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CANINE EPILEPSY

The role of functional brain imaging and vagus nerve stimulation

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"He remembered that he always had one minute just before the epileptic fit when suddenly in the midst of sadness, spiritual darkness and oppression, there seemed at moments a flash of light in his brain, and with extraordinary impetus all his vital forces suddenly began working at their highest tension. The sense of life, the consciousness of self, were multiplied ten times at these moments which passed like a flash of lightning. His mind and heart were flooded with extraordinary light... But these moments, these flashes, were only the prelude of that final second in which the fit began."

From 'The Idiot' by F. Dostoevsky

voor Daan en Arno

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LIST OF ABBREVIATIONS

AEDs	antiepileptic drugs
AI	asymmetry index
BBB	blood brain barrier
BRASS	brain registration and automated SPET semiquantification
Co-SCo	cortical-subcortical index
CSF	cerebrospinal fluid
CSV	cerebrospinaal vocht
CT	Computed Tomography
DA	dopamine
ECG	electrocardiography
EEG	electroencephalography
GABA	Gamma-aminobutyric acid
5HT	Serotonin
IM	intramuscular
IV	intravenously
LC	Locus Coeruleus
LChr	Liquid Chromatography
MOA	mechanism of action
MRI	Magnetic Resonance Imaging
fMRI	functional MRI
NA	noradrenaline
NE	norepinephrine
NST	nucleus of the solitary tract
NVS	nervus vagus stimulatie
PET	Positron Emission Tomography
PH	phenobarbital
PI	perfusion index
PTZ	Pentylenetetrazole
R-C	rostro-caudal gradient
RN	raphe nucleus
ROR	radius of rotation
RX	radiography
rCBF	regional cerebral blood flow
SD	standard deviation
SPECT	Single Photon Emission Computed Tomography
µ-SPECT	micro-SPECT
^{99m}Tc-ECD	^{99m}technetium-ethyl cysteinate dimer
US	ultrasound
VNS	vagus nerve stimulation
VOI	volume of interest

General Introduction

Adapted from:

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1. Canine epilepsy

1.1 Terminology and classification

Epilepsy is a disease characterized by recurrent seizures originating from the brain. It is the most common chronic neurologic disorder both in humans (Sander and Shorvon, 1996; Brodie et al., 1997) and dogs (Chandler, 2006). The prevalence of epilepsy in dogs is estimated between 0.5 and 5.7% (Bielfelt et al., 1971; Schwartz-Porsche, 1994; Knowles, 1998; Patterson et al., 2005a; Chandler, 2006). Epilepsy is a general term covering different disorders that can be classified according to cause. The classification scheme for human epilepsy has recently been updated by the International League Against Epilepsy (Berg et al., 2010). In veterinary medicine, a universally accepted epilepsy terminology and species specific classification does not exist, and a classification system, based upon its human counterpart, is most often followed. This classification includes three categories: idiopathic, symptomatic and probable symptomatic epilepsy (see Table 1) (Berendt, 2008; Muñana, 2013a). The majority of dogs with recurrent seizures are classified as having idiopathic epilepsy which implies that no underlying cause for the seizures can be identified and a familial or genetic predisposition for the development of epilepsy is presumed(Schwartz-Porsche, 1994; Berendt, 2008). Recently, an update of descriptive terminology and of the diagnostic classification scheme for the use in veterinary patients has been proposed (Mariani, 2013), and one of its suggestions is to replace the term idiopathic by genetic epilepsy. Since this has just recently been proposed and definitive conclusions of veterinary neurologists have not yet been published, we still use the term idiopathic epilepsy in this thesis.

Table 1: Commonly used epilepsy classification in veterinary medicine

Epilepsy Type	Definition	Synonyms
Idiopathic epilepsy	Chronic seizures with no underlying cause other than a presumed genetic predisposition	*Primary epilepsy* *Genetic epilepsy*
Symptomatic epilepsy	Identifiable lesion in the brain associated with an increased risk of developing seizures	*Structural epilepsy* *Secondary epilepsy*
Probable symptomatic epilepsy	Suspected underlying cause of the seizures is as yet unidentified/unknown	*Cryptogenic epilepsy / Epilepsy of unknown origin*

Human neurologists also classify seizures depending on their clinical and electroencephalographic (EEG) presentation (Chandler, 2006; Berg et al., 2010) with the two major categories being generalized and focal seizures, based on the origin and spreading of the epileptogenic activity. Applying this classification system to veterinary patients is difficult due to several reasons (Thomas, 2010; Muñana, 2013a). First of all, the seizure description is delivered by the owner, who is just an observer (Chandler, 2006). Secondly, it is possible that not all human seizure types exist in dogs (Thomas, 2010). Finally, EEG data are usually not available for veterinary patients and are difficult to interpret due to possible confounding factors, such as muscle and motion artifacts and the influence of sedation (Pellegrino and Sica, 2004; Thomas, 2010). Despite these disadvantages, several attempts to classify seizure types, based on their semiology, in dogs have been made (Schwartz-Porsche, 1994; Berendt and Gram, 1999; Licht et al., 2002; Podell, 2004).

Historically, focal seizures have been considered rare in dogs, and most dogs with idiopathic epilepsy were considered to have generalized tonic-clonic seizures (Schwartz-Porsche, 1994). However, now it is known that focal seizures occur more frequently in dogs than previously thought (Jaggy and Bernardini, 1998; Berendt and Gram, 1999; Licht et al., 2002) and there is increasing evidence that focal seizures can be idiopathic (Heynold et al., 1997; Patterson et al., 2003, 2005a; Berendt et al., 2004a).

1.2 Standard diagnostic work-up

A detailed and accurate history is an essential part of the diagnosis of canine epilepsy. Other diseases in dogs can cause episodic seizure-like events, which have to be distinguished from epilepsy. The most important differential diagnoses are syncope, narcolepsy-cataplexy, episodic neuromuscular weakness, vestibular disorders, generalized tremor syndromes and compulsive behavior problems (Shell, 1993; Thomas, 2010). An epileptic seizure rarely occurs in the consultation room and therefore, a detailed description of the seizure, possibly together with a video of the event has to be critically analyzed.

A presumptive diagnosis of idiopathic epilepsy can be made based on the history, signalment of the dog and lack of other clinical or neurological signs. A definitive diagnosis of idiopathic epilepsy additionally needs an extensive blood work, structural brain imaging (Magnetic Resonance Imaging (MRI) or Computed Tomography (CT)) and analysis of cerebrospinal fluid (CSF) to exclude underlying causes for the seizures (Chandler, 2006).

1.3 Standard medical treatment

The ultimate goal of antiepileptic treatment is to reach seizure freedom without side effects, but a more realistic goal is to reduce the seizure frequency and severity to an acceptable level for the pet and his owner while avoiding serious side effects (Berendt, 2004b; Thomas, 2010). The decision on when to start treatment with antiepileptic drugs (AEDs) follows general guidelines (see Table 2), but additional factors such as the general health of the dog, the owner's lifestyle, financial limitations and comfort with the proposed therapy play an important role as well. So, a final decision has to be taken on an individual basis (Muñana, 2013a).

Table 2: General criteria to start AED treatment in canine epilepsy

Seizure frequency ≥ 1 /month
Sudden increase in seizure frequency/severity
History of cluster seizures / status epilepticus
Presence of underlying, progressive brain disorder responsible for the seizures
Severe seizures / severe postictal signs
Owner has a strong desire to treat regardless of frequency / severity

adapted from Thomas, 2010; Muñana, 2013a

Owner compliance is fundamental for a successful treatment of canine epilepsy, so good client education is imperative. The dog owner has to be informed about the disease, the realistic goals of therapy and possible side effects of the AEDs. The owner must also

understand the need for regular AED administration and avoid dose alterations based on his/her own assessment of seizure control (Berendt, 2004b; Thomas, 2010; Muñana, 2013a).

Until recently, treatment options for dogs with epilepsy were limited to phenobarbital (PB) and potassium bromide, both relatively safe, effective and inexpensive AEDs (Al-Tahan, 1985; Podell, 1993). PB is usually the initial drug of choice as it is effective in approximately 60 – 80% of epileptic dogs when plasma concentrations are maintained within the therapeutic range (Farnbach, 1984; Schwartz-Porsche et al., 1985). Bromide is thought to be less efficacious than PB as first-line AED in dogs (Boothe et al., 2012) and is most often added to PB when insufficient seizure control is reached. About 25% of PB resistant canine epilepsy patients become seizure free on polytherapy with PB and bromide (Podell, 1993). Side effects can be an important downside of these AEDs and, although some are reversible and resolve within the first weeks of therapy, others are more serious. A detailed monitoring with regular general blood work and serum concentration measurements is therefore advised for both AEDs.

In Europe, a new AED, imepitoin, has been recently approved for the treatment of canine idiopathic epilepsy. The effectiveness seems comparable to PB, but the major advantage is that fewer side effects are expected (Löscher et al., 2013; Rundfeldt et al., 2014). Although this new AED seems promising, future larger and comparative studies in dogs are warranted.

1.4 Refractory epilepsy

In human medicine, refractory or drug resistant epilepsy has recently been defined as ‘a failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom’ (Kwan et al., 2010). A similar definition has not been established yet in veterinary medicine. The term refractory epilepsy describes a condition in which an animal fails to attain satisfactory seizure control or suffers intolerable side effects despite appropriate therapy with conventional AEDs (Muñana, 2013b). Throughout literature, refractory epilepsy has also been described as drug resistant, medically intractable or pharmacoresistant. Refractory epilepsy is an important problem in small animal practice

as it occurs in up to 30% of dogs with idiopathic epilepsy (Farnbach, 1984; Lane and Bunch, 1990). Interestingly, the incidence of refractory epilepsy is comparable in humans (Kwan and Brodie, 2000).

The underlying pathogenesis of refractory epilepsy is unclear, but three major theories leading to drug refractoriness have been described: 1) a change in the neuronal network , 2) a reduced sensitivity of drug targets to AEDs in epileptogenic brain tissue and 3) an overexpression of multidrug transporters leading to removal of AEDs from the epileptogenic tissue (Kwan and Brodie, 2006; Volk, 2008). Humans with refractory epilepsy are often resistant to multiple AEDs with a different MOA, which mainly supports the third theory (Löscher, 2007). Considerable data have been generated in support of all hypotheses, which reflects that the etiology of pharmacoresistance is likely multifactorial and varies between individuals (Kwan and Brodie, 2002). Inherent disease severity has recently been proposed to play a role as well (Rogawski and Johnson, 2008), which could be reflected in breed-related differences in seizure severity in dogs (Muñana, 2013b). The contribution of the different hypotheses to canine refractory epilepsy remains to be elucidated (Volk, 2008).

Many novel AEDs have been developed for the use in human epilepsy over the past 20 years. Several of these drugs are unsuitable for use in dogs, because they are metabolized too quickly or because they are toxic (Löscher, 1993; Podell, 1998; Govendir et al., 2005). The most important human AEDs that can be safely used as add-on treatment in dogs with refractory epilepsy are gabapentin, pregabalin, zonisamide and levetiracetam. They seem to have a wide therapeutic index and offer the potential of minimizing adverse effects, although the optimum use and their efficacy have not yet been fully determined in veterinary patients (Muñana, 2013a). Only retrospective or prospective open-label studies, including a small number of dogs, have been described for most of these AEDs (Dewey et al., 2004; Govendir et al., 2005; Platt et al., 2006; Volk et al., 2008; Muñana et al., 2012) so, it is possible that their efficacy is overestimated due to an important placebo effect recognized in canine epilepsy (Muñana et al., 2010). Similar as in human epilepsy trials, a reduction in seizure frequency has also been demonstrated in a considerable part of dogs receiving placebo treatment. This placebo response might be related to the natural waxing and waning course of the disease, but the owners expectations of a new treatment probably play an important role as well (Muñana et al., 2010).

1.5 Limitations of canine epilepsy

The diagnosis, classification and treatment of epilepsy are slowly advancing fields in dogs compared to humans due to several reasons. Regarding the diagnosis and classification, a lot of subjectivity and misinterpretation can be present in the clinical description of the seizure, as it is provided by the owner instead of the patient. As mentioned before, EEG is not routinely used in dogs due to the lack of availability and expertise, due to a lot of artefacts and due to the need for sedation (Pellegrino and Sica, 2004; Chandler, 2006). Another important limitation is that brain imaging, although improving and expanding also in veterinary medicine, is not at the same state-of-the-art level as in humans. Since micro-structural changes of the cortex are often identified as causes of symptomatic epilepsy in humans (Raymond et al., 1995), it is possible that these lesions are overlooked in dogs. Consequently, a proportion of dogs could be misclassified as having idiopathic epilepsy.

