EPs

VNS was associated with a decrease in fEPSP slope, an increase in population spike amplitude, and an increase in population spike latency (**Fig. 5.2**). Comparison of VNS conditions based on the average effect in the last hour of the VNS/SHAM period revealed a main effect of VNS condition on fEPSP slope (**Fig. 5.2b**; $F(6) = 7.6$; $p < 0.001$), population spike amplitude (**Fig. 5.2c**; $\chi^2(6) = 29.7$; $p < 0.001$), and population spike latency (**Fig. 5.2d**; $\chi^2(6) = 39.5$; $p < 0.001$). Relative to SHAM, a significant decrease in fEPSP slope was observed in the R250 and R1000 conditions. Further, decreases in fEPSP slope in R250 and R1000 were significantly more prominent than observed in any of the other conditions, including the standard-cycle conditions with equivalent or higher output currents. Similarly, significant increases in population spike amplitude, relative to SHAM, were only observed in the R250 and R1000 conditions. Compared with SHAM, population spike latency was significantly increased in the RSUB, R250, S1000, and R1000 conditions. Rapid-cycle conditions R250 and R1000 more prominently increased population spike latency than S250 and S1000 conditions. Relative to SHAM, no effect of VNS on any of the measured dentate field EP parameters in the R25 or S250 sessions could be demonstrated.

The Effect of Various VNS Parameters on Hippocampal EEG

VNS was associated with a general reduction in broadband 2–100 Hz hippocampal power, which was most prominent in the theta frequency band (4–12 Hz) and gamma frequency ranges (>30 Hz), while little change was observed in an intermediate 15–30 Hz band (**Fig. 5.3a, b**). VNS further reduced the theta peak frequency by 2 Hz (**Fig. 5.3b**). Shifting the entire 2–100 Hz hippocampal power spectrum obtained during VNS towards higher frequencies, to compensate for this reduction in theta peak frequency, conserved the above-described decreases in power (**Fig. 5.3b**). An effect of stimulation condition was found on hippocampal (2–100 Hz) EEG power (**Fig. 5.3c**; $F(6) = 5.8$; $p < 0.001$). Relative to SHAM, a significant decrease in hippocampal EEG power was observed in R250, S1000, and R1000 conditions. The reduction in hippocampal EEG power observed in the R1000 condition was significantly more prominent than observed in the R25, RSUB, and S250 conditions. Stimulation condition also had a significant effect on theta peak frequency (**Fig. 5.3d**; $F(6) = 10.9$; $p < 0.001$). Relative to SHAM and R25 conditions, a significant reduction in theta peak frequency was observed in the RSUB, S250, R250, S1000, and R1000 conditions. The hippocampal EEG displayed a strong coherence between the phase of theta oscillations in the 4–8-Hz range and the amplitude of fast gamma oscillations in the 75–150 Hz range (**Fig. 5.4a, b**). We found that the power of the fast gamma oscillation was highest at

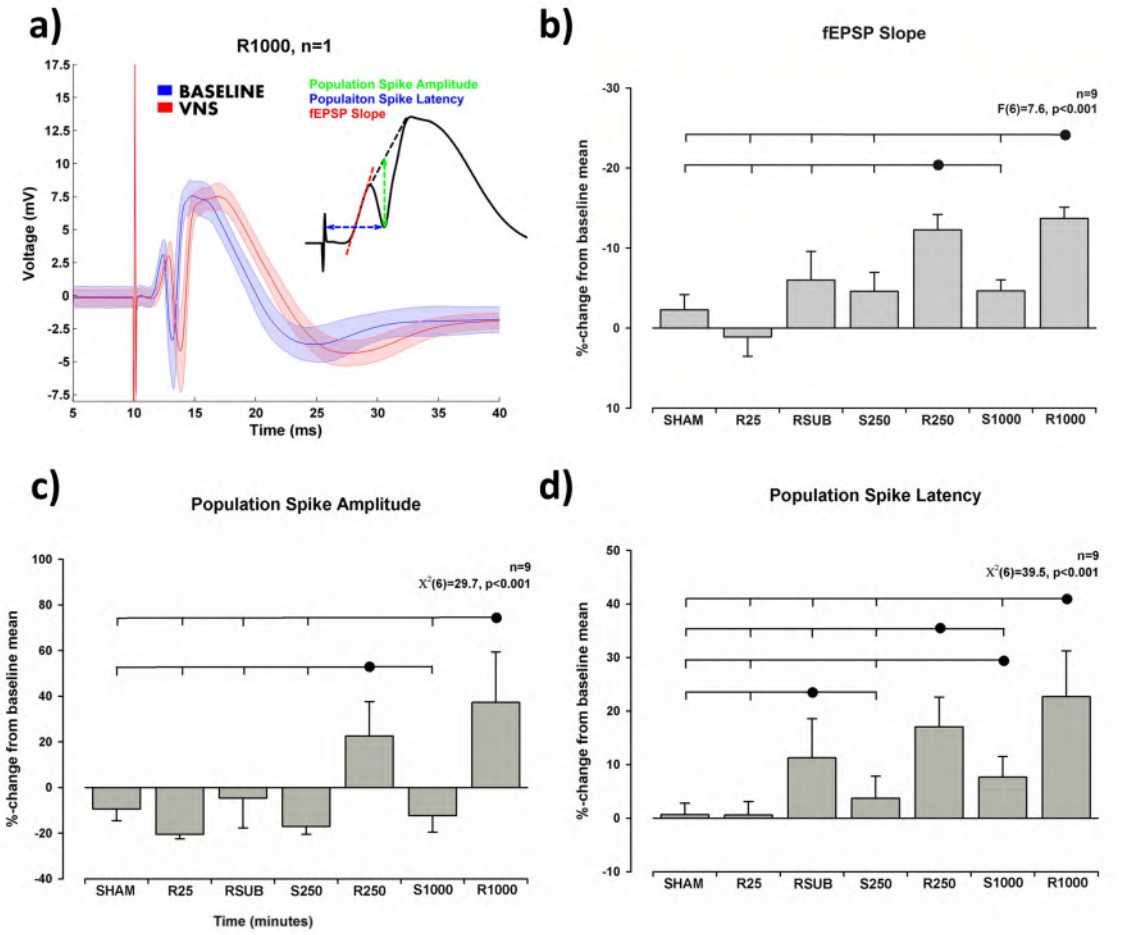

Figure 5.2: Effects of vagus nerve stimulation (VNS) on field-evoked potentials (EP) in dentate gyrus in response to electrical stimulation of the perforant path with an intensity that at baseline evoked a population spike of 75% of the maximum amplitude. In **(a)**, an EP from a representative rat is averaged over the 1-h baseline period (blue) and the last hour of VNS (red) in the rapid cycle 1000 μA condition (R1000). The shaded error bars denote the 95% confidence intervals. The 3 measured outcomes—field excitatory post synaptic potential (fEPSP) slope, population spike amplitude, and latency—are graphically illustrated in the upper right corner of **(a)**. In **(b)**, **(c)**, and **(d)**, fEPSP slope, population spike amplitude, and latency outcomes have been averaged over the last hour of the VNS period for comparison of VNS conditions. The outcome of the 1-way repeated-measures ANOVA is noted in the upper right corner of these plots. The standard cycle (30 s on/300 s off) VNS intensities included were 250 μA (S200) and 1000 μA (S1000). Further, rapid cycle (7 s on/18 s off) VNS conditions with intensities of 25 μA (R25), an intensity below the behavioral threshold (RSUB), 250 μA (R250), and 1000 μA (R1000) were included. Lines connecting bars denote post-hoc significant differences between the VNS conditions. Bars represent means ± SEM. Note that for plot **(b)**, the direction of the y-axis has been reversed.

the positive peak of the theta oscillation. This coupling was well above what can be expected at random, when comparing the data with surrogate data computed by randomly shuffling the data (**Fig. 5.4c**; $t(8) = -5$; $p < 0.001$). Compared with SHAM, VNS significantly reduced the theta–gamma PAC but only in the R250 and R1000 conditions, with the most prominent reduction observed in the R1000 condition. The PAC in the R250 and R1000 condition was also significantly reduced compared with the other stimulation conditions.

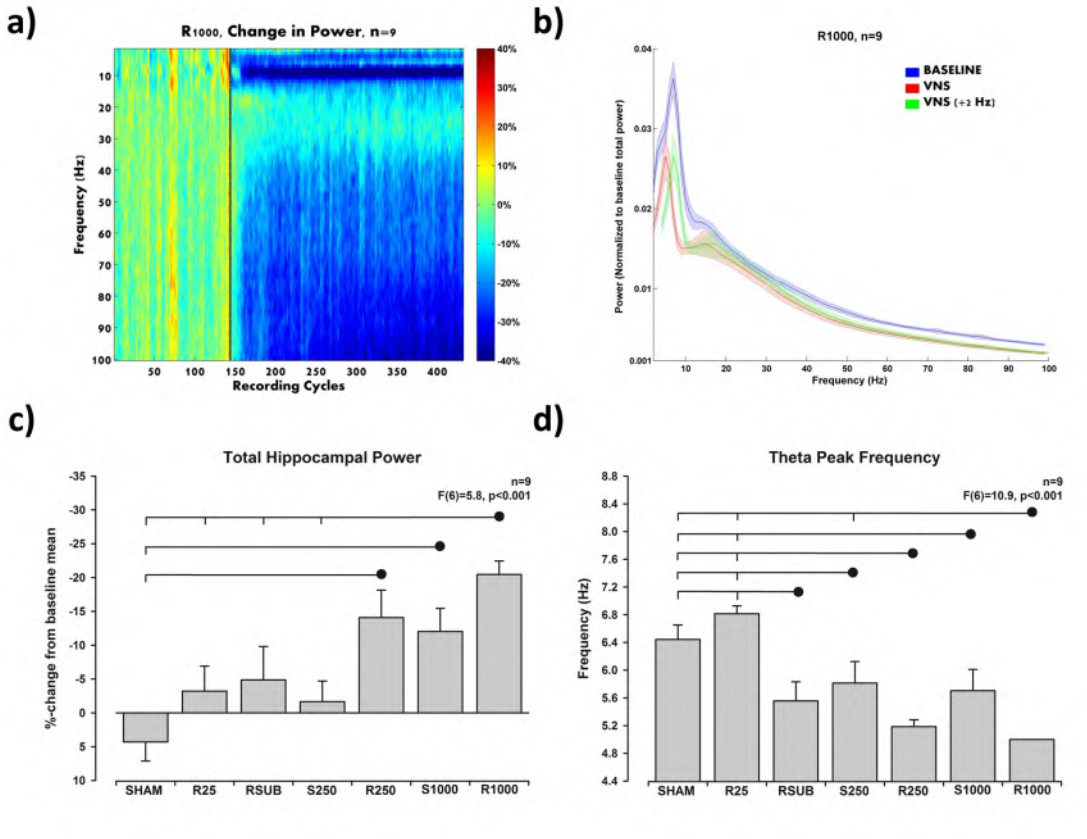

Figure 5.3: Effects of vagus nerve stimulation (VNS) on hippocampal electroencephalography. In **(a)**, the normalized change from baseline mean power within 1-Hz frequency bins has been calculated. Warm colors and cold colors represent an increase and decrease in power, respectively. The matrix from which the plot has been created has been subjected to a 3 × 3 Gaussian smoothing filter with a sigma of 0.7 prior to creation. In **(b)**, the 2–100-Hz hippocampal power spectrum has been averaged across the 1-h baseline period (blue) and the last hour of the rapid cycle 1000 μA VNS condition (red) to demonstrate the change in theta peak frequency. In order to compensate for the shift in frequency observed during VNS, an additional plot has been added where the VNS spectrum has been shifted +2 Hz (green), which shows that despite compensation for the shift in frequency, reductions in power during VNS are conserved. The shaded bars represent the 95 % confidence intervals. In **(c)** and **(d)**, the hippocampal electroencephalography power (2–100 Hz) and theta peak frequency outcomes have been averaged over the last hour of the VNS period for comparison of VNS conditions. The outcome of the 1-way repeated measures ANOVA is given in the upper right corner of these plots. The standard cycle (30 s on/300 s off) VNS intensities included were 250 μA (S200) and 1000 μA (S1000). Further, rapid cycle (7 s on/18 s off) VNS conditions with intensities of 25 μA (R25), an intensity below the behavioral threshold (RSUB), 250 μA (R250), and 1000 μA (R1000) were included. Post-hoc significant differences between VNS conditions are marked with lines between the bars. All bars denote means ± SEM. Note that for plots **(c)** and **(d)** the direction of the y-axis has been reversed.

To more accurately assess the influence of VNS intensity,specifically, on the hippocampal EEG outcomes, a VNS intensity input/output relationship was generated in a subset of 6 rats for the rapid-cycle VNS condition. Stimulation intensity had a significant impact on both hippocampal EEG power ($F(16)=9.6$; $p<0.001$), theta peak frequency ($F(16)=24.6$; $p<0.001$), and theta–gamma PAC ($F(16) = 5.1$; $p < 0.001$). On average, maximal effect on all parameters was

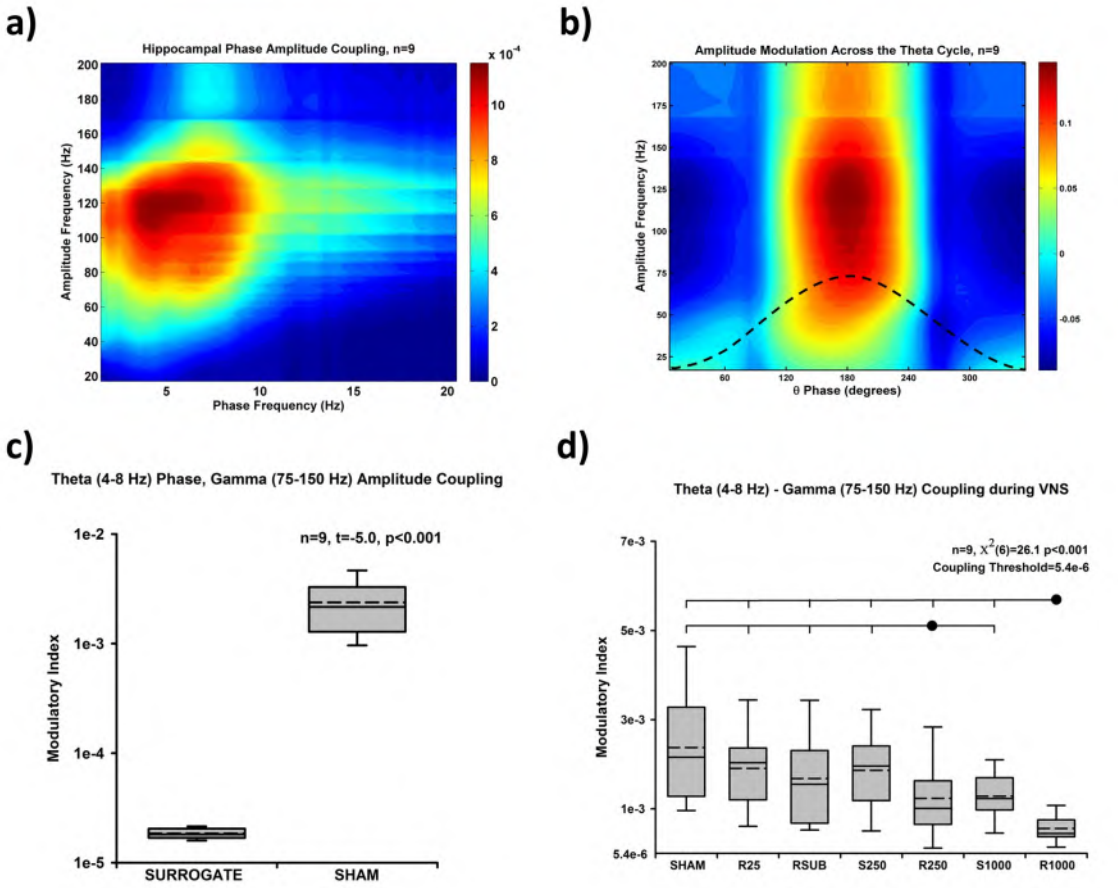

Figure 5.4: Phase amplitude coupling between theta and high gamma activity is reduced in response to vagus nerve stimulation (VNS). To explore the coupling between theta and gamma activity in the hippocampal electroencephalography (EEG) initially, a comodulogram based on EEG from the entire SHAM condition was computed **(a)**. The phase frequencies studied were from 2 to 20 Hz in steps of 0.5 Hz and were extracted with a band pass filter with a filter width of 1 Hz. The amplitude frequencies were filtered from 15 to 200 Hz in steps of 2 Hz and with a filter width of 4 Hz. The amplitude distributions were averaged across rats before calculating a representative modulatory index value for the group for each frequency pairing. Following identification of coupling between 4 and 8 Hz theta activity and high-frequency oscillations, the connected high-frequency components were studied with regard to which phase of the cycle that displayed the largest amplitude **(b)**. Only 1 component in the 75–150 Hz frequency range was found and used for further analysis. In **(c)**, the robustness of the identified theta–gamma coupling has been studied by comparing the coupling in the SHAM condition to shuffled EEG data from the same condition. Note that in **(c)**, the y axis has a logarithmic scaling. In **(d)**, the modulatory index over the last hour of the VNS period (PERI) has been calculated and compared between VNS conditions in a 1-way repeated measures ANOVA. Post-hoc significant differences between VNS conditions are marked with lines between the bars. Solid lines in the box plots denote the medians and dashed lines the means.

observed around 300 μA, beyond which further increases in VNS intensity did not yield further effect (**Fig. 5.5**).

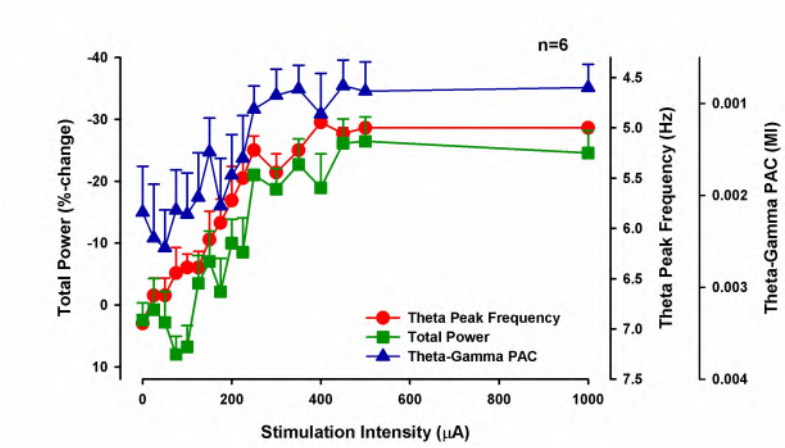

Figure 5.5: Rapid-cycle vagus nerve stimulation (VNS) output currents were titrated from 25 μA to 1000 μA in a subset of 6 rats. The figure shows effects of increasing VNS intensities on hippocampal electroencephalography power (2–100 Hz), theta peak frequency, and theta–gamma coupling. The effect of VNS on the outcomes were averaged over the last hour of the VNS period. Error bars indicate the SEM. Note that the y-axis has been reversed to reflect increasing effects of VNS as the output current is increased. PAC = phase amplitude coupling.

Discussion

This study shows that, besides modulating dentate field EPs and reducing hippocampal EEG power and theta peak frequency, VNS also reduces theta–gamma phase amplitude coupling of hippocampal EEG. The present data also demonstrate that the extent by which VNS modulates hippocampal field activity depends on the type of VNS paradigm. Effects observed with rapid-cycle VNS were more pronounced compared with the standard-cycle VNS. Effects of rapid-cycle VNS at 250 μA had a greater impact on dentate field EP parameters than standard-cycle VNS at 1000 μA, while standard-cycle VNS at 250 μA induced no change. Finally, effects of rapid-cycle VNS on hippocampal EEG parameters reached maximal levels at output currents of approximately 300 μA, with no additional effect of increasing the output current.

A reduction in hippocampal EEG power during VNS is in accordance with the early finding of the desynchronizing and power-suppressing effect of vagal afferent stimulation on cortical EEG in cats, though the effect at that time was not quantified with spectral analysis [25]. Owing to the association of a reduction in EEG power and the suppression of strychnine induced convulsive spike activity [25], EEG desynchronization or reduction in EEG power was believed to reflect the anticonvulsant efficacy of VNS [26]. In the present study, the simultaneous slowing of hippocampal theta rhythm could suggest that a general shift in power towards lower frequencies could produce the general reduction in broadband 2–100 Hz power found in the present study. However, the observation of reduced power was most pronounced around the theta peak and at gamma frequencies (>30 Hz), while little effect was observed in the 15–30-Hz range. If the drop in power during VNS alone should be explained by a shift in the spectrum, one would expect a uniform reduction in power across the whole spectrum, which is not the case. Indeed, when shifting the spectrum to compensate for the slowing observed, reductions in power remained.

The idea that decreased EEG power could reflect a decrease in excitability is supported by the notion that EEG predominantly is an aggregate of synaptic potentials [27]. This may be coupled with the decrease in fEPSP slope of the dentate field EP, which indicates decreased synaptic efficacy. Decreased synaptic efficacy further suggests decreased excitability and could thus ex-

plain an anticonvulsive effect. Shorter population spike latencies have further been described in epileptic rats [28], while VNS increased the population spike latency, constituting another effect likely to reflect anticonvulsive efficacy of VNS. The simultaneously observed increase in population spike amplitude, however, contradicts a uniform reduction in excitability during VNS, as it rather indicates the opposite and thus is unlikely to relate to any anticonvulsant effect.

PAC between theta and gamma oscillations has been described previously in different hippocampal regions of the rat [16, 23, 24]. This study shows for the first time a reduced PAC between theta and gamma oscillations during rapid-cycle VNS. Although, in theory, the normalized MI is insensitive to absolute changes in power of theta or gamma frequency bands, previous studies reported a correlation between theta power and strength of theta-gamma PAC [16, 23]. This suggests that the amount of theta activity in the hippocampus determines to what degree gamma frequencies are modulated by this theta rhythm. The fact that the coupling strength is reduced during VNS is thus most likely an indirect consequence of the VNS-induced decrease in theta power [29, 30].

Theta–gamma PAC is considered to be supportive for memory encoding and retrieval in the hippocampus [17, 18]. The reduction of PAC in response to VNS was an unexpected finding, as several studies have reported that VNS can enhance hippocampus-dependent memory and hippocampal long-term potentiation, though mainly in an intermediate output current regimen [11, 31–33]. However, none of these studies continuously cycled VNS, as in our study. If theta–gamma PAC does, indeed, play an important role in memory, our findings suggest that continuous cycling of VNS, in particular rapid cycling, might interfere with hippocampus-dependent memory, although such an effect has not yet been reported. However, the reduced theta–gamma PAC in response to rapid cycle VNS could be an important mechanism for suppression of seizure activity. As reduced theta–gamma PAC indicates that local neuronal populations are less recruited by the more global theta rhythm, one could imagine that VNS also prevents their recruitment by pathological seizure rhythms. Although this needs to be proven. However, our data could suggest that VNS-associated anticonvulsant and memory enhancing effects may require different VNS parameters.

A typical clinical approach to adjustment of VNS parameters involves a continuous adjustment of the stimulator output current just below maximally tolerated levels and a "trial and error" adaptation of the duty cycle, which is typically intensified in poorly responding patients [26]. This concept means that both duty cycle and stimulation intensity are increased, which results in larger amounts of energy requirements, leading to an increased load on the stimulator battery. In the present study, however, it was apparent that lower VNS intensities can already affect brain functioning when using a heavier duty cycle, that is, the rapid cycle. For example, compared with standard-cycle VNS at 1000 μA, rapid-cycle VNS at 250 μA resulted in a similar reduction of EEG power and even more pronounced effects on dentate field EP parameters and theta–gamma PAC in the hippocampal EEG. The active stimulation period of the rapid duty cycle covers 28% of the time, while the standard cycle, used in the present study, is on for only 9% of the time. This ratio of 3.1/1 is compensated for by the respective currents of 250 μA and 1000 μA, yielding a power ratio of 1/16 ($Watts = Current^2 * Resistance$), and, finally, an energy ratio of $3.1/16 = 0.19$ in favor of the low current rapid cycle, not to mention the more pronounced effects observed. Electrode safety, biocompatibility, and, especially, battery life of implanted stimulators would be obvious benefits.

A titration of rapid-cycle VNS intensities revealed that reduction of EEG power, slowing of theta rhythm, and reduction in theta–gamma PAC seem to appear at an intensity threshold and then saturate within the range of very few intensity increments. On average, saturation was reached around 300 μA. This observation aligns with a previous observation from our laboratory

that VNS at 250 μA was sufficient to reduce cortical excitability [20], while further increases did not yield additional effects. The observation that further increases in stimulation intensity produces no further effects supports the involvement of thick myelinated vagal type A afferents, which generally exhibit a more homogeneous threshold for activation than slowly conducting and less densely myelinated fiber types [34, 35]. Thick myelinated vagal afferents additionally exhibit a threshold for electrical activation similar to that of efferent motor fibers innervating the laryngeal muscle, and laryngeal muscle-evoked potentials have thus been proposed as a marker for effective recruitment of vagal afferents during VNS [36]. Interestingly, a recent study found a median recruitment threshold for the laryngeal muscle-evoked potential of 300 μA [19], which is similar to the intensity where effects of VNS on EEG saturate in the present study. Evidence from dogs further supports that maximal recruitment of thick myelinated vagal afferents is achieved at intensities around 350 μA using pulse widths of 300 μs [35]. Thus, the presented data strongly support that VNS-associated reduction of EEG power is due to recruitment of thickly myelinated A-fibers and that a saturation of A-fiber recruitment likely reflects a saturation of the observed effects on hippocampal EEG at higher VNS intensities. With no further benefit of increasing the stimulation intensity, this suggests that the current approach to adjustment of VNS parameters should be optimized by using noninvasive measurements to estimate A-fiber recruitment. This finding could further suggest that VNS output current only plays a role in effectively recruiting A-fiber afferents, which implies that beyond this point the "stimulation dose" can only be increased by intensifying the duty cycle, such as applying a rapid cycle instead of a standard cycle. Our findings do, indeed, support that intensification of duty cycle constitutes an option to obtain additional efficacy.

It should be noted, that the present study assessed effects of VNS in the healthy brain, which motivates attempts to replicate results in an epileptic state, to further correlate observed effects to anticonvulsant efficacy of VNS. In relation to this, it is relevant to assess how concomitant antiepileptic drugs influence the outcomes, as this could give information on the possible synergistic effects of antiepileptic drugs and VNS. Additionally, it is noteworthy that the present study only analyzed acute neurophysiological changes during VNS, while the chronic effects could possibly differ or be more pronounced, as it is well known that VNS efficacy tends to increase over time [1, 6, 37]. Nevertheless, these results stress the importance of reevaluating the current approach to VNS parameter selection. Using neurophysiological parameters, as in the present study, it may be possible to develop sophisticated algorithms to adapt VNS parameters to a specific desired clinical outcome for given individual patients.

References

[1] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[2] T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, I. Heuser, M. Bajbouj, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. Van den Abbeele, Z. Stanga, and M. Hasdemir. Vagus nerve stimulation for depression: efficacy and safety in a european study. *Psychol Med*, 38(5):651–61, 2008.

[3] Cyberonics. Cyberonics images and captions, 2013.

[4] A. J. Rush, L. B. Marangell, H. A. Sackeim, M. S. George, S. K. Brannan, S. M. Davis, R. Howland, M. A. Kling, B. R. Rittberg, W. J. Burke, M. H. Rapaport, J. Zajecka, A. A. Nierenberg, M. M. Husain, D. Ginsberg, and R. G. Cooke. Vagus nerve stimulation for treatment-resistant depression: a randomized, controlled acute phase trial. *Biol Psychiatry*, 58(5):347–54, 2005.

[5] D. Labar. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. *Seizure*, 13(6):392–8, 2004.

[6] C. M. DeGiorgio, J. Thompson, P. Lewis, S. Arrambide, D. Naritoku, A. Handforth, D. Labar, P. Mullin, and C. Heck. Vagus nerve stimulation: analysis of device parameters in 154 patients during the long-term xe5 study. *Epilepsia*, 42(8):1017–20, 2001.

[7] H. Ura, Y. Sugaya, H. Ohata, I. Takumi, K. Sadamoto, T. Shibasaki, and E. Maru. Vagus nerve stimulation induced long-lasting enhancement of synaptic transmission and decreased granule cell discharge in the hippocampal dentate gyrus of urethane-anesthetized rats. *Brain Res*, 1492:63–71, 2013.