The optimization of existing and the development of new treatment options for canine refractory epilepsy are limited by different reasons. First of all, owner compliance is fundamental to reach a successful AED treatment in dogs. Secondly, the efficacy of recent AEDs has not yet been established using well-controlled canine clinical trials (Muñana et al., 2010) and the number of AEDs with an acceptable metabolization is limited in dogs. Financial concerns cannot be ignored in veterinary medicine as well.

The possibility exists that some dogs with difficult-to-control seizures have no true refractory epilepsy. False pharmacoresistance also occurs in human epilepsy (Smith et al., 1999) and is not easily recognizable (Pati and Alexopoulos, 2010). The frequency of this phenomenon could even be higher in dogs than in humans, due to overlooked underlying causes in the diagnostic work-up or due to bad owner compliance in the treatment.

2. Brain SPECT

2.1 Introduction

Single photon emission computed tomography (SPECT) is, besides positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), a non-invasive imaging technique that can be used to evaluate the functional state of the brain *in vivo*. In contrast to structural brain imaging techniques, such as CT or MRI, functional brain imaging offers the advantage to look beyond structural abnormalities and detect disturbances at the molecular level. Therefore, the use of SPECT provides an ideal application for understanding the pathophysiology of brain disorders (e.g. epilepsy) and for testing certain therapeutic strategies (e.g. vagus nerve stimulation (VNS)) in a preclinical setting (Khalil et al., 2011). SPECT can evaluate the regional cerebral blood flow (rCBF) which is an indirect reflection of brain metabolism and regional neuronal activity (Roy and Sherrington, 1890; Warwick, 2004). The main principle of SPECT is that a photon emitting radiopharmaceutical is injected in a subject (Abraham and Feng, 2011). As the isotope decays, photons (gamma rays) are emitted from the body, which are then detected and recorded by one or more detectors rotating around the patient (Figure 1). In this way, an image of the distribution of the radioligand in the target organ is obtained (Vermeire et al., 2011).

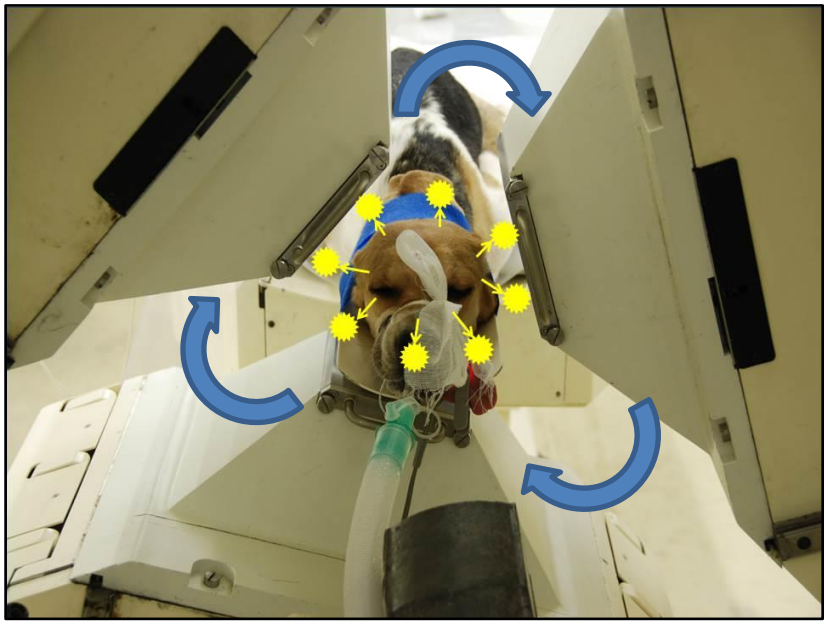

Figure 1: Photons emitted from the dog's brain (yellow dots) are registered by 3 detectors rotating around the head of the dog

Each detector consists of a collimator, a crystal and photomultiplier tubes. Collimators will only allow photons from a specific direction to pass through and will absorb scattered photons. Consequently, scattered photons will not reach the crystal. The crystal is an essential part of the detector that registers the photons and transforms them into a light signal. Finally, the light signals will be converted by the photomultiplier tubes into an electric current. This results in a three-dimensional image that can be presented as multiple two-dimensional images in the 3 spatial planes (Figure 2) (Vermeire et al., 2011).

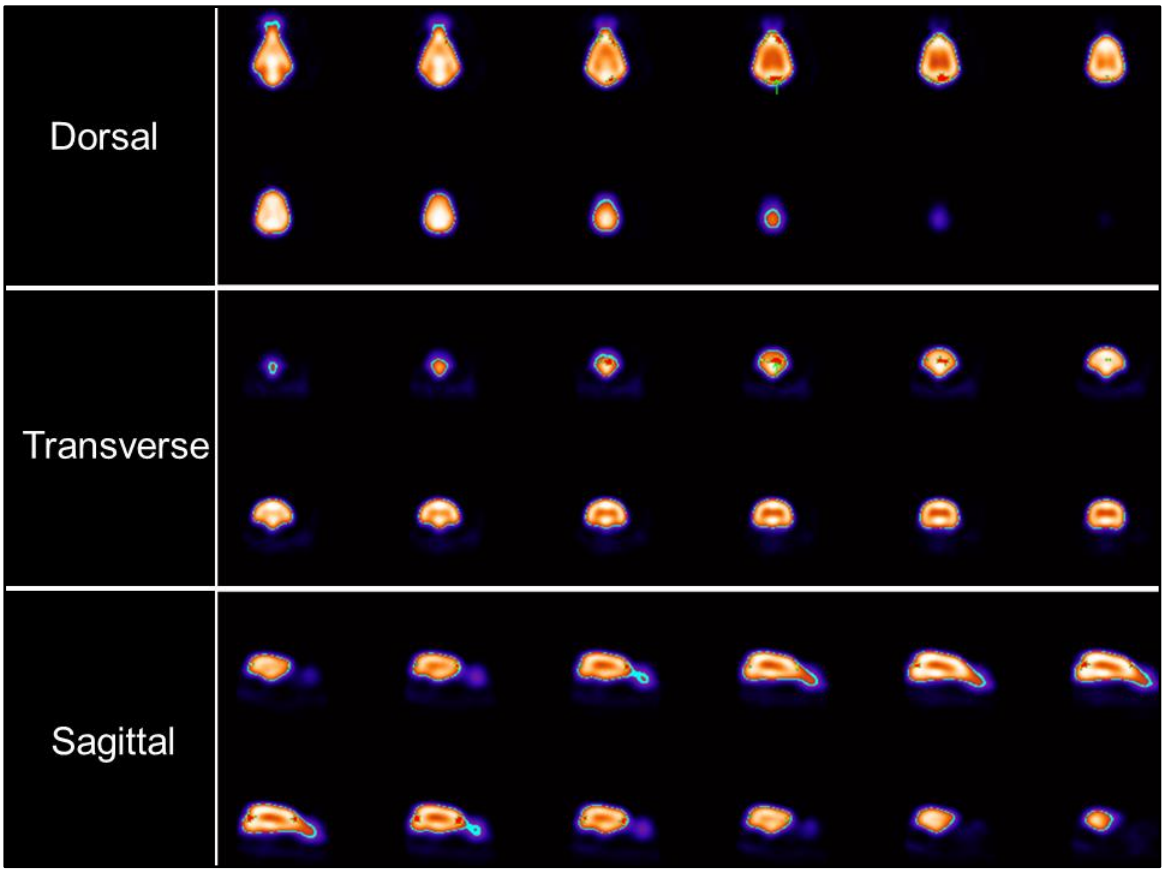

Figure 2: Conventional SPECT study of a dog represented in the 3 planes

An important advantage of using nuclear medicine techniques for experimental research is that repeated longitudinal studies can be performed in the same animal, so that each animal can serve as its own control (Groch and Erwin, 2000). Also, human dedicated cameras can be used for canine or feline brain research, because their brains are of sufficient size compared to rodents. An important limitation of SPECT is that attenuation,

scatter and partial volume effects can influence the images (Peremans et al., 2005). Consequently, the registered photons are not derived with certainty from the actual volume of interest (VOI), which precludes absolute quantification methods (Peremans et al., 2003). A semiquantitative analysis of brain perfusion in different VOIs has become the preferred method of quantification (Catafau, 2001). For each VOI a perfusion index (PI) is obtained by normalizing the radioactivity of a certain VOI to the radioactivity of the total brain or the cerebellum. This allows an estimation of the relative rCBF distribution within the brain (Peremans et al., 2001).

2.2 Tracer

A radiopharmaceutical systematically has two compounds: (1) a chemical substance (e.g. ethyl cysteinate dimer (ECD)) which directs the radiopharmaceutical to a certain target and (2) a radioactive marker (e.g. ^{99m}technetium) which enables the radiopharmaceutical to be visualized (Vermeire et al., 2011).

In the present work, ^{99m}technetium-ethyl cysteinate dimer (^{99m}Tc-ECD; Neurolite, Lamepro, the Netherlands) was used as a tracer to assess the rCBF. This technetium labeled lipophilic tracer crosses the blood brain barrier rapidly after intravenous (IV) injection. Once intracellular, it is converted into a hydrophilic compound and therefore is trapped within the brain (Figure 3), with a regional distribution proportional to the rCBF (Leonard et al., 1986). This frozen image of tracer distribution arises within 2 minutes after injection and remains stable for at least two hours in the human and canine brain, independent of rCBF changes occurring after the fixation time (Leveille et al., 1992; Ichise et al., 1997; Catafau, 2001; Peremans et al., 2002). This means that the SPECT images reflect brain activity at the time of tracer injection which leads to certain opportunities using this technique e.g. the tracer can be injected at the onset of an epileptic fit, while the image acquisition can be done later once the seizure is controlled. Furthermore, it is possible in veterinary medicine to obtain a frozen image reflecting regional brain perfusion in the awake animal, by injecting the tracer before acquisition under general anesthesia (Waelbers et al., 2010).

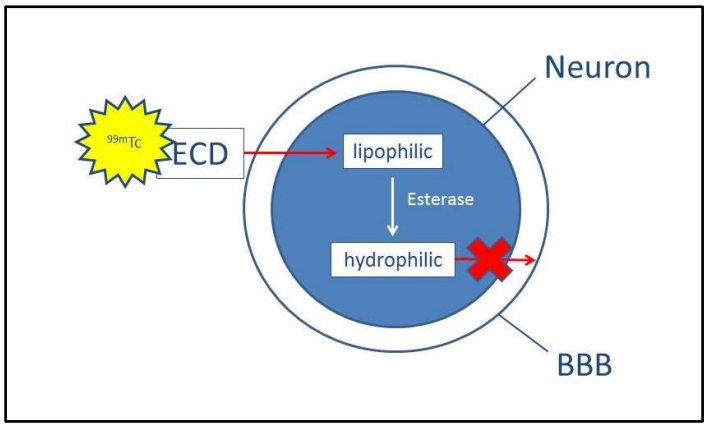

Figure 3: Schematic representation of entrapment of ^{99m}Tc-ECD into a neuron

(BBB = Blood Brain Barrier)

2.3 Conventional versus high-resolution micro-SPECT

Even with the use of a triple head gamma camera equipped with low-energy ultra-high resolution collimators positioned as close as possible to the animal's head, resolution restriction remains one of the most important limitations of conventional SPECT (Vermeire, 2010). Certainly compared to PET, SPECT cameras suffer from lower spatial resolution and lower detection sensitivity (Peremans et al., 2005), and the partial volume effect can disable the precise anatomical localization of small brain structures and hampers absolute quantification methods (Peremans et al., 2003; Vermeire, 2010). The spatial resolution of the SPECT systems used in animals should be higher than in humans, because the subjects under investigation are smaller. This should result in a quality of SPECT images in animals at least equivalent to human studies (Peremans et al., 2005).

Efforts have been made to improve the resolution of conventional SPECT systems by co-registration with CT or MRI, motion correction, modeling of the detectors response and by the use of smaller pixel sizes (Beekman et al., 2001; Soret et al., 2007). The spatial resolution can further be increased by using multi-pinhole collimators (Figure 4) placed in front of a conventional gamma camera (= micro-SPECT, µ-SPECT or HiSPECT), which leads to a magnification of the image compared to conventional SPECT (Peremans et al., 2005; Beekman and van der Have, 2007). The smaller the distance between the object and pinhole, the higher the magnification and resolution (Young et al., 1997).

A downside of the µ-SPECT system with multi-pinhole collimation is its decreased sensitivity so that more radioactivity or longer acquistion times are required (Peremans et al., 2005).

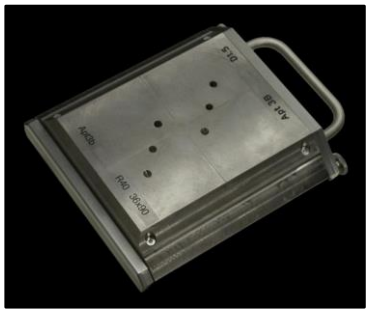

Figure 4: Example of a multi-pinhole collimator *(Bioscan, Inc.)*

Especially for heart and brain imaging, pinhole µ-SPECT is thought to be useful as small lesions may be masked by over- and underlying tissue radioactivity in conventional SPECT (Peremans et al., 2005). Dedicated small rodent µ-SPECT systems obtain resolutions of < 1mm using pinhole collimators. These systems, built for use in small rodents, cannot be used in dogs and cats due to a limited gantry opening (Beekman and van der Have, 2007; Peremans et al., 2011). Therefore, Bioscan developed a system in which a conventional triple-head gamma camera is equipped with three multi-pinhole collimators (so called HiSPECT). The feasibility of this technique, co-registered with MRI, has been confirmed to evaluate the rCBF of the feline brain (Waelbers et al., 2013), but has not yet been evaluated for canine brain imaging.

2.4 SPECT and epilepsy

Noninvasive functional brain imaging methods, including SPECT and PET are powerful tools used in the evaluation of human patients suffering from refractory focal seizures (Jayalakshmi et al., 2011; Kim and Mountz, 2011). These imaging modalities are mainly applied in the process of epileptogenic focus localization, but they can also help in elucidating the neurobiology of epilepsy (Van Paesschen et al., 2007). An accurate

localization of the ictal onset zone can be challenging, but is essential in the presurgical evaluation of human patients with refractory epilepsy (Kim and Mountz, 2011; von Oertzen et al., 2011). Of the different functional neuroimaging techniques, only SPECT has the unique capacity to image blood flow changes that occur at the onset of seizures due to the frozen image obtained at the moment of tracer injection. This allows postponement of the acquisition until the seizure is under control without any change in obtained perfusion image (Kim and Mountz, 2011). A true ictal image can never be obtained with PET, because compared to SPECT, the temporal resolution is lower due to a longer tracer uptake period (30-45 minutes). Therefore, PET is only feasible to use during the interictal phase. Functional MRI of seizures has been described more recently, but deals with specific challenges like patient safety and motion and noise artifacts that are unavoidable during seizures (Chaudhary et al., 2013).

The use of functional brain imaging in dogs with epilepsy is a rather unexplored field, however, since not much is known about the pathophysiology and localization of canine epilepsy, these techniques could be promising. PET has been used to evaluate brain metabolism alterations in a cohort of epileptic Lagotto Romagnolo (Jokinen et al., 2013) and Finnish Spitz dogs (Viitmaa et al., 2014) and regional blood flow abnormalities, visible on SPECT, have been described in one epileptic Boxer dog (Lass et al., 2006).

3. Vagus nerve stimulation

3.1 Introduction

In the past decades, there has been a considerable expansion of treatment options for human refractory epilepsy. Although a substantial number of new AEDs has become available (Beghi, 2004), several long-term studies revealed that only few human patients became seizure free after initiating a third AED when two AEDs had failed (Kwan and Brodie, 2000; Mohanraj and Brodie, 2005, 2006). Therefore, non-pharmacological treatment options are becoming increasingly important in humans and are being considered earlier in the course of therapy. Different treatment modalities are currently available including the ketogenic diet, epilepsy surgery and neurostimulation. The ketogenic diet, which contains a high amount of fat and a low amount of carbohydrates, is used mainly for the treatment of refractory childhood epilepsy (Kossoff et al., 2009). The efficacy is rather limited and it is difficult to remain compliant in the long-term (Levy et al., 2012). Epilepsy surgery leads to seizure freedom in a considerable number of treated patients, but requires a strict patient selection based on an extensive pre-surgical work-up (Noachtar and Borggraefe, 2009). In the past decade, various types of neurostimulation have emerged as promising treatment modalities for humans with refractory epilepsy (Ben-Menachem, 2012).

Only a limited number of the newer treatment strategies in humans have been investigated for canine epilepsy. The use of the ketogenic diet has been investigated in a small group of dogs with refractory epilepsy, but its effectiveness remains undetermined (Patterson et al., 2005b). Also, it has to be recognized that it is more difficult to induce ketosis in dogs in comparison with humans (Crandall, 1941; Patterson et al., 2005b). The surgical treatment of canine idiopathic epilepsy is still in its infancy. The main reason why this treatment modality in veterinary medicine has not yet been further developed is probably the difficulty in accurately localising the origin of canine seizures. Only the disconnective technique of callosotomy has been investigated in normal dogs and in a few dogs with idiopathic epilepsy. Short-term results seemed promising, but long-term results and larger studies are lacking (Bagley et al., 1995, 1996). Currently, many dogs with refractory epilepsy are eventually euthanized or die during uncontrollable seizures (Arrol

et al., 2012; Monteiro et al., 2012). For this group of dogs, it would be useful to have alternative treatment options.

Detailed information on the different non-pharmacological treatment modalities and their use in the treatment of human and canine epilepsy is beyond the scope of this introduction, but can be read elsewhere (Martlé et al., 2014). In the present work we will focus on neurostimulation and more specifically on vagus nerve stimulation as an alternative treatment for refractory epilepsy.

3.2 Neurostimulation

A substantial part of humans suffering refractory epilepsy, will benefit from surgical resection. However, as many patients are not suitable candidates for resection (Duncan, 2011), there is a need for alternative third-line therapies such as neurostimulation (Theodore and Fisher, 2004; Kotagal, 2011; Fridley et al., 2012; Rolston et al., 2012; Wu and Sharan, 2013). Neurostimulation refers to a group of treatment modalities in which electrical or magnetic pulses are delivered directly to or around nerve tissue in order to influence a pathological substrate and to achieve a therapeutic effect (Vonck et al., 2004; Boon et al., 2009).

The following neurostimulatory treatments have been described for the treatment of human refractory epilepsy: vagus nerve stimulation (VNS), deep brain stimulation, trigeminal nerve stimulation and transcranial magnetic stimulation. These treatments are usually delivered independent of seizure activity, but more recently responsive neurostimulation has been developed (Gigante and Goodman, 2011).

The electrical or magnetic pulses can be delivered either directly to the brain (intracranial stimulation) or indirectly, i.e. by stimulating cranial nerves or by transcranial magnetic stimulation (extracranial stimulation; (Boon et al., 2009)). Also, two major strategies can be followed regarding the target of stimulation. Firstly, the stimulation can be targeted towards crucial central nervous system structures with a triggering or propagating role in the epileptogenic network, such as the thalamus. Secondly, the stimulation can be targeted towards the epileptogenic focus itself, which of course implies prior knowledge on its localisation (Boon et al., 2009).

Although a substantial reduction in seizure frequency combined with an improved quality of life can be achieved in a considerable number of patients, these neurostimulation treatments are considered palliative, because seizure freedom is rarely achieved (Theodore and Fisher, 2004; Fisher, 2012). A seizure suppressing effect has been proven for most of these treatments, but the scientific and clinical applications have mainly preceded the knowledge of the mechanism of action (MOA) (Albert et al., 2009; Boon et al., 2009). The effectiveness of neurostimulation often improves over time, which could indicate a long-lasting neuromodulatory effect of these treatments, either at a molecular level or by reorganisation of certain brain circuits (Vonck et al., 2013; Wu and Sharan, 2013). Other advantages are that stimulation parameters can be customised and adapted individually for each patient and that the treatment is possibly reversible (Theodore and Fisher, 2004; Gigante and Goodman, 2011; Wu and Sharan, 2013). The efficacy in controlling seizures seems quite similar between different types of neurostimulation and side effects are moderate, although comparative trials are warranted (Ben-Menachem, 2012). The optimal targets and stimulation parameters for the different types of neurostimulation remain unknown. According to Ben-Menachem (2012), the biggest challenge for the future is to find out which types of patients and epilepsy syndromes are most sensitive to a specific type of neurostimulation.

In this PhD thesis we will only focus on VNS. More detailed information about the other types of neurostimulation can be read elsewhere (e.g. Martlé et al., 2014).

3.3 Vagus nerve stimulation

3.3.1 Definition

VNS is the intermittent electrical stimulation of the left cervical vagus nerve using an implantable VNS device. The device, which is surgically implanted in the left cervical region, consists of spiral-shaped electrodes that are wrapped around the vagus nerve and a pulse generator or neurostimulator that is subcutaneously implanted below the clavicle in humans and in the left cervical region in dogs (Figure 5). Subcutaneously tunnelled wires connect the stimulator with the electrodes. A program wand is used to obtain wireless communication between the stimulator and the programmer (Landy et al., 1993; Ben-Menachem, 2002; Vonck et al., 2004).

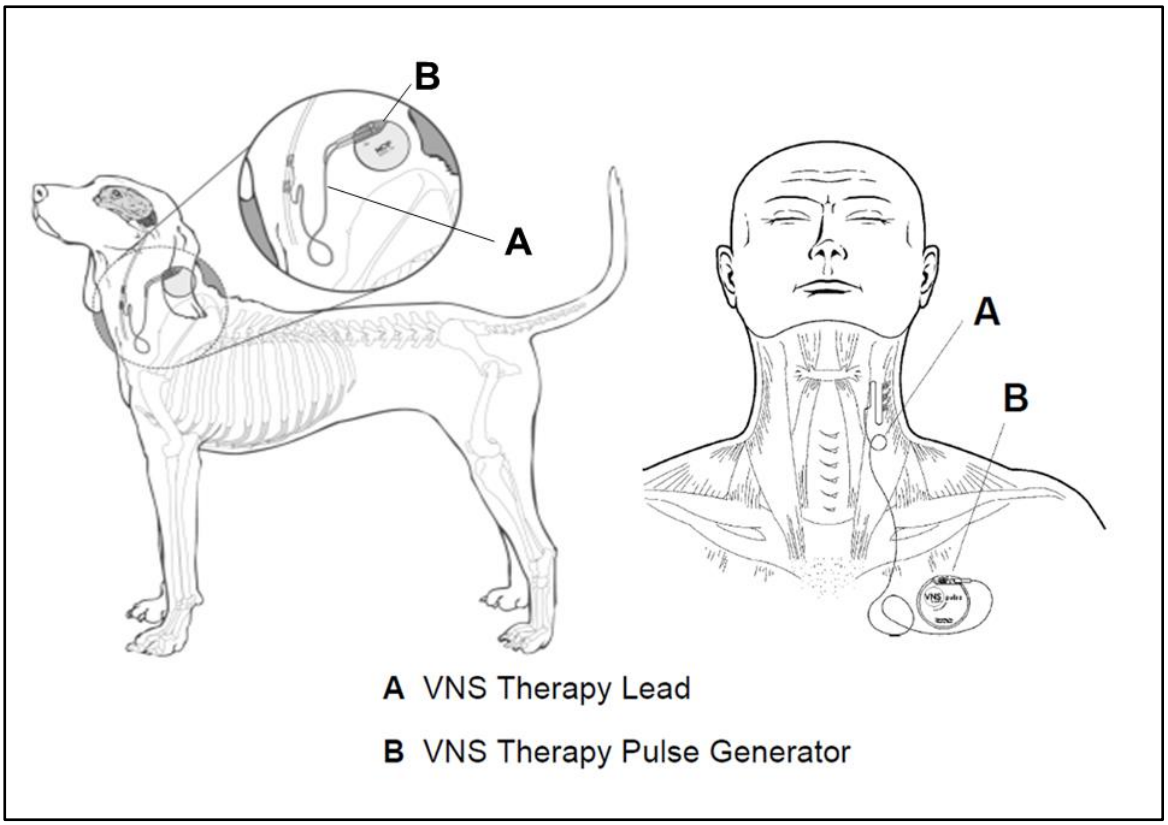

Figure 5: A VNS Therapy® System implanted in the left cervical region in a dog and a man
(with permission of Cyberonics, Inc. and Prof. K Muñana)

3.3.2 Anatomy of the vagus nerve

The vagus nerve is the longest cranial nerve and traditionally was considered to be a parasympathetic efferent nerve. Now it is known that it is actually a mixed nerve with the majority of vagal nerve fibres (± 80%) being visceral afferents projecting widely throughout the central nervous system and providing the brain with visceral sensation from the head, neck, thorax and abdomen (Foley and DuBois, 1937; Agostoni et al., 1957; Rutecki, 1990). The efferent fibres (± 20%) provide parasympathetic innervation of the heart, aorta, lungs and gastrointestinal tract, and also innervate the voluntary striated musculature of the larynx and pharynx (Agostoni et al., 1957). Different animal experiments have demonstrated that the efferent fibres are not required for the antiepileptic effect of VNS (Zabara, 1992; Hassert et al., 2004; Osharina et al., 2006). The afferent vagal fibres have their cell body in the nodose and jugular ganglia which relay ascending information predominantly to the nucleus of the solitary tract (NST) in the

caudal brainstem. The NST has widespread direct and indirect projections to a lot of areas in the brain (Groves and Brown, 2005; Fanselow, 2012) (Figure 6). Importantly, monoamine nuclei in the brainstem (locus coeruleus (LC) and the raphe nuclei (RN)) receive direct and/or indirect projections from the NST. Also, forebrain and limbic structures receive NST projections (Henry, 2002; Krahl and Clark, 2012).