[8] M. Y. Shen, Y. L. Luo, C. H. Yang, T. Ruan, and C. J. Lai. Hypersensitivity of lung vagal c fibers induced by acute intermittent hypoxia in rats: role of reactive oxygen species and trpa1. *Am J Physiol Regul Integr Comp Physiol*, 303(11):R1175–85, 2012.

[9] N. D. Engineer, J. R. Riley, J. D. Seale, W. A. Vrana, J. A. Shetake, S. P. Sudanagunta, M. S. Borland, and M. P. Kilgard. Reversing pathological neural activity using targeted plasticity. *Nature*, 470(7332):101–4, 2011.

[10] J. A. Nichols, A. R. Nichols, S. M. Smirnakis, N. D. Engineer, M. P. Kilgard, and M. Atzori. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience*, 189:207–14, 2011.

[11] Y. Zuo, D. C. Smith, and R. A. Jensen. Vagus nerve stimulation potentiates hippocampal ltp in freely-moving rats. *Physiol Behav*, 90(4):583–9, 2007.

[12] L. E. Larsen, W. J. Wadman, P. van Mierlo, J. Delbeke, A. Grimonprez, B. Van Nieuwenhuyse, J. Portelli, P. Boon, K. Vonck, and R. Raedt. Modulation of hippocampal activity by vagus nerve stimulation in freely moving rats. *Brain Stimul*, 9(1):124–32, 2016.

[13] B. L. McNaughton and C. A. Barnes. Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. *J Comp Neurol*, 175(4):439–54, 1977.

[14] D. A. Prince, B. W. Connors, and L. S. Benardo. Mechanisms underlying interictal-ictal transitions. *Adv Neurol*, 34:177–87, 1983.

[15] J. E. Lisman and O. Jensen. The theta-gamma neural code. *Neuron*, 77(6):1002–16, 2013.

[16] A. B. Tort, R. Komorowski, H. Eichenbaum, and N. Kopell. Measuring phase-amplitude coupling between neuronal oscillations of different frequencies. *J Neurophysiol*, 104(2):1195–210, 2010.

[17] N. Axmacher, M. M. Henseler, O. Jensen, I. Weinreich, C. E. Elger, and J. Fell. Cross-frequency coupling supports multi-item working memory in the human hippocampus. *Proc Natl Acad Sci U S A*, 107(7):3228–33, 2010.

[18] J. Y. Park, Y. R. Lee, and J. Lee. The relationship between theta-gamma coupling and spatial memory ability in older adults. *Neurosci Lett*, 498(1):37–41, 2011.

[19] A. Grimonprez, R. Raedt, L. De Taeye, L. E. Larsen, J. Delbeke, P. Boon, and K. Vonck. A preclinical study of laryngeal motor-evoked potentials as a marker vagus nerve activation. *Int J Neural Syst*, page 1550034, 2015.

[20] L. Mollet, A. Grimonprez, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. *Acta Neurol Scand*, 128(6):391–6, 2013.

[21] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[22] J. Liporace, D. Hucko, R. Morrow, G. Barolat, M. Nei, J. Schnur, and M. Sperling. Vagal nerve stimulation: adjustments to reduce painful side effects. *Neurology*, 57(5):885–6, 2001.

[23] A. B. Tort, R. Scheffer-Teixeira, B. C. Souza, A. Draguhn, and J. Brankack. Theta-associated high-frequency oscillations (110-160hz) in the hippocampus and neocortex. *Prog Neurobiol*, 100:1–14, 2013.

[24] J. B. Bott, M. A. Muller, J. Jackson, J. Aubert, J. C. Cassel, C. Mathis, and R. Goutagny. Spatial reference memory is associated with modulation of theta-gamma coupling in the dentate gyrus. *Cereb Cortex*, 2015.

[25] A. Zanchetti, S. C. Wang, and G. Moruzzi. The effect of vagal afferent stimulation on the eeg pattern of the cat. *Electroencephalogr Clin Neurophysiol*, 4(3):357–61, 1952.

[26] C. Heck, S. L. Helmers, and C. M. DeGiorgio. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. *Neurology*, 59(6 Suppl 4):S31–7, 2002.

[27] G. Buzsaki, C. A. Anastassiou, and C. Koch. The origin of extracellular fields and currents–eeg, ecog, lfp and spikes. *Nat Rev Neurosci*, 13(6):407–20, 2012.

[28] C. M. Queiroz, J. A. Gorter, F. H. Lopes da Silva, and W. J. Wadman. Dynamics of evoked local field potentials in the hippocampus of epileptic rats with spontaneous seizures. *J Neurophysiol*, 101(3):1588–97, 2009.

[29] A. B. Tort, M. A. Kramer, C. Thorn, D. J. Gibson, Y. Kubota, A. M. Graybiel, and N. J. Kopell. Dynamic cross-frequency couplings of local field potential oscillations in rat striatum and hippocampus during performance of a t-maze task. *Proc Natl Acad Sci U S A*, 105(51):20517–22, 2008.

[30] R. Scheffer-Teixeira, H. Belchior, F. V. Caixeta, B. C. Souza, S. Ribeiro, and A. B. Tort. Theta phase modulates multiple layer-specific oscillations in the ca1 region. *Cereb Cortex*, 22(10):2404–14, 2012.

[31] K. B. Clark, D. K. Naritoku, D. C. Smith, R. A. Browning, and R. A. Jensen. Enhanced recognition memory following vagus nerve stimulation in human subjects. *Nat Neurosci*, 2(1):94–8, 1999.

[32] K. B. Clark, S. E. Krahl, D. C. Smith, and R. A. Jensen. Post-training unilateral vagal stimulation enhances retention performance in the rat. *Neurobiol Learn Mem*, 63(3):213–6, 1995.

[33] K. B. Clark, D. C. Smith, D. L. Hassert, R. A. Browning, D. K. Naritoku, and R. A. Jensen. Posttraining electrical stimulation of vagal afferents with concomitant vagal efferent inactivation enhances memory storage processes in the rat. *Neurobiol Learn Mem*, 70(3):364–73, 1998.

[34] S. E. Krahl, S. S. Senanayake, and A. Handforth. Destruction of peripheral c-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. *Epilepsia*, 42(5):586–9, 2001.

[35] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

[36] L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, D. E. Herdt V, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Electrophysiological responses from vagus nerve stimulation in rats. *Int J Neural Syst*, 23(6):1350027, 2013.

[37] C. M. DeGiorgio, S. C. Schachter, A. Handforth, M. Salinsky, J. Thompson, B. Uthman, R. Reed, S. Collins, E. Tecoma, G. L. Morris, B. Vaughn, D. K. Naritoku, T. Henry, D. Labar, R. Gilmartin, D. Labiner, I. Osorio, R. Ristanovic, J. Jones, J. Murphy, G. Ney, J. Wheless, P. Lewis, and C. Heck. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. *Epilepsia*, 41(9):1195–200, 2000.

Chapter 6

Vagus Nerve Stimulation Induces Hypothermia in Freely Moving Rats

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Abstract

Vagus nerve stimulation is a widely used neuromodulation technique that is currently used or being investigated as therapy for a wide array of human diseases such as epilepsy, depression, Alzheimer's disease, tinnitus, inflammatory diseases, pain, heart failure and many others. Here we report a pronounced decrease in brain and core temperature during vagus nerve stimulation in rats. This hypothermic effect is associated with a pronounced change in brain physiology and is induced by tail vasodilation which is an active heat release mechanism. Despite previous evidence indicating an important role of the locus coeruleus-noradrenergic system in therapeutic effects of vagus nerve stimulation, lesioning this system did not attenuate the hypothermic effect. Since body and brain temperature affect most physiological processes, this finding is of substantial importance for interpretation of several previously published studies and for the future direction of research on vagus nerve stimulation.

Introduction

The early finding of vagus nerve stimulation (VNS)-induced desynchronization of spontaneous brain rhythms [1, 2] inspired researchers to initiate preclinical trials, which documented anticonvulsant efficacy of VNS in multiple species [3–5]. Convincing outcomes of these studies subsequently prompted a rapid decision to initiate full clinical development, which led to regulatory clearance of VNS for drug resistant epilepsy [6]. At that time, however, relatively little was known about the underlying therapeutic mechanisms.

Now, there is accumulating evidence for an important role of the locus coeruleus noradrenergic (LC-NA) system in the anticonvulsant and antidepressant mechanisms of VNS [7–11]. Evidence from our laboratory has demonstrated a causal link between the anticonvulsant efficacy and the influence of VNS on noradrenergic signaling in rats [10]. In epilepsy patients, an effect of VNS on an event related potential, the P300, known to be sensitive to the activity of the LC-NA system, was further found to predict positive VNS response [12], suggesting translational value of the accumulated preclinical evidence.

Next to epilepsy, VNS is now also approved for use in drug resistant depression [13] and has been suggested as a possible treatment for conditions such as memory disorders [14], pain and headache [15, 16] as well as inflammatory disorders [17]. Numerous effects are now associated

with VNS as a result of thousands of studies conducted worldwide, many of which have been conducted in rats. In a recent study of ours [18], we observed a pronounced slowing of hippocampal theta rhythm and dentate field evoked potentials [18] during VNS, which resembled changes previously described in hypothermic rats [19, 20]. We therefore hypothesized that the finding of electrographic slowing resulted from a decrease in brain temperature during VNS. In the present study, we studied effects of VNS on brain and rectal temperature in freely moving rats.

Methods

A total of 18 male Sprague Dawley rats (Harlan, the Netherlands) were used in the present experiment. During the experiment, rats were housed under controlled conditions at a constant light/dark cycle of 12 hours per phase (lights on at 8 am.). All procedures were approved by the local animal experimental committee (ECD 15/89, Ghent University Hospital, Ghent, Belgium) and were in accordance with the European directive 2010/63/EU.

Surgery

Following initial habituation to the laboratory environment of two weeks, rats were operated under isoflurane anesthesia (mixture of medical oxygen and isoflurane, 5% isoflurane for induction, 2% for maintenance). According to procedures described elsewhere [10], a custom-made cuff electrode for VNS was wrapped around the cervical division of the left vagus nerve. The rat was then placed in a stereotactic frame and a bipolar recording electrode, consisting of two twisted polyimide coated 70 μm diameter stainless steel wires (0.9 mm tip separation), was placed in the hilus of the right dentate gyrus (3.8 mm posterior and 1.9 mm lateral to bregma, *sim*3 mm ventral to brain surface, using electrophysiological feedback). A guide cannula was placed at the contralateral side at the corresponding coordinates, but was only lowered 2 mm, as the thermocouple, later to be inserted into the guide cannula, protruded the cannula by 1 mm. A ground and reference electrode, consisting of a stainless steel micro-screw was placed in the right frontal bone. All electrodes were assembled in a connector block, which along with the guide cannula was fixed to the skull with additional anchor screws and dental acrylic cement. Post-surgery, rats were given a dose of buprenorphine to minimize post-surgical pain. The rats recovered from surgery for at least a month before being included in any subsequent recordings. During this period, the rats were handled regularly by experimenters to accustom them to human interaction as required for the experiment.

Experiments

Rats with an intact VNS system (VNS-electrode impedance < 10 kΩ) were connected to the video-EEG setup, while permitting free movement via an electrical commutator. Any recordings commenced after habituation to the setup for at least a day. Effective VNS delivery in rats was assessed by confirming an effect of VNS on hippocampal EEG power, in accordance with previously published methods [18, 21].

Rats were included in two initial experimental sessions: a SHAM session and a VNS session, scheduled on consecutive days in a randomized order. Each session consisted of a two hour baseline period, a two hour VNS period, and a four hour post VNS period. VNS was applied with a rapid duty cycle of 7 seconds on, 18 seconds off, which previously was associated with pronounced effects on hippocampal electrophysiology [18]. In the SHAM condition, the VNS output current in the VNS period was 0, while it was 1000 μA in the VNS condition. VNS was

delivered at a frequency of 30 Hz and with a pulse width of 250 μs. To assess brain temperature, the obturator of the guide cannula was replaced by a copper/constantan thermocouple (HYPO-33-1-T-G-60-SMPW-M, OMEGA), which was connected to a thermometer to sample brain temperature and send data to a computer every 30 seconds. In addition to temperature, 10 seconds of hippocampal EEG was recorded in the middle of the 'off' phase of each VNS duty cycle to assess the coherence between effects of VNS on electrophysiology and brain temperature. This resulted in 144 sweeps of EEG per hour of recording.

The effect of VNS on rectal temperature was assessed in two separate experimental sessions, a SHAM session and VNS session, on two consecutive days in randomized order. The experimental setup was identical to the initial brain temperature experiment, except from the addition of rectal temperature measurements every 20 minutes, starting from the 10th minute of each session, which marked the middle of 20 minute epochs over which other outcomes were averaged for analysis. Rectal temperature was measured with a standard handheld thermometer.

To assess the influence of repetitive VNS iterations on brain temperature, an additional recording condition was included by extending the original recording design. Thus, an extra two hour VNS period was added following the four hour post VNS period and concluded by a second four hour recording period following the second VNS period making up a 14 hour recording session. VNS was only applied with an output current of 1000 μA in this experiment.

Effects of varying VNS parameters on the brain temperature outcome were studied by varying duty cycle intensity and VNS output current in four rats used for the previous recordings. In addition to the rapid duty cycle used in the previous experiments, a standard cycle of 30 seconds on, 300 seconds off condition, resembling a frequently used clinically applied treatment regimen in epilepsy and depression, was added. The effect of various VNS output currents was studied by including additional rapid cycle VNS conditions with output currents of 100 μA, 200 μA, 400 μA, 600 μA and 800 μA. This data was then added to the preexisting 0 μA (SHAM) and 1000 μA data.

To screen for behavior associated with cold or heat five minute epochs of video, acquired continuously during the recording sessions, were extracted at time points of interest: five minutes prior to VNS, during the first five minutes of VNS, five minutes half an hour into the VNS period, during the last five minutes of VNS and the first five minutes following VNS. Behaviors associated with cold stress include shivering and rolling in a ball to decrease body surface area [22]. Behaviors associated with heat stress include body extension, salivation and grooming [22].

To study the effect of VNS on tail vasomotor tone, a thermographic camera (FLIR E60, FLIR Instruments, resolution of 320 x 240 pixels) was used to assess tail surface temperature, which increases in case of tail vasodilation [22]. Rapid and standard cycle VNS was applied for two hours following a one hour baseline period and followed up by a one hour post VNS period. An image was taken every 20 minutes starting from the 10th minute. The thermographic images were processed in FLIR tools by drawing rectangular regions of interest onto the tail of the rat. For each image, the average tail surface temperature was calculated as the mean temperature of all regions of interest. Rectal temperature was measured in parallel. This experiment was done in two rats also used in the previous experiments.

Four rats, which were used for the previous recordings, were in a final experiment injected twice with the selective noradrenergic neurotoxin DSP-4 in order to lesion the noradrenergic system [23]. The first injection (60 mg/kg, dissolved in 1 ml of saline, i.p), was administered two weeks prior to testing, while the second injection (50 mg/kg, dissolved in 1 ml of saline, i.p) was administered one week prior to testing. The influences of DSP-4 on the effect of VNS on

temperature were examined in a recording session consisting of a two hour baseline period and a two hour VNS period. Effects were compared to a recording obtained the day before the first injection. After completing the experiment, an overdose of sodium pentobarbital (180 mg/kg, i.p.) was used to deeply anesthetize the rats following which they were cardially perfused with a paraformaldehyde solution (4%, pH 7.4). The brains were isolated and post-fixed with the same paraformaldehyde solution for at least 24 hours before they were snap-frozen in isopentane cooled in liquid nitrogen and subsequently stored at -20 ^{o}C. Brains were coronally sectioned (40 μm slices) with a cryostat (Leica). Slices were washed twice for 5 minutes in phosphate buffered saline (PBS) and subsequently incubated in 0.6% hydrogen peroxide at room temperature for 30 minutes. Slices were again washed for in PBS and incubated in 3% donkey serum and 0.25% triton X for 30 minutes. Slices were then initially incubated for one hour at room temperature with the primary antibody, anti-dopamine-β-hydroxylase (1:1000, Merck Millipore, MAB308) and subsequently overnight at 4 ^{o}C. The following day, slices were again initially washed with PBS, before being incubated for two hours with the secondary antibody, biotinylated donkey-anti-mouse (1:1000, Jackson ImmunoResearch lab). Following washing with PBS the dopamine-β-hydroxylase in the slices was detected with an avidine biotin conjugate (Vectastain ABC kit, Vector Laboratories) and stained with 3,3-diaminobenzidine (DAB; brown precipitate). For inspection and photography, slices were mounted on glass slides and fixed with cover slips using Entellan.

Data Analysis

Hippocampal EEG was recorded by referencing each tip of the hippocampal recording electrode to a stainless steel screw placed epidurally through the right frontal bone. EEG was high pass filtered at 0.1 Hz, amplified 500 times and digitized at 1 kHz with a 16 bit dynamic (3.05 μV amplitude resolution). Digitization was performed with a USB-6259 National Instruments Device (National Instruments, Austin, Texas, USA) and stored offline for analysis. Analysis was performed with Matlab (The MathWorks, Inc., Natick, US). To isolate local hippocampal activity, the two hippocampal signals were subtracted. Sweeps excessively affected by artifacts were removed automatically by detecting sweeps with a total power deviating more than 3 standard deviations from the mean. The EEG was then filtered offline between 2 and 100 Hz with a first order Butterworth filter. Two parameters were estimated in subsequent analysis: the total power of the 2 to 100 Hz spectrum and the theta peak frequency. To determine the total power of the 2 to 100 Hz spectrum, the 10 second EEG sweeps were split into one second windows, overlapping by 50%, resulting in a total of 19 windows. The power spectrum was calculated from the fast Fourier transform and the 19 windows were averaged into one representative power spectrum of that 10 second EEG sweep. Total power was then calculated by integrating power in the 2 to 100 Hz spectrum. Theta peak frequency was determined by splitting the 10 second EEG sweep into three 5 second windows overlapping by 50%. Larger windows were used to increase the frequency resolution (0.2 Hz in this case). The power spectrum was then calculated from the fast Fourier transform and the spectrum was smoothed with a running average of 2 Hz. The resultant spectra were averaged over 20 minute periods and theta peak frequency was determined as the frequency bin containing the most power in the averaged spectrum.

For statistical analysis, outcomes were averaged into 20 minute epochs. In initial brain and rectal temperature experiments, all outcomes were processed in two way repeated measures ANOVAs with the factors time and stimulation condition. The effect of repetitive VNS application was then assessed by examining the difference between the last 20 minute of the two VNS periods with the baseline in a one way repeated measures ANOVA. In all cases Turkey's post hoc

test was applied to identify differences between specific conditions in specific time points. To examine and compare the dynamics of changes in brain and rectal temperature during VNS, the group averages of the two parameters were correlated in a Pearson correlation analysis. Further, exponential decay functions (formula $T(t) = T(\infty) + \Delta T \times e^{-\frac{t}{\tau}}$) were fitted to the mean decrease in temperature during VNS and mean increase in temperature following VNS, using the dynamic curve fitting tool available in SigmaPlot. The starting point ($T(\infty) + \Delta T$) was constrained to the mean baseline value before the onset of VNS. For the exponential recovery towards baseline temperature following VNS, a similar function (formula $T(t) = T(\infty) - \Delta T \times e^{-\frac{t}{\tau}}$), was fitted. Here, the starting point ($T(\infty) - \Delta T$) was constrained as the value at the end of the VNS period and $T(\infty)$ was constrained as the mean baseline temperature. The effect of standard cycle VNS was compared to rapid cycle VNS by comparing the change in brain temperature over the last 20 minutes of the VNS periods relative to the last 20 minutes of the baseline period in a two way repeated measures ANOVA, with the factors time and stimulation. To examine the association between change in temperature and EEG parameters using various VNS intensities, mixed models were built with VNS intensity as repeated measures factor. The model fit was optimized using Akaike's information criterion. The effect of DSP-4 injection on outcomes was assessed with paired t-tests, examining pre and post injection effects.

Results

VNS decreases brain and rectal temperature

VNS was applied via a custom-made cuff electrode which was implanted around the left vagus nerve at least one month prior to any measurements. Brain temperature was measured with an intracranial copper constantan thermocouple, plugged into a compatible guide cannula, which was stereotactically placed over the left dentate gyrus in the hippocampal formation. In the freely moving rat, application of two hours of rapid cycle VNS (7 s on / 18 s off, 1 mA, 250 μs, 30 Hz) was associated with a decrease in brain temperature compared to SHAM, when no active stimulation was applied (**Fig. 6.1a**, time x condition: $F(1,23)=32.8$, $p<0.001$). There were no baseline differences in brain temperature between the SHAM and VNS condition. Brain temperature continued to decrease throughout the two hour VNS period reaching a value of -2.3 $\pm$ 0.3 ^{o}C at the end of the VNS period, relative to the temperature at the onset of VNS ($t(9)=8.1$, $p<0.001$). Following the VNS period, brain temperature gradually returned to baseline, stabilizing after four hours. In addition to measuring brain temperature, hippocampal EEG was acquired from a bipolar depth electrode implanted at the level of the right dentate gyrus. VNS was associated with the previously described shift in theta peak frequency of the hippocampal EEG from 6.5 to 5.2 Hz (**Fig. 6.1b**, time x condition: $F(1,23)=5.6$, $p<0.001$) and a 20% reduction in hippocampal EEG power (**Fig. 6.1c**, time x condition: $F(1,23)=10.1$, $p<0.001$) [18]. The VNS induced shift in theta peak frequency followed a time profile similar to the effect of VNS on brain temperature (**Fig. 6.1d**). It had a slow onset, reached statistical significance in the second 20-minute epoch of VNS and returned gradually to baseline. The reduction in hippocampal EEG power had a fast onset, reaching statistical significance within the first 20 minute epoch of VNS and quickly returned to baseline. When a second VNS iteration was applied four hours after the end of the first VNS period, VNS induced similar effects, with reductions in brain temperature (**Fig. 6.1e**), theta peak frequency (**Fig. 6.1f**) and hippocampal EEG power (**Fig. 6.1g**).

In separate experimental sessions, where brain and rectal temperature were assessed concomitantly, VNS decreased rectal temperature ($F(1,23)=15.1$, $p<0.001$) to a similar extent as brain temperature (**Fig. 6.2a**) with the outcomes being almost perfectly correlated (**Fig. 6.2b**,

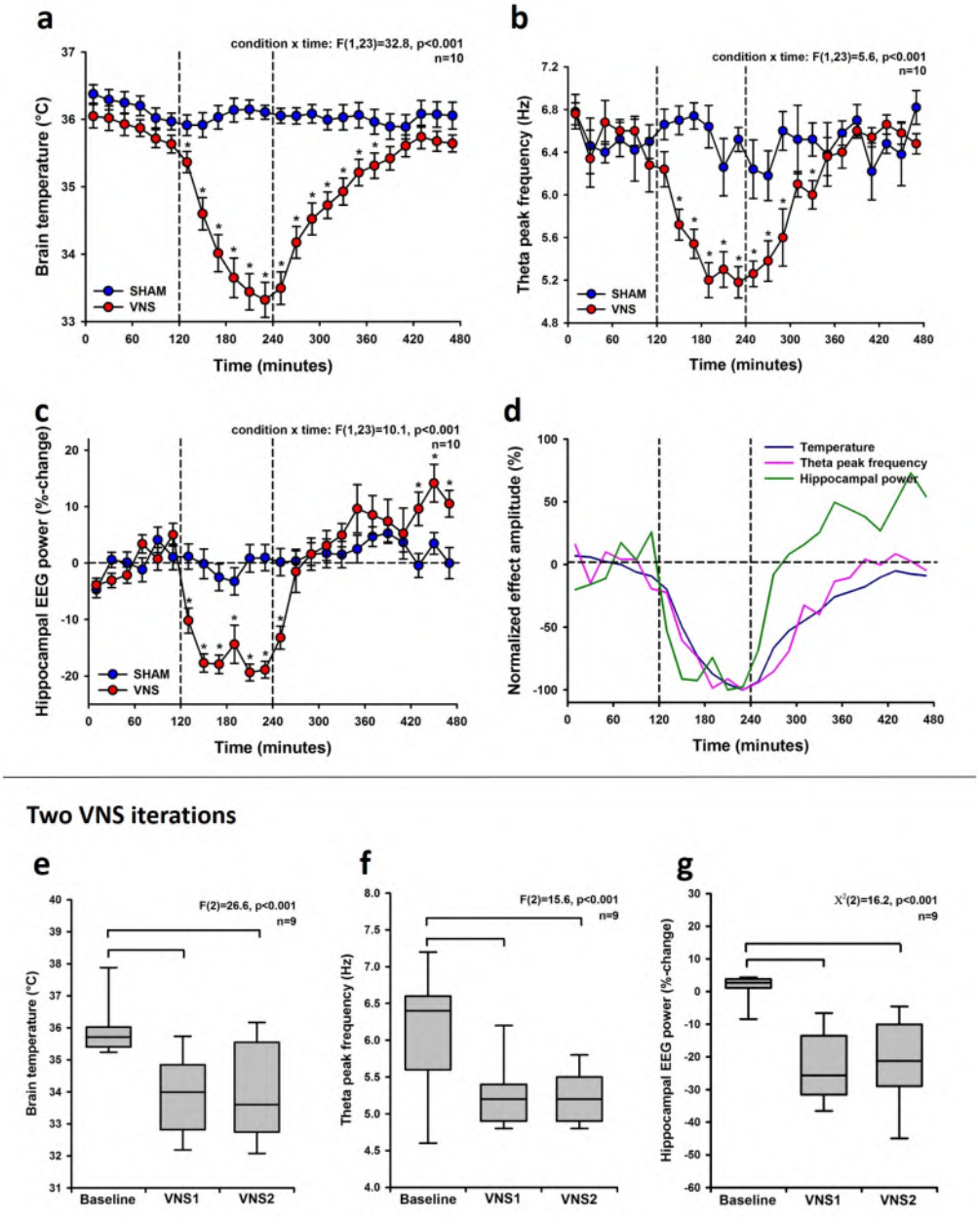

Figure 6.1: VNS reduces brain temperature in freely moving rats. Rapid Cycle VNS was observed to decrease brain temperature **(a)**, reduce theta peak frequency of the hippocampal EEG **(b)** and reduce hippocampal EEG power **(c)**. The effect of VNS on theta peak frequency followed a time-profile similar to that of brain temperature, while effects of VNS on hippocampal EEG power had a more rapid onset and faster decay **(d)**. Dashed vertical lines denote the beginning and end of the VNS period. Repeated application of VNS was associated with similar reductions in brain temperature **(e)**, theta peak frequency **(f)** and hippocampal EEG power **(g)**. Outcomes for **(e)**, **(f)** and **(g)** have been averaged over the last 20 minutes of the two hour VNS periods or baseline period. In **(a)**, **(b)** and **(c)** data is presented as mean ± standard error of the mean. In **(e)**, **(f)** and **(g)**, data is presented in box plots, with dashed lines denoting the means and the solid lines denoting the median. Significant differences ($p<0.05$) is denoted with an asterisk in **(a)**, **(b)**, and **(c)**, and with bars in **(e)**, **(f)** and **(g)**.