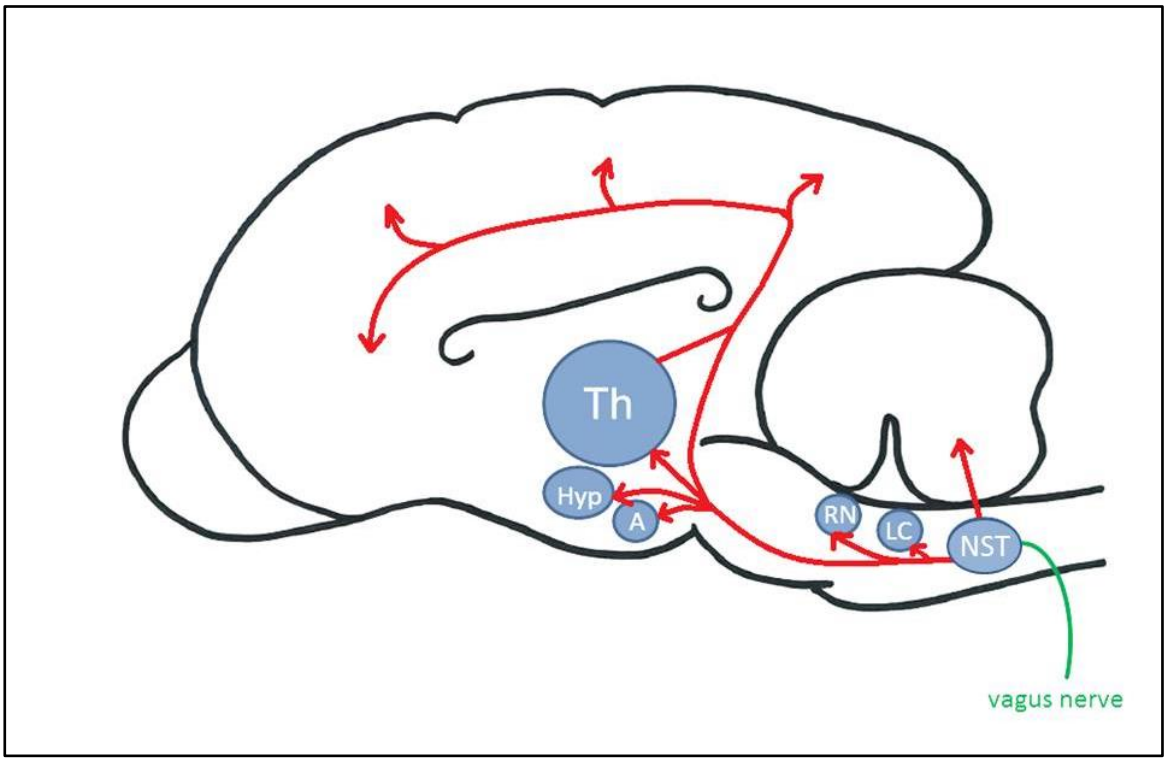

Figure 6: Schematic representation of the most important central afferent projections of the vagus nerve (NST: nucleus of the solitary tract; LC: locus coeruleus; RN: dorsal raphe nucleus; A: amygdala; Hyp: hypothalamus; Th: thalamus)

The cervical part of the vagus nerve is accessible and the electrodes for VNS are usually implanted there. In the cervical region, each vagus nerve lies within the carotid sheath, between the carotid artery and the internal jugular vein. VNS systems used for the treatment of epilepsy are preferably implanted in the left cervical region to avoid cardiac side effects (Ben-Menachem, 2002).

Important anatomical differences exist between the cervical vagus nerve in humans and dogs (Mizeres, 1955). In the human cervical region the vagus and sympathetic nerve are 2 separate nerves, whereas in the canine cervical region, both nerves are fused, forming the vagosympathetic trunk. Both nerves are encapsulated in a common epineurial sheath and

the degree of fusion is dependent on the region. In the upper portion of the neck, just distal to the nodose and cranial cervical ganglion, the vagus and sympathetic nerves are completely fused and intermingled. In the middle cervical region a thin common perineurial septum can be visualised between both on histological examination. In the distal cervical and cranial thoracic region both nerves actually separate completely again, but often are still embedded within a common epineurial sheath (Mizeres, 1955). This has the important consequence that the VNS electrodes are usually wrapped around the vagosympathetic trunk as a whole in dogs.

3.3.3 VNS and human epilepsy

VNS is the oldest and most frequently used neurostimulation modality for refractory epilepsy in humans (Ben-Menachem, 2012; Wu and Sharan, 2013). VNS was officially approved in 1994 in Europe and in 1997 in the USA for the treatment of focal-onset refractory seizures in patients > 12 years who are not candidates for surgical resection (Fisher and Handforth, 1999). This was mainly based on class I evidence gathered from 2 large, randomized, placebo controlled, double-blinded, multi-centre studies (Ben-Menachem et al., 1994; Handforth et al., 1998). Additional studies have also shown the effectiveness of VNS to treat refractory epilepsy in children and in certain generalised epilepsy types (Hauptman and Mathern, 2012; Thompson et al., 2012; Ryzi et al., 2013). In general, almost half of treated VNS patients have a reduction in seizure frequency of > 50%, but only a small amount of treated patients become seizure-free (Vonck et al., 2004; Connor et al., 2012;). It has been demonstrated extensively now that the efficacy of VNS improves over time, which is probably caused by an additional chronic antiepileptic effect developing after several months of treatment (Morris and Mueller, 1999; Vonck et al., 1999; Ben-Menachem, 2002; Boon et al., 2002; Rolston et al., 2012; Wu and Sharan, 2013).

There is substantial evidence indicating that modulation of brain activity occurs with VNS through stimulation of the afferent vagal fibres. Ascending fibres reach the NST and from there, widespread projections reach the limbic, reticular, and autonomic regions of the brain as well as other brainstem nuclei like the LC and RN (Ben-Menachem et al., 1995). Different potential mechanisms that might mediate the antiepileptic effect of VNS have been investigated during the past decades. VNS induced EEG changes in animals

(Zanchetti et al., 1952; Chase et al., 1966, 1967), but human studies could not reproduce these results in an acute setting (Hammond et al., 1992a; Salinsky and Burchiel, 1993). However, Koo (2001) showed that long-term VNS caused a reduction in interictal spike discharges and a prolongation of the interspike interval in human patients. Crucial brainstem and intracranial structures that are influenced by VNS, such as the LC, the NST, the thalamus and limbic structures have been identified (Naritoku et al., 1995; Krahl et al., 1998; Osharina et al., 2006; Cunningham et al., 2008). The LC is thought to play a key role in the antiepileptic effect of VNS (Krahl et al., 1998; Fornai et al., 2011). VNS might exert its antiepileptic effect by modulating the release of certain neurotransmitters in the brain. Excitatory and inhibitory neurotransmission and the balance between both is often altered in the epileptic brain and antiepileptic therapies may target these neurotransmitters or their receptors (Meldrum, 1995). Therefore, the MOA of VNS may be based on regional gamma-aminobutyric acid (GABA) increases or glutamate and aspartate decreases. It has also been demonstrated that $GABA_A$ receptor density in the hippocampus was increased after a year of VNS in the responsive patients (Marrosu et al., 2003). Besides the influence on amino acids, VNS might also affect the concentration of monoamines, such as serotonin (5HT), dopamine (DA) and norepinephrine (NE), which have been previously associated with seizure suppressing effects (Starr, 1996; Boon et al., 2002; Giorgi et al., 2004;). Neurochemical studies quantified neurotransmitter concentrations in human CSF before and after VNS and an increase in metabolites of 5HT and DA, an increase in GABA and a decrease in aspartate levels were detected. Whether these findings were associated with the antiepileptic effects of VNS remained uncertain (Hammond et al., 1992b; Ben-Menachem et al., 1995). Norepinephrine, released by the LC, appears to play a pivotal role in the MOA of VNS as different animal studies found an increase of NE after VNS (Roosevelt et al., 2006; Raedt et al., 2011). Moreover, a causal link between increased NE levels in the hippocampus and the anticonvulsant effect of VNS has recently been shown in a limbic seizure model in rats (Raedt et al., 2011). A variety of functional neuroimaging studies using PET, SPECT or fMRI have shown that VNS causes acute and longer-term changes in brain regions with vagus innervation, which have been involved in depression, as well as in epilepsy (for a review: (Chae et al., 2003)). Despite all these investigations, there is still much to learn about the exact MOA of VNS. On-going research is mainly focused on revealing optimal stimulation parameters by comparing different stimulation paradigms and on the identification of predictive factors for responsiveness (Wu and Sharan, 2013). Recently, a

non-invasive, transcutaneous method of VNS - by stimulating the auricular branch of the vagus nerve at the left ear - was evaluated in humans with refractory epilepsy and this technique appeared to be safe and practicable in the long term (Stefan et al., 2012). VNS is in general very well tolerated and side effects, although quite frequently seen, are most of the time mild and reversible (Schachter and Saper, 1998). The most commonly reported side effects of VNS in humans are presented in Table 3. Although refractory epilepsy was the original application for VNS, it is now also an approved additional treatment for depression and is currently under investigation for other applications like heart failure, anxiety, obesity, migraine, eating disorders and Alzheimer's disease (Groves and Brown, 2005; Beekwilder and Beems, 2010).

Table 3: Reported side effects of vagus nerve stimulation (VNS) in humans

Acute, surgery related	Chronic, often stimulation related	Chronic, not related with stimulation
Infection	Coughing	Lead breakage
Hemorrhage	Voice alteration – hoarseness	Infection
Left vocal-cord paresis	Throat pain	Gastro-esophageal reflux disease
Lower facial weakness	Dyspnea – shortness of breath	
Bradycardia and asystole during device test	Dyspepsia – vomiting	
Pain at the implant site	Swallowing difficulties – aspiration (children)	
Seroma formation	Contractions of sternocleidomastoid muscle	
Paresthesia	Paresthesia	
Headache	Gagging	
Coughing	Drooling	
Voice alteration - hoarseness	Sleep apnea	
Nausea		

3.3.4 Vagus nerve stimulation and canine epilepsy

An experimental study in dogs (Zabara, 1992) provided essential information to initiate clinical trials with VNS for refractory epilepsy in humans. Zabara (1992) demonstrated that chemically induced seizures in dogs could be aborted by stimulation of the cervical vagus nerve. Ocular compression, which is an indirect way of stimulating the vagus nerve, has been beneficial for controlling seizures in dogs (Speciale and Stahlbrodt, 1999). Only one randomised, placebo controlled, double-blinded cross-over study evaluated the safety and efficacy of VNS in 10 dogs with refractory epilepsy using a similar implantable device as in humans (Muñana et al., 2002). No significant difference in seizure frequency, duration and severity was detected between the treatment and control period of 13 weeks, but when the final 4 weeks of both periods were compared, a significant decrease in seizure frequency was found during the treatment period. VNS appears to be safe in dogs with comparable minimal side effects as in humans. Furthermore, owner satisfaction was relatively high (Muñana et al., 2002). Obviously, additional studies are needed. A major advantage of using VNS in dogs is its independence of the localisation of the epileptogenic focus and of owner compliance.

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Scientific Aims

The continuous search for and development of improved diagnostic techniques have led to a major evolution in human epilepsy. The introduction of structural and functional imaging modalities such as high-field MRI and nuclear imaging techniques (PET and SPECT) were important milestones. The past decade, functional imaging techniques such as SPECT have been introduced in veterinary research as well.

The specific aims of the **first part of this PhD thesis** were:

1/ to assess interictal changes in regional cerebral blood flow and neuronal activity in dogs with spontaneous idiopathic epilepsy using SPECT

2/ to investigate the feasibility of µ-SPECT to evaluate the regional brain perfusion in healthy Beagle dogs and to define the normal regional brain perfusion pattern based on MRI

The last 15 years, different neuromodulatory treatments have been developed to treat human refractory epilepsy, of which VNS is the most established. Further research is still needed to elucidate the precise MOA, to identify predictive factors for response and to determine the most optimal stimulation parameters of VNS. Since human and canine epilepsy share a lot of similarities, the second part of this PhD thesis is dedicated to the examination of the MOA of VNS in healthy Beagle dogs. Hereby, a new experimental stimulation paradigm, microburst VNS, was evaluated and compared with standard VNS.

The specific aims of the **second part of this PhD thesis** were:

1/ to describe the surgical implantation technique of a VNS Therapy® System in healthy Beagle dogs

2/ to evaluate regional brain perfusion changes using µ-SPECT after acute standard and microburst VNS in healthy Beagle dogs

3/ to evaluate changes in the CSF monoamine concentrations induced by acute standard and microburst VNS in healthy Beagle dogs

4/ to assess the influence of acute standard and microburst VNS on the seizure threshold in a canine PTZ model

5/ to evaluate cardiac rhythm, using Holtermonitoring, in healthy Beagle dogs during standard and microburst VNS

Research studies

PART I:

SPECT AND CANINE IDIOPATHIC EPILEPSY

CHAPTER 1

REGIONAL BRAIN PERFUSION IN EPILEPTIC DOGS EVALUATED BY TECHNETIUM-99M-ETHYL CYSTEINATE DIMER SPECT

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Sources and manufacturers

[a] Airis Mate, Hitachi Med.Corp., Tokyo, Japan

[b] Domitor, Pfizer, Louvain la Neuve, Belgium

[c] Propovet, Abbott, Queensborough, UK

[d] Isoflo, Abbott, Queensborough, UK

[e] Triad, Trionix, Twinsburg, OH, USA

[f] Gardenal, Sanofi-Aventis, Diegem, Belgium

[g] Epikal, ASTfarma, Oudewater, the Netherlands

[h] Mysoline, AstraZeneca, Brussels, Belgium

Summary

We evaluated the feasibility of interictal Single Photon Emission Computed Tomography (SPECT) to detect alterations in regional cerebral blood flow and neuronal activity in dogs with idiopathic epilepsy. Twelve dogs with idiopathic epilepsy underwent interictal ^{99m}technetium-ethyl cysteinate dimer (^{99m}Tc-ECD) SPECT of the brain. Different cortical volumes of interest (VOIs), 1 VOI at the cerebellum and 1 VOI at the subcortical area were evaluated by semiquantitative analysis and compared with a control group (18 dogs). Significant hypoperfusion (P = 0.02) was present in the subcortical area, which includes the thalamus, of epileptic dogs. This hypoperfusion was not associated with seizure frequency, age at onset of seizures, duration of epilepsy, or time since the last seizure. Interictal SPECT did not reveal cortical or cerebellar perfusion alterations. The subcortical area may play an important role in the pathophysiology of canine idiopathic epilepsy.

Introduction

In dogs with primary, idiopathic epilepsy, structural brain imaging such as with computed tomography or magnetic resonance (MR) imaging are typically within normal limits except for occasional reversible postictal changes that can be visible on MR images (Mellema et al., 1999). Functional brain imaging could provide valuable information regarding neuronal activity in these dogs. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) can be used to assess the functional state of the brain. These techniques enable three-dimensional visualization of functional parameters such as cerebral blood flow (CBF) and brain metabolism (Peremans et al., 2001). Measurement of regional CBF by SPECT can allow detection of impaired neuronal function, which is not visualized by conventional MR imaging (Podreka et al., 1997). It is known that neuronal activity and metabolism are reflected by brain perfusion (Roy and Sherrington, 1890). In this regard, brain perfusion SPECT is a useful technique to evaluate neuronal activity indirectly.

In humans, SPECT is used for presurgical evaluation of most refractory types of partial epilepsies to identify the epileptogenic area (Boon et al., 1989; Nehlig et al., 2004). Epileptogenic foci are usually characterized by decreased interictal and increased ictal blood flow (Oommen et al., 2004; Buch et al., 2008). SPECT provides a unique opportunity to investigate interictal CBF (Yune, 1998), however interictal SPECT imaging has a lower sensitivity for localizing the seizure onset site compared with ictal SPECT (Devous et al., 1998).

Since little is known about brain perfusion in epileptic dogs, this study was performed to evaluate brain perfusion alterations on interictal SPECT images in dogs with idiopathic epilepsy and to investigate the feasibility of interictal SPECT to detect foci of abnormal neuronal activity which could be related to seizures in the dog.

Materials & Methods

Animals, clinical variables and diagnostic evaluation

Dogs evaluated between February and October 2007 for recurrent generalized or partial seizures, or presented in status epilepticus, were studied if they were suspected to have primary, idiopathic epilepsy. The neurologic evaluation in the dogs presented in status epilepticus was repeated after recovery from the seizure to avoid errors in interpretation caused by postictal deficits. The age at the first seizure had to be between 6 months and 5 years. Exclusion criteria were other clinical or neurologic signs and abnormalities on neurologic examination. Underlying causes for the seizures were evaluated by a complete blood examination (cell blood count, biochemistry including liver enzymes, BUN, creatinine, albumin, total protein, glucose, ammonia or bile acids, and serum electrolytes [Na, K, Ca]), MR imaging of the brain (0.2 T[a]) and a cerebrospinal fluid (CSF) examination.

The age at the time of SPECT had to exceed 1 year, because young dogs have alterations in brain perfusion related to brain maturation (K. Peremans, personal communication). The time between the last seizure and SPECT did not exceed 30 days, because recent seizure activity potentially increases the sensitivity to detect perfusion alterations (Giobbe and Castellano, 1997). Therefore, the SPECT scan was scheduled as soon as possible after the last seizure. This interval was mainly determined by practical issues like tracer and SPECT availability, possibility of the owner to bring the dog to the faculty,…

SPECT

For evaluation of brain perfusion ^{99m}Tc-ECD (±925 MBq) was injected intravenously (IV) before sedation or anesthesia. Premedication was 25–50 μg/kg medetomidin hydrochloride[b] given IV 10 min after injection of the radiopharmaceutical. General anesthesia was induced with IV propofol[c] and maintained with isoflurane[d] in 100% oxygen to effect. All dogs were in ventral recumbence and the detectors positioned as closely as possible to the head. Acquisition was started 20–30 min after radiopharmaceutical injection. SPECT was performed using a triple head gamma camera[e] equipped with ultra-high-resolution parallel hole collimators (tomographic resolution

FWHM=8 mm). Data were acquired for 20 min in step-and-shoot mode (120 steps, 10 s/step, 3° steps), over a circular 360° rotation on a 128 × 128 matrix. Images were processed using filtered back projection and a Butterworth filter. Pixel size was 1.72 mm.

The perfusion images were automatically registered to a template using BRASS software (Brain Registration and Automated SPET Semiquantification, Nuclear Diagnostics), after which the different volumes of interest (VOIs) were semiquantified using a predefined region map. VOIs were drawn over the different cortical regions (olfactory, frontal, temporal, parietal, and occipital lobes), 1 VOI over the cerebellum and 1 VOI over the subcortical area (Peremans et al., 2001). The latter VOI mainly contained the thalamus and basal ganglia. Further differentiation of this subcortical area was not possible because of the small size of the individual structures and the limited resolution of the SPECT system. Average counts per pixel were calculated for all regions. A perfusion index (PI) was obtained by normalizing the average regional counts to total counts of all VOIs.

Control group

The PI was compared for all regions with the PIs of a normal data base that consisted of 18 normal control dogs (11 males and seven females) of different breeds with a mean (±SD) age of 41.4±22.9 months and a mean (±SD) body weight of 20.1±7.1 kg (Table 1). These dogs had no history of seizures or neurological or behavioral disease, and were not receiving medication. The SPECT data of these normal control dogs were previously collected in a normal data base and were available on site at the moment of this study.

Statistical analysis

Nonparametric statistical analysis was performed using SPSS (Statistical Software Package for the Social Sciences, v.15). The Wilcoxon Signed Ranked test and the Pearson Chi-Square test were used to compare respectively the age and the gender of the epileptic group with the control group. To evaluate regional brain perfusion alterations and to look for associations with clinical variables (recent seizure frequency, age at onset of seizures, duration of the epilepsy, and time since the last seizure) a Mann–Whitney *U*-test and a Spearman correlation test were respectively used. Level of significance was set at $P \leq 0.05$.

Table 1: demographics of the control dogs (n=18)

Breed	Sex	Age at presentation*
Kooiker Dog	F	60
Mongrel	M	18
Dachshund	M	78
Foxhound	F	45
Kooiker Dog	M	56
Border Collie	F	64
Mongrel	M	18
German Shepherd	M	60
Mongrel	M	36
Mongrel	M	36
Border Collie	M	72
Border collie	M	24
German Shepherd	F	56
German Shepherd	F	70
Beagle	M	14
Beagle	M	14
Beagle	F	12
Beagle	F	12

*: expressed in months

Results

Twelve dogs met the criteria. There were 11 males and 1 female with a mean (±SD) age of 36.8±11.4 months at presentation and a mean (±SD) body weight of 32.1±15.5 kg. All dogs had a history of generalized seizures and 1 dog was presented in status epilepticus. In 10 dogs some signs of a focal seizure onset had been noticed by the owner before the seizure, such as anxiety and restlessness (4 dogs), seeking attention (2 dogs), rhythmic contractions of a hind limb (2 dogs), tonic jaw opening (6 dogs), and salivation (6 dogs). In 2 dogs a focal seizure onset was not described by the owner. The demographics and clinical variables of the epileptic dogs are summarized in Table 2.

Blood examination was normal in all dogs. MR imaging did not reveal any significant abnormality in any dog. Four dogs had mild ventriculomegaly, and 2 had mild asymmetry of the lateral ventricles without a mass effect or other visible lesion. White blood cell count of the CSF was within limits in all dogs (<8 cells/µl). Two dogs had a mildly elevated CSF protein level (41.8 and 40.2 mg/dl, reference <30 mg/dl), due to iatrogenic blood contamination. It was concluded that all dogs had primary, idiopathic epilepsy.

The duration of epilepsy before presentation was variable, with a mean of 9.8 months (range, 0.5–39 months). The mean (±SD) age at the first seizure was 27±9.6 months. Four dogs (33.3%) had ≤1 seizure a month, 6 dogs (50%) had 2–4 seizures a month, 1 dog (8.3%) had 5–10 seizures a month and 1 dog (8.3%) more than 10 seizures a month. Eleven dogs (91.7%) received antiepileptic treatment at the moment of the study. Eight dogs (66.7%) received phenobarbital (PB)[f], 2 dogs (16.7%) received PB and potassium bromide[g] and 1 dog (8.3%) received primidone[h]. The duration of treatment was variable (mean: 262 days; range, 12–1224 days).