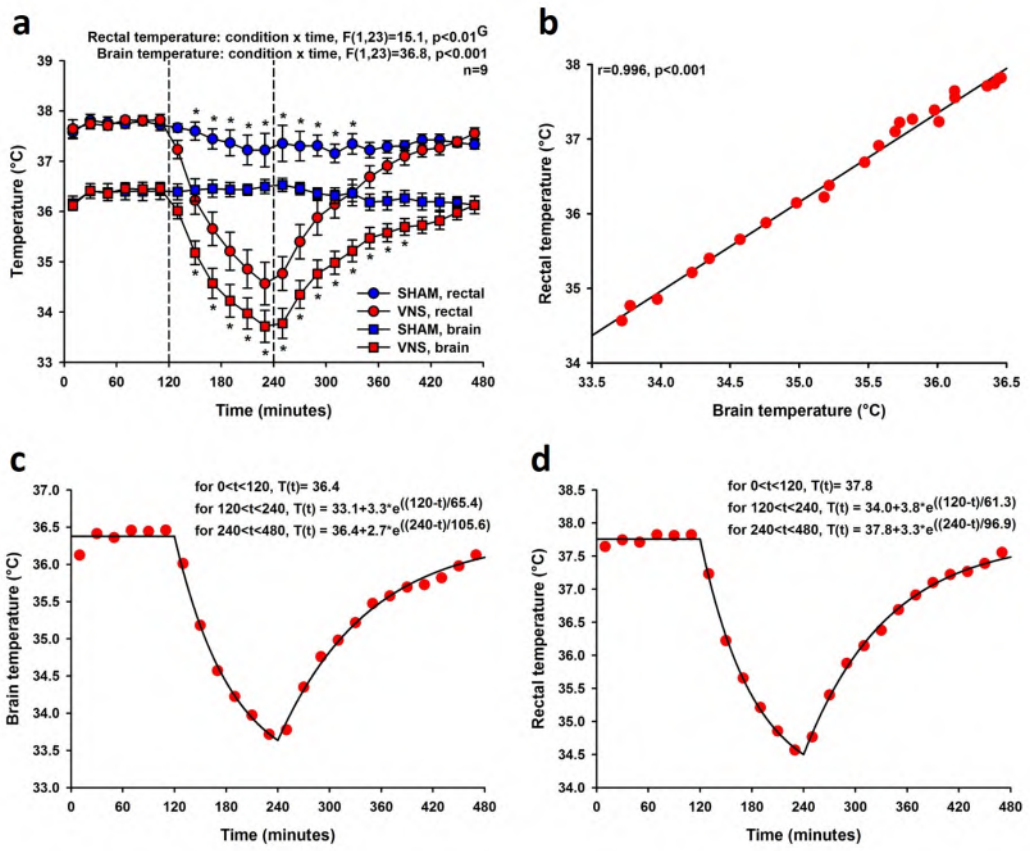

Figure 6.2: VNS reduces brain and rectal temperature with similar dynamics. In a separate experiment, where rectal temperature was additionally measured every 20 minutes, VNS was observed to decrease both brain and rectal temperature **(a)**. Dashed vertical lines denote the beginning and end of the VNS period. The change in rectal temperature was observed to be linearly correlated to the changes in brain temperature **(b)**. Using curve fitting tools, additionally revealed a strong fit for exponential decay functions following the formula $T(t) = T(\infty) + \Delta T \times e^{-\frac{t}{\tau}}$ and revealed similar dynamics of brain temperature **(c)** and rectal temperature **(d)**. In **(a)**, data is expressed as means ± standard error of the mean. Significant differences ($p<0.05$) are denoted with an asterisk. In the remaining plots, data represents group means (n=9).

r=0.996, $p<0.001$). The effect of VNS on brain and rectal temperature displayed similar dynamics, decreasing in an exponential manner (**Fig. 6.2c** and **6.2d**). Using a curve fitting tool, exponential decay functions were fitted to the mean temperature decrease during VNS according to the formula

$$T(t) = T(\infty) + \Delta T \times e^{-\frac{t}{\tau}}$$

where t is the time after the start of VNS, $T(\infty)$ is the temperature towards which an infinitely long VNS session tends to cool the body and brain, and ΔT is the maximal change in temperature. The time constant, τ, determines the effect dynamics. The time constants during VNS were 65.4 ± 12.1 (coefficient ± standard error) minutes and 61.3 ± 7.2 minutes for brain and rectal temperature, respectively. The recovery towards baseline temperature levels similarly

followed an exponential shape for brain as well as rectal temperature but with slightly larger time constants of 105.6 ± 13.2 and 96.9 ± 7.3 minutes, respectively. Similarly to the experiments when only the brain temperature was measured, VNS again induced a shift in theta peak frequency ($F(1,23)=2.4$, $p<0.001$) and hippocampal EEG power ($F(1,23)=2.0$, $p<0.01$).

VNS Parameter Dependence

In a subset of four rats, also used in the previous recordings, effects of varying stimulation parameters were studied. A more commonly applied clinical VNS duty cycle, the standard duty cycle (30 s on/300 s off, 1000 μA, 250 μs, 30 Hz), was also associated with a decrease in brain temperature relative to baseline of -0.83 ± 0.14 ^{o}C ($p<0.05$), though the reduction was less pronounced than during rapid cycle VNS (**Fig. 6.3a** and **6.3b**, time x duty cycle: $F(1,1)=75.3$, $p<0.01$), where a temperature decrease of -3.01 ± 0.21 ^{o}C ($p<0.001$) was observed. In addition to the effects of duty cycle, we examined the influence of VNS output currents in the range 0 to 1000 μA. As previously reported [21], VNS was observed to shift the theta peak frequency (**Fig. 6.3d**) and hippocampal EEG power (**Fig. 6.3e**) also at VNS intensities below 1000 μA. Effects of VNS on temperature (**Fig. 6.3c**) were found at the same intensities that affected hippocampal electrophysiology. To study whether a change in brain temperature could predict a change in theta peak frequency and hippocampal EEG power and to correct for repeated measures, a mixed model analysis was applied (**Fig. 6.3f** and **6.3g**). The analysis revealed that at any given VNS intensity, a change in brain temperature during VNS was accompanied by a change in both hippocampal theta frequency ($p<0.001$ for slope) and hippocampal EEG power ($p<0.001$ for slope), demonstrating that the effects are recruited at similar VNS intensities.

VNS provokes active release of heat

The relatively rapid reduction in temperature during VNS suggests an active heat release, through the regulation of peripheral vasomotor tone or evaporative heat loss, the latter being a result of coordinated salivation and grooming in rats [22]. Additionally, rats attain extended body posture to increase the body surface area and in that way facilitate heat loss [22, 24]. To study these phenomena, we used an infrared thermographic camera to assess the tail vasomotor response in addition to filming the behavior of rats during VNS with regular video cameras.

Rapid cycle VNS was associated with a pronounced increase in tail surface temperature, an indication of vasodilation, within the first hour of VNS (**Fig. 6.4a**, **6.4b** and **6.4c**), whereas vasoconstriction, evidenced by decreased tail surface temperature, was observed towards the end of the two hour VNS period. The decrease in rectal temperature during rapid cycle VNS (**Fig. 6.4d**) was tightly associated with a rapid increase in tail surface temperature (**Fig. 6.4e**). Standard cycle VNS induced a small decrease in temperature, while there was no major change in tail surface temperature (**Fig. 6.4g** and **6.4h**).

Behavioral signs of heat or cold stress were assessed by inspection of video acquired during the initial recordings presented in **Fig. 6.1**. At the onset of the VNS session, 10 of 10 rats reacted to the induced sensation and initially moved around in the cage. Later during VNS, 6 out of 10 rats became more immobile and extended their body while resting in a prone position, generally at the edge of the cage. There was no change in behavior after stopping VNS. No shivering, an indicator of cold stress, was observed at any point.

Effects of lesioning the locus coeruleus

Lesioning noradrenergic axons with systemic injection of the selective neurotoxin DSP-4 (**Fig. 6.5a** and **6.5b**) did not attenuate hypothermic effects of VNS, but slightly increased them (**Fig.**

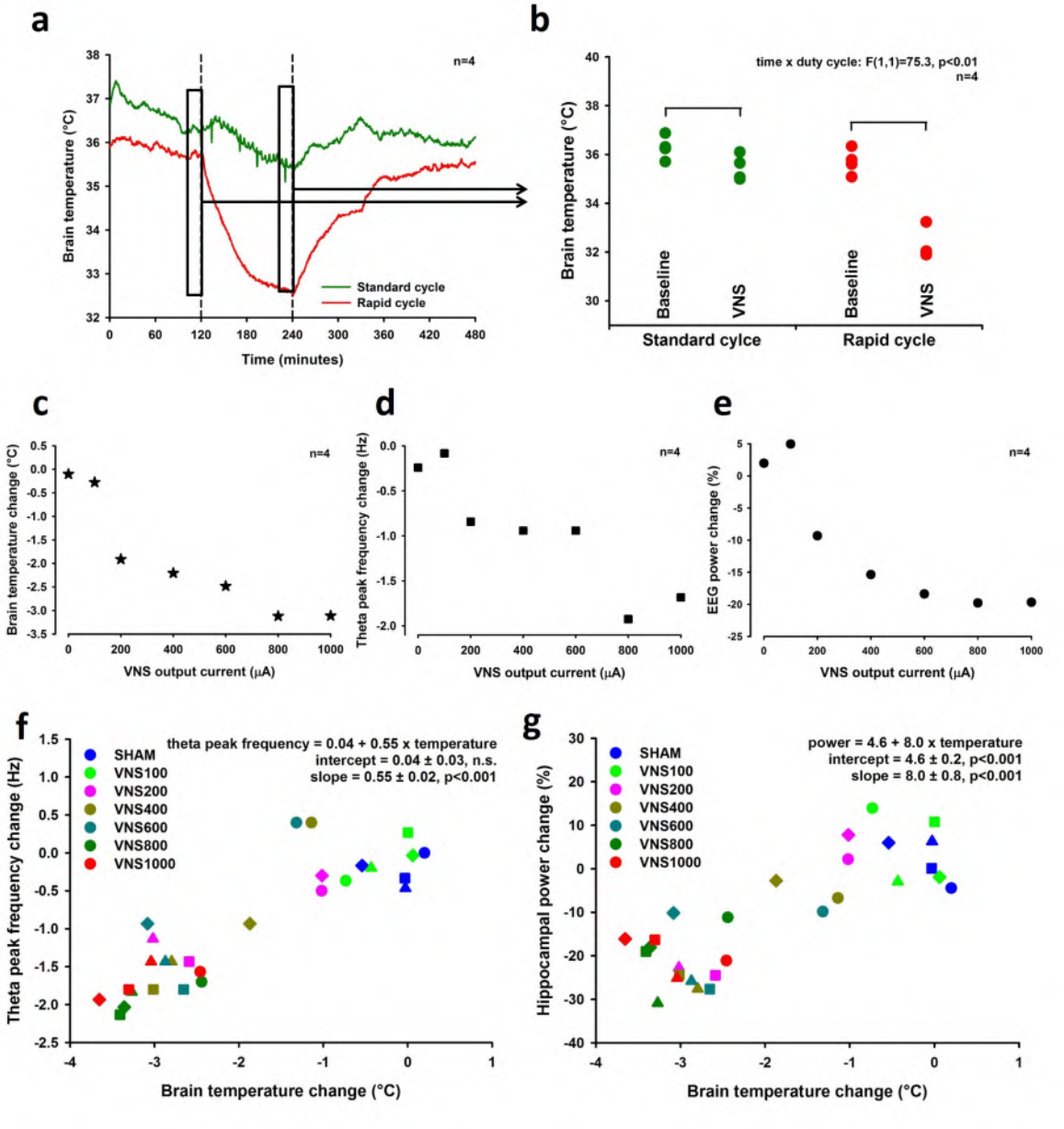

Figure 6.3: VNS associated reduction in temperature is parameter dependent. Application of standard cycle VNS was also associated with a decrease in brain temperature **(a)**, though the effect was more moderate than seen during rapid cycle VNS **(b)**. The effect of rapid cycle VNS on brain temperature, theta peak frequency and hippocampal EEG power was further observed to depend on the VNS output current used **(c, d, e)**. Across different VNS output currents, a change in brain temperature was further found to be associated with changes in both theta peak frequency **(f)** and hippocampal EEG power **(g)** suggesting that effects on brain temperature and EEG parameters are recruited at similar intensities. In **(b)**, effects have been averaged over the last 20 minutes of the two hour baseline period and the two hour VNS period. Significant differences ($p<0.05$) from baseline within conditions are denoted with a bar and asterisk. In the remaining plots, effects have been averaged over the last hour of the VNS period.

6.5c). DSP-4 further had no influence on the effect on the effect of VNS on hippocampal EEG parameters (**Fig. 6.5d** and **6.5e**).

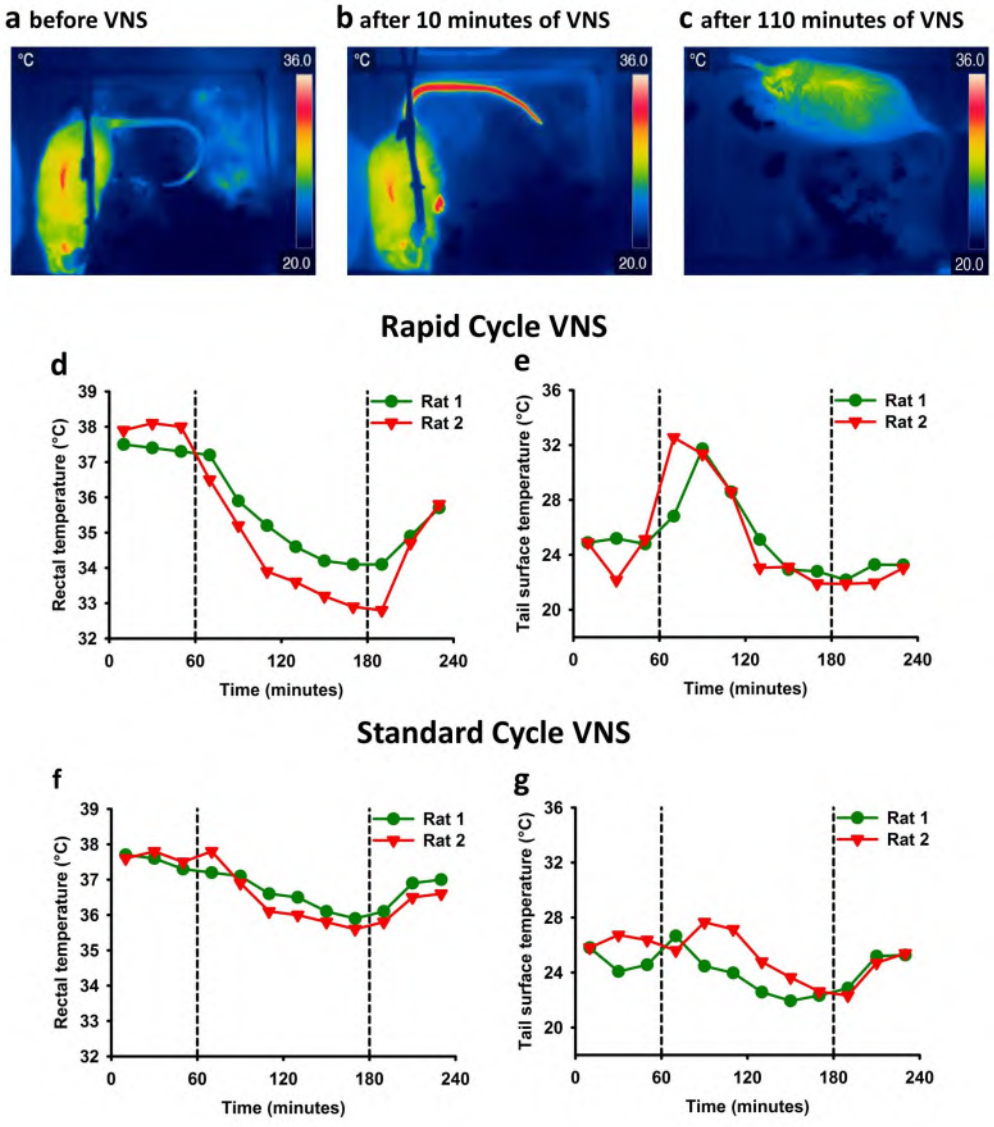

Figure 6.4: VNS reduces temperature via active heat release induced by a tail vasomotor response. Application of VNS was observed to induce a transient increase in tail surface temperature **(b)**, compared to baseline **(a)**, measured with a thermographic camera. Towards the end of the two hour VNS period **(c)**, tail temperature was reduced or back at baseline levels. Rapid cycle VNS resulted in a reduction in rectal temperature **(d)** associated with a rapid increase in tail surface temperature **(e)**, reflecting tail vasodilation. Standard cycle was associated with a small decrease in rectal temperature **(f)** and only minor effects on tail surface temperature **(g)**.

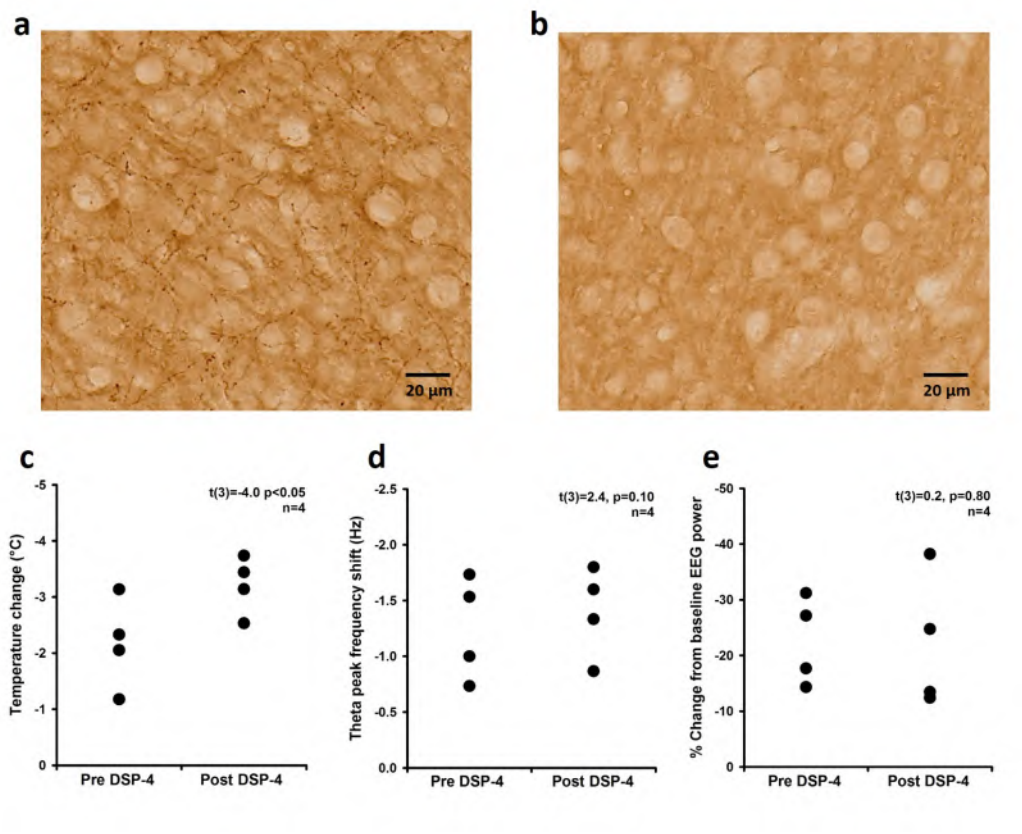

Figure 6.5: DSP-4 has no influence on hypothermic effects of VNS. Administration of DSP-4 eliminated noradrenergic axons in the prefrontal cortex, confirmed by negative dopamine-β-hydroxylase staining **(a)** vs. untreated control rats **(b)**. Comparing effects of VNS on brain temperature **(c)**, theta peak frequency of the hippocampal EEG **(d)** and hippocampal EEG power **(e)** before and after DSP-4 injection revealed no effect of DSP-4 treatment on the theta peak frequency or EEG power, while effects on brain temperature were slightly increased. Significant differences were noted at $p<0.05$ or less.

Discussion

The present study substantiates the novel observation that VNS profoundly decreases both brain and body temperature in freely moving rats. Internal temperature is predominantly regulated by a population of temperature sensitive neurons in the medial preoptic area of the hypothalamus [25, 26], which regulate the so called internal set point. The absence of any behavioral signs of cold stress but rather indications of heat stress, our findings would indicate that VNS reduces the internal set point, which will give the rat the perception of feeling too warm until the new set temperature has been reached [22]. This is associated with activation of heat release mechanisms such as peripheral vasodilation [22, 26], which was observed in our study. Due to the involvement of parasympathetic vagal efferents in the vasomotor response, it is further likely that direct of stimulation of these could lead to the same vasodilation response as observed without central hypothalamic involvement. A reduction in temperature without regulation of the internal set point, however, would constitute a homeostatic challenge which is counteracted by hypothalamic activation of heat production mechanisms such as shivering [22]. As this was not observed in any cases, this scenario is considered unlikely. Further, the threshold for activation of parasympathetic vagal efferents is substantially higher than the VNS intensities

used in the present study [28]. The vagus nerve has previously been described to be involved in the regulation of temperature, though mostly in relation to fever, which can be induced by chemical activation of subdiaphragmatic vagal afferents [27]. The vagal afferents responsible for this effect, however, are mainly thinly or unmyelinated c-fibers [27], requiring substantially higher VNS output currents to be recruited than the stimuli used in the present study [28]. This suggests an alternative mechanism mediated by thick myelinated vagal afferents, which has not previously been identified. The vagus nerve has been described as being connected to thermoregulatory centers of the hypothalamus via several pathways, including the LC-NA system, which in fact has been associated with induction of hypothermia [29]. However, the observation that DSP-4 did not attenuate hypothermic effects of VNS suggests that the observed effect is not noradrenergically mediated. Alternatively, second order vagal afferents in the nucleus of the solitary tract, project to the hypothalamic areas via the parabrachial nucleus [30], which is thus a more likely pathway for the body cooling.

Using varying VNS intensities, we also found that the effect of VNS on temperature is strongly associated with the modulation of both EEG power and theta peak frequency of the hippocampal EEG. The fact that effects of VNS on hippocampal EEG previously have been reported to reach maximal amplitude at intensities as low as 200-300 μA [21], suggest that these effects are due to recruitment of thick myelinated type A afferents. Our findings thus indicate that the same fibers mediate the effect on temperature. Thick myelinated type A fibers are those recruited at the lowest output currents [28, 31], which implies that at any given effective VNS intensity, VNS will affect temperature in rats. While more intense duty cycling, i.e. rapid cycling, produced the most prominent body temperature decrease, the standard cycle, resembling frequently clinically used duty cycles was also associated with a temperature decrease. This is in accordance with our previous findings [21], showing a more prominent slowing of dentate field evoked potentials in response to rapid cycle VNS than standard cycle VNS. Our previous study [21] suggested that at any effective intensity, associated with full A-fiber recruitment, only the duty cycle can be increased in order to increase the stimulation efficacy and consequently the resulting therapeutic effect. The data of the present study supports that magnitude of the hypothermic effect of VNS is a function of the %-on time of the VNS duty cycle, with higher %-on times resulting in a more pronounced effect.

The implications of the present findings depend on their translational relevance. Despite more than 20 years of clinical experience with VNS the effect on temperature has never been reported in the clinic [32]. This question remains to be investigated in a controlled setting. If it appears that the decrease in temperature during VNS is a rat or species specific phenomenon, one should question the translational value of preclinical VNS research in rats. Temperature exerts an extensive influence on the dynamics of neural transmission [33, 34] in addition to general effects on cellular metabolism [35] and may thus confound any given study outcome if not accounted for. It must be noted, however, that there is no direct evidence that hypothermic effects of VNS are related to anticonvulsant effects previously reported in rats [7, 10]. In fact, as noted previously, an LC-NA lesion strongly attenuated anticonvulsant effects of VNS in an acute rat seizure model [7], while it had no influence on the hypothermic effects of VNS. On the other hand, hypothermia has been associated with anticonvulsant effects in in vitro slice models [36], in vivo seizure models [37, 38], and clinical observations indicate that cooling can be effective in treating cases of refractory status epilepticus [39]. If our findings prove translationally relevant, several clinical perspectives open. Obvious applications of VNS would include status epilepticus, where cooling is associated with dual anticonvulsive and neuroprotective effects [40], as well as in stroke, where cooling has a proven neuroprotective effect [41]. Stroke could then become an additional indication for vagus nerve stimulation, which preclinical evidence indeed supports [42]. This perspective is particularly interesting if VNS indeed affects temperature via a regulation

of the internal set point, in contrast to application of external cooling, seeing as once the new set temperature is reached, minor thermal discomfort would be expected.

References

[1] M. H. Chase, M. B. Sterman, and C. D. Clemente. Cortical and subcortical patterns of response to afferent vagal stimulation. *Exp Neurol*, 16(1):36–49, 1966.

[2] A. Zanchetti, S. C. Wang, and G. Moruzzi. The effect of vagal afferent stimulation on the eeg pattern of the cat. *Electroencephalogr Clin Neurophysiol*, 4(3):357–61, 1952.

[3] D. M. Woodbury and J. W. Woodbury. Effects of vagal stimulation on experimentally induced seizures in rats. *Epilepsia*, 31 Suppl 2:S7–19, 1990.

[4] J. S. Lockard, W. C. Congdon, and L. L. DuCharme. Feasibility and safety of vagal stimulation in monkey model. *Epilepsia*, 31 Suppl 2:S20–6, 1990.

[5] J. Zabara. Inhibition of experimental seizures in canines by repetitive vagal stimulation. *Epilepsia*, 33(6):1005–12, 1992.

[6] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[7] S. E. Krahl, K. B. Clark, D. C. Smith, and R. A. Browning. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia*, 39(7):709–14, 1998.

[8] A. E. Dorr and G. Debonnel. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *J Pharmacol Exp Ther*, 318(2):890–8, 2006.

[9] R. W. Roosevelt, D. C. Smith, R. W. Clough, R. A. Jensen, and R. A. Browning. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res*, 1119(1):124–32, 2006.

[10] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[11] H. Furmaga, A. Shah, and A. Frazer. Serotonergic and noradrenergic pathways are required for the anxiolytic-like and antidepressant-like behavioral effects of repeated vagal nerve stimulation in rats. *Biol Psychiatry*, 70(10):937–45, 2011.

[12] L. De Taeye, K. Vonck, M. van Bochove, P. Boon, D. Van Roost, L. Mollet, A. Meurs, V. De Herdt, E. Carrette, I. Dauwe, S. Gadeyne, P. van Mierlo, T. Verguts, and R. Raedt. The p3 event-related potential is a biomarker for the efficacy of vagus nerve stimulation in patients with epilepsy. *Neurotherapeutics*, 11(3):612–22, 2014.

[13] Z. Nahas, C. Burns, M. J. Foust, B. Short, T. Herbsman, and M. S. George. Vagus nerve stimulation (vns) for depression: what do we know now and what should be done next? *Curr Psychiatry Rep*, 8(6):445–51, 2006.

[14] K. B. Clark, D. K. Naritoku, D. C. Smith, R. A. Browning, and R. A. Jensen. Enhanced recognition memory following vagus nerve stimulation in human subjects. *Nat Neurosci*, 2(1):94–8, 1999.

[15] A. Mauskop. Vagus nerve stimulation relieves chronic refractory migraine and cluster headaches. *Cephalalgia*, 25(2):82–6, 2005.

[16] G. Lange, M. N. Janal, A. Maniker, J. Fitzgibbons, M. Fobler, D. Cook, and B. H. Natelson. Safety and efficacy of vagus nerve stimulation in fibromyalgia: a phase i/ii proof of concept trial. *Pain Med*, 12(9):1406–13, 2011.

[17] L. V. Borovikova, S. Ivanova, M. Zhang, H. Yang, G. I. Botchkina, L. R. Watkins, H. Wang, N. Abumrad, J. W. Eaton, and K. J. Tracey. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*, 405(6785):458–62, 2000.

[18] L. E. Larsen, W. J. Wadman, P. van Mierlo, J. Delbeke, A. Grimonprez, B. Van Nieuwenhuyse, J. Portelli, P. Boon, K. Vonck, and R. Raedt. Modulation of hippocampal activity by vagus nerve stimulation in freely moving rats. *Brain Stimul*, 9(1):124–32, 2016.

[19] I. Q. Whishaw and C. H. Vanderwolf. Hippocampal eeg and behavior: effects of variation in body temperature and relation of eeg to vibrissae movement, swimming and shivering. *Physiol Behav*, 6(4):391–7, 1971.

[20] E. I. Moser, M. B. Moser, and P. Andersen. Potentiation of dentate synapses initiated by exploratory learning in rats: dissociation from brain temperature, motor activity, and arousal. *Learn Mem*, 1(1):55–73, 1994.

[21] L. E. Larsen, W. J. Wadman, D. Marinazzo, P. van Mierlo, J. Delbeke, S. Daelemans, M. Sprengers, L. Thyrion, W. Van Lysebettens, E. Carrette, P. Boon, K. Vonck, and R. Raedt. Vagus nerve stimulation applied with a rapid cycle has more profound influence on hippocampal electrophysiology than a standard cycle. *Neurotherapeutics*, 2016.

[22] C. J. Gordon. Thermal biology of the laboratory rat. *Physiol Behav*, 47(5):963–91, 1990.

[23] A. Grimonprez, R. Raedt, J. Portelli, I. Dauwe, L. E. Larsen, C. Bouckaert, J. Delbeke, E. Carrette, A. Meurs, V. De Herdt, P. Boon, and K. Vonck. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. *J Psychiatr Res*, 68:1–7, 2015.

[24] E. Satinoff. *Thermoregulation*, chapter 21. Oxford University Press, New York, 2004.

[25] J. A. Boulant and H. N. Demieville. Responses of thermosensitive preoptic and septal neurons to hippocampal and brain stem stimulation. *J Neurophysiol*, 40(6):1356–68, 1977.

[26] K. C. Bicego, R. C. Barros, and L. G. Branco. Physiology of temperature regulation: comparative aspects. *Comp Biochem Physiol A Mol Integr Physiol*, 147(3):616–39, 2007.