Table 2: Demographics and clinical variables of the epileptic dogs (n=12)

Breed	Sex	Weight (kg)	Age at presentation*	Age at first seizure*	Duration Epilepsy*	Seizure Frequency†	Time since last seizure
German Shepherd	M	36	46	45.5	0.5	B	5-30 days
Golden Retriever	M	36	52	13	39	A	5-30 days
Border collie	F	13	25	19	6	D	1-5 days
English Bulldog	M	35	34	29	5	B	< 24 hours
Golden Retriever	M	37	46	34	12	A	5-30 days
German Pointer	M	29	35	34.5	0.5	B	1-5 days
German Shepherd	M	29	57	37	20	B	5-30 days
Malinois	M	30	34	28	6	C	1-5 days
English Staff.Bullterrier	M	23	39	21	18	A	< 24 hours
Bordeaux Dog	M	74	20	15	5	B	5-30 days
Miniature Schnauzer	M	8	33	31	2	B	1-5 days
White Swiss Shepherd	M	35	20	17	3	A	5-30 days

*: expressed in months

†: A: ≤ 1 seizure/month, B: 2-4 seizures/months, C: 5-10 seizures/month, D: > 10 seizures/month

There was no significant difference in age (P = 0.67) or gender (P = 0.1) between the epileptic dogs and the control group. SPECT was performed within 24 h after the last seizure in 2 dogs (16.7%), between 1 and 5 days after the last seizure in 4 dogs (33.3%) and between 5 and 30 days after the last seizure in 6 dogs (50%). The PIs of all the VOIs of the epileptic group were compared with the PIs of the control group (Figure 1). A significant hypoperfusion (P = 0.02) was present in the subcortical, thalamic area of the epileptic dogs compared with control dogs. In this VOI the epileptic dogs had a mean (±SD) PI of 1.078±0.090 and the mean (±SD) PI of the control group in that area was 1.151±0.058. There were no cortical or cerebellar differences in regional CBF between the two groups. There was no significant association between the degree of perfusion in the subcortical region of the epileptic dogs and the clinical variables (seizure frequency, age at onset of seizures, duration of the epilepsy, and time since last seizure).

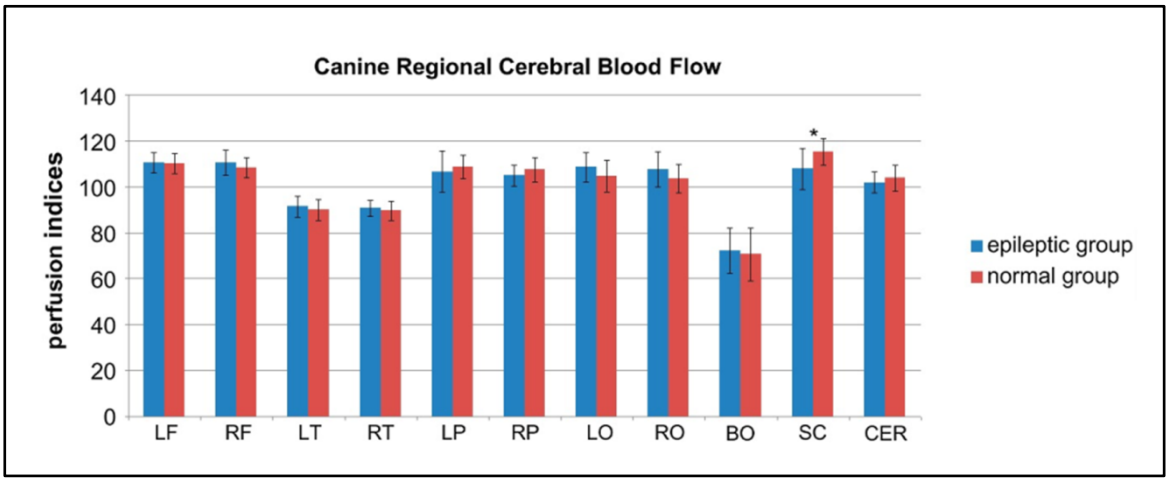

Figure 1: Mean perfusion indices (±SD) of the epileptic and the control dogs from the different VOIs. LF: left frontal lobe; RF: right frontal lobe; LT: left temporal lobe; RT: right temporal lobe; LP: left parietal lobe; RP: right parietal lobe; LO: left occipital lobe; RO: right occipital lobe; BO: olfactory bulb; SC: subcortical region; CER: cerebellum
* significant difference between both groups, $P \leq 0.05$.

Discussion

The gold standard for seizure localization in humans has been invasive intracranial EEG recording, but this carries some operative risks (Spencer et al., 1997). Recent advantages in neuroimaging offer the possibility of localizing seizures safely and noninvasively (Spencer and Bautista, 2000). The major practical application is the use of SPECT to detect the epileptogenic focus, but functional imaging can also be an important tool to better understand the neurobiology of epilepsy (Van Paesschen et al., 2007). This was also one of the goals of this study, because there are many unanswered questions about the pathophysiology, classification, and nomenclature of epilepsy in dogs.

All the dogs in the study had generalized seizures. Generalized seizures in dogs can have a focal onset, described previously as the aura, so searching for an epileptogenic focus can be useful (Berendt and Gram, 1999). A major limitation of epilepsy in dogs is the dependency on the owner to observe and describe the seizures and a number of subtle clinical signs in humans that give a clue to the origin of the epileptic fit, can never be confirmed in animals (Berendt and Gram, 1999). Most dogs in this study experienced some clinical signs at the beginning of the seizure which could be a sign of a focal onset of the seizure. These signs included seeking attention from the owner, rhythmic contractions of a specific limb, tonic opening of the jaw, anxiety, salivation, and restlessness. A focal onset of the seizures was not observed in two dogs, but could have been missed due to the lack of supervision during the seizures.

In this study, ^{99m}Tc-ECD was used as a tracer. ^{99m}Tc-ECD is lipophilic and is rapidly cleared from blood. In the human and canine brain, the lipophilic complex ^{99m}Tc-ECD crosses the intact blood brain barrier and is trapped intracellular within 2 min in proportion to CBF, probably by de-esterification to polar complexes (Walovitch et al., 1994; Peremans et al., 2001). The uptake and distribution of the tracer, which are a reflection of the rCBF, remain unchanged for 2 h (Ichise et al., 1997; Peremans et al., 2001; Waelbers et al., 2012). The image obtained when performing SPECT is a static image of brain perfusion at the time of injection. This has the major advantage that any influence of sedative or anesthetic agents on brain perfusion can be avoided by injecting the tracer before sedation or anesthesia.

Evaluation of the SPECT images was done by semiquantification with normalization of regional counts to total counts of the individual brain, which is more objective than visual analysis (Slomka et al. 1997; McNally et al., 2005). Visual comparison of images, as performed in the past, has some disadvantages, including difficulty in making comparisons between corresponding slices and the lack of quantitative assessment (Russo, 1981). Automated registration of the perfusion data to a template combined with an automatically applied region map results in a more reliable analysis of brain perfusion images, as compared to manual registration and visual interpretation (Slomka et al., 1997). Therefore, semiquantitative analysis was the evaluation method in this study.

Significant hypoperfusion was found in the subcortical region of the epileptic dogs. An important part of this subcortical area consists of the thalamus, although it was not possible to visualize the thalamus in detail due to the resolution limits. The function of the thalamus in seizures could be causative. In the centrencephalic theory it is proposed that most generalized seizure activity originates in deep structures of the thalamus and brain stem and is thereafter projected to the cortex (Russo, 1981; Bagley et al., 1996). Some interictal SPECT studies in humans with temporal lobe epilepsy allowed identification of thalamic hypoperfusion and a primary role of the thalamus in the initiation or propagation of seizures in several types of epileptic disorders was proposed (Henry et al., 1990; Yune et al., 1998). It is possible that idiopathic epilepsy in dogs is most comparable to idiopathic generalized epilepsy in people, for example the childhood absence seizures. Using PET in this type of epilepsy in children, focal activation in the thalamus during absences was found, emphasizing the key role of this structure in the pathogenesis of absence seizures (Prevett et al., 1995). The significant hypoperfusion in the subcortical, thalamic area of epileptic dogs could also be explained as a consequence of seizures. In this regard, corticothalamic diaschisis is a possible contributing factor. The diaschisis phenomenon is hypoperfusion and hypometabolism in a portion of the brain distant from the site of damage due to an interruption of its afferent axonal supply (Yune et al., 1998). Further investigation of thalamic dysfunction and alterations in blood flow is needed in dogs, and also in humans, before drawing more definitive conclusions. Significant cortical perfusion alterations were not detected in the epileptic group, however, it is possible that by evaluating the mean regional PIs of the epileptic dogs as a group, perfusion alterations in individual epileptic dogs or side differences might not have been

noticed. It is probable that, also in dogs, interictal SPECT is less sensitive than ictal SPECT to detect cortical epileptogenic foci.

We evaluated interictal SPECT in epileptic dogs. In humans, interictal SPECT is part of a multimodality work-up for refractory epilepsy. Combining imaging modalities integrates the strengths of various modalities and at the same time eliminates one or more weaknesses of an individual modality (Van Paesschen et al., 2007; Goffin et al., 2008). Although interictal brain SPECT can be useful for localization of the seizure focus (Yune et al., 1998), interictal SPECT is less sensitive in identifying epileptogenic foci than either ictal or immediate postictal studies (Matsuda et al., 1997; Devous et al., 1998; Goffin et al., 2008). The highest sensitivity is obtained by combining ictal with interictal perfusion SPECT (Spanaki et al., 1999). However, there are practical difficulties in performing routine ictal studies and also the interpretation can be difficult (Menzel et al., 1997). Ictal SPECT perfusion is only available in tertiary care human hospitals (Goffin et al., 2008). A true ictal injection is required, but not always achieved and early secondary generalization of a seizure can lead to difficulties in interpreting an ictal SPECT (Rowe et al., 1997; Wiest et al., 2005). Ictal SPECT in dogs could provide useful information, but this would be practically difficult. It is also possible that ictal SPECT in dogs would provide more information about seizure propagation than about the epileptogenic focus, because seizures in dogs tend to generalize rapidly.

In 2 dogs, the SPECT scan was performed within 24 h of the last seizure. In humans, a safety margin of 24 h after the last seizure is usually allowed before performing interictal SPECT to reduce the effect of prior seizures (Oommen, 2004). To our knowledge, no exact definition of the timing when a postictal state switches to an interictal state exists. In humans, postictal perfusion changes lasted only up to 3 h after the seizure (Oommen et al., 1993). Therefore, postictal perfusion changes, although they cannot be excluded, were probably not of great influence in these 2 dogs, because the time between the last seizure and the SPECT exceeded 12 h in both dogs. In the future, it would be interesting to compare immediate postictal and interictal brain perfusion changes in epileptic dogs.

As part of a multimodality work-up, it would have been useful to include interictal EEG in our dogs. However, some limitations accompany the use of scalp EEG in dogs. The large temporal muscles can cause muscle artifacts and may also attenuate the amplitude of the brain waves and mask true spike activity. Also the effect of sedation, which is

almost indispensable in dogs, can influence the EEG results (Russo, 1981). Other limitations of our study are the small number of patients and the heterogenicity in the demographics and clinical variables of the dogs. Furthermore, it is important to recognize that because all epileptic dogs except 1 received antiepileptic drugs at the moment of the SPECT scan, an influence of these drugs on brain perfusion cannot be excluded. This is also a possible confounding factor in many human SPECT studies, but unfortunately there are only few human studies in which the influence of these drugs on brain perfusion and metabolism has been investigated. For example, PB and phenytoin can cause diffuse cerebral hypoperfusion (Jibiki et al., 1993). To minimize these effects on global brain perfusion in our study, PIs where used. These are ratios of the regional brain perfusion normalized to the total counts of the brain. Of course, it is possible that antiepileptic drugs could alter regional brain perfusion depending on their mechanism. There is a need for further studies that evaluate the influence of antiepileptic drugs on brain perfusion before conclusions can be drawn.

Conclusion

In conclusion, using interictal SPECT in epileptic dogs we identified alterations in subcortical brain perfusion. The subcortical area, including the thalamus may play an important role in the pathophysiology of canine idiopathic epilepsy. Interictal SPECT did not allow identification of cortical alterations in brain perfusion. Future studies on larger patient groups are needed and comparison of interictal with ictal SPECT should be attempted if practically achievable.

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CHAPTER 2

HIGH-RESOLUTION MICRO-SPECT TO EVALUATE THE REGIONAL BRAIN PERFUSION IN THE ADULT BEAGLE DOG

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Sources and manufacturers

[a] HiSPECT, Bioscan, Paris, France

[b] Airis Mate, Hitachi Med.Corp., Tokyo, Japan

[c] Neurolite, Lamepro, Raamdonksveer, The Netherlands

[d] Dolorex, MSD AH, Brussels, Belgium

[e] Propovet, Abbott Laboratories, Queensborough, UK

[f] Isoflo, Abbott Laboratories, Queensborough, UK

[g] Triad, Trionix, Twinsburg, OH, USA

[h] Multimodality, Hermes, Nuclear Diagnostics

Summary

Conventional Single Photon Emission Computed Tomography (SPECT) precludes a detailed evaluation of the subcortical region. Micro-SPECT (µ-SPECT) has a higher resolution, but has not been used to evaluate the dog's brain until now. In this study, µ-SPECT of the brain was evaluated in 10 Beagle dogs. Magnetic Resonance Imaging (MRI) of the brain was used to draw a new region map containing 19 volumes of interest (VOIs). Semi-quantitative analysis of the µ-SPECT data was performed and the regional cerebral perfusion was represented by the perfusion indices (PIs).

The highest perfusion was found in the parietal cortex and the lowest in the piriform cortex. An asymmetry towards the left hemisphere in general and a regional asymmetry in the frontal, temporal and parietal cortex were found.

This study shows that functional imaging of the canine brain is possible using µ-SPECT and it describes the normal regional brain perfusion in the adult Beagle dog.

Introduction

Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) evaluate the functional state of the brain in a non-invasive way. SPECT assesses neuronal function indirectly by measuring the regional cerebral blood flow (rCBF) (Roy and Sherrington, 1890). Moreover, impaired neuronal function which is not visualized by conventional MRI can be detected (Podreka et al., 1997).

In human medicine, SPECT functional brain imaging is currently used in the diagnostic work-up for a wide variety of neurological disorders such as dementia, cerebrovascular disorders and epilepsy (Masdeu et al., 2006). In humans with refractory epilepsy, functional neuroimaging by SPECT or PET is frequently used as part of a multimodality work-up in the presurgical evaluation to identify the epileptogenic focus (Boon and Williamson, 1989; Matsuda et al., 1997; Menzel et al., 1997; Podreka et al., 1997; la Fougère et al., 2009). Although long-term intracranial EEG evaluation remains the gold standard to localize the epileptogenic focus, functional brain imaging techniques have the advantage that they are less invasive and not primarily dependent on electrical brain activity. Furthermore, functional brain imaging can also help to gain a better insight into the neurobiology of epilepsy (la Fougère et al., 2009). Also in dogs, where refractory epilepsy occurs commonly (Chandler, 2006), brain SPECT could become a useful diagnostic tool. The classification and localization of canine epilepsy remain largely unknown, so it is important to optimize techniques, such as SPECT, that could contribute to the classification and localization of canine epilepsy.

The regional brain perfusion evaluated by conventional fan-beam SPECT in normal dogs has been described previously (Peremans et al., 2001) and could be used as a reference atlas to evaluate different canine pathophysiological changes (Peremans et al., 2002; Martlé et al., 2009; Vermeire et al., 2009a). The same type of SPECT study has been performed by our group interictally in dogs with idiopathic epilepsy and revealed a significant hypoperfusion in the subcortical region (Martlé et al., 2009). Until now, the resolution of the conventional gamma camera system used in canine SPECT studies (Peremans et al., 2002; Martlé et al., 2009; Vermeire et al., 2009a; Waelbers et al., 2011) was insufficient to evaluate different structures within the subcortical region (hippocampus, thalamus and corpus striatum) and the brainstem. Furthermore, the VOI map, which is used to evaluate the regional brain perfusion, was previously created based

on Computed Tomography (CT) which provides less anatomical detail than MRI (Peremans et al., 2001). Therefore it would be useful to increase the resolution capacity of this technique.

To increase resolution, µ-SPECT systems have been developed (Wirrwar et al., 2001). With this technique, the image is magnified and the resolution increased compared to conventional SPECT, by using pinhole collimators (Young et al., 1997). Micro-SPECT imaging systems in rodents have been developed because of the existing need for high spatial resolution techniques in preclinical studies. The combined sensitivity and spatial resolution achieved by µ-SPECT make a detailed in vivo imaging feasible in small laboratory animals (Weber et al., 1994). Since most of the µ-SPECT systems are specifically built for use in small rodents, the limited gantry opening precludes their use in dogs. The HiSPECT system[a] is a multi-pinhole collimated µ-SPECT system for use on conventional gamma camera systems and can be adapted to larger animals (Peremans et al., 2011). This system consists of pinhole apertures fixed on a multi-detector gamma camera and has already been used successfully on the feline brain (Dobbeleir et al., 2006; Waelbers et al., 2012a; Waelbers et al., 2013). To the author's knowledge, there are no studies describing brain perfusion evaluated by µ-SPECT in the normal dog. Reliable and high-resolution normal reference SPECT data of the canine brain are necessary. Therefore, the aims of this study were (1) to investigate the feasibility of µ-SPECT of the canine brain, (2) to create a new, MRI-based region map and (3) to define the normal regional brain perfusion pattern in the adult Beagle dog.

Materials & Methods

Subjects

Ten male castrated healthy, drug naïve Beagle dogs, aged between 1.5 and 2 years, weighing between 14 and 19 kg were included in this study. The dogs had no history of neurological or other diseases and were trained to being handled for imaging procedures. The study was approved by the local Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/020).

Magnetic Resonance Imaging

A permanent, 0.2 T magnet[b] was used to perform MRI of the brain in all dogs to rule out pathology and to create a new VOI map. The dogs were positioned in dorsal recumbence with the head and neck extended and a human knee coil was used. Sagittal, transverse and dorsal T1- and T2-weighted spin echo sequences were performed in all dogs under general anaesthesia. Slice thickness was 4mm in all planes with no interslice gap in all studies.

Tracer

After placing a 20G intravenous catheter in the cephalic vein, the dogs were allowed to relax for five minutes. Then, Technetium-99m-Ethyl Cysteinate Dimer (^{99m}Tc-ECD)[c] (injected activity: range 555-792 MBq, mean ± SD: 730.6 ± 68.9 MBq) was injected IV in the awake dog.

Anesthetic protocol

Twenty min after tracer injection, the dogs were premedicated with butorphanol[d] 0.3 mg/kg IV. Anaesthesia was induced 10 min after premedication, using 2-5 mg/kg propofol[e] IV to effect and maintained, after endotracheal intubation, with isoflurane[f] in oxygen. Acquisition started 5 min after induction meaning that there was a time span of 35 min between tracer injection and the start of the acquisition in all dogs. Dogs were allowed to breathe spontaneously during acquisition and heart rate and peripheral hemoglobin saturation (SpO_2%) were monitored with a pulse oximeter. During the acquisition SpO_2 remained above 92% in all subjects.

µ-SPECT

All dogs were positioned in ventral recumbence. Micro-SPECT was performed using a conventional triple head gamma camera[g], equipped with three multi-pinhole collimators[a] (6 multi-focused holes, 3 mm Ø). The radius of rotation (ROR) was set at 21.5 cm. The resolution of the system was 2.25 mm (Dobbeleir et al., 2006). Data were acquired in step-and-shoot mode (10 steps, 36° angular step, 120 s/step) and the total acquisition time was 20 min for each dog. Images were reconstructed using a dedicated ordered subset-expectation maximization (OSEM) algorithm (nine iterations, five subsets) (Scivis) and a Butterworth filter was applied (order 5, cut-off frequency 2.5 cycles/cm). The µ-SPECT data were fitted to the MRI images with the help of fusion software.

Semi-quantitative analysis

A VOI map was created by 3 investigators (VM, KP and SV) based on the MR images of one dog. Nineteen VOIs were manually delineated: left and right frontal, temporal, parietal, piriform and occipital cortical regions (LF, RF, LT, RT, LP, RP, LPy, RPy, LO, RO); left and right thalamus (LTh, RTh), hippocampus (LHi, RHi) and corpus striatum (LCs, RCs) and one VOI was drawn over the cerebellum (CER), the olfactory bulbs (BO) and the brainstem (BS). For the delineation of the hippocampal VOI it was impossible to include all parts of the hippocampus because the VOI would otherwise have been too small at certain areas (tail and head of the hippocampus), which could have created additional partial volume effects. Therefore, the hippocampal body was delineated. Additionally, the µ-SPECT images of all dogs were fused to the µ-SPECT image of the dog used to create the VOI map, using BRASS software from Hermes (Brain Registration and Automated SPET Semi-quantification, Nuclear Diagnostics). Using this automated registration method, a compensation for size and shape differences was applied. A new mean dog µ-SPECT template image was created which can be used for future µ-SPECT studies. From the fitted µ-SPECT data, the counts and voxels of every VOI were determined and perfusion indices (PIs), representing the regional perfusion of every brain region, were calculated by normalizing the counts/voxel of each VOI to the counts/voxel of the whole brain (all VOIs). The perfusion within one brain hemisphere was represented by the left and right hemispheric PI which was calculated by normalizing the left or right hemispheric counts/voxel to the counts/voxel of all VOIs. Regional asymmetry was furthermore evaluated by comparing the PIs of the 8 bilateral VOIs. A bilateral frontal

(rostral = RPI) and occipital PI (caudal =CPI) was calculated in each dog by normalizing the bilateral frontal counts/voxel to the counts/voxel of all VOIs and respectively the bilateral occipital counts/voxel to the counts/voxel of all VOIs. A rostro-caudal gradient was defined as (R-C/R+C) x 100 where R is the bilateral frontal PI and C the bilateral occipital PI. A cortical PI (CoPI) was calculated in each dog by normalizing the counts/voxel of all cortical VOIs (= LF, RF, LT, RT, LP, RP, LPy, RPy, LO, RO) to the counts/voxel of all VOIs and a subcortical PI (SCoPI) was calculated by normalizing the counts/voxel of all subcortical regions (LTh, RTh, LHi, RHi, LCs, RCs) to the counts/voxel of all VOIs. A cortical-subcortical index was calculated and defined as (CoPI/SCoPI) x 100.

Statistical analysis

The Statistical Package for Social Sciences (PASW Statistics 19, SPSS) was used to perform the analyses. Due to the lack of normal distribution of data non-parametric tests were used. The related samples Wilcoxon signed ranked test was applied to evaluate regional and hemispheric left-right differences. The significance of the R-C gradient (H_0: R-C=0) and the Co-SCo index (H_0: Co-SCo=1) was evaluated using the one-sample Wilcoxon Signed Rank Test. The significance level was set at $P < 0.05$.

Results

The MRI of the brain was normal in each dog. The brain of all dogs fitted within the field of view of the µ-SPECT. Figure 1 shows a dorsal view of a part of the VOI map and a mean µ-SPECT image at the same level. The mean of the obtained perfusion images of all dogs is shown in Figure 2 in the 3 planes (sagittal, dorsal and transverse). A dorsal view after fitting of the µ-SPECT images on the MRI images of one dog is shown in Figure 3. The mean PIs for the different VOIs ranged between 0.72 and 1.21 and are outlined in Figure 4. The highest PI was found in the parietal cortex and the lowest in the piriform cortex.

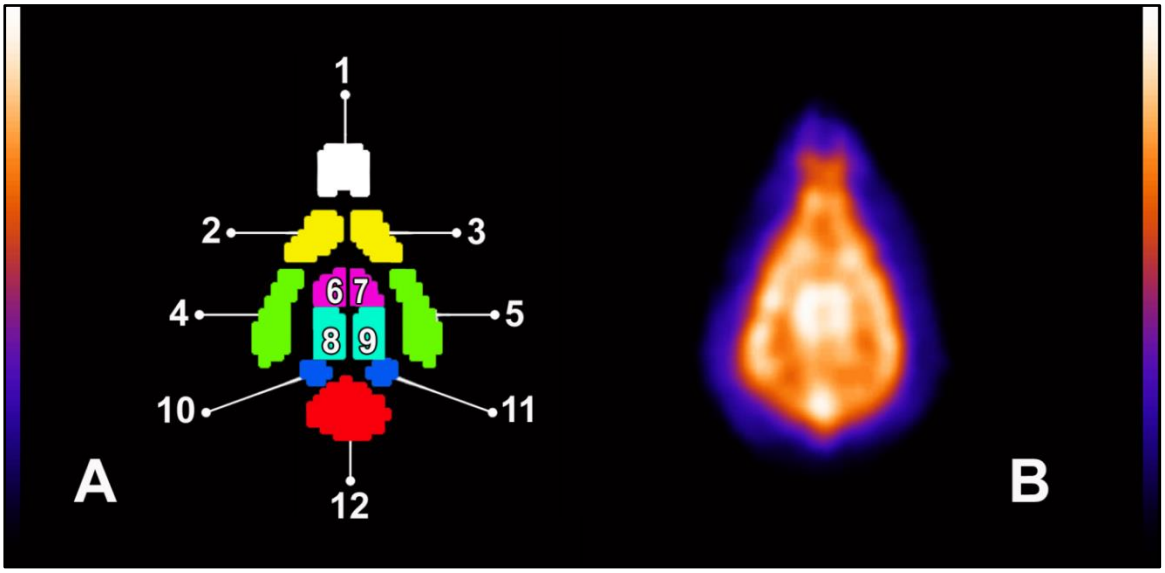

Figure 1: Dorsal view of a part of the VOI map (A) and the mean dorsal µ-SPECT image (B) of the canine brain. Different volumes of interest are outlined: olfactory bulbs (1), frontal lobe (2, 3), temporal lobe (4, 5), corpus striatum (6, 7), thalamus (8, 9), hippocampus (10, 11) and the cerebellum (12)