[27] A. A. Romanovsky. Thermoregulatory manifestations of systemic inflammation: lessons from vagotomy. *Auton Neurosci*, 85(1-3):39–48, 2000.

[28] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

[29] V. M. Kumar, R. Vetrivelan, and H. N. Mallick. Noradrenergic afferents and receptors in the medial preoptic area: neuroanatomical and neurochemical links between the regulation of sleep and body temperature. *Neurochem Int*, 50(6):783–90, 2007.

[30] H. R. Berthoud and W. L. Neuhuber. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci*, 85(1-3):1–17, 2000.

[31] L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, D. E. Herdt V, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Electrophysiological responses from vagus nerve stimulation in rats. *Int J Neural Syst*, 23(6):1350027, 2013.

[32] G. H. Vijgen, N. D. Bouvy, L. Leenen, K. Rijkers, E. Cornips, M. Majoie, B. Brans, and W. D. van Marken Lichtenbelt. Vagus nerve stimulation increases energy expenditure: relation to brown adipose tissue activity. *PLoS One*, 8(10):e77221, 2013.

[33] S. M. Thompson, L. M. Masukawa, and D. A. Prince. Temperature dependence of intrinsic membrane properties and synaptic potentials in hippocampal ca1 neurons in vitro. *J Neurosci*, 5(3):817–24, 1985.

[34] M. Volgushev, T. R. Vidyasagar, M. Chistiakova, and U. T. Eysel. Synaptic transmission in the neocortex during reversible cooling. *Neuroscience*, 98(1):9–22, 2000.

[35] A. C. Guyton and J.E. Hall. *Thextbook of Medical Physiology*. Elsevier Sauders, Philadelphia, 11th edition edition, 2006.

[36] G. K. Motamedi, P. Salazar, E. L. Smith, R. P. Lesser, W. R. Webber, P. I. Ortinski, S. Vicini, and M. A. Rogawski. Termination of epileptiform activity by cooling in rat hippocampal slice epilepsy models. *Epilepsy Res*, 70(2-3):200–10, 2006.

[37] F. C. Schmitt, K. Buchheim, H. Meierkord, and M. Holtkamp. Anticonvulsant properties of hypothermia in experimental status epilepticus. *Neurobiol Dis*, 23(3):689–96, 2006.

[38] X. F. Yang, J. H. Chang, and S. M. Rothman. Long-lasting anticonvulsant effect of focal cooling on experimental neocortical seizures. *Epilepsia*, 44(12):1500–5, 2003.

[39] A. B. Kowski, H. Kanaan, F. C. Schmitt, and M. Holtkamp. Deep hypothermia terminates status epilepticus–an experimental study. *Brain Res*, 1446:119–26, 2012.

[40] S. Legriel, M. Resche-Rigon, and A. Cariou. Dual anticonvulsant and neuroprotective effects of therapeutic hypothermia after status epilepticus. *Clin Neurol Neurosurg*, 131:87–8, 2015.

[41] T. S. Olsen, U. J. Weber, and L. P. Kammersgaard. Therapeutic hypothermia for acute stroke. *Lancet Neurol*, 2(7):410–6, 2003.

[42] T. Hiraki, W. Baker, and J. H. Greenberg. Effect of vagus nerve stimulation during transient focal cerebral ischemia on chronic outcome in rats. *J Neurosci Res*, 90(4):887–94, 2012.

Chapter 7

Modulation of Electrophysiological Parameters by Vagus Nerve Stimulation: a Translational Study

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Abstract

The recent finding of hypothermia during vagus nerve stimulation (VNS) in rats potentially has several translational implications for the future of research in the field. In the present study, we compared the dynamics of VNS associated effects on EEG and temperature in rats, to estimate which effects are most likely to be related to changes in temperature. Secondly, we examined effects of VNS on EEG in epilepsy patients in order to assess the potential to translate the effects observed on EEG in rats. In rats, EEG was recorded from the hippocampal formation, the prefrontal cortex and from the superficial frontal cortex. Intracranial temperature was additionally registered in a subset of these rats. In patients, scalp EEG was recorded, focusing on midline electrodes from the frontal to the occipital pole. In rats, effects of VNS on EEG were assessed within the first 5 minutes of VNS and a 5 minute period from the 25th to the 30th minute of VNS and compared to a 5 minute period before VNS. In patients, a 5 minute epoch of EEG acquired during a period without VNS was compared to a 5 minute period following 30-40 minutes of VNS. In both patients and rats, a rapid VNS duty cycle (7s ON/18s OFF) was used. In rats, VNS reduced theta band (4-12 Hz) EEG power in both the hippocampus and the frontal cortex during the first 5 minutes of VNS. Effects of VNS on temperature and theta peak frequency were only observed following 30 minutes of VNS. In patients, VNS had no effect on EEG power or the frequency of the alpha rhythm. These findings suggest that the translational barrier between rats and humans in VNS research may be larger than previously anticipated.

Introduction

Vagus Nerve Stimulation (VNS) is an alternative treatment for pharmacoresistant epilepsy and depression [2, 23]. It is estimated that more than 70,000 patients have undergone surgical implantation of a VNS device [5]. Despite wide-spread use, the therapeutic response remains highly variable and there are currently no factors to predict response prior to implantation of the device [8]. Among other factors, this is partly a consequence of a lack of knowledge on mechanisms underlying the therapeutic effects of VNS, despite it being the subject of intense investigation in recent years [13, 14, 25].

We recently discovered a pronounced hypothermic effect of VNS in freely moving rats, with brain and rectal temperature decreasing as much as 3 ^oC during a two hour rapid cycle VNS treatment regimen [15]. Effects were additionally attributed to stimulation of thick myelinated A-fibers [15], and because A-fibers exhibit the lowest threshold for electrical recruitment [18, 27], the implication was that at any given effective VNS intensity, an effect on temperature in rats can be expected. The fact that this effect has not previously been reported, despite decades of research, and due to the extensive influences of temperature on general physiology [10], this effect has likely had unintended influence on several previously published studies in rats. Further, hypothermia has never been reported in patients treated with VNS [24], which prompts questions on whether hypothermic and other effects observed during VNS in rats are translatable.

Hypothermia was previously found to induce a slowing of perforant path dentate field evoked potentials [19] and slowing of hippocampal theta rhythm [26]. Our observation that VNS was associated with a similar modulation [17] thus also initially prompted the hypothesis of VNS induced hypothermia in rats. Hypothermia, however, has been reported to be without effect on theta peak power or general spectral power [26]. In our previous study, nevertheless, we observed a reduction in EEG power across the entire hippocampal EEG power spectrum during VNS [17]. The VNS effect dynamics previously reported [17], could indeed indicate that effects of VNS on particularly theta power occurs faster than effects of VNS on temperature, suggesting an effect without relation to hypothermia. To further investigate this question, data from three previous rat studies (data presented in [15–17]) were aggregated to increase the sample size and increase the precision of the otherwise variable EEG outcomes. We additionally investigated whether similar effects could be observed in cortical regions, such as the prefrontal cortex and superficial frontal cortex, as EEG obtained from the scalp in patients mainly reflects superficial cortical activity [3].

It remains a question whether EEG effects observed during VNS in rats can be traced in a comparable manner in patient EEG. To study this, we examined EEG of naive patients not previously treated with VNS, which has been recorded as a part of another larger VNS study running at Ghent University Hospital (Ghent, Belgium). The analysis focused both on influences of VNS on power of specific frequency bands as well as on the alpha peak frequency. Even minor changes in temperature are associated with shifts in EEG frequency components [6], such as previously reported slowing of hippocampal theta rhythm [26]. Simulations on human EEG data similarly indicate that a decrease in brain temperature, such as observed during VNS in rats, should shift the alpha activity towards a lower frequency [6].

Methods

Rats

A total of 34 rats were included in the present study, which collects data from three different studies [15–17]. An overview of the electrode locations in each of these studies is given in **Table 7.1** and described in the following.

Rats underwent surgery under isoflurane anesthesia (5% induction, 2% maintenance). In all rats, a custom-made cuff electrode for VNS was wrapped around the left vagus nerve according to procedures described in further detail elsewhere [17, 21]. Electrode leads were tunneled subcutaneously to an incision made at the scalp. In all rats, a bipolar depth electrode, made from two twisted 70 μm thick polyimide coated stainless steel wires (tip distance of 0.9 mm), was implanted in hilus of the dentate gyrus (3.8 mm posterior, 1.9 mm lateral to bregma, ~3

	n	Electrode Locations
Study 1	8	VNS, left DG rec, left PFC rec
Study 2	16	VNS, left DG rec, left PFC rec
Study 3	10	VNS, right DG rec, left frontal cortical rec

Table 7.1: An overview of the rats included for this study and the locations of electrodes. DG rec: a bipolar recording electrode placed with the deepest tip in the hilus of the dentate gyrus, PFC rec: a bipolar recording electrode placed in prefrontal cortex, frontal cortical rec: a scalp electrode placed superficially over the frontal cortex.

mm ventral to brain surface). The implantation was based on electrophysiological feedback. In 10 rats, the electrode was placed on the right side while it was placed on the left side in 24 rats. In 18 rats, an identical bipolar depth electrode was placed in the left prefrontal cortex (PFC, 3.2 mm anterior and 0.6 mm lateral to bregma, 3.8 mm ventral to brain surface). In 10 rats, where the dentate gyrus recording electrode was placed on the right side, because a guide cannula was placed over the left dentate gyrus (3.8 mm posterior, 1.9 mm lateral to bregma, ∼2 mm ventral to brain surface). The guide cannula was compatible with a thermocouple probe, which was used for registration of intracranial temperature. These same rats additionally had an electrode placed epidurally over the right frontal cortex (∼3 mm anterior and ∼2 mm lateral to bregma). The epidural frontal cortex recording electrode was a stainless steel microscrew (diameter of 1.57 mm) placed through the right frontal bone. In all rats an identical stainless steel microscrew electrode placed over the left frontal cortex was used as a reference and ground. All electrodes were assembled in a connector block, which was fixed to the skull with additional anchor screws and acrylic cement. Upon conclusion of the surgery, rats were treated with buprenorphine (0.03 mg/kg, subcutaneously) and additional daily doses were given on the following days in case of signs of pain. The rats recovered for at least one month before inclusion in any experiments. All procedures were approved by the ethical committee for animal experiments at Ghent University Hospital (ECD 12/63, ECD 15/89) and were in accordance with the European directive 2010/63/EU.

Recordings in Rats

After at least one month of post surgery recovery, rats were attached to a custom made video-EEG setup, with free movement allowed through an electrical commutator. Rats were initially screened for response to VNS, and in accordance with previously published methods [16, 17], only rats showing a reduction in hippocampal EEG power following VNS (7s ON/18s OFF, 1000 μA, 250 μ, 30 Hz) were included for further study. All rats were included for two experimental sessions: a VNS (7s ON/18s OFF, 1000 μA, 250 μs, 30 Hz) session and a SHAM session, where no active stimulation was applied. In 10 rats, an additional similar VNS session, with VNS applied at an intensity of 250 μA instead of 1000 μA, was included. All sessions had an initial one hour baseline recording period, followed by a two hour VNS recording period. In each "OFF" phase of the VNS duty cycle, a 10 s sweep of EEG was obtained. EEG was single-ended recorded, using a microscrew electrode placed over left frontal cortex as reference and ground. All EEG signals were high-pass filtered at 0.1 Hz and was sampled at 1kHz using a USB-6259 National Instruments data acquisition device (National Instruments, Austin, Texas, USA) with a 16 bit dynamic resulting in a $\pm$3.05 μV amplitude resolution for the $\pm$ 10 mV input range.

In study 3 (**Table 7.1**), intracranial temperature was registered alongside hippocampal and cortical EEG recordings. Temperature was registered in 10 rats with a copper-constantan thermocouple (HYPO-33-1-T-G-60-SMPW-M, OMEGA), which was plugged into the intracra-

	Gender	Age	AEDs
Patient 1	M	24	TPM, LEV, PGB
Patient 2	M	51	VPA, PGB, LCZ, CZP
Patient 3	F	54	CZP, CBZ, TPM, LCZ
Patient 4	M	51	LEV, PGB, CZP, LCZ
Patient 5	F	54	CLB, LTG, LCZ, TPM
Patient 6	F	52	LEV, LTG, CZP, CBZ, DZP, LZP
Patient 7	F	59	LCZ, LEV, LZP, PZP
Patient 8	M	29	VPA, TPM, LCZ
Patient 9	F	59	LCZ, LEV
Patient 10	F	63	LTG, CZP, TPM, LCZ
Patient 11	M	36	PRP, LEV, VPA
Patient 12	M	46	CBZ, VPA, PHT, PGB
Patient 13	M	28	OXC, VPA, LCZ
Patient 14	F	45	PGB, CBZ
Patient 15	F	24	LCZ, TPM
Patient 16	M	69	CBZ, LEV, VPA, LCZ
Patient 17	F	28	LEV, PGB, CZP, LCZ, APZ

Table 7.2: An overview of the patients included in the study. F: Female, M: Male, AED: Antiepileptic drug, APZ: Alprazolam; CBZ: Carbamazepine, CZP: Clonazepan, DZP: Diazepam, LCZ: Lacosamide, LEV: Levetiracetam, LTG: Lamotrigine, LZP: Lorazepam, OXC: Oxcarbazepine, PGB: Pregabalin, PRP: Perampanel, PHT: Phenytoin, PZP: Prazepam, TPM: Topiramate, VPA: Valproic Acid.

nial guide cannula placed over the left dentate gyrus. Temperature was sampled every 30 s to a computer.

Patients

Seventeen patients (9 women, 8 men) with drug resistant epilepsy and a VNS device implanted were included for the study. Patients had an age of 45.4 $\pm$ 14.5 (mean $\pm$ standard deviation) years at the time of the study (**Table 7.2**). The VNS device (Cyberonics, Houston, TX, USA) was implanted 2-3 weeks prior to the experimental procedure. The VNS device consisted of a pulse generator implanted under the left clavicle and 2 helical electrodes placed around the cervical trunk of the left vagus nerve. The data presented is a part of a larger VNS study, which took place at the Reference Center for Refractory Epilepsy, Ghent University Hospital, during a video-EEG monitoring session. Patients included were all older than 18 years of age and had an IQ>70 on the Wechsler Adult Intelligence Scale, Third Edition. The patients included were all naive patients, being treated with VNS for the first time in the course of this study. The study was approved by the ethics committee of Ghent University Hospital. All patients gave written informed consent according to the Helsinki Declaration, following thorough explanation and understanding of the experimental procedure.

Recordings in Patients

During the EEG recordings, patients were engaged in an auditory oddball task, whereby the behavioral state of the patient was fixed. The study included at least 30 minutes of EEG during VNS (VNS ON; 7s ON/18s OFF, 250 μA, 250 μ, 30 Hz) and 30 minutes of EEG with no VNS applied (VNS OFF). The order of these periods were randomized. EEG was recorded with a Micromed System Plus (Micromed, Mogliano Veneto, Italy), using gold plated electrodes placed according to the 27 standard locations proposed by the extended international 10-20 system [12].

All electrodes were referenced to an electrode placed on the right mastoid. The ground electrode was placed on the left mastoid. VNS artifacts were detected using an electrode placed in the anterior neck area. Signals were sampled at 1024 Hz, low-pass filtered at 250 Hz, high-pass filtered at 0.1 Hz and amplified 316 times. Recording impedances were all below 10 kΩ.

Analysis EEG

All processing of EEG was performed in Matlab (The MathWorks, Inc., Natick, US). In rats, local hippocampal and PFC activity was isolated by subtracting the two signals acquired from each of these two regions. Ten second EEG sweeps affected by large artifacts were detected based on raw power and were removed if the raw power of the EEG sweep deviated more than 3 standard deviations from the mean power of all samples. For power spectral analysis, all 10 s EEG sweeps were initially subjected to digital filtering between 2 and 100 Hz, using a first order Butterworth filter. Additionally, a 3rd order bandstop filter between 48 and 52 Hz was applied to attenuate any 50 Hz contamination of signals and to yield the analysis comparable to the analysis later performed on the patient EEG, where the same procedure was applied. The 10 s EEG sweeps were then split into 1 s windows overlapping by 0.5 s, resulting in 19 windows. The windows were detrended before applying a Fast Fourier Transform and the power spectrum was extracted by calculating the modulus of each frequency component. The 19 windows of each 10 s EEG sweep were then averaged to yield one representative power spectrum with a frequency resolution of 1 Hz. To calculate theta band (4-12 Hz) and gamma band (30-100 Hz) power, power of frequency components within these frequency bands was integrated.

Theta peak frequency of the hippocampal EEG was calculated by initially bandpass filtering the signals between 3 and 20 Hz with a 1st order Butterworth filter. Each 10 s EEG signal was then split into 5 s windows overlapping by 2.5 seconds, yielding 3 windows. Following detrending, application of the Fast Fourier Transform and averaging, this resulted in a power spectrum with a frequency resolution of 0.2 Hz. The spectrum was further smoothed with a running average of 2 Hz. The theta peak was determined as the frequency component in the resulting spectrum displaying the most power.

For the patient EEG, 12 s EEG samples were extracted in each VNS OFF period. EEG samples were removed if the raw power deviated more than 3 standard deviations from the mean power of all samples. All analysis focused on the midline electrodes (Fpz, Fz, Cz, Pz and Oz). For power spectral analysis, EEG was filtered between 2 and 100 Hz with a 1st order Butterworth bandpass filter and between 48 and 52 Hz with a 3rd order Butterworth bandstop filter. The 12 s EEG samples were split into 23 windows of 1 s overlapping by 0.5 s. Following detrending, calculation of the power spectrum using the Fast Fourier Transform and averaging the 23 power spectra for each 12 s EEG sample, the resulting power spectrum had a frequency resolution of 1 Hz. The power of Delta ($<$4 Hz), Theta (4-7 Hz), Alpha (8-13 Hz), Beta (14-30 Hz) and Gamma (30-100) frequency bands was calculated by integrating power of the frequency components within the respective frequency bands.

Alpha peak frequency was analyzed by initially filtering between 5 and 16 Hz using a 1st order Butterworth bandpass filter and then splitting the 12 s EEG sample into 4 second windows overlapping by 2 s, resulting in 5 windows. Following detrending, calculation of the power spectrum using the Fast Fourier Transform and averaging of the 5 windows of each 12 s EEG sample, this resulted in a frequency resolution of 0.25 Hz. The power spectrum was further smoothed with a running average over 2.5 Hz. The alpha peak frequency was determined as the frequency component displaying the highest power value.

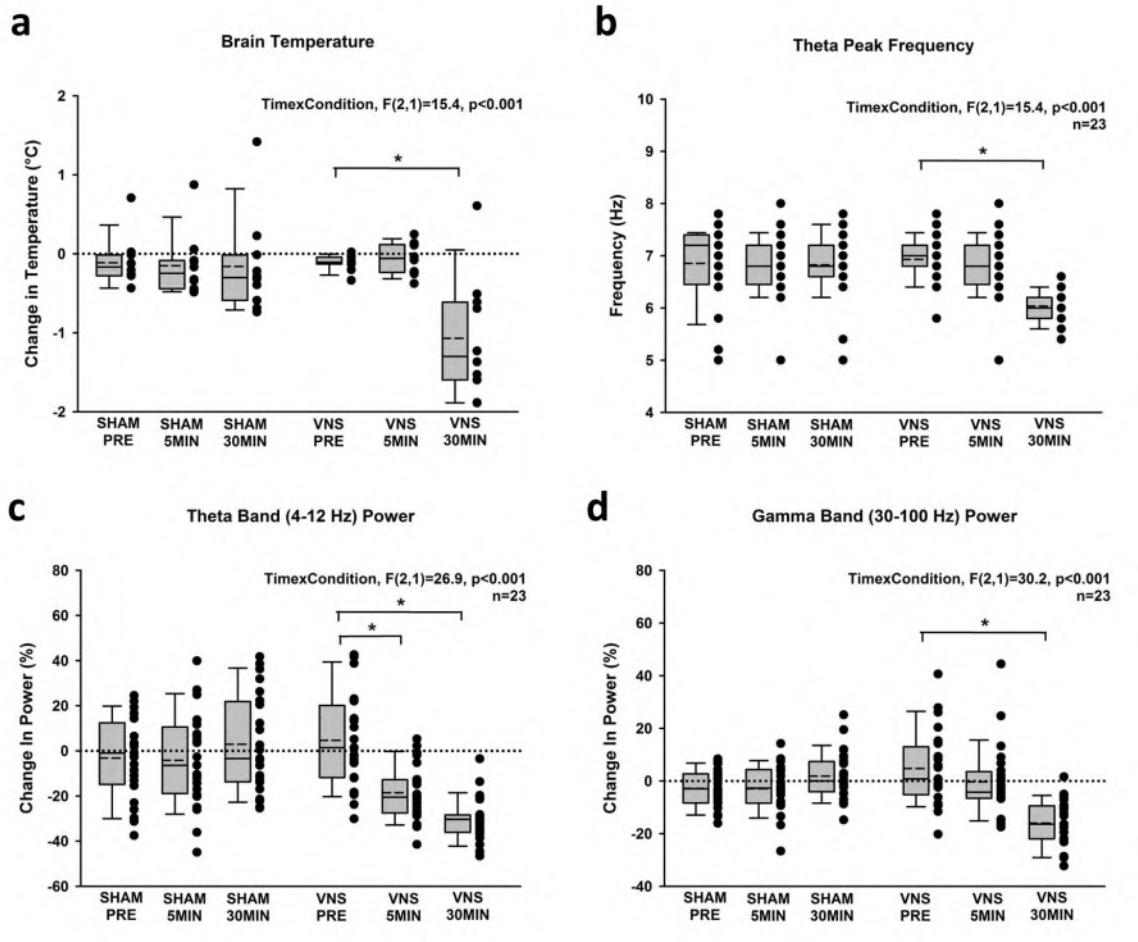

Figure 7.1: Effects of VNS applied at an intensity of 1000 μA, on brain temperature and hippocampal EEG outcomes in rats. Outcomes have been averaged over 5 minute periods: 5 minutes prior to VNS or SHAM onset (PRE), the first 5 minutes of VNS or SHAM (5MIN) and the 5 minutes from the 25th and 30th minute of VNS or SHAM (30MIN). Outcomes included were brain temperature **(a)**, theta peak frequency **(b)**, theta band (4-12 Hz) power **(c)**, and gamma band (30-100 Hz) power **(d)**. Solid lines of box plots denote medians, while dashed lines denote means. Significant differences ($p<0.05$) between time points within conditions are marked with a bar and an asterisk.

Statistical Analysis

All parameters from rats were averaged into 5 minute epochs: the 5 minutes just prior to VNS, the first 5 minutes during VNS and 5 minutes from the 25th to the 30th minute of the VNS period. Similarly, in patients, the EEG parameters were averaged into 5 minute epochs at the end of the VNS ON and VNS OFF periods, respectively. All outcomes, with exception of the theta peak frequency and alpha peak frequency outcomes, were normalized to the mean of the whole baseline in rats, and to the mean of the 5 minute VNS OFF epoch in patients. For rats, the full baseline period was a full hour of recordings before VNS onset, whereas for patients it was the whole approximately 30 minute VNS OFF period. For rats, where a SHAM session was included, a two way repeated measures ANOVA, with factors time and stimulation condition was used to analyze all outcomes. Turkey's post hoc test was used to identify differences between

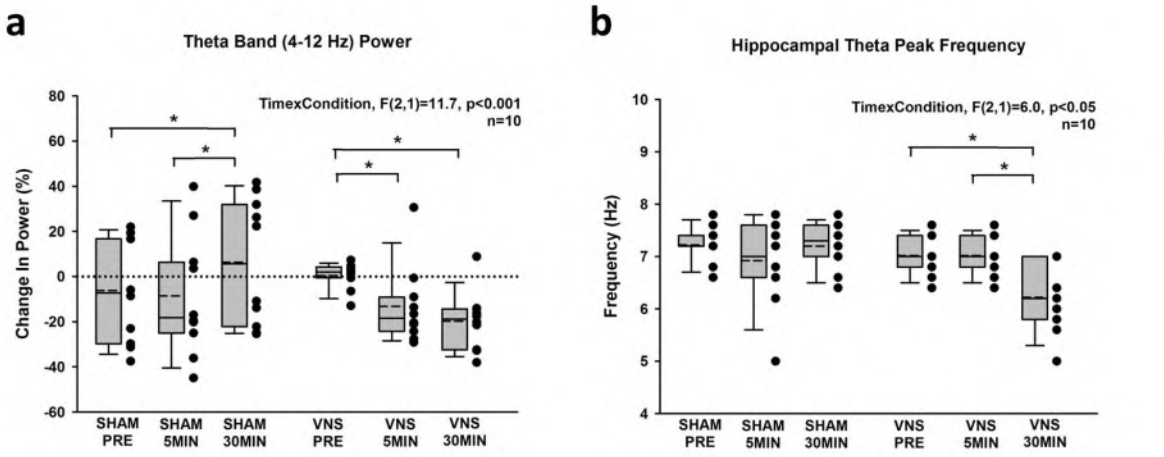

Figure 7.2: Effects of VNS applied at an intensity of 250 μA on hippocampal EEG outcomes in rats. Outcomes have been averaged over 5 minute periods: 5 minutes prior to VNS or SHAM onset (PRE), the first 5 minutes of VNS or SHAM (5MIN) and the 5 minutes from the 25th and 30th minute of VNS or SHAM (30MIN). Outcomes assessed were hippocampal theta band (4-12 Hz) power and theta peak frequency. Solid lines of box plots denote medians, while dashed lines denote means. Significant differences ($p<0.05$) between time points within conditions are marked with a bar and an asterisk.

specific time points. For patients, difference between VNS ON and OFF periods were assessed in paired t-tests.

Results

Modulation of the EEG in the Rat

VNS applied with an output current of 1000 μA significantly reduced brain temperature (**Fig. 7.1a**, time x condition: $F(2,1)=15.4$, $p<0.001$), the theta peak frequency of hippocampal EEG (**Fig. 7.1b**, time x condition: $F(2,1)=15.4$, $p<0.001$), hippocampal theta band (4-12 Hz) power (**Fig. 7.1c**, time x condition: $F(2,1)=17.0$, $p<0.001$) and gamma band (30-100 Hz) power (**Fig. 7.1d**, time x condition: $F(2,1)=30.5$, $p<0.001$). There were no effects of VNS on brain temperature within the first 5 minutes of VNS, though a significant effect was observed following 30 minutes of VNS. The same pattern was observed for theta peak frequency and gamma band power, while an immediate effect of VNS was observed on theta band power within the first 5 minutes of VNS. Application of VNS at an intensity of 250 μA similarly shifted theta peak frequency to a lower frequency (**Fig. 7.2b**, time x condition: $F(2,1)=6.0$, $p<0.001$), reduced theta band power (**Fig. 7.2a**, time x condition: $F(2,1)=11.7$, $p<0.001$) and reduced gamma band power (time x condition: $F(2,1)=19.8$, $p<0.001$, data not shown). As when VNS was applied at an intensity of 1000 μA, VNS was associated with an immediate decrease in theta band power, while the shift in theta peak frequency and reduction in gamma band power only was observed following 30 minutes of VNS.

In addition to modulating the hippocampal EEG, VNS was found to modulate theta band power in both the prefrontal EEG (**Fig. 7.3d**, time x condition: $F(2,1)=7.1$, $p<0.001$) and epidural frontal EEG (**Fig. 7.3d**, time x condition: $F(2,1)=20.4$, $p<0.001$). As in the hippocampal EEG,

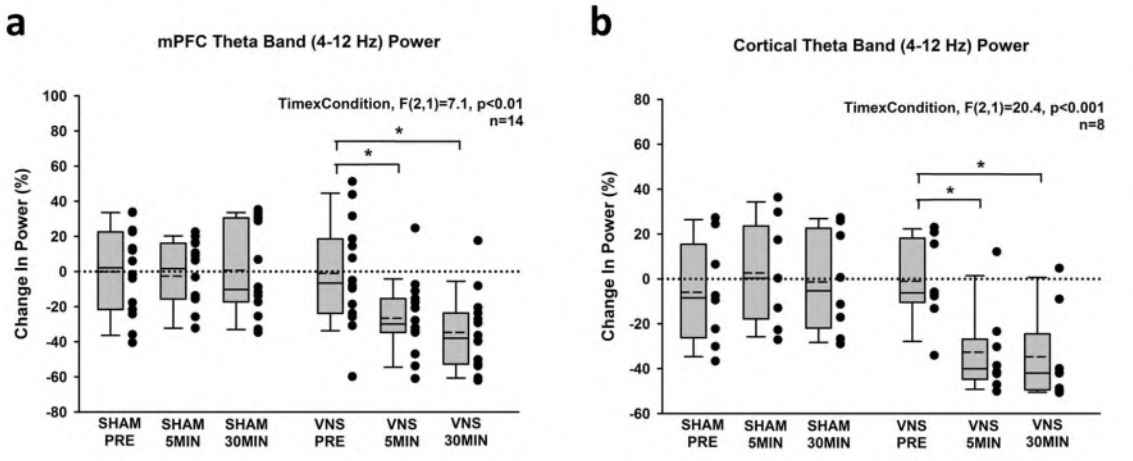

Figure 7.3: Effects of VNS applied at an intensity of 1000 μA on prefrontal depth EEG and epidural frontal EEG in rats. Outcomes have been averaged over 5 minute periods: 5 minutes prior to VNS or SHAM onset (PRE), the first 5 minutes of VNS or SHAM (5MIN) and the 5 minutes from the 25th and 30th minute of VNS or SHAM (30MIN). In **(a)**, effects of VNS on prefrontal depth EEG are presented, while effects on epidural frontal EEG are shown in **(b)**. Solid lines of box plots denote medians, while dashed lines denote means. Significant differences ($p<0.05$) between time points within conditions are marked with a bar and an asterisk.

reductions in theta band power were observed during the first 5 minutes of VNS. No significant effects of VNS were observed on gamma band power.

Modulation of EEG in Epilepsy Patients

VNS was not associated with any consistent changes in general EEG power from the frontal to occipital poles (**Fig. 7.4**). There were further no changes in power within individual frequency bands. As seen in **Fig. 7.4**, an alpha peak was increasingly present in the EEG power spectra towards the posterior scalp and was only found to be robust at Pz and Oz electrodes. VNS was found to be without effect on the frequency of the alpha power peak (**Fig. 7.5**).

Discussion

The main outcomes of the present study were: 1) effects of VNS on low frequency EEG power in rats do not depend on temperature as low frequency EEG power decreased before any effect on temperature was observed; 2) effects of VNS on low frequency EEG power may be a global cortical phenomenon as it is also observed both in the prefrontal depth EEG and epidural frontal EEG; 3) no comparable effects of VNS were observed in the EEG of epilepsy patients.

Following the finding of VNS associated hypothermia in rats, the question of whether VNS also induces hypothermia in patients has been pending. For ethical reasons it is not possible to measure brain temperature in patients invasively as was done in rats. Hypothermia generally has been reported to slow neural potentials, manifesting among other effects as a slowing of hippocampal theta frequency [26]. We thus sought to use the alpha frequency, which is a commonly observed rhythm in posterior cortical regions, as a surrogate marker for a decrease in brain temperature [6]. However, we did not find any comparable effect of VNS on alpha peak

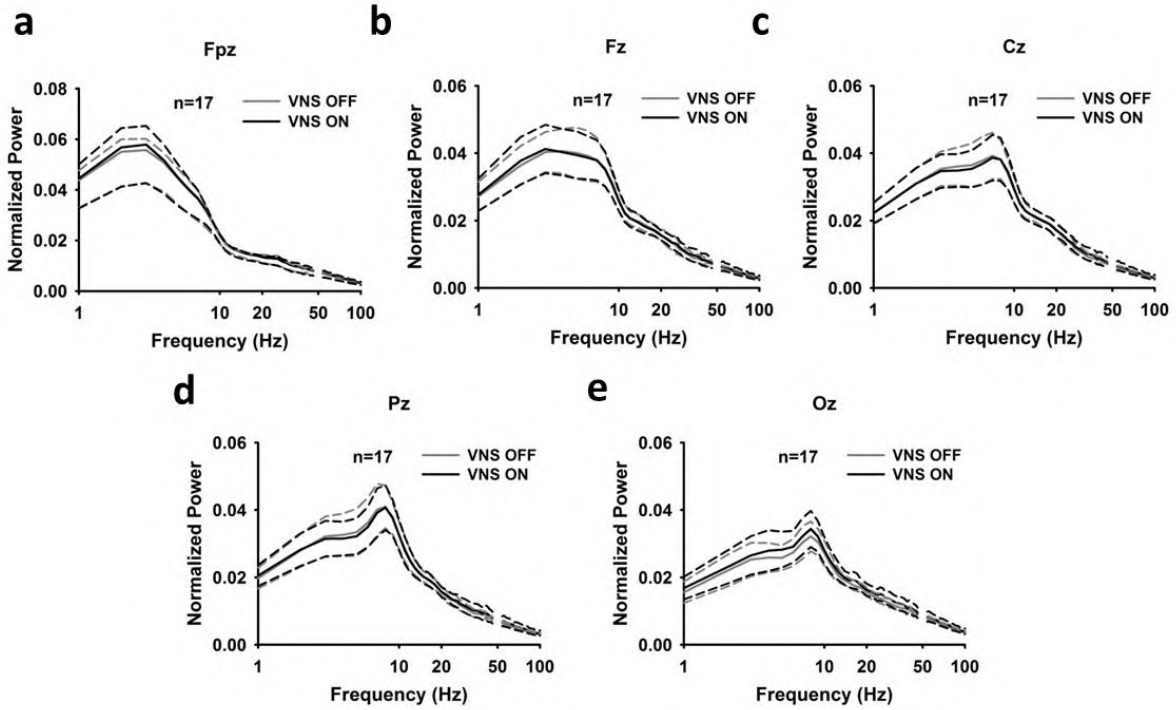

Figure 7.4: VNS was not associated with any effect on scalp EEG power in epilepsy patients. Electrodes included were the Fpz **(a)**, Fz **(b)**, Cz **(c)**, Pz **(d)** and Oz **(e)**. The EEG power spectra have been averaged over 5 minute periods during VNS OFF and ON periods, respectively, and further averaged for all 17 patients included. The dashed lines denote the 95% confidence intervals. Note that the x-axis is scaled logarithmically.

frequency, which contradicts any prominent effect of VNS on brain temperature in patients and further suggests that VNS induced hypothermia may be a species specific phenomenon.

The findings of the present study, however, also suggest that VNS may trigger multiple mechanisms simultaneously in rats, with some mechanisms not being directly related to a change in temperature. This includes a reduction in theta EEG power, which occurred before any hypothermic effects of VNS. The effect of VNS on theta EEG may be comparable to the "desynchronizing" effects of VNS on low frequency field activity observed in early studies in cats [4, 28]. The conclusions of these studies were however not based on quantitative power spectral analysis, but rather extracted simply from visual inspection of the EEG. Our findings may similarly reflect the same phenomena of decreased power, quantified by power spectral analysis, which has been reported for VNS applied during slow wave sleep in the auditory cortex of rats [7, 20]. In the present study, we extend these reports, showing that VNS reduces theta power of EEG in the frontal cortex, which could suggest a global cortical effect.

Because we observed these effects in the cortical EEG, we hypothesized that we would see similar effects of VNS on scalp EEG power in patients. However, VNS was without effect on patient EEG in any frequency bands at any of the electrodes studied, extending from the frontal to the occipital pole. Notably, the patients studied had not previously been treated with VNS, excluding the influence of a potential chronic neuromodulatory effect. Our observation is thus in line with previous studies reporting no effects of VNS on EEG in patients [11, 22]. The observation of reduced low frequency EEG power in rats may thus be another species specific

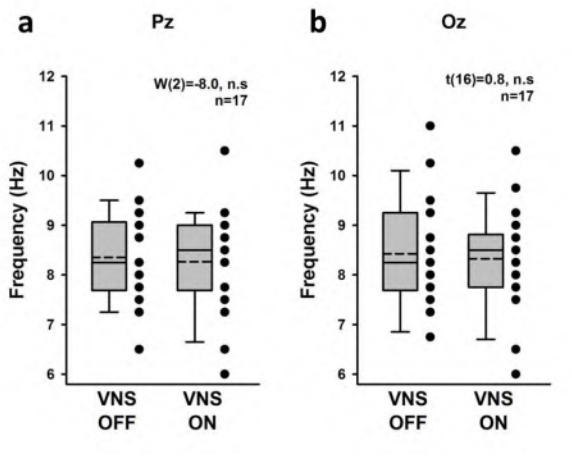

Figure 7.5: Effects of VNS on alpha peak frequency in patients. . Effects of VNS on EEG acquired from the Pz (a) and Oz (b) electrodes are presented. Solid lines of box plots denote medians, while dashed lines denote means.

phenomenon and potentially implies that all of the effects we observed on the EEG in rats, both temperature dependent and independent effects, are not to be traced in a comparable manner in patients. This prompts obvious questions regarding the extent to which rats constitute an appropriate translational model to study mechanisms of VNS.

Apart from the obvious translational barrier of species differences, some substantial differences between the recording conditions in the rats and patients studied should be noted. First of all, there are differences in the quality of EEG from patients and rats. In patients, EEG was recorded from the scalp. In rats, however, EEG was recorded invasively, which limits the loss of signal amplitude through the poorly conducting skull. Secondly, the thermoregulatory dynamics in rats and humans are likely to differ due to differences in body surface/volume ratios, which is lower in humans and thus could result in a slower release of heat during a thermoregulatory response, such as observed during VNS in rats. Thirdly, the patients studied were drug-resistant epilepsy patients, who in the effort to achieve maximal seizure control were treated with high doses of anti-epileptic drugs likely to interfere with VNS associated effects.

It may additionally be difficult to assess precisely whether we stimulate the vagus nerve in the same way, i.e. with regard to fiber type recruitment, in rats and patients. In the majority of the rat data reported, an intensity of 1000 μA was used with a pulse width of 250 μs, while the patients were only stimulated with an intensity of 250 μA. A VNS intensity of 250 μA in rats, however, induced a similar modulation of hippocampal EEG as 1000 μA. Lower intensities in the range of 200-300 μA have additionally been reported as sufficient to modulate temperature [15], which overall indicates predominant involvement of thick myelinated A-fibers [27]. Simultaneous activation of thick myelinated efferent motor fibers, innervating the laryngeal muscles, results in a recordable muscle potential, the laryngeal muscle evoked potential (LMEP), which thus has been proposed as a marker for effective vagal nerve activation [9]. Importantly, the LMEP displays a threshold similar to the threshold for recruitment of thick myelinated type A-afferents in both rats [18] and dogs [27], where the size of the vagus nerve resembles the vagus nerve of humans [27]. This overall suggests that VNS intensities are comparable across species. Preliminary evidence from our group further indicates that LMEPs can also be recorded in patients, at intensities as low as 250 μA (unpublished observations). This would suggest that at

least a proportion of the patients included in the present study would have been stimulated at an intensity sufficient to effectively recruit thick myelinated type-A afferents, whereby similar effects could be expected in the patient EEG. VNS additionally induced voice alterations in some patients, which is also attributed to stimulation of thick-myelinated A-fibers [1] and thus another indication that sufficient current was used.

In this context, it should be mentioned that rats without any effects of VNS on EEG were excluded from the study in accordance with previously published methods [7, 17, 20], with the idea being that this reflects ineffective VNS delivery and that no comparable approach was applied to the patient data. In our previous rat study [17], however, we described how a cluster analysis clearly could identify two clusters of rats, with one cluster displaying a clear reduction in EEG power, while no effects were observed in the other cluster. Even when combining these clusters, the overall group mean still showed a clear reduction in EEG power, while no such effect were observed in patients of the present study.

The study illustrates that the translational barrier between rats and humans may be larger than previously anticipated in VNS research. It should be noted that none of the electrophysiological effects in rats described above have been related to anticonvulsant effects of VNS. The present study, however, questions the relevance of such comparisons and may instead encourage further study of VNS in species that more appropriately represent the human scenario, which could include larger animals as pigs or dogs, or where possible, in patients.

References

[1] J. J. Ardesch, J. R. Sikken, P. H. Veltink, H. E. van der Aa, G. Hageman, and H. P. Buschman. Vagus nerve stimulation for epilepsy activates the vocal folds maximally at therapeutic levels. *Epilepsy Res*, 89(2-3):227–31, 2010.

[2] E. Ben-Menachem. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol*, 1(8):477–82, 2002.

[3] G. Buzsaki, C. A. Anastassiou, and C. Koch. The origin of extracellular fields and currents–eeg, ecog, lfp and spikes. *Nat Rev Neurosci*, 13(6):407–20, 2012.

[4] M. H. Chase, M. B. Sterman, and C. D. Clemente. Cortical and subcortical patterns of response to afferent vagal stimulation. *Exp Neurol*, 16(1):36–49, 1966.

[5] Cyberonics. Cyberonics images and captions, 2013.

[6] T. Deboer. Brain temperature dependent changes in the electroencephalogram power spectrum of humans and animals. *J Sleep Res*, 7(4):254–62, 1998.

[7] N. D. Engineer, J. R. Riley, J. D. Seale, W. A. Vrana, J. A. Shetake, S. P. Sudanagunta, M. S. Borland, and M. P. Kilgard. Reversing pathological neural activity using targeted plasticity. *Nature*, 470(7332):101–4, 2011.

[8] D. J. Englot, E. F. Chang, and K. I. Auguste. Vagus nerve stimulation for epilepsy: a meta-analysis of efficacy and predictors of response. *J Neurosurg*, 115(6):1248–55, 2011.

[9] A. Grimonprez, R. Raedt, L. De Taeye, L. E. Larsen, J. Delbeke, P. Boon, and K. Vonck. A preclinical study of laryngeal motor-evoked potentials as a marker vagus nerve activation. *Int J Neural Syst*, page 1550034, 2015.

[10] A. C. Guyton and J.E. Hall. *Thextbook of Medical Physiology*. Elsevier Sauders, Philadelphia, 11th edition edition, 2006.

[11] E. J. Hammond, B. M. Uthman, S. A. Reid, and B. J. Wilder. Electrophysiologic studies of cervical vagus nerve stimulation in humans: Ii. evoked potentials. *Epilepsia*, 33(6):1021–8, 1992.

[12] Herbert Henri Jasper. The ten twenty electrode system of the international federation. *Electroencephalography and Clinical Neurophysiology*, 10:371–375, 1958.

[13] S. E. Krahl. Vagus nerve stimulation for epilepsy: A review of the peripheral mechanisms. *Surg Neurol Int*, 3(Suppl 1):S47–52, 2012.

[14] S. E. Krahl and K. B. Clark. Vagus nerve stimulation for epilepsy: A review of central mechanisms. *Surg Neurol Int*, 3(Suppl 4):S255–9, 2012.

[15] L. E. Larsen, W. Van Lysebettens, S. Carrette, S. Daelemans, M. Sprengers, L. Thyrion, W. J. Wadman, J. Delbeke, P. Boon, K. Vonck, and R. Raedt. Vagus nerve stimulation induces hypothermia in freely moving rats. *(In Preparation)*, 2016.

[16] L. E. Larsen, W. J. Wadman, D. Marinazzo, P. van Mierlo, J. Delbeke, S. Daelemans, M. Sprengers, L. Thyrion, W. Van Lysebettens, E. Carrette, P. Boon, K. Vonck, and R. Raedt. Vagus nerve stimulation applied with a rapid cycle has more profound influence on hippocampal electrophysiology than a standard cycle. *Neurotherapeutics*, 2016.

[17] L. E. Larsen, W. J. Wadman, P. van Mierlo, J. Delbeke, A. Grimonprez, B. Van Nieuwenhuyse, J. Portelli, P. Boon, K. Vonck, and R. Raedt. Modulation of hippocampal activity by vagus nerve stimulation in freely moving rats. *Brain Stimul*, 9(1):124–32, 2016.

[18] L. Mollet, R. Raedt, J. Delbeke, R. El Tahry, A. Grimonprez, I. Dauwe, D. E. Herdt V, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Electrophysiological responses from vagus nerve stimulation in rats. *Int J Neural Syst*, 23(6):1350027, 2013.

[19] E. I. Moser, M. B. Moser, and P. Andersen. Potentiation of dentate synapses initiated by exploratory learning in rats: dissociation from brain temperature, motor activity, and arousal. *Learn Mem*, 1(1):55–73, 1994.

[20] J. A. Nichols, A. R. Nichols, S. M. Smirnakis, N. D. Engineer, M. P. Kilgard, and M. Atzori. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience*, 189:207–14, 2011.

[21] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[22] M. C. Salinsky and K. J. Burchiel. Vagus nerve stimulation has no effect on awake eeg rhythms in humans. *Epilepsia*, 34(2):299–304, 1993.

[23] T. E. Schlaepfer, C. Frick, A. Zobel, W. Maier, I. Heuser, M. Bajbouj, V. O'Keane, C. Corcoran, R. Adolfsson, M. Trimble, H. Rau, H. J. Hoff, F. Padberg, F. Muller-Siecheneder, K. Audenaert, D. Van den Abbeele, Z. Stanga, and M. Hasdemir. Vagus nerve stimulation for depression: efficacy and safety in a european study. *Psychol Med*, 38(5):651–61, 2008.

[24] G. H. Vijgen, N. D. Bouvy, L. Leenen, K. Rijkers, E. Cornips, M. Majoie, B. Brans, and W. D. van Marken Lichtenbelt. Vagus nerve stimulation increases energy expenditure: relation to brown adipose tissue activity. *PLoS One*, 8(10):e77221, 2013.

[25] K. Vonck, R. Raedt, J. Naulaerts, F. De Vogelaere, E. Thiery, D. Van Roost, B. Aldenkamp, M. Miatton, and P. Boon. Vagus nerve stimulation...25 years later! what do we know about the effects on cognition? *Neurosci Biobehav Rev*, 45:63–71, 2014.

[26] I. Q. Whishaw and C. H. Vanderwolf. Hippocampal eeg and behavior: effects of variation in body temperature and relation of eeg to vibrissae movement, swimming and shivering. *Physiol Behav*, 6(4):391–7, 1971.

[27] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

[28] A. Zanchetti, S. C. Wang, and G. Moruzzi. The effect of vagal afferent stimulation on the eeg pattern of the cat. *Electroencephalogr Clin Neurophysiol*, 4(3):357–61, 1952.

Part III

Discussion and Conclusions

Chapter 8

Questioning the Status Quo: Revision of Current Ideas about Vagus Nerve Stimulation

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Abstract

In recent years, our understanding of Vagus Nerve Stimulation (VNS) has improved substantially. Recently, however, we showed that VNS induces hypothermia in freely moving rats, decreasing brain and rectal temperature by as much as 3 ^{o}C. Following a revision of current literature on VNS, it is apparent that hypothermia is likely to have exerted influence on the outcome of more than 40 previously studied studies on VNS. Because of the vast physiological influences of temperature it is further likely that the finding has implications for our current understanding of VNS, which calls for a critical review of the currently dominant theories within the field of VNS research.

Background

Over the past decades, the use of Vagus Nerve Stimulation (VNS) has expanded from initial clinical application and regulatory clearance for drug resistant epilepsy [1], to regulatory clearance for drug resistant depression [2]. VNS has additionally been experimentally applied in pain disorders [3, 4], stroke syndromes [5, 6], inflammatory disorders [7, 8] and cardiac disorders [9], with several other potential applications in the pipeline. This development first of all reflects the result of intense investigation counting thousands of preclinical and clinical studies. The multitude of effects associated with VNS further relies on the widespread systemic influences of the vagus nerve [10].

The understanding of working mechanisms underlying therapeutic effects of VNS has substantially improved in recent years and what once were mere hypotheses are now well documented theories supported by abundant experimental evidence. Here, the aim is to critically address some of the more dominant theories. The first part aims to discuss the implications of recent findings demonstrating hypothermia in freely moving rats subjected to continuously cycled VNS to our understanding of VNS [11]. This is relevant as temperature influences the dynamics of several physiological processes and because this effect previously has gone unnoticed, which makes it likely that changes in temperature can have influenced the outcome of several previously published studies. The final part additionally includes a critical assessment of other dominant theories in the field of VNS research.

Conditions under which VNS is likely to induce hypothermia

Decreased brain and rectal temperature was found following application of continuously cycled VNS in awake freely moving rats [11]. Application of a rapid duty cycle (7s ON/18 s OFF, 28% ON-time) for two hours was associated with a decrease in brain temperature of around 3 ^{o}C, while a standard cycle (30 s ON/300 s OFF, 9% ON-time) applied for two hours was associated with a decrease in brain temperature of around 1 ^{o}C. This led to the idea that the extent to which temperature is affected depends on the %-ON-time, with higher %-ON times resulting in a larger effect. Exponential fits applied to the temperature decrease additionally indicated that temperature would decrease even further if VNS would be applied for longer durations than two hours. The finding raised an important question regarding the extent to which hypothermic effects of VNS in rats are relevant in a translational context. Despite more than 20 years of clinical application, VNS has never been described to affect temperature or induce thermal discomfort in patients [12]. Assessment of electroencephalographic parameters as a surrogate for changes in brain temperature further did not indicate major changes in brain temperature [13]. Though the question should be examined under controlled conditions, VNS-induced hypothermia is very likely to be species specific, which would raise a series of questions regarding the translational value of VNS research conducted in rats. Nevertheless, the only circumstance under which we can be sure VNS affects temperature is in rats under the conditions already described [11].

To get an idea about how many studies that are likely to be affected by unintended temperature changes, the PUBMED database was searched using the keywords "vagus nerve stimulation AND rat"[1]. Studies were classified according to whether VNS has been likely or unlikely to have affected temperature. The majority of VNS studies have been conducted under anesthesia, which typically are temperature controlled, due to which these were classified as unlikely affected. Remaining studies, conducted in awake rats, were classified as likely affected if VNS was applied with a standard duty cycle (30 s ON/300 s OFF, 9%) or cycles with a higher %-ON time for a duration of an hour or more. Because effects of VNS on temperature were observed at output currents as low as 200 μA, using a pulse width of 250 μs, only studies stimulating above this threshold were included. Additionally, only studies using a VNS pulse frequency of 20-30 Hz, the most commonly used frequency range [14], were included. Based on these criteria 40 studies were selected where VNS-induced hypothermia is likely to have occurred and affected the study outcome. In addition, four other studies, where VNS was described as applied continuously, i.e. no cycle, for 20 minutes or more, are discussed as likely affected due to the intense VNS regimen (100% ON-time) applied. All studies are summarized in **Table 8.1** and form the basis of the discussion in the following sections.

Physiological consequences of hypothermia

Temperature has the potential to influence all known physiological processes, which is why experimentalists mostly want to control it in order to avoid unaccounted influence on the experimental outcome. The degree to which even minor changes of a few degrees Celsius is capable of affecting the fundamental physiology or pathophysiology is relatively well studied [15, 16]. Fever is perhaps the most commonly studied phenomenon, where an increase in temperature facilitates the rate of molecular interactions and as a result increases the activity of the immune system in the presence of pathogens [17]. Reducing temperature, contrarily, attenuates the immune response and slows the general metabolic rate of cells [18], decreasing requirements for oxygen and other nutritional requirements. In the brain, hypothermia has been found to be neuroprotective [19],

[1] The search yielded 1965 publications on April 6th, 2016

decreasing the release and increasing the reuptake of excitatory neurotransmitters [20, 21], in addition to increasing the integrity of the blood-brain-barrier [22]. Hypothermia thus influences several neurophysiological correlates, manifesting in a general slowing of neural transmission [23, 24] and neural oscillations [25, 26], a disruption of synchrony [27] and an alteration of the responsivity of the neural tissue to electrical stimuli [28]. It was indeed the finding of slowed neural transmission during VNS [29] which initially prompted the hypothesis that VNS might be associated with hypothermia. If not accounted for, even a minor change in temperature may lead to false interpretation of study outcomes.

Table 8.1: Studies in which hypothermic effects may have occurred as a result of continuously cycled VNS in freely moving rats. A rapid duty cycle (7s ON/18s OFF, 28%) applied for two hours was associated with a larger reduction in temperature of around 3 ^{o}C, compared to a standard cycle (30s ON/300s OFF, 9%), which reduced temperature by around 1 ^{o}C [11]. Applications of VNS for longer than two hours are likely to decrease temperature even further [11]. For these reasons, the table reports the duty cycle used in addition to the duration over which VNS was applied in the studies. The %-value reported in the duty cycle column refers to the %-ON time of VNS relative to the total duration of the duty cycle. The table additionally reports the major outcomes of the studies. It should be noted that only studies using intensities of 200 μA with a pulse width of at least 250 μs are included. Further, with exception of 4 studies, where continuous VNS was applied, only studies applying VNS for one hour or longer are included.

Study	**Duty Cycle**	**Duration**	**Study Outcome**
Samniang 2016 [30]	14s ON/48s OFF (23%)	12 weeks	↓ plasma insulin, cholesterol, triglycerides, ↓ body weight
Agarwal 2016 [31]	10s ON/60s OFF (14%)	2 weeks	↓ heart size/weight
Larsen 2016 [32]	7s ON/18s OFF (28%)	2 hours	Modulation of hippocampal electrophysiology
Larsen 2016 [29]	7s ON/18s OFF (28%)	2 hours	Modulation of hippocampal electrophysiology
Xiang 2015 [33]	7s ON/66s OFF (10%)	4 weeks	↓ cerebral infarct size, ↓ inflammation
Grimonprez 2015 [34]	7s ON/18s OFF (28%)	1 hour	↓ depressive symptoms
Grimonprez 2015 [35]	7s ON/18s OFF (28%)	2 weeks	↓ depressive symptoms
Annoni 2015 [36]	30s ON/300s OFF (9%)	2 weeks	↓ blood pressure, ↓ cardiac arrhythmia
Xie 2014 [37]	7s ON/60s OFF (10%)	12 weeks	↑ cardiac function following ischemia
Perez 2014 [38]	30s ON/300s OFF (9%)	4 weeks	↓ schizophrenic symptoms
Kawada 2014 [39]	10s ON/60s OFF (14%)	6 weeks	↑ baroreflex function following cardiac ischemia
Carreno 2014 [40]	30s ON/300s OFF (9%)	2 weeks	↑ phosphorylation of transcription markers associated with antidepressant effects
Thrivikraman 2013 [41]	30s ON/300s OFF (9%)	2 weeks	reversal of hormonal imbalances in stress
Sun 2013 [42]	30s ON/300s OFF (9%)	3 hours	↓ colonic inflammation
Mollet 2013 [43]	30s ON/108s OFF (22%)	1 hour	↓ cortical excitability
Manta 2013 [44]	30s ON/300s OFF (9%)	2 weeks	↑ cortical and hippocampal norepinephrine, ↑ cortical dopamine,↓ firing dopaminergic ventral tegmental neurons
Gebhardt 2013 [45]	30s ON/300s OFF (9%)	3 weeks	↑ hippocampal neurogenesis
Manta 2012 [46]	30s ON/300s OFF (9%)	2 weeks	↑ firing serotonergic raphe neurons
Furmaga 2012 [47]	30s ON/300s OFF (9%)	2 weeks	↑ brain neurotrophic factors
Banni 2012 [48]	30s ON/300s OFF (9%)	4 weeks	↓ body weight
Alexander 2012 [49]	30s ON/300s OFF (9%)	1 week	anticonvulsant effects
Raedt 2011 [50]	7s ON/18s OFF (28%)	4 hours	↑ hippocampal NE, anticonvulsant effects

Liu 2011 [51]	30s ON/300s OFF (9%)	1 hour	↓ heroin seeking behavior
De Herdt 2010 [52]	30s ON/108s OFF (22%)	1 hour	↓ cortical excitability
Sahin 2009 [53]	Continuous (100%)	1 hour	anticonvulsant effects, ↓ seizure associated pathological cardiac activity
Manta 2009 [54]	30s ON/108s OFF (22%)	2 weeks	↑ firing serotonergic raphe neurons
De Herdt 2009 [55]	30s ON/108s OFF (22%)	1 hour	↑ plasma corticosterone
Biggio 2009 [56]	30s ON/300s OFF (9%)	4 weeks	↑ cellular markers of neuroplasticity
Revesz 2008 [57]	30s ON/300s OFF (9%)	2 days	↑ proliferation of dentate progenitor cells
Cunningham 2008 [58]	30s ON/300s OFF (9%)	3 weeks	↑ cfos in vagal afferent pathways
Follesa 2007 [59]	30s ON/300s OFF (9%)	3 hours	↑ hippocampal and cortical expression of brain derived neurotrophic factor and fibroblast growth factor, ↑ cortical NE
Dorr 2006 [60]	30s ON/300s OFF (9%)	2 weeks	↑ firing of noradrenergic locus coeruleus neurons, ↑ firing of serotonergic raphe neurons
Dedeurwaerdere 2006 [61]	30s ON/66s OFF (31%)	2 hours	anti- and proconvulsive effects
Dedeurwaerdere 2005 [63]	30s ON/300s OFF (9%)	1 week	↓ hippocampal and striatal glucose uptake
Dedeurwaerdere 2005 [64]	12s ON/60s OFF (17%)	2 weeks	no anticonvulsive effects
Deurwaerdere 2004 [65]	30s ON/300s OFF (9%)	3 hours	no anticonvulsive effects
Bohotin 2003 [66]	20s ON/18s OFF (53%)	1 day	antinociceptive effects
Bohotin 2003 [67]	20s ON/18s OFF (53%)	1 day	antinociceptive effects
Takaya 1996 [68]	Continuous (100%)	1 hour	anticonvulsant effects
Krahl 2004 [69]	Continuous (100%)	∼20 min	anticonvulsant effects
Krahl 2004 [70]	Continuous (100%)	∼30 min	antidepressant effects
Krahl 2003 [71]	Continuous (100%)	∼20 min	anticonvulsant effects
Krahl 2001 [72]	Continuous (100%)	∼20 min	anticonvulsant effects

Therapeutic effects of vagus nerve stimulation in the context of hypothermia

Hypothermia has been shown to disrupt epileptic activity in in vitro slice models [73, 73] and has been associated with anticonvulsant effects in experimental animal models of status epilepticus [74] as well as in patients [75, 76]. Hypothermia may have been induced by VNS in at least eight studies reporting anticonvulsant or seizure-suppressing effects of VNS in rats [43, 49, 50, 52, 53, 61, 68]. Anticonvulsant effects were similarly reported when VNS was applied for only a few minutes prior to injection of a chemoconvulsant [71, 72]. The fact that VNS was applied for another 15 minutes following injection, with an intense VNS treatment regimen (continuous VNS, i.e. no cycling), however, makes it difficult to exclude a potential effect of hypothermia in these studies. Two studies, nevertheless, did report acute anticonvulsant effects of a single 30 second train or a few minutes of VNS [77, 78], which are unlikely to have induced changes in temperature [11, 13]. This suggests that although hypothermic effects may have contributed to anticonvulsant effects reported in many previous studies, VNS possesses anticonvulsant efficacy in rat seizure models beyond a mere effect of hypothermia.

Experimental studies have further shown protective effects of hypothermia in both stroke [19] and cardiac ischemia [79]. VNS, applied in freely moving rats, has also been associated with favorable outcomes in experimental models of both stroke [33] and cardiac ischemia [37]. The stimulation parameters used in these studies clearly support induction of hypothermia, and thus a likely contribution of hypothermia to the study outcome. However, the described effects have also been demonstrated under temperature controlled anesthetic conditions [6, 80], suggesting that VNS has additional neuro- and cardioprotective effects that do not depend on hypothermic effects of VNS. Neuroprotective effects have also been attributed, at least partially, to activation

of anti-inflammatory pathways by VNS [33]. VNS has been described to lower levels of pro-inflammatory cytokines as TNF-α, IL-6 by activation of the efferent cholinergic pathway and as a result suppress colonic inflammation [42]. It should be mentioned that hypothermia has direct anti-inflammatory effects [81]. Anti-inflammatory effects mediated by the efferent cholinergic pathway has, however, also been reported under anesthesia and thus temperature controlled conditions [8]. It has additionally been reported in studies using VNS parameters unlikely to induce previously described hypothermic effects [82] and anti-inflammatory effects are thus likely not to depend on hypothermia. In general, VNS administered with parameters likely to induce hypothermia, has been associated with several additional biological [38, 41, 44–46, 54–57, 59, 60, 64] and behavioral [34, 35, 51] effects that have not been confirmed under temperature controlled conditions, for which reason care should be taken when interpreting these results.

The role of the noradrenergic system

The influence of VNS on noradrenergic signaling has been shown through several experimental studies, mostly performed in rats. VNS has been shown to facilitate the neuronal firing rate of noradrenergic locus coeruleus (LC) neurons both acutely [83] and following weeks of VNS [60], and as a result increase both limbic and cortical concentrations of norepinephrine [50, 84, 85]. Administration of an α2-adrenoceptor antagonist was further observed to attenuate anticonvulsant effects of VNS, which were otherwise correlated to VNS-induced increases in hippocampal norepinephrine concentrations [50]. VNS-induced hypothermia is however likely to have influenced several of the studies suggesting an effect of VNS on noradrenergic signaling. In fact, noradrenergic signaling is also known to be directly involved in regulation of temperature [86], suggesting that noradrenergic signaling also could modulate hypothermic effects of VNS. However, lesioning the LC-noradrenergic system with the neurotoxin DSP-4 was found to be without effect on hypothermic effects of VNS [11]. This is in contrast to previous studies showing a clear attenuation or elimination of anticonvulsant and antidepressive effects of VNS following LC-lesioning [34, 78]. Thus, apart from indicating that VNS-induced hypothermia is independent from noradrenergic signaling, this also clearly suggests that anticonvulsant and antidepressive effects previously described in rats are not necessarily related to hypothermic effects of VNS. Anticonvulsant effects of VNS, which were eliminated by LC-lesioning, were however induced by a short 30 second VNS burst. Thus, acute anticonvulsant mechanisms of VNS in rats may be due to the result of an acute activation of noradrenergic signaling, while anticonvulsant effects associated with chronic VNS in rats may be the result of multiple simultaneously triggered effects, including anti-inflammatory and hypothermic effects. A previous study indeed showed that anticonvulsant effects evoked by two hours of VNS are only attenuated, but not completely eliminated upon local α2-adrenoceptor blockade [50]. This suggests that local noradrenergic signaling is not necessarily responsible for the full anticonvulsant effect associated with continuously cycled VNS.

Furthermore, it is worth questioning how robust the evidence of an effect of VNS on noradrenergic signaling is. Acute VNS has indeed been described to facilitate the activity of LC neurons, measured under urethane anesthesia [83], though other salient sensory stimuli are known to do the same [87]. Two weeks of VNS have been shown to substantially increase the tonic LC-firing rate [60]. In this context, however, it should be noted that cooling has been shown to similarly increase the firing rate of the LC, both in vivo [88, 89] and in vitro [90]. This opens the possibility that an effect on the LC-noradrenergic system in fact could occur secondary to a hypothermic effect. If VNS-induced hypothermia then is a rat- or species-specific phenomenon, one could question whether the noradrenergic system is likely to play a role in clinical use of VNS. What contradicts this critique, however, is the observation that a specific

component of oddball evoked event related potential, the P300, which is known to be sensitive to noradrenergic signaling, is found modulated by VNS in patients [91, 92]. Therefore, while the recent finding of VNS-induced hypothermia may give rise to questions concerning the strength of the theory of LC-noradrenergic involvement in therapeutic mechanisms of VNS, the evidence is still overall in favor. It is however also clear that additional work is needed to specify to which extent effects of VNS on the LC-noradrenergic system can be attributed to hypothermic effects of VNS and to which extend the LC-noradrenergic system is involved in chronic anticonvulsant effects of VNS.

Cortical desynchronization

Cortical desynchronization has commonly been reported as a likely anticonvulsant mechanism of VNS [14]. Desynchronization may refer to the desynchronization of neuronal discharges, though these are not detected by local field potential recordings such as EEG [93]. On the other hand, desynchronization could refer to the disentangling of oscillatory activity in the local field [93]. The concept of desynchronization has, however, often been applied to observations where the data obtained has not provided substantial evidence to support such claim, at least not by modern standards. A common reference is made to an early study by Zanchetti and colleagues [94], who showed "desynchronization" during VNS in encephale isole preparations of cats. These observations were subsequently reproduced in immobilized cats, where even synchronization was reported in some cases, depending on VNS parameters [95, 96]. With the lack of today's digital systems and computerized analyses, however, the data was not subjected to any kind of quantitative analysis, but only interpreted by visual inspection. The effects described could thus simply imply a reduction in the amplitude of low frequency oscillations, which has been described in several more recent rat studies in cortical and hippocampal regions [11, 13, 29, 32, 97, 98]. Though it is possible, there is still no evidence that such reduction in low frequency amplitude represents an anticonvulsant effect of VNS. In fact, while lesioning the noradrenergic system eliminated acute anticonvulsant effects of VNS [78], reductions in hippocampal EEG amplitude during VNS were retained following a similar lesion [11]. Further, studies attempting to trace similar effects in patient EEG have been unsuccessful [13, 99]. Meanwhile, there is some evidence to support actual desynchronizing effects of VNS, though this is not necessarily related to the described effect on EEG amplitude. VNS was indeed found to desynchronize unit activity in the auditory cortex in anesthetized rats [97] and disentangle the synchrony of cortical oscillatory activity in epilepsy patients [100]. It is clear, however, that more work is warranted in order to couple any of these effects to clinical outcomes. In the light of the finding of VNS-induced hypothermia in rats [11], it is encouraged that such work should be done either in patients or at least in species that more appropriately represent VNS in humans.

Inverted U-shape relation between output current and VNS outcome

In recent years, several studies have addressed potential effects of VNS on cognition (extensively reviewed in [101]). Though chronic or continuously cycled VNS is generally found to exert little influence on cognitive functions [101], several studies have found that transient bursts of VNS, delivered at the right moment, can enhance performance on various memory tasks [102–105]. More specifically, delivery of a short VNS burst stimulus (0.5-1.5s) just before a stimulus can enhance consolidation of that stimulus [102–107]. What is intriguing, however, is that this effect has been mainly described when VNS has been applied with an intensity in an intermediate intensity window, which typically ranges from 0.4 mA-0.8 mA [104, 105], using pulse widths of 250-500 μs and a pulse frequency of 20-30 Hz. At higher intensities

of VNS (>1mA), no effect is observed [104, 105]. Other neurophysiological or neurobiological outcomes, such as hippocampal LTP or hippocampal neurogenesis, have been described to follow the same pattern with regard to how different output currents differentially affect the outcome [57, 108, 109]. It has been argued that moderate intensities of VNS may moderately recruit endogenous neuromodulatory systems, such as the LC, whereas higher VNS intensities lead to a higher recruitment [101]. This hypothesis has been inspired by the idea that modulation of cognitive functions by endogenous neuromodulatory systems generally follows an inverted U-shape relationship [110]. However, this is not coherent with what we know about recruitment of vagal fibers as a function of stimulation intensity. The recruitment of vagal fibers is far from linear, which partially contradicts the idea that moderate VNS intensities results in a moderate recruitment and high VNS intensities in a high recruitment. More specifically, most evidence indicates that thick myelinated A-fibers display a homogenous threshold for electrical recruitment and are fully recruited within few intensity increments around a threshold of 0.2 mA – 0.6 mA, using pulse widths of 250-500 μs, though some intersubject variability is observed [43, 111, 112]. Due to the homogeneous threshold of these fibers, the chances of recruiting only a proportion of thickly myelinated vagal fibers at an intermediate intensity are rather low. In a case where crude intensity increments of for example 0.2 mA are used, it is much more likely that either no fibers are recruited, or all of them are recruited. The only likely mechanism underlying the observed phenomena would be that recruitment of less densely myelinated B-fibers fibers at higher intensities eliminates the otherwise beneficial effects of stimulating thick myelinated A-fibers, though evidence suggests that this requires intensities >1.5 mA [112]. The many reports supporting the phenomenon, nevertheless, clearly support the validity of the theory. Due to the controversy discussed, however, it is clear that additional data is needed to elucidate the underlying mechanisms and support our understanding of the phenomenon. This should include evidence on peripheral mechanisms, such as which vagal fibers that mediate the observed effects.

Future directions

Following a critical review of currently dominant theories in the field of VNS research, it is clear that a general questioning of the status quo of the field is needed, particularly in a time where new studies on VNS are published almost on a weekly basis. In this context, the recent finding of VNS-induced hypothermia, which is likely to be a species specific phenomenon, has further led to the questioning of the true translational value of VNS research in rats. There are, however, several arguments that support the validity of current ideas of VNS, including the likely involvement of noradrenergic signaling in therapeutic mechanisms of VNS. Nevertheless, it must still be regarded that most mechanistic research has been performed in rats. Though hypothermia is unlikely to explain all previous effects associated with VNS, it illustrates a potential functional difference in the vagal system between rats and humans, which can lead to the questioning of other effects observed in relation to VNS in rats. For example, reduced low frequency EEG power reported in rats cannot be traced in a similar way in patients either [13]. From this it is clear that future VNS studies in rats should examine likely differences between rats and humans and at least account for potential changes in temperature. This should include both registration of temperature, and in case any change in temperature is observed, a compensation strategy, such as application of external heating. Counteracting any change in temperature which is likely mediated by regulation of the thermoregulatory control centers of the hypothalamus should be treated with extreme care, however, as it will likely constitute a homeostatic challenge and thus trigger homeostatic control responses.

References

[1] FDA. July 1997 pma approvals, 1997.

[2] FDA. July 2005 pma approvals, 2005.

[3] E. D. Hord, M. S. Evans, S. Mueed, B. Adamolekun, and D. K. Naritoku. The effect of vagus nerve stimulation on migraines. *J Pain*, 4(9):530–4, 2003.

[4] G. Lange, M. N. Janal, A. Maniker, J. Fitzgibbons, M. Fobler, D. Cook, and B. H. Natelson. Safety and efficacy of vagus nerve stimulation in fibromyalgia: a phase i/ii proof of concept trial. *Pain Med*, 12(9):1406–13, 2011.

[5] N. Khodaparast, M. P. Kilgard, R. Casavant, A. Ruiz, I. Qureshi, P. D. Ganzer, 2nd Rennaker, R. L., and S. A. Hays. Vagus nerve stimulation during rehabilitative training improves forelimb recovery after chronic ischemic stroke in rats. *Neurorehabil Neural Repair*, 2015.

[6] Z. Sun, W. Baker, T. Hiraki, and J. H. Greenberg. The effect of right vagus nerve stimulation on focal cerebral ischemia: an experimental study in the rat. *Brain Stimul*, 5(1):1–10, 2012.

[7] F. A. Koopman, P. R. Schuurman, M. J. Vervoordeldonk, and P. P. Tak. Vagus nerve stimulation: a new bioelectronics approach to treat rheumatoid arthritis? *Best Pract Res Clin Rheumatol*, 28(4):625–35, 2014.

[8] L. V. Borovikova, S. Ivanova, M. Zhang, H. Yang, G. I. Botchkina, L. R. Watkins, H. Wang, N. Abumrad, J. W. Eaton, and K. J. Tracey. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*, 405(6785):458–62, 2000.

[9] G. M. De Ferrari. Vagal stimulation in heart failure. *J Cardiovasc Transl Res*, 7(3):310–20, 2014.

[10] H. R. Berthoud and W. L. Neuhuber. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci*, 85(1-3):1–17, 2000.

[11] L. E. Larsen, W. Van Lysebettens, S. Carrette, S. Daelemans, M. Sprengers, L. Thyrion, W. J. Wadman, J. Delbeke, P. Boon, K. Vonck, and R. Raedt. Vagus nerve stimulation induces hypothermia in freely moving rats. *(In Preparation)*, 2016.

[12] G. H. Vijgen, N. D. Bouvy, L. Leenen, K. Rijkers, E. Cornips, M. Majoie, B. Brans, and W. D. van Marken Lichtenbelt. Vagus nerve stimulation increases energy expenditure: relation to brown adipose tissue activity. *PLoS One*, 8(10):e77221, 2013.

[13] L. E. Larsen, W. Van Lysebettens, S. Carrette, S. Daelemans, M. Sprengers, L. Thyrion, E. Carrette, V. De Herdt, W. J. Wadman, J. Delbeke, P. Boon, K. Vonck, and R. Raedt. Modulation of electrophysiological parameters by vagus nerve stimulation: a translational study. *(In Preparation)*, 2016.

[14] C. Heck, S. L. Helmers, and C. M. DeGiorgio. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. *Neurology*, 59(6 Suppl 4):S31–7, 2002.

[15] A. C. Guyton and J.E. Hall. *Thextbook of Medical Physiology*. Elsevier Sauders, Philadelphia, 11th edition edition, 2006.

[16] Kathryn L McCance and Sue E Huether. *Pathophysiology: The biologic basis for disease in adults and children*. Elsevier Health Sciences, 2015.

[17] C. B. Saper. Neurobiological basis of fever. *Ann N Y Acad Sci*, 856:90–4, 1998.

[18] P. M. Schulte. The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J Exp Biol*, 218(Pt 12):1856–66, 2015.

[19] T. S. Olsen, U. J. Weber, and L. P. Kammersgaard. Therapeutic hypothermia for acute stroke. *Lancet Neurol*, 2(7):410–6, 2003.

[20] K. Nakashima and M. M. Todd. Effects of hypothermia on the rate of excitatory amino acid release after ischemic depolarization. *Stroke*, 27(5):913–8, 1996.

[21] A. J. Baker, M. H. Zornow, M. R. Grafe, M. S. Scheller, S. R. Skilling, D. H. Smullin, and A. A. Larson. Hypothermia prevents ischemia-induced increases in hippocampal glycine concentrations in rabbits. *Stroke*, 22(5):666–73, 1991.

[22] H. Karibe, G. J. Zarow, S. H. Graham, and P. R. Weinstein. Mild intraischemic hypothermia reduces postischemic hyperperfusion, delayed postischemic hypoperfusion, blood-brain barrier disruption, brain edema, and neuronal damage volume after temporary focal cerebral ischemia in rats. *J Cereb Blood Flow Metab*, 14(4):620–7, 1994.

[23] E. I. Moser, M. B. Moser, and P. Andersen. Potentiation of dentate synapses initiated by exploratory learning in rats: dissociation from brain temperature, motor activity, and arousal. *Learn Mem*, 1(1):55–73, 1994.

[24] G. Tegzes-Dezso and G. Czeh. Effect of cooling on latency of monosynaptic discharges evoked in motoneurons of the frog. *Acta Biochim Biophys Acad Sci Hung*, 16(1-2):95–100, 1981.

[25] I. Q. Whishaw and C. H. Vanderwolf. Hippocampal eeg and behavior: effects of variation in body temperature and relation of eeg to vibrissae movement, swimming and shivering. *Physiol Behav*, 6(4):391–7, 1971.

[26] T. Deboer. Brain temperature dependent changes in the electroencephalogram power spectrum of humans and animals. *J Sleep Res*, 7(4):254–62, 1998.

[27] S. P. Javedan, R. S. Fisher, H. G. Eder, K. Smith, and J. Wu. Cooling abolishes neuronal network synchronization in rat hippocampal slices. *Epilepsia*, 43(6):574–80, 2002.

[28] E. de la Pena, A. Malkia, H. Vara, R. Caires, J. J. Ballesta, C. Belmonte, and F. Viana. The influence of cold temperature on cellular excitability of hippocampal networks. *PLoS One*, 7(12):e52475, 2012.

[29] L. E. Larsen, W. J. Wadman, D. Marinazzo, P. van Mierlo, J. Delbeke, S. Daelemans, M. Sprengers, L. Thyrion, W. Van Lysebettens, E. Carrette, P. Boon, K. Vonck, and R. Raedt. Vagus nerve stimulation applied with a rapid cycle has more profound influence on hippocampal electrophysiology than a standard cycle. *Neurotherapeutics*, 2016.

[30] B. Samniang, K. Shinlapawittayatorn, T. Chunchai, W. Pongkan, S. Kumfu, S. C. Chattipakorn, B. H. KenKnight, and N. Chattipakorn. Vagus nerve stimulation improves cardiac function by preventing mitochondrial dysfunction in obese-insulin resistant rats. *Sci Rep*, 6:19749, 2016.

[31] R. Agarwal, E. Mokelke, S. B. Ruble, and C. M. Stolen. Vagal nerve stimulation evoked heart rate changes and protection from cardiac remodeling. *J Cardiovasc Transl Res*, 9(1):67–76, 2016.

[32] L. E. Larsen, W. J. Wadman, P. van Mierlo, J. Delbeke, A. Grimonprez, B. Van Nieuwenhuyse, J. Portelli, P. Boon, K. Vonck, and R. Raedt. Modulation of hippocampal activity by vagus nerve stimulation in freely moving rats. *Brain Stimul*, 9(1):124–32, 2016.

[33] Y. X. Xiang, W. X. Wang, Z. Xue, L. Zhu, S. B. Wang, and Z. H. Sun. Electrical stimulation of the vagus nerve protects against cerebral ischemic injury through an anti-infammatory mechanism. *Neural Regen Res*, 10(4):576–82, 2015.

[34] A. Grimonprez, R. Raedt, J. Portelli, I. Dauwe, L. E. Larsen, C. Bouckaert, J. Delbeke, E. Carrette, A. Meurs, V. De Herdt, P. Boon, and K. Vonck. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. *J Psychiatr Res*, 68:1–7, 2015.

[35] A. Grimonprez, R. Raedt, I. Dauwe, L. Mollet, L. E. Larsen, A. Meurs, V. De Herdt, W. Wadman, J. Delbeke, K. Vonck, and P. Boon. Vagus nerve stimulation has antidepressant effects in the kainic acid model for temporal lobe epilepsy. *Brain Stimul*, 8(1):13–20, 2015.

[36] E. M. Annoni, X. Xie, S. W. Lee, I. Libbus, B. H. KenKnight, J. W. Osborn, and E. G. Tolkacheva. Intermittent electrical stimulation of the right cervical vagus nerve in salt-sensitive hypertensive rats: effects on blood pressure, arrhythmias, and ventricular electrophysiology. *Physiol Rep*, 3(8), 2015.

[37] X. Xie, S. W. Lee, C. Johnson, J. Ippolito, B. H. KenKnight, and E. G. Tolkacheva. Intermittent vagal nerve stimulation alters the electrophysiological properties of atrium in the myocardial infarction rat model. *Conf Proc IEEE Eng Med Biol Soc*, 2014:1575–8, 2014.

[38] S. M. Perez, F. R. Carreno, A. Frazer, and D. J. Lodge. Vagal nerve stimulation reverses aberrant dopamine system function in the methylazoxymethanol acetate rodent model of schizophrenia. *J Neurosci*, 34(28):9261–7, 2014.

[39] T. Kawada, M. Li, C. Zheng, S. Shimizu, K. Uemura, M. J. Turner, H. Yamamoto, and M. Sugimachi. Chronic vagal nerve stimulation improves baroreflex neural arc function in heart failure rats. *J Appl Physiol (1985)*, 116(10):1308–14, 2014.

[40] F. R. Carreno and A. Frazer. Activation of signaling pathways downstream of the brain-derived neurotrophic factor receptor, trkb, in the rat brain by vagal nerve stimulation and antidepressant drugs. *Int J Neuropsychopharmacol*, 17(2):247–58, 2014.

[41] K. V. Thrivikraman, F. Zejnelovic, R. W. Bonsall, and M. J. Owens. Neuroendocrine homeostasis after vagus nerve stimulation in rats. *Psychoneuroendocrinology*, 38(7):1067–77, 2013.

[42] P. Sun, K. Zhou, S. Wang, P. Li, S. Chen, G. Lin, Y. Zhao, and T. Wang. Involvement of mapk/nf-kappab signaling in the activation of the cholinergic anti-inflammatory pathway in experimental colitis by chronic vagus nerve stimulation. *PLoS One*, 8(8):e69424, 2013.

[43] L. Mollet, A. Grimonprez, R. Raedt, J. Delbeke, R. El Tahry, V. De Herdt, A. Meurs, W. Wadman, P. Boon, and K. Vonck. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. *Acta Neurol Scand*, 128(6):391–6, 2013.

[44] S. Manta, M. El Mansari, G. Debonnel, and P. Blier. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. *Int J Neuropsychopharmacol*, 16(2):459–70, 2013.

[45] N. Gebhardt, K. J. Bar, M. K. Boettger, G. Grecksch, G. Keilhoff, R. Reichart, and A. Becker. Vagus nerve stimulation ameliorated deficits in one-way active avoidance learning and stimulated hippocampal neurogenesis in bulbectomized rats. *Brain Stimul*, 6(1):78–83, 2013.

[46] S. Manta, M. El Mansari, and P. Blier. Novel attempts to optimize vagus nerve stimulation parameters on serotonin neuronal firing activity in the rat brain. *Brain Stimul*, 5(3):422–9, 2012.

[47] H. Furmaga, F. R. Carreno, and A. Frazer. Vagal nerve stimulation rapidly activates brain-derived neurotrophic factor receptor trkb in rat brain. *PLoS One*, 7(5):e34844, 2012.

[48] S. Banni, G. Carta, E. Murru, L. Cordeddu, E. Giordano, F. Marrosu, M. Puligheddu, G. Floris, G. P. Asuni, A. L. Cappai, S. Deriu, and P. Follesa. Vagus nerve stimulation reduces body weight and fat mass in rats. *PLoS One*, 7(9):e44813, 2012.

[49] G. M. Alexander and J. O. McNamara. Vagus nerve stimulation elevates seizure threshold in the kindling model. *Epilepsia*, 53(11):2043–52, 2012.

[50] R. Raedt, R. Clinckers, L. Mollet, K. Vonck, R. El Tahry, T. Wyckhuys, V. De Herdt, 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(3):461–9, 2011.

[51] H. Liu, Y. Liu, J. Yu, M. Lai, H. Zhu, A. Sun, W. Chen, and W. Zhou. Vagus nerve stimulation inhibits heroin-seeking behavior induced by heroin priming or heroin-associated cues in rats. *Neurosci Lett*, 494(1):70–4, 2011.

[52] V. De Herdt, J. De Waele, R. Raedt, T. Wyckhuys, R. El Tahry, K. Vonck, W. Wadman, and P. Boon. Modulation of seizure threshold by vagus nerve stimulation in an animal model for motor seizures. *Acta Neurol Scand*, 121(4):271–6, 2010.

[53] D. Sahin, G. Ilbay, M. Imal, O. Bozdogan, and N. Ates. Vagus nerve stimulation suppresses generalized seizure activity and seizure-triggered postictal cardiac rhythm changes in rats. *Physiol Res*, 58(3):345–50, 2009.

[54] S. Manta, J. Dong, G. Debonnel, and P. Blier. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. *J Psychiatry Neurosci*, 34(4):272–80, 2009.

[55] V. De Herdt, L. Puimege, J. De Waele, R. Raedt, T. Wyckhuys, R. El Tahry, C. Libert, W. Wadman, P. Boon, and K. Vonck. Increased rat serum corticosterone suggests immunomodulation by stimulation of the vagal nerve. *J Neuroimmunol*, 212(1-2):102–5, 2009.

[56] F. Biggio, G. Gorini, C. Utzeri, P. Olla, F. Marrosu, I. Mocchetti, and P. Follesa. Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus. *Int J Neuropsychopharmacol*, 12(9):1209–21, 2009.

[57] D. Revesz, M. Tjernstrom, E. Ben-Menachem, and T. Thorlin. Effects of vagus nerve stimulation on rat hippocampal progenitor proliferation. *Exp Neurol*, 214(2):259–65, 2008.

[58] J. T. Cunningham, S. W. Mifflin, G. G. Gould, and A. Frazer. Induction of c-fos and deltafosb immunoreactivity in rat brain by vagal nerve stimulation. *Neuropsychopharmacology*, 33(8):1884–95, 2008.

[59] P. Follesa, F. Biggio, G. Gorini, S. Caria, G. Talani, L. Dazzi, M. Puligheddu, F. Marrosu, and G. Biggio. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of bdnf and bfgf in the rat brain. *Brain Res*, 1179:28–34, 2007.

[60] A. E. Dorr and G. Debonnel. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *J Pharmacol Exp Ther*, 318(2):890–8, 2006.

[61] S. Dedeurwaerdere, K. Gilby, K. Vonck, J. Delbeke, P. Boon, and D. McIntyre. Vagus nerve stimulation does not affect spatial memory in fast rats, but has both anti-convulsive and pro-convulsive effects on amygdala-kindled seizures. *Neuroscience*, 140(4):1443–51, 2006.

[62] S. Dedeurwaerdere, K. Vonck, V. De Herdt, L. Waterschoot, T. De Smedt, R. Raedt, T. Wyckhuys, B. Legros, P. Van Hese, K. Van Laere, J. Delbeke, W. Wadman, and P. Boon. Neuromodulation with levetiracetam and vagus nerve stimulation in experimental animal models of epilepsy. *Acta Neurol Belg*, 106(2):91–7, 2006.

[63] S. Dedeurwaerdere, K. Vonck, P. Van Hese, W. Wadman, and P. Boon. The acute and chronic effect of vagus nerve stimulation in genetic absence epilepsy rats from strasbourg (gaers). *Epilepsia*, 46 Suppl 5:94–7, 2005.

[64] S. Dedeurwaerdere, B. Cornelissen, K. Van Laere, K. Vonck, E. Achten, G. Slegers, and P. Boon. Small animal positron emission tomography during vagus nerve stimulation in rats: a pilot study. *Epilepsy Res*, 67(3):133–41, 2005.

[65] S. Dedeurwaerdere, K. Vonck, P. Claeys, P. Van Hese, M. D'Have, T. Grisar, D. Naritoku, and P. Boon. Acute vagus nerve stimulation does not suppress spike and wave discharges in genetic absence epilepsy rats from strasbourg. *Epilepsy Res*, 59(2-3):191–8, 2004.

[66] C. Bohotin, M. Scholsem, V. Bohotin, R. Franzen, and J. Schoenen. Vagus nerve stimulation attenuates heat- and formalin-induced pain in rats. *Neurosci Lett*, 351(2):79–82, 2003.

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

[68] M. Takaya, W. J. Terry, and D. K. Naritoku. Vagus nerve stimulation induces a sustained anticonvulsant effect. *Epilepsia*, 37(11):1111–6, 1996.

[69] S. E. Krahl, F. C. Martin, and A. Handforth. Vagus nerve stimulation inhibits harmaline-induced tremor. *Brain Res*, 1011(1):135–8, 2004.

[70] S. E. Krahl, S. S. Senanayake, A. E. Pekary, and A. Sattin. Vagus nerve stimulation (vns) is effective in a rat model of antidepressant action. *J Psychiatr Res*, 38(3):237–40, 2004.

[71] S. E. Krahl, S. S. Senanayake, and A. Handforth. Right-sided vagus nerve stimulation reduces generalized seizure severity in rats as effectively as left-sided. *Epilepsy Res*, 56(1):1–4, 2003.

[72] S. E. Krahl, S. S. Senanayake, and A. Handforth. Destruction of peripheral c-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. *Epilepsia*, 42(5):586–9, 2001.

[73] G. K. Motamedi, P. Salazar, E. L. Smith, R. P. Lesser, W. R. Webber, P. I. Ortinski, S. Vicini, and M. A. Rogawski. Termination of epileptiform activity by cooling in rat hippocampal slice epilepsy models. *Epilepsy Res*, 70(2-3):200–10, 2006.

[74] F. C. Schmitt, K. Buchheim, H. Meierkord, and M. Holtkamp. Anticonvulsant properties of hypothermia in experimental status epilepticus. *Neurobiol Dis*, 23(3):689–96, 2006.

[75] A. B. Kowski, H. Kanaan, F. C. Schmitt, and M. Holtkamp. Deep hypothermia terminates status epilepticus–an experimental study. *Brain Res*, 1446:119–26, 2012.

[76] A. E. Bennett, R. E. Hoesch, L. D. DeWitt, P. Afra, and S. A. Ansari. Therapeutic hypothermia for status epilepticus: A report, historical perspective, and review. *Clin Neurol Neurosurg*, 126:103–9, 2014.

[77] K. Rijkers, M. Aalbers, G. Hoogland, L. van Winden, J. Vles, H. Steinbusch, and M. Majoie. Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat. *Brain Res*, 1319:155–63, 2010.

[78] S. E. Krahl, K. B. Clark, D. C. Smith, and R. A. Browning. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia*, 39(7):709–14, 1998.

[79] M. J. Herring, S. L. Hale, W. Dai, P. M. Oskui, and R. A. Kloner. Hypothermia in the setting of experimental acute myocardial infarction: a comprehensive review. *Ther Hypothermia Temp Manag*, 4(4):159–67, 2014.

[80] Q. Wang, R. P. Li, F. S. Xue, S. Y. Wang, X. L. Cui, Y. Cheng, G. P. Liu, and X. Liao. Optimal intervention time of vagal stimulation attenuating myocardial ischemia/reperfusion injury in rats. *Inflamm Res*, 63(12):987–99, 2014.

[81] M. A. Yenari and H. S. Han. Neuroprotective mechanisms of hypothermia in brain ischaemia. *Nat Rev Neurosci*, 13(4):267–78, 2012.

[82] F. Garcia-Oscos, D. Pena, M. Housini, D. Cheng, D. Lopez, R. Cuevas-Olguin, N. Saderi, R. Salgado Delgado, L. Galindo Charles, H. Salgado Burgos, S. Rose-John, G. Flores, M. P. Kilgard, and M. Atzori. Activation of the anti-inflammatory reflex blocks lipopolysaccharide-induced decrease in synaptic inhibition in the temporal cortex of the rat. *J Neurosci Res*, 93(6):859–65, 2015.

[83] D. A. Groves, E. M. Bowman, and V. J. Brown. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. *Neurosci Lett*, 379(3):174–9, 2005.

[84] R. W. Roosevelt, D. C. Smith, R. W. Clough, R. A. Jensen, and R. A. Browning. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res*, 1119(1):124–32, 2006.

[85] D. L. Hassert, T. Miyashita, and C. L. Williams. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. *Behav Neurosci*, 118(1):79–88, 2004.

[86] V. M. Kumar, R. Vetrivelan, and H. N. Mallick. Noradrenergic afferents and receptors in the medial preoptic area: neuroanatomical and neurochemical links between the regulation of sleep and body temperature. *Neurochem Int*, 50(6):783–90, 2007.

[87] S. J. Sara and S. Bouret. Orienting and reorienting: The locus coeruleus mediates cognition through arousal. *Neuron*, 76(1):130–41, 2012.

[88] M. J. Mana and A. A. Grace. Chronic cold stress alters the basal and evoked electrophysiological activity of rat locus coeruleus neurons. *Neuroscience*, 81(4):1055–64, 1997.

[89] H. P. Jedema, J. M. Finlay, A. F. Sved, and A. A. Grace. Chronic cold exposure potentiates crh-evoked increases in electrophysiologic activity of locus coeruleus neurons. *Biol Psychiatry*, 49(4):351–9, 2001.

[90] H. P. Jedema and A. A. Grace. Chronic exposure to cold stress alters electrophysiological properties of locus coeruleus neurons recorded in vitro. *Neuropsychopharmacology*, 28(1):63–72, 2003.

[91] L. De Taeye, K. Vonck, M. van Bochove, P. Boon, D. Van Roost, L. Mollet, A. Meurs, V. De Herdt, E. Carrette, I. Dauwe, S. Gadeyne, P. van Mierlo, T. Verguts, and R. Raedt. The p3 event-related potential is a biomarker for the efficacy of vagus nerve stimulation in patients with epilepsy. *Neurotherapeutics*, 11(3):612–22, 2014.

[92] A. H. Neuhaus, A. Luborzewski, J. Rentzsch, E. L. Brakemeier, C. Opgen-Rhein, J. Gallinat, and M. Bajbouj. P300 is enhanced in responders to vagus nerve stimulation for treatment of major depressive disorder. *J Affect Disord*, 100(1-3):123–8, 2007.

[93] G. Buzsaki, C. A. Anastassiou, and C. Koch. The origin of extracellular fields and currents–eeg, ecog, lfp and spikes. *Nat Rev Neurosci*, 13(6):407–20, 2012.

[94] A. Zanchetti, S. C. Wang, and G. Moruzzi. The effect of vagal afferent stimulation on the eeg pattern of the cat. *Electroencephalogr Clin Neurophysiol*, 4(3):357–61, 1952.

[95] M. H. Chase, Y. Nakamura, C. D. Clemente, and M. B. Sterman. Afferent vagal stimulation: neurographic correlates of induced eeg synchronization and desynchronization. *Brain Res*, 5(2):236–49, 1967.

[96] M. H. Chase, M. B. Sterman, and C. D. Clemente. Cortical and subcortical patterns of response to afferent vagal stimulation. *Exp Neurol*, 16(1):36–49, 1966.

[97] J. A. Nichols, A. R. Nichols, S. M. Smirnakis, N. D. Engineer, M. P. Kilgard, and M. Atzori. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience*, 189:207–14, 2011.

[98] N. D. Engineer, J. R. Riley, J. D. Seale, W. A. Vrana, J. A. Shetake, S. P. Sudanagunta, M. S. Borland, and M. P. Kilgard. Reversing pathological neural activity using targeted plasticity. *Nature*, 470(7332):101–4, 2011.

[99] M. C. Salinsky and K. J. Burchiel. Vagus nerve stimulation has no effect on awake eeg rhythms in humans. *Epilepsia*, 34(2):299–304, 1993.

[100] C. Bodin, S. Aubert, G. Daquin, R. Carron, D. Scavarda, A. McGonigal, and F. Bartolomei. Responders to vagus nerve stimulation (vns) in refractory epilepsy have reduced interictal cortical synchronicity on scalp eeg. *Epilepsy Res*, 113:98–103, 2015.

[101] K. Vonck, R. Raedt, J. Naulaerts, F. De Vogelaere, E. Thiery, D. Van Roost, B. Aldenkamp, M. Miatton, and P. Boon. Vagus nerve stimulation...25 years later! what do we know about the effects on cognition? *Neurosci Biobehav Rev*, 45:63–71, 2014.

[102] J. V. Corwin and J. S. Slaughter. Effects of vagal stimulation on the learning of specific and diffuse conditioned suppression. *Behav Neural Biol*, 25(3):364–70, 1979.

[103] J. S. Slaughter and W. W. Hahn. Effects on avoidance performance of vagal stimulation during previous fear conditioning in rats. *J Comp Physiol Psychol*, 86(2):283–7, 1974.

[104] K. B. Clark, D. C. Smith, D. L. Hassert, R. A. Browning, D. K. Naritoku, and R. A. Jensen. Posttraining electrical stimulation of vagal afferents with concomitant vagal efferent inactivation enhances memory storage processes in the rat. *Neurobiol Learn Mem*, 70(3):364–73, 1998.

[105] K. B. Clark, S. E. Krahl, D. C. Smith, and R. A. Jensen. Post-training unilateral vagal stimulation enhances retention performance in the rat. *Neurobiol Learn Mem*, 63(3):213–6, 1995.

[106] C. T. Engineer, N. D. Engineer, J. R. Riley, J. D. Seale, and M. P. Kilgard. Pairing speech sounds with vagus nerve stimulation drives stimulus-specific cortical plasticity. *Brain Stimul*, 8(3):637–44, 2015.

[107] N. Khodaparast, S. A. Hays, A. M. Sloan, T. Fayyaz, D. R. Hulsey, 2nd Rennaker, R. L., and M. P. Kilgard. Vagus nerve stimulation delivered during motor rehabilitation improves recovery in a rat model of stroke. *Neurorehabil Neural Repair*, 28(7):698–706, 2014.

[108] Y. Zuo, D. C. Smith, and R. A. Jensen. Vagus nerve stimulation potentiates hippocampal ltp in freely-moving rats. *Physiol Behav*, 90(4):583–9, 2007.

[109] M. S. Borland, W. A. Vrana, N. A. Moreno, E. A. Fogarty, E. P. Buell, P. Sharma, C. T. Engineer, and M. P. Kilgard. Cortical map plasticity as a function of vagus nerve stimulation intensity. *Brain Stimul*, 9(1):117–23, 2016.

[110] C. W. Berridge and B. D. Waterhouse. The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev*, 42(1):33–84, 2003.

[111] A. Grimonprez, R. Raedt, L. De Taeye, L. E. Larsen, J. Delbeke, P. Boon, and K. Vonck. A preclinical study of laryngeal motor-evoked potentials as a marker vagus nerve activation. *Int J Neural Syst*, page 1550034, 2015.

[112] P. B. Yoo, N. B. Lubock, J. G. Hincapie, S. B. Ruble, J. J. Hamann, and W. M. Grill. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. *J Neural Eng*, 10(2):026003, 2013.

Chapter 9

Conclusions & Future Perspectives

Conclusions

Based on the experimental work presented in **Part II**, the following conclusions can be drawn:

Chapter 4 - In freely moving rats, Vagus Nerve Stimulation (VNS) is associated with several neurophysiological effects in the hippocampal formation. VNS decreases the power of the hippocampal EEG across the entire hippocampal spectrum, with the exception of a narrow 20-35 Hz spectrum, and shifts the hippocampal theta rhythm from around 7 Hz to 5 Hz. VNS additionally modulates dentate field evoked potentials evoked by electrical stimulation of the perforant path. The field excitatory post synaptic potential (fEPSP) slope decreases, while both the amplitude and latency of the population spike increased following VNS.

Chapter 5 - VNS further modulates phase amplitude coupling between theta phase and gamma amplitude in the hippocampal EEG. The modulation of hippocampal electrophysiology is highly dependent on the stimulation parameters used. Rapid cycle VNS (28% ON-time) is associated with greater modulation of all parameters than standard cycle VNS (9% ON-time). By titrating VNS intensities, effects of rapid cycle VNS on hippocampal EEG parameters were further found to saturate around 300 μA (pulse width of 250 μs). Further increases in stimulation intensity did not yield additional effect.

Chapter 6 - VNS produces hypothermia in freely moving rats, which explains many of the previously described electrophysiological effects, including slowing of dentate field evoked potentials and hippocampal theta rhythm. Both brain and rectal temperature decreases during VNS. Rapid cycle VNS decreases brain temperature by as much as 3 ^oC, while standard cycle VNS decreases brain temperature by a bit less than 1 ^oC. VNS further leads to a fast tail vasodilation response, which suggests an active release of heat during VNS. Lesioning the noradrenergic system, which attenuates both anticonvulsant and antidepressive effects of VNS, has no effect on temperature or EEG outcomes.

Chapter 7 - VNS is associated with a rapid decrease in low frequency power of both the hippocampal and prefrontal EEG, which happens before any changes in brain temperature occurs. The shift in theta peak frequency is more likely related to hypothermic effects of VNS based on effect dynamics. In epilepsy patients, VNS is not associated with comparable effects on low frequency EEG power or alpha peak frequency, suggesting that both temperature dependent and independent effects observed in the rat EEG are not translatable to the human setting.

Future Perspectives, Thoughts and Ideas

Ultimately, the goal of all VNS research is to improve the outcome for patients treated with VNS in the clinic. This was the motivation for initiating many of the studies presented in **Part II**. In my view, studies on VNS fall into three categories:

1. Fundamental studies searching for novel effects of VNS, with potential to be introduced to the clinic
2. Studies aiming to optimize delivery of VNS (e.g. optimization of stimulation parameters)
3. Studies aiming to optimize appropriate selection of treatment candidates

In the present thesis, the overall goal was to investigate neurophysiological mechanisms of VNS. In particular, it was of interest to assess effects of VNS on excitability, which is a crucial element in the treatment of epileptic disorders, where hyperexcitable neuronal circuits are typically observed [1]. The idea was specifically to identify concrete neurophysiological effects, which could be linked to anticonvulsant effects of VNS (later to be studied in appropriate disease models), whereby these effects could be used as biomarkers to optimize delivery of VNS, i.e. high-throughput investigation and priming of stimulation parameters. Thereby the work initially fell into the second category listed above. The conclusions, however, differ substantially from our initial ideas and though it may prove to be a setback in terms of reaching a better understanding of VNS, it is nevertheless of substantial importance to the future direction of research in the field.

Following this work, the first question requiring clarification is the question of whether hypothermic and other effects of VNS observed in rats are translatable. While data from **Chapter 7** would indicate that many of the effects found in rats are possibly not translatable to the human setting, there are still some potentially important factors that complicate direct comparison between the results obtained in rats and those obtained in patients. First of all patient study populations display a greater diversity than typically very homogenous study populations in preclinical experiments. Secondly, as noted in **Chapter 7**, the patients studied are typically drug resistant patients that are treated with large doses of antiepileptic medication, which is likely to interfere with a potential effect of VNS. To overcome this barrier, it is likely that patient studies will require substantially larger study populations in order to factor in concomitant medication and subtypes of epileptic disorders. Furthermore, studying effects of VNS in naive patients, as in **Chapter 7**, may be a key consideration, as years of VNS treatment is likely to induce chronic neuroplastic changes. This hinders comparison to preclinical studies, where VNS is typically not applied for longer durations in a comparable way.

If, following conduction of rigidly designed studies, such as suggested above, effects of VNS observed in preclinical studies in rats prove not to be translatable in any way, it will be important to realize that many of the commonly accepted theories on VNS (discussed in **Chapter 8**), which largely are based on rat studies, may require substantial revision. Further it will be necessary to question whether rats constitute a feasible model for further study of VNS. It is thus encouraged to either investigate VNS in other species more likely to resemble humans, or to spend all efforts on clinical research, despite the obvious limits such research has. Though patient studies do not present the same opportunities as preclinical studies, the development of still more sophisticated technology continues to open several possibilities for future VNS research in the clinical setting.

What first of all is warranted is to look for means to assess effective delivery of VNS, i.e. effective recruitment of relevant fibers of the vagus nerve and subsequent conduction of effects towards the brain or other organs of interest. Currently, the basis for selecting stimulation parameters in the clinic is based mainly on a perception that the more stimulation the better, which means that

the stimulation parameters are typically adjusted to the maximally tolerable level. However, one thing is clear from preclinical studies, which is very likely to be a general principle of electrical stimulation of peripheral nerves: application of suprathreshold currents will not yield additional effect [2]. Thus, identification of this threshold is key to ensuring appropriate application of VNS as currents below this level likely results in no effect, whereas suprathreshold currents is a waste of battery energy. Effective vagal nerve activation may be assessed either by finding means of measuring compound action potentials of the vagus nerve, or using surrogate measures, such as laryngeal muscle evoked potentials (LMEPs) or afferent upstream sensory potentials (e.g. evoked activity in the brain). LMEPs have indeed been measured in patients (unpublished observations). Designing feedback-back driven closed loop systems, where the current levels are automatically adjusted to such feedback, would be a potentially substantial improvement.

What secondly must be a goal is to identify markers that represent specific clinical effects. Here, it is important to distinguish between a mere effect, e.g. reduced excitability, and a therapeutic effect, e.g. reduction in seizure frequency. To assess excitability it may be possible to use transcranial magnetic stimulation (TMS) to evoke activity in specific circuits and record evoked potentials. As such studies require expensive specialized equipment it would further be of interest to look for parallels between potential effects of VNS on excitability and effects on more easily acquired parameters, for example EEG or other imaging modalities, which would greatly improve the applicability of such measures in a standard clinical setting.

As mentioned, though VNS may be associated with specific effects like reduced excitability, it is still likely that no therapeutic effect is observed. This may ultimately depend on patient specific characteristics such as underlying disease etiology and genetics. Thus, it is highly relevant to systematically conduct studies with the aim of identifying patients most likely to respond to VNS. Due to the heterogeneity of epilepsies, it will be necessary to acquire massive amounts of data, meaning thousands of patients, using a comprehensive and standardized data acquisition approach for each patient. Data acquired should include pretreatment anamnesis, EEG, structural MRI and other relevant measures. Acquisition of such data sets would depend on a combined effort of as several study centers worldwide and establishment of a common data base to which all data can be uploaded and shared. Such an initiative will be incredible expensive and challenging both at a political and logistic level. Nevertheless, such approaches are likely required to progress.

Mining and arranging data set of this size constitutes a secondary challenge, where the scientific community however has progressed significantly in recent years with the development of modern machine learning protocols. The idea would be to attempt to cluster as many patient characteristics as possible in order to establish patient subtypes. This may be done on the basis of EEG features ranging from simple power features, connectivity measures, interictal spikes and localization of the source of these, structural MRI abnormalities and patient anamnesis. The ultimate goal is to find means of identifying patient clusters that based on characteristics prior to VNS application with high probability respond to VNS, which can ensure that only patients who are most likely to respond to VNS will undergo surgical implantation.

References

[1] D. A. Prince, B. W. Connors, and L. S. Benardo. Mechanisms underlying interictal-ictal transitions. *Adv Neurol*, 34:177–87, 1983.

[2] Joseph D Bronzino. *Biomedical engineering handbook*, volume 2. CRC press, 1999.

ENGLISH SUMMARY

Vagus Nerve Stimulation (VNS) is an invasive neurostimulation technique where electrical stimulation is typically delivered to the cervical trunk of the vagus nerve. In 1994, VNS received regulatory approval for the treatment of drug resistant epilepsy in Europe, and in 2005 VNS was further approved for the treatment of drug resistant depression. Today, it is estimated that more than 70,000 patients have been treated with VNS. Despite more than 20 years of clinical experience, VNS is associated with a variable response rate and factors predicting therapeutic response are lacking. Among other factors, this is partially due to a lack of knowledge on mechanisms underlying therapeutic effects of VNS.

In the present thesis, the focus of the experimental work was on assessing neurophysiological mechanisms underlying VNS, using predominantly electrophysiology in freely moving rats. In the first study the aim was to assess the effect of VNS on excitability, with hyperexcitability being a key element in many epileptic syndromes. To study excitability, dentate field potentials, evoked by stimulation of the perforant path, were studied, in addition to the hippocampal electroencephalogram (EEG). Application of VNS was found to slow the dentate field evoked potentials indicating decreased efficiency of excitatory neurotransmission, while it increased the population spike amplitude, reflecting a greater and more synchronous dentate granule cell discharge in response to a certain input. In the EEG, VNS decreased both low and high frequency power and led to a slowing of the hippocampal theta rhythm. This study showed that VNS did not uniformly reduce excitability, associated with a traditional anticonvulsant treatment, but instead exerted a more complex modulation of hippocampal neurotransmission.

In the second study, the influence of varying VNS parameters was examined. VNS was applied with either a rapid cycle (7s ON/18s OFF) or with a standard cycle (30s ON/300s OFF) and varying output currents. In addition to previously studied hippocampal parameters, effects on cross frequency coupling in the hippocampal EEG were studied. Strong coupling was found between phase of theta (4-8 Hz) oscillations and amplitude of fast gamma (75-150 Hz) oscillations. VNS was found to decrease this coupling. In addition, rapid cycle VNS was associated with a stronger modulation of all parameters than standard cycle VNS. Titrating VNS output currents from 0-1000 μA, effects of VNS were observed to reach maximal amplitude around 300 μA, beyond which further increases in VNS intensity did not yield additional effect. The study indicated that effects of VNS on hippocampal EEG were due to recruitment of thick myelinated A fibers. The implications were that VNS output current only plays a role with regard to recruitment of thick myelinated A fibers, following which additional effect only can be gained by increasing the intensity of the VNS duty cycle.

In the third study, we examined the effect of VNS on brain and body temperature in freely moving rats, as the slowing of dentate field potentials and the theta rhythm of the hippocampal EEG previously has been reported under hypothermic conditions. VNS was indeed found to decrease both brain and rectal temperature. Rapid cycle VNS decreased brain temperature by as much as 3 ^{o}C, while standard cycle VNS decreased brain temperature by a bit less than 1 ^{o}C. Data further indicated that hypothermic effects of VNS were recruited at the same VNS output current levels as effects of VNS on hippocampal EEG parameters, again supporting the involvement of thick myelinated A-fibers. VNS was further found to induce a fast peripheral vasodilation response indicating an active release of heat triggered by VNS. A noradrenergic

lesion, which previously has eliminated both anticonvulsant and antidepressive effects of VNS were found to be without effect on both temperature and EEG outcomes. Due to the extensive and widespread physiological influences of temperature and due to the fact that hypothermic effects previously have gone unnoticed, it was clear that hypothermia could have widespread implications for interpretation of several previously published rat VNS studies.

In the fourth study, we examined the dynamics of effects of VNS on temperature and EEG and found that VNS reduced theta EEG power before any effects on temperature occurred. Slowing effects of VNS on the theta frequency, however, followed a profile similar to the effects of VNS on temperature, indicating a greater likelihood for these to be related, which other studies indeed previously have shown. VNS was further found to reduce theta power of prefrontal depth EEG and of superficial cortical EEG, suggesting that reductions in theta power is a potential global cortical phenomenon, which should be similarly traceable in patient scalp EEG. Thus, following this observation, we compared effects of VNS on EEG in rats with EEG obtained from epilepsy patients, in order to assess the translatability the previous observations. VNS, however, had no comparable effect on low frequency EEG power, or on alpha peak frequency of scalp EEG, suggesting that both effects independent on temperature changes and effects likely related to temperature changes in rats were not translatable to the human setting.

These studies indicate that the translational barrier between rats and human in VNS research may be bigger than previously assumed. Though VNS has been applied clinically for more than 20 years, there are no reports of VNS affecting temperature in patients and there are no reports of thermal discomfort induced by VNS. Added with data from the fourth study, it is possible that effects of VNS on temperature is a species specific phenomenon. A revision of literature revealed that at least 40 studies applied VNS in a manner which supports induction of hypothermia. Because many of these studies are important to our current understanding of VNS, it is necessary to question the extent to which this knowledge is relevant in a translational context.

Efforts should still be dedicated to conduct rigidly designed larger translational studies to assess the translatability of VNS associated effects across species. Meanwhile, as the ultimate goal of all VNS research is to improve application in the clinical setting, the present work highly encourages that future efforts predominantly should be dedicated to clinical VNS investigations, or at least to studies in species where effects of VNS are to a larger extent comparable to effects observed in patients.

NEDERLANDSE SAMENVATTING

Nervus vagus stimulatie (NVS) is een invasieve neurostimulatie behandeling waarbij elektrische stimulatie wordt toegediend aan het cervicale deel van de nervus vagus. In 1994 ontving NVS goedkeuring voor de behandeling van medicatie-resistente epilepsie in Europa, en in 2005 werd goedkeuring gegeven voor de behandeling van medicatie-resistente depressie. Meer dan 70 000 patiënten worden reeds behandeld met NVS. Ondanks 20 jaar van klinische ervaring blijft NVS kampen met een variabele respons en ontbreken biomerkers voor het voorspellen van een positieve respons op de therapie. Dit is onder andere door het gebrek aan kennis over de werkingsmechanismen die aan de basis liggen van de therapeutische effecten van NVS. In deze thesis lag de focus van het experimenteel werk op het onderzoeken van de neurofysiologische mechanismen die aan de basis liggen van NVS, gebruik makend van elektrofysiologische technieken bij vrij bewegende ratten. Het doel van de eerste studie was het bepalen van het effect van NVS op de exciteerbaarheid, gezien hyperexiteerbaarheid een sleutelelement is in de meeste epileptische syndromen. Om exciteerbaarheid te bestuderen werden veldpotentialen in de dentate gyrus uitgelokt door het stimuleren van het perforante pad, gecombineerd met het evalueren van het hippocampale elektro-encefalogram (EEG). Toediening van NVS vertraagde de veldpotentialen in de dentate gyrus wat wijst op een verlaagde efficiëntie van de excitatoire neurotransmissie, terwijl gelijktijdig de amplitude van de population spike vergrootte, wat wijst op een grotere en meer gesynchroniseerde dentate gyrus korrelcel ontlading als antwoord op een gegeven stimulus. In het EEG verlaagt NVS de power van zowel lage als hoge frequenties. Ook leidt NVS tot het vertragen van het hippocampale theta-ritme. Deze studie toonde aan dat NVS geen uniforme daling van de exciteerbaarheid veroorzaakt, zoals gezien bij traditionele anticonvulsieve behandelingen. In plaats daarvan wordt er een complexe modulatie van de hippocampale neurotransmissie gezien.

In een tweede studie werd de invloed van variërende NVS parameters bestudeerd. NVS werd toegediend via ofwel een 'Rapid cycle' (7s aan/18s uit) of een standaard cycle (30s aan/300s uit), met variërende output intensiteiten. Naast de boven beschreven hippocampale parameters werd hier ook het effect op de koppelingen tussen verschillende frequenties bestudeerd in het hippocampale EEG. Er werd een sterke koppeling gevonden tussen de fase van de theta (4-8 Hz) oscillatie en de amplitude van de snelle gamma (75-150 Hz) oscillaties. NVS werd bevonden deze koppeling te verzwakken. Hiernaast werd gevonden dat rapid cycle NVS geassocieerd wordt met een sterkere modulatie van alle parameters dan standaard NVS. Bij het titreren van de NVS output intensiteiten met een bereik van 0 tot 1000 µA bereikten de effecten van NVS een plateau van maximale amplitude rond 300 µA, waarbij het verder stijgen van de intensiteit geen extra effecten vertoonden. Deze studie wijst op de mogelijkheid dat de effecten van NVS op het hippocampale EEG bekomen worden door het rekruteren van sterk gemyeliniseerde A-vezels. De implicaties zijn dat NVS output intensiteit enkel een rol speelt bij het rekruteren van deze vezels, waarbij additionele effecten enkel kunnen bekomen worden door het verhogen van de intensiteit van de NVS arbeidscyclus.

In de derde studie werd het effect van NVS op hersenen-en lichaamstemperatuur bestudeerd in vrij bewegende ratten, gezien het vertragen van de dentate veldpotentialen en het hippocampaal theta ritme reeds gerapporteerd werd onder hypotherme omstandigheden. NVS was in staat zo-

wel de temperatuur van het lichaam als van de hersenen te verlagen. Rapid cycle NVS verlaagde de hersentemperatuur met 3^oC, terwijl standaard NVS de hersentemperatuur verlaagt met bijna $1\ ^oC$. Verder toonde de data aan dat de hypotherme effecten van NVS gerekruteerd worden bij dezelfde intensiteiten waarbij de effecten van NVS op de hippocampale EEG parameters werden gezien, wat opnieuw een bewijs is voor de rekrutering van de sterk gemyeliniseerde A-vezels. Verder werd ontdekt dat NVS een snelle perifere vasodilatatie veroorzaakt wat wijst op een actieve warmtevrijlating geïnduceerd door NVS. Een noradrenerge laesie, waarbij in vroeger onderzoek zowel het anticonvulsieve als antidepressieve effect van NVS verdween, had geen effect op zowel de temperatuur als de EEG resultaten. Gezien de wijdverspreide fysiologische invloeden van temperatuur en gezien het feit dat hypotherme effecten nog niet eerder werden opgepikt, was het duidelijk dat de hypothermie een grote implicatie kan hebben voor de interpretatie van verschillende vooraf gepubliceerd studies over NVS bij ratten.

In de vierde studie werden de dynamische effecten van NVS op temperatuur en EEG bekeken en werd gevonden dat NVS theta EEG power verlaagde voor enige temperatuurseffecten bekomen worden. De vertragende effecten van NVS op de theta frequentie echter volgen hetzelfde profiel als het effect van NVS op temperatuur, wat wijst op een grote kans dat deze gerelateerd zijn, wat vorige studies reeds aantoonden. NVS werd verder bevonden theta power de reduceren van prefrontaal diepte-EEG en oppervlakkig corticaal EEG, wat indiceert dat de reductie van theta power een globaal corticaal fenomeen is, wat detecteerbaar zou moeten zijn in het scalp EEG van patiënten. Gezien deze observatie werden de effecten van NVS op het EEG van ratten vergeleken met EEG van epilepsie patiënten, om de transleerbaarheid van voorgaande observaties te bepalen. NVS had echter geen vergelijkbaar effect op laagfrequent EEG power noch op de alfa piek frequentie van het scalp EEG, wat erop wijst dat beide effecten onafhankelijk zijn van temperatuursveranderingen en de effecten gerelateerd aan de temperatuursveranderingen in ratten niet transleerbaar zijn naar de humane conditie.

Deze studies tonen aan dat de translationele barrière tussen ratten en mensen in NVS onderzoek groter is dan vroeger aangenomen. Hoewel NVS reeds meer dan 20 jaar klinisch wordt toegepast, zijn er geen rapporteringen die wijzen dat NVS de temperatuur van patiënten beïnvloedt, en er zijn geen rapporteringen van thermisch discomfort geïnduceerd door NVS. Gecombineerd met de data van de vierde studie is het mogelijk dat de effecten van NVS op temperatuur een species-specifiek fenomeen is. Een revisie van de literatuur toonde dat minstens 40 studies NVS bij ratten toedienden op een manier waarbij inductie van hypothermie plausibel is. Gezien vele van deze studies belangrijk zijn voor ons huidig begrip van NVS, is het noodzakelijk om zich of te vragen in hoeverre deze informatie relevant is in een translationele context. Verder onderzoek is nodig voor het uitvoeren van robuuste en grotere translationele studies voor de translatie van NVS geassocieerde effecten tussen species te bepalen. Ondertussen, gezien het ultieme doel van NVS onderzoek het verbeteren van de applicatie in de klinische setting is, moedigt het huidige werk sterk aan dat de toekomstige inspanningen toegewijd worden aan klinische onderzoeken, of op zijn minst studies in species waarbij de effecten van NVS vergelijkbaar zijn met de effecten gezien in patiënten.

RÉSUMÉ FRANÇAIS

La stimulation du nerf vague (SNV) est une stimulation invasive électrique du tronc cervical du nerf vague. En 1994, la SNV a reçu l'approbation réglementaire pour le traitement de l'épilepsie réfractaire aux médicaments en Europe, et en 2005 la SNV a reçu l'approbation supplémentaire pour le traitement des dépressions résistantes aux médicaments. A ce jour, on estime que 70 000 patients ont été traités par SNV. Malgré plus de 20 ans d'expérience clinique, la SNV est associée à une variabilité du taux de réponses et il y a un manque de facteurs prédictifs concernant la réponse thérapeutique. Entre autres, ceci est partiellement dû à un manque de connaissances des mécanismes sous-jacents les effets thérapeutiques du traitement SNV.

L'accent du travail expérimental de cette thèse de doctorat est mis sur l'analyse des mécanismes neurophysiologiques sous-jacents de la SNV, en utilisant principalement l'électrophysiologie de rats en mouvement libre. L'objectif de la première étude était d'analyser les effets de la SNV sur l'excitabilité, étant donné que l'hyperexcitabilité est un élement-clé dans de nombreaux syndromes épileptiques. Afin d'analyser l'hyperexcitabilité, en outre de l'analyse de l'électroencéphalogramme, la potentialité de champs dentaires évoquée par stimulation de la voie perforante a été étudiée. L'application de la SNV a décéléré la potentialité évoquée par le champ dentaire, indiquant une efficacité réduite de la neurotransmission excitatrice, tout en augmentant le pic d'amplitude de la population, ce qui reflète une décharge de cellules granulées plus importante et plus synchronisée en réponse à certaines entrées. La SNV a réduit la puissance de haute tout comme de basse fréquence de l'électroencéphalogramme, conduisant ainsi à une décélération des rythmes Théta de l'hippocampe. Cette étude a montré que la SNV ne réduit pas l'excitabilité de manière uniforme, attribuée au traitement anticonvulsivant traditionnel, mais exerce plutôt une modulation plus complexe de la neurotransmission hippocampique.

La seconde étude analyse la variation des paramètres de la SNV. Le traitement SNV a été appliqué soit avec un cycle rapide (7s ON/18s OFF), soit avec un cycle standard (30s ON/300s OFF), et des courants de sortie variables. En plus des paramètres hippocampiques étudiés préalablement, les effets sur le couplage croisé des fréquences d'électroencéphalogramme hippocampique ont été étudiés. Un couplage fort a été détecté entre la phase d'oscillations théta (4-8 Hz) et l'amplitude des oscillations (75-150 Hz) rapides gamma. Le traitement SNV s'est avéré diminuer ce couplage. De plus, la SNV par cycle rapide a été associé à une modulation renforcée de tous les paramètres du cycle SNV standard. En titrant les courants de sortie SNV de 0 à 1000 µA, les effets de SNV ont été observés à amplitude maximale autour de 300 µA, au-delà cette amplitude de nouvelles augmentations d'intensité de la SNV n'ont pas entraîné d'effets additionnels. L'étude indique que les effets de la SNV sur l'électroencéphalogramme hippocampique sont dûs au recrutement de fibres nerveuses myélinisées type A, après quoi des effets additionnels peuvent seulement être obtenus en augmentant l'intensité du cycle de service SNV.

Dans la troisième étude nous examinons les effets de la SNV sur la température du cerveau et du corps de rats en mouvement libre, parce que le ralentissement de la potentialité de champs dentaires et du rythme Théta de l'électroencéphalogramme hippocampique furent jusqu'ici rapportés dans des conditions hypothermiques. La SNV a effectivement entraîné une réduction de la température du cerveau et du corps. La SNV par cycle rapide a réduit la température du

cerveau de 3 ^{o}C, tandis que la SNV par cycle standard a réduit la température du cerveau d' un peu moins de 1 ^{o}C. Par ailleurs, les données indiquent que les effets hypothermiques de la SNV ont été déployés sur les mêmes courants de sortie SNV que les effets de la SNV sur les paramètres de l'électroencéphalogramme hippocampique, appuyant l'implication d'épaisses fibres nerveuses myélinisées type A. De plus, la SNV a provoqué une réponse de vasodilatation périphérique indiquant une libération active de chaleur déclenchée par la SNV. Une lésion noradrénergique, qui a antérieurement éliminé les effets anticonvulsivants et antidépressifs de la SNV, n'a pas eu d'effets sur les résultats concernant la température et l'électroencéphalogramme. Etant donné l'impact des effets approfondis et généralisés de la température et en considérant que les effets hypothermiques sont jusqu'ici restés inaperçus, il est clair que l'hypothermie pourrait avoir des implications graves pour l'interprétation de beaucoup d'études antérieurement publiées sur le traitement SNV chez les rats.

La quatrième étude analyse les effets de la SNV sur la température et l'électroencéphalogramme et révèle que la SNV réduit la puissance Théta de l'électroencéphalogramme avant que n'apparaissent des effets sur la température. Les effets ralentissants de la SNV sur la fréquence Théta suivent pourtant un profil similaire à celui des effets de la SNV sur la température, indiquant une probabilité accrue que ces effets sont liés, ce qui a effectivement été démontré par d'autres études antérieurement. De plus, la SNV a réduit la puissance Théta de l'électroencéphalogramme préfontal profond et de l'électroencéphalogramme superficiel cortical, suggérant que les réductions de la puissance Théta sont un phénomène global, qui devrait être identifiable sur l'électroencéphalogramme du scalp d'un patient. Par conséquent, en raison de cette observation, nous avons comparé les effets de la SNV sur l'électroencéphalogramme du rat avec des électroencéphalogrammes de patients épileptiques, afin d'évaluer la traductibilité de ces observations. Cependant, SNV n'a pas eu d'effet comparable sur la puissance de fréquence basse de l'électroencéphalogramme, ni sur le pic de fréquence alpha de l'électroencéphalogramme du scalp, suggérant que les effets indépendants du changement de température ainsi que les effets reliés aux changements de température chez les rats ne sont pas traductibles sur l'environnement humain. Ces études indiquent que la barrière translationnelle entre rats et humains dans la recherche SNV pourrait être plus importante que supposée jusqu'ici. Bien que la SNV soit appliquée cliniquement depuis plus de 20 ans, aucun effet de la SNV sur la température des patients et aucun inconfort thermique provoqué par SNV n'ont été signalés. En ajoutant les données de la quatrième étude, il semble probable que les effets de la SNV sont un phénomène propre à l'espèce. Une analyse bibliographique a révélé qu'un minimum de 40 études ont appliqué la SNV d'une façon qui conforte l'induction d'hypothermie. Parce que beaucoup de ces études sont importantes pour notre compréhension actuelle de la SNV, il est nécessaire de mettre en cause la mesure dans laquelle ces connaissances sont pertinentes dans un contexte translationnel.

Des efforts doivent être faits afin de mener des études translationnelles à grande échelle pour évaluer la traduisibilité des effets de la SNV d'une espèce à l'autre. Cependant, considérant que l'objectif ultime de toute recherche SNV est d'améliorer l'application clinique, les présents résultats encouragent fortement à principalement dédier les efforts à venir aux études SNV cliniques, ou du moins à des études sur des espèces aux effets SNV comparables à ceux observés chez les patients.

DANSK RESUMÉ

Vagus Nerve Stimulation (VNS) er en ikke invasiv neurostimulationsteknik, hvor elektriske stimulation administreres til den cervikale del af vagusnerven. I 1994 blev VNS godkendt i Europa til behandling af epilepsiske syndromer som ikke kan behandles effektivt med tilgængelige lægemidler. Senere, i 2005, blev VNS endvidere godkendt til behandlingen af behandlingsresistente depressioner. I dag vurderes det at mere end 70.000 patienter er blevet behandlet med VNS. Selvom VNS har været klinisk anvendt i mere end 20 år er VNS stadig associeret med varierende behandlingssucces og samtidigt er der ingen biomarkører tilgængelige som kan forudsige hvor vidt en patient kan effektivt behandles med VNS. Dette skyldes først og fremmest at det stadig ikke er nok viden om hvordan VNS virker.

Målet med denne afhandling var først og fremmest at undersøge underliggende mekanismer som kan forklare terapeutiske effekter, som f.eks. antikonvulsive effecter, der er forbundet med VNS. For at undersøge dette, er der primært blevet anvendt elektrofysiologi i vågne frit bevægende rotter. I det første studie var målet at undersøge hvordan VNS påvirker hjernens responsivitet og aktivitet, da hyperresponsivitet og hyperaktivitet er typiske kerneelementer i mange epileptiske syndromer. For at undersøge dette blev der målt på elektriske potentialer i gyrus dentatus, en underdel af hippocampus-strukturen, som findes på indersiden af tindingelappen. Der blev målt både på spontan aktivitet lokalt i gyrus dentatus, såkaldt elektroencefalografi (EEG), såvel som synkrone potentialer (også kaldet "evoked potentials") fremprovokeret ved at levere elektrisk stød til en nervebane som projekterer til gyrus dentatus. VNS påvirkede både EEG såvel som evoked potentials. Effekten af VNS på evoked potentials indikerede at VNS hæmmede eksitatorisk neurotransmission, reflekteret af langsommere evoked potentials, hvilket typisk ses for lægemidler der bruges i behandlingen af epilepsi. Men det samtidige øgede de cellulære respons i gyrus dentatus under VNS indikerede en mere kompleks effekt af VNS på hjernens responsivitet. VNS påvirkede ydermere det hippocampale EEG, hvilket afspejledes ved en reduktion i signalstyrken (power). Derudover var den mest prominente hippocampale EEG rytme, theta rytmen, langsommere under VNS behandlingen. Konklusionen på studiet var at effekten af VNS på hjernens responsivitet og aktivitet er mere kompleks end ellers observeret for andre antikonvulsive behandlingsformer.

I studie nummer to var målet at undersøge hvordan forskellige VNS parametre (hvor meget strøm og hvor længe der stimuleres ad gangen) havde indflydelse på de effekter der blev observeret i det første studie. To forskellige VNS cykluser blev undersøgt: en hurtig cyclus, hvor VNS var blev administreret i 7 sekunder efterfulgt af en periode på 18 sekunder uden stimulation, samt en standard cyclus, som oftest bruges i klinikken, hvor VNS blev administreret i 30 sekunder efterfulgt af 300 sekunder uden stimulation. Derudover undersøgte vi hvorledes strømstyrken havde indflydelse på de effekter vi obseverede. Udover tidligere studerede effekter, blev der ydermere analyseret hvorledes forskellige spontane rytmer i det hippocampale EEG interagerede og hvorledes VNS påvirkede denne interaktion. Observationerne viste at hurtige EEG rytmer var stærkt koblet til langsomme EEG rytmer. Denne kobling var stærkt reduceret under VNS behandling. Den hurtige cyclus havde en større effekt end standard cyclus. Desuden indikerede observationerne at maximal effekt kunne opnås ved et bestemt tærskelniveau. Ved at øge strømstyrken yderlige forbi dette tærskelniveau kunne yderligere effekt ikke observeres.

Dette er i overenstemmelse med eksisterende viden omkring hvorledes forskellige strømstyrker har indflydelse på hvilke nervefibertyper som aktiveres af elektriske stød. Mere specifikt indikerede strømstyrken anvendt i dette studie at de observerede effekter skyldes aktivering af såkaldte type-A fibre, som er de tykkeste og hurtigtst transmiterende fibre.

Det tredje studie var primært en opfølgning på det første studie hvor indflydelsen af VNS på hippocampal aktivitet blev beskrevet. Observationerne at både evoked potentials, såvel som theta rytmen i det hippocampale EEG var langsommere under VNS indikerede at VNS muligvis havde en indflydelse på hjernetemperaturen fordi de samme observationer tidligere var blevet gjort i afkølede rotter. I overenstemmelse med denne hypotese viste resultaterne en afkøling på så meget som 3 ^{o}C under VNS behandling med en hurtig cyclus, hvorimod standard cyclus sænkede hjernetemperaturen med en smule mindre end 1 ^{o}C. VNS sænkede både temperaturen i hjernen såvel som i resten af kroppen reflekteret af en lavere rektal temperatur. Resultaterne var desuden i overenstemmelse med tidligere resultater og indikerede at effekten af VNS på hjernetemperatur krævede samme strømstyrkeniveau som tidligere observerede effekter på det hippocampale EEG. Ved brug af et thermografisk kamera kunne der ligeledes observeres hvordan VNS inducerede en udvidelse af blodkar i periferien, primært i halen, hvorledes varmen blev afledt til omgivelserne. Tidligere studier har ydermere vist at det centrale noradrenerge system er ansvarlig for både antikonvulsive såvel som antidepressive effekter associeret med VNS. Effekten af VNS på temperatur, derimod, var intakt selv efter en læsion af det centrale noradrenerge system. Fordi temperatur har stor inflydelse på mange fysiologiske processer var konklusionen på dette studie at den observerede effekt muligvist vil have haft stor inflydelse på mange tidligere studier, såvel som på den fremtidige retning af forskning vedrørende VNS.

I det fjerde studie var målet at undersøge hvorvidt tidligere observerede effekter af VNS på hjerneaktivitet såvel som temperatur i rotter på lignende vis kan observeres i patienter under behandling med VNS. Indledningsvis blev det undersøgt hvorvidt tidsprofilen af de observerede effekter af VNS på hjerneaktivitet var i overenstemmelse med effekten af VNS på hjernetemperatur. Her viste analyserne at effekterne på signalstyrken af EEG fra både hippocampale regioner såvel som EEG fra pandelappen kun krævede få minutter VNS, hvorimod effekten af VNS på temperatur først kunne observeres efter en halv times VNS behandling. Den sløvende effekt af VNS på den hippocampale theta rytme, derimod, fulgte en tidsprofil i overenstemmelse med effekten af VNS på temperature, hvilket indikerer at disse effekter muligvist er forbundet. I epilepsipatienter implanteret med et VNS system kunne ingen tilsvarende ændringer i EEG observeres, hvorfor det konkluderes at mange af de tidligere observerede effekter i rotter muligvist ikke er klinisk relevante. Dette er en vigtig observation fordi den samtidigt stiller spørgsmål ved den kliniske relevans af mange tidligere observerede effekter af VNS i rotter og fordi den eksisternede viden om VNS primært kommer fra rottestudier.

Den overordnede konklusion på disse studier er derfor at barrieren mellem prækliniske rotteforsøg og kliniske forsøg er større end hidtil antaget for forskning vedrørende VNS. Selvom VNS har været anvendt i mere end 20 år i klinikken er der endnu ingen rapporter om effekter af VNS på temperatur. Dette indikerer at effekten af VNS på temperatur er et art-specifikt fænomen. En gennemgang af litteraturen viste at mindst 40 tidligere udgivede rottestudier har anvendt VNS på en måde hvor en effekt på temperatur kan forventes. Fordi at temperatur har en inflydelse på almen fysiologi, og fordi mange af disse studier ikke har taget højde for denne effekt, er det derfor nødvendigt at nuværende ideer om VNS revurderes. Det er først og fremmest vigtigt at undersøge hvilke af disse effekter som er klinisk relevante i patienter. Det står derfor klart at fremtidens VNS-studier først og fremmest bør udføres i arter som representerer mennesket bedre, eller hvor muligt udføres i patienter.

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I will additionally direct my gratitude to some of my closest friends of whom I have enjoyed the company over these years. I will especially thank **Ruben**. The only Belgian friend I had before I came to Belgium. Our conversations, sometimes serious and sometimes not so serious, have always meant a lot to me. I additionally thank **James** and **Christoph**, my football buddies. We have had many wonderful moments together and it feels as if we have formed the great foundation for a future long-lasting friendship. I also thank the friends at home - those I left behind to live in Belgium. **Peter**, my childhood friend. **Lydia**, since the early uni-years. It means a great deal that I have been able to keep close contact to you. Thanks also to **Dennis** for the efforts in maintaining our friendship. We could forever talk about our past adventures in Erlangen back in 2011 and 2012.

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Curriculum Vitae

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Publications (A1)

Larsen LE, Van Lysebettens W, Wadman WJ, Delbeke J, Boon P, Vonck K, Raedt R (2016) Questioning the status quo: is it time to revise our current ideas of vagus nerve stimulation? *In Preparation*

Larsen LE, Van Lysebettens W, Carrette S, Daelemans S, Dauwe I, Sprengers M, De Taeye L, Thyrion L, Wadman WJ, Delbeke J, Carrette E, Boon P, Vonck K, Raedt R (2016) Modulation of Electrophysiological Parameters by Vagus Nerve Stimulation: a Translational Study. *In Preparation*

Larsen LE, Van Lysebettens W, Carrette S, Daelemans S, Sprengers M, Thyrion L, Wadman WJ, Delbeke J, Boon P, Vonck K, Raedt R (2016) Vagus Nerve Stimulation Induces Hypothermia in Freely Moving Rats. *Submitted*

Thyrion L, Portelli J, Raedt R, Glorieux, G, **Larsen LE**, Sprengers M., Van Lysebettens W, Carrette E, Delbeke J., Carrette, E, Vonck K, Boon P (2016) Disruption, but not overexpression of urate oxidase alters susceptibility to pentylenetetrazole and pilocarpine induced seizures in mice. *In Press, Epilepsia*

Larsen LE, Wadman WJ, Marinazzo D, van Mierlo P, Delbeke J, Daelemans S, Sprengers M, Thyrion L, Van Lysebettens W, Carrette E, Boon P, Vonck K, Raedt R (2016) Vagus Nerve Stimulation Applied with a Rapid Cycle Has More Profound Influence on Hippocampal Electrophysiology than Standard Cycle. *Neurotherapeutics*, DOI: 10.1007/s13311-016-0432-8

Larsen LE, Wadman WJ, van Mierlo P, Delbeke J, Grimonprez A, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R (2016) Modulation of Hippocampal Activity by Vagus Nerve Stimulation in Freely Moving Rats. *Brain Stimul*, 9:124-32

Grimonprez A, Raedt R, De Taeye L, **Larsen LE**, Delbeke J, Boon P, Vonck K (2015). Laryngeal motor-evoked potentials mark vagus nerve activation: a preclinical study. *Int J Neural Syst*, 8, 1550034.

Grimonprez A, Raedt R, Portelli J, Dauwe I, **Larsen LE**, Bouckaert C, Delbeke J, Carrette E, Meurs A, De Herdt V, Boon P, Vonck K (2015). *J Psychiatr Res*, 68, 1-7.

Grimonprez A, Raedt R, Dauwe I, Mollet L, **Larsen LE**, Delbeke J, Meurs A, De Herdt V, Wadman WJ, Boon P, Vonck K (2015). Vagus Nerve Stimulation has Antidepressant Effects in the Kainic Acid Model for Temporal Lobe Epilepsy. *Brain Stimul*, 8(1), 13-2015.

Larsen LE, Jakobsen LA, Jensen A, Lambden BM, Sørensen MR, Ellrich J (2015). Inhibition of soleus Hoffmann reflex by ankle-foot orthosis application in healthy volunteers. *Prosthet Orthot Int*, 39(6), 444-53.

Jung K, **Larsen LE**, Rottmann S, Ellrich J (2011). Heterotopic low-frequency stimulation induces nociceptive LTD within the same central receptive field in man. *Exp Brain Res*, 212(2), 189-98.

Jakobsen LA, Jensen A, **Larsen LE**, Sørensen MR, Hoeck HC, Arendt-Nielsen L, Gazerani P (2011). Effect of cutaneous blood flow on absorption of insulin: a methodological study in healthy male volunteers. *Int J Physiol Pathophysiol Pharmacol*, 3(4), 257-65

Conference Abstracts (C3)

Larsen LE, van Mierlo P, Staljanssens W, Wadman WJ, Delbeke J, Grimonprez A, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R (2014) Vagus nerve stimulation decreases hippocampal and prefrontal EEG power in freely moving rats: a biomarker for effective stimulation? *Proceedings for Belgian Brain Council, Front Hum Neurosci.*, DOI:10.3389/conf.fnhum.2014.214.00035.

Larsen LE, Jung K, Ellrich J (2010) Heterotopic low-frequency stimulation induces nociceptive LTD within the same central receptive field in man. *Acta Physiologica*, 198, Suppl 677: P-MON-116, 2010.

Participation in Congresses and Meetings

- Joint Meeting of the Scandinavian and German Physiological Societies, Copenhagen, Denmark, March, 2010
- 7th Annual Meeting of the German Society for Neuromodulation (DGNM), Aachen, Germany, November 2011
- 51st Annual Meeting of the German Society for Epileptology, Stuttgart, Germany, March, 2012
- International Epilepsy Workshop. Ghent, Belgium, October, 2012
- Second Ghent Institute for Neuroscience Symposium, Ghent, Belgium, December, 2012
- 15th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2013
- 1st PhD Day of the Institute for Neuroscience, Ghent, Belgium, December, 2013
- 12th Dutch Endo-Neuro-Psycho Meeting (EPN), Lunteren The Netherlands, May, 2014
- Belgian Brain Council, Ghent, Belgium, October, 2014
- PhD Workshop, Ghent, Belgium, February, 2015
- Science Day, Ghent, Belgium, March, 2015
- SWO Midwintermeeting, Amsterdam, The Netherlands, March, 2015
- The Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, March, 2015
- 16th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2015
- 2nd PhD Day of the Institute for Neuroscience, Ghent, Belgium, April, 2015
- Symposium: Functional Neuroimaging in Epilepsy and Neruomodulation, Ghent, Belgium, November, 2015
- 16th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2016

Oral Presentations

- Vagus Nerve Stimulation Modulates Hippocampal Electrophysiology in Freely Moving Rats. *International Peer Review*, Ghent, Belgium, February, 2015
- Vagus Nerve Stimulation Modulates Hippocampal Electrophysiology in Freely Moving Rats. *Science Day*, Ghent, Belgium, February, 2015
- Vagus Nerve Stimulation Modulates Hippocampal Electrophysiology in Freely Moving Rats. *SWO Midwintermeeting*, Amsterdam, The Netherlands, March, 2015
- Vagus Nerve Stimulation Modulates Hippocampal Electrophysiology in Freely Moving Rats. *The Göttingen Meeting of the German Neuroscience Society*, Göttingen, Germany, March, 2015
- Changes in Hippocampal Electrophysiology during Vagus Nerve Stimulation in the Rat. *Symposium: Functional Neuroimaging in Epilepsy and Neruomodulation*, Ghent, Belgium, November, 2015

Poster Presentations

Larsen LE, Jung K, Ellrich J. Heterotopic low-frequency stimulation induces nociceptive LTD within the same central receptive field in man. Joint Meeting of the Scandinavian and German Physiological Societies, Copenhagen, Denmark, March, 2010.

Larsen LE, van Mierlo P, Wadman WJ, Delbeke J, Grimonprez A, Mollet L, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R. Vagus nerve stimulation decreases hippocampal and prefrontal EEG power in freely moving rats: a biomarker for effective stimulation? 12th Dutch Endo-Neuro-Psycho Meeting (EPN), Lunteren The Netherlands, May, 2014

Larsen LE, van Mierlo P, Wadman WJ, Delbeke J, Grimonprez A, Mollet L, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R. Vagus nerve stimulation decreases hippocampal and prefrontal EEG power in freely moving rats: a biomarker for effective stimulation? Belgian Brain Council, Ghent, Belgium, October, 2014

Larsen LE, van Mierlo P, Wadman WJ, Delbeke J, Grimonprez A, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R. Effects of Vagus Nerve Stimulation on Hippocampal Neurophysiology in Freely Moving Rats, SWO Midwintermeeting, Amsterdam, The Netherlands, March, 2015

Larsen LE, van Mierlo P, Wadman WJ, Delbeke J, Grimonprez A, Van Nieuwenhuyse B, Portelli J, Boon P, Vonck K, Raedt R. Effects of Vagus Nerve Stimulation on Hippocampal Neurophysiology in Freely Moving Rats, 16th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2015

Larsen LE, Wadman WJ, van Mierlo P, Delbeke J, Daelemans S, Sprengers M, Thyrion L, Van Lysebettens W, Carrette E, Boon P, Vonck K, Raedt R. Modulation of Hippocampal Activitiy by Vagus Nerve Stimulation Depends on Stimulation Parameters, 17th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2016

Teaching and Supervision of Students

- Master Thesis of Eline Devos: Establishment and optimization of the attentional set shifting task for rats (2013-2014)
- Master Thesis of Chloë De Ruyck: Investigation of cognitive flexibility in the rat kindling mobel of temporal lobe epilepsy (2014-2015)
- Lessons on behavioral animal sciences, for biomedical sciences students, February-March, 2015
- Master Thesis of Charlotte Germonpré: The effect of vagus nerve stimulation on thermoregulation in the rat (2015-2016)
- Lesson on epilepsy research at Ghent University, for biology students, February, 2016
- Introduction to digital signal processing in the context of electrophysiology, for biomedical sciences students, April, 2016

Etc.

- FELASA-C license for animal experimentation
- Member of organizing committee for the PhD day for the Institute for Neurosciences in 2013 and 2015
- Awarded best technical research contribution at the 16th Annual International Clinical Symposium Kempenhaeghe, Kempenhaeghe Heeze, March, 2015