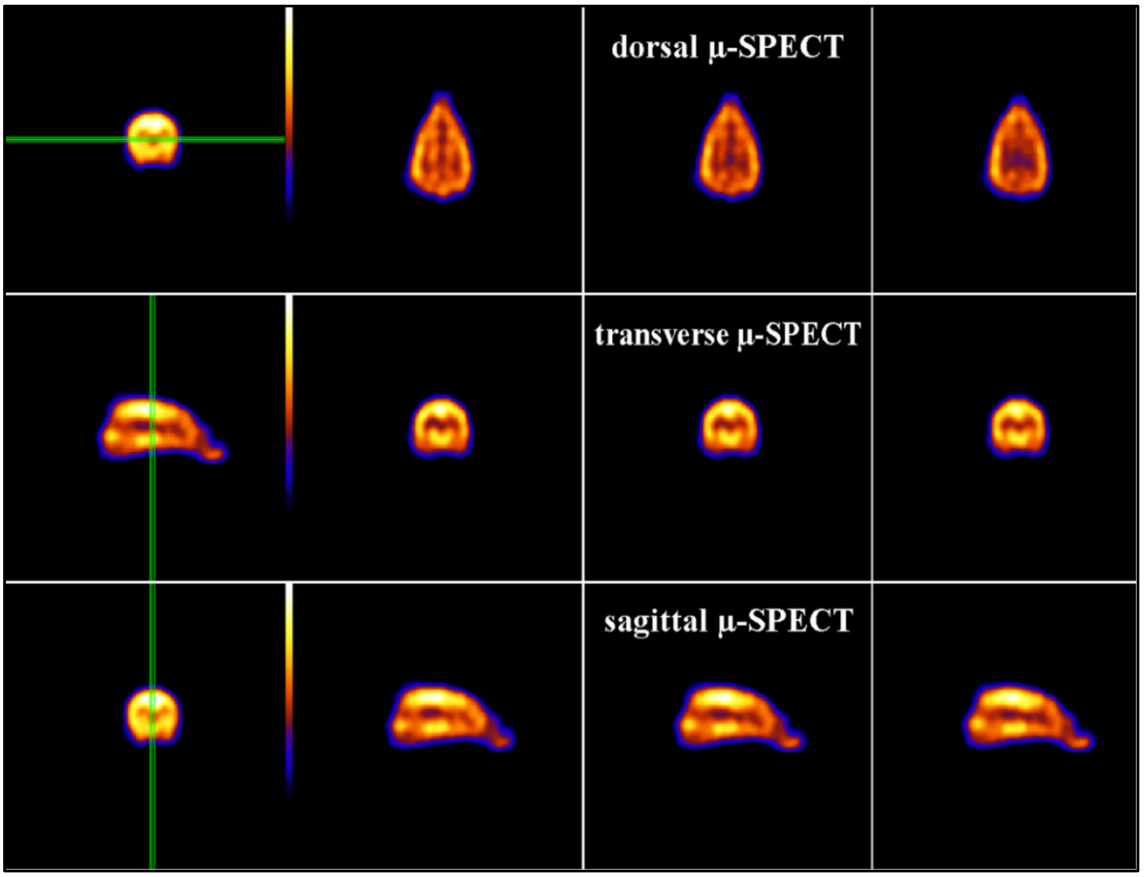

Figure 2: The µ-SPECT images (mean of all dogs) represented in the 3 planes (dorsal, transverse and sagittal)

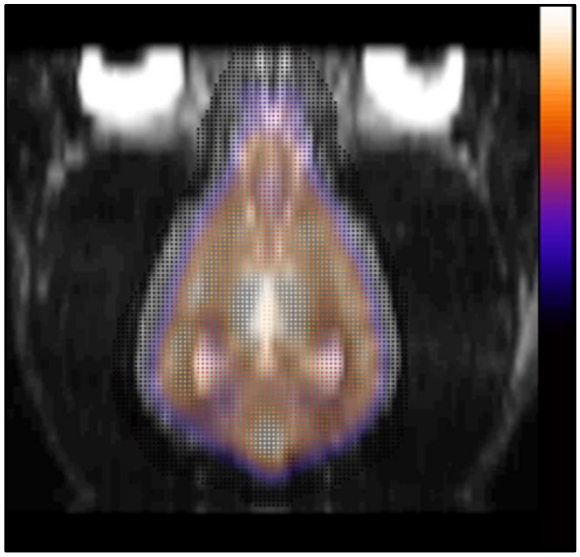

Figure 3: A dorsal µ-SPECT image fitted on a corresponding dorsal MR image of one dog

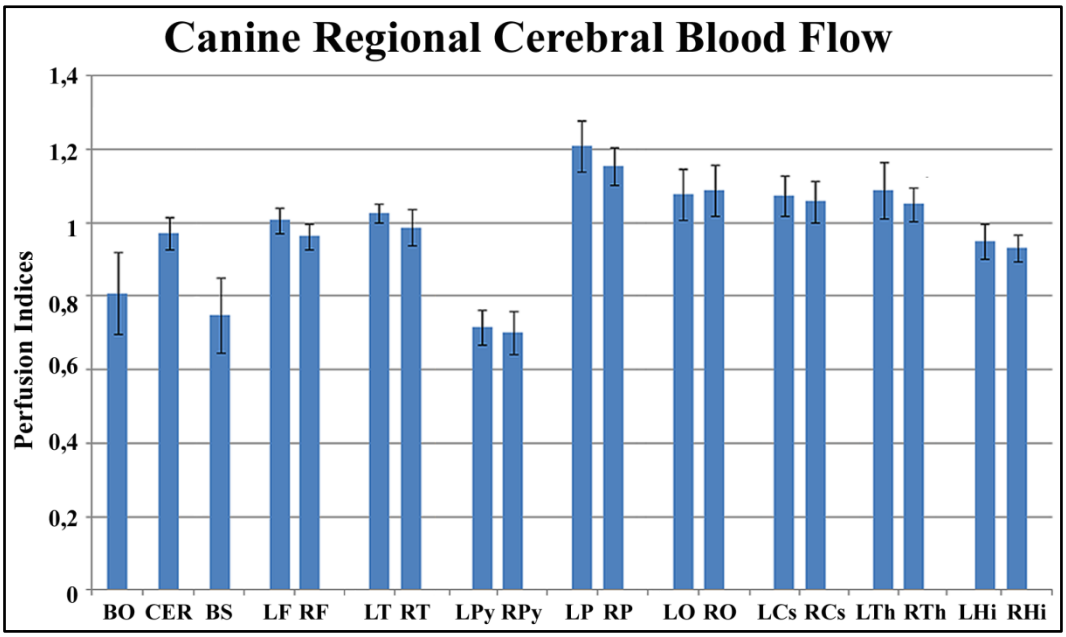

Figure 4: The mean perfusion indices (± SD) per VOI (BO: olfactory bulbs; CER: cerebellum; BS: brainstem; LF, RF: left and right frontal cortex; LT, RT: left and right temporal cortex; LPy, RPy: left and right piriform cortex; LP, RP: left and right parietal cortex; LO, RO: left and right occipital cortex; LCs, RCs: left and right corpus striatum; LTh, RTh: left and right thalamus; LHi, RHi: left and right hippocampus)

A significant difference between the right and left hemispheric PI was detected (P = 0.005) with an overall tracer uptake asymmetry towards the left (Table 1). A significant regional asymmetry towards the left was found in the temporal (P = 0.037), frontal (P = 0.028) and parietal cortex (P = 0.005) (Table 1).

A significant negative rostro-caudal gradient (P = 0.005) was present in all dogs (mean: -5.2%, SD: 1.9%) meaning that there existed a higher perfusion in the occipital versus the frontal cortices (Table 2). No obvious differences were found between the cortical and subcortical PI which was also reflected in a mean (±SD) Co-SCo index of 99.4% ± 5.6% (P = 0.721) (Table 2).

Table 1: The regional and hemispheric bilateral Perfusion Indices (mean ± SD)

VOI	L	R	*P* (L vs. R)*
piriform	0.72 ± 0.05	0.70 ± 0.06	0.285
temporal	1.03 ± 0.03	0.99 ± 0.05	0.037
frontal	1.01 ± 0.03	0.96 ± 0.04	0.028
hippocampus	0.95 ± 0.05	0.93 ± 0.03	0.093
thalamus	1.09 ± 0.08	1.05 ± 0.05	0.074
corpus striatum	1.07 ± 0.05	1.06 ± 0.06	0.508
occipital	1.08 ± 0.07	1.09 ± 0.07	0.445
parietal	1.21 ± 0.07	1.15 ± 0.05	0.005
hemispheric	1.07 ± 0.02	1.03 ± 0.02	0.005

* Perfusion indices of the left side (L) are compared with the perfusion indices of the corresponding VOI on the right side (R). *P*-values are reported.

The piriform, temporal, frontal, occipital and parietal VOIs are cortical regions; the hippocampus, thalamus and corpus striatum VOIs are subcortical regions.

Table 2: The individual and mean (± SD) Cortical Perfusion Indices (CoPI), the Subcortical Perfusion Indices (SCoPI), the Cortical-Subcortical (Co-SCo) Indices, the frontal Perfusion Indices (RPI), the occipital Perfusion Indices (CPI) and the Rostro-Caudal Gradients (R-C).

Dog	CoPI	SCoPI	Co-SCo (%)	RPI	CPI	R-C (%)
1	1.05	1.13	93.0	1.00	1.08	-3.9
2	1.05	1.09	96.3	1.01	1.10	-4.4
3	1.04	1.06	97.9	1.01	1.05	-1.8
4	1.05	1.05	100.1	0.98	1.08	-5.1
5	1.04	1.12	93.2	0.98	1.08	-5.1
6	1.03	1.07	96.9	1.01	1.21	-9.0
7	1.04	1.08	96.5	0.99	1.15	-7.1
8	1.08	0.99	109.2	1.03	1.15	-5.2
9	1.06	1.02	104.2	1.03	1.16	-6.0
10	1.05	0.99	106.6	1.04	1.14	-4.7
mean	1.05	1.06	99.4	1.01	1.12	-5.2
SD	0.01	0.05	5.6	0.02	0.05	1.9

Discussion

This study is, to our knowledge, the first to describe the regional brain perfusion in the dog evaluated with ^{99m}Tc-ECD µ-SPECT. The results of this study show that with a system using multi-pinhole collimation for a conventional gamma camera system (=HiSPECT) the perfusion of the complete brain can be evaluated in Beagle dogs. With the ROR set at 21.5 cm a resolution of 2.25 mm could be obtained (Dobbeleir et al., 2006). This resolution is less than that of dedicated small rodent micro-systems (<1 mm) (Beekman and van der Have, 2007), however, this is compensated by the larger size of the object under investigation. The obtained resolution of 2.25 mm is much better than the resolution of the conventional gamma camera SPECT (7-8 mm) (Peremans et al., 2005). This improved resolution allowed us to evaluate the regional perfusion in 19 different regions: 10 cortical regions, 6 subcortical regions, the brainstem, olfactory bulbs and cerebellum, whereas only 10 regions could be evaluated with the conventional fan-beam SPECT (Peremans et al., 2001). A previous interictal conventional SPECT study of our group (Martlé et al., 2009) found a significant hypoperfusion in the subcortical region, but the limited resolution of this system precluded a more detailed evaluation of this subcortical hypoperfusion. Therefore, performing µ-SPECT of the brain in epileptic dogs could contribute to the detection of epileptogenic foci and to the classification of canine epilepsy. The currently available pinhole plates, which were used in the past for mouse, rat and cat studies, seem appropriate to evaluate the canine brain. However, the field of view is limited and may hamper its use in the evaluation of the brain of larger dog breeds (> 20 kg).

The regional perfusion of the canine brain is represented in this study by the regional perfusion indices. The highest perfusion index was found in the parietal lobes and the lowest in the piriform lobes. In a previous conventional canine SPECT study (Peremans et al., 2001) the highest regional perfusion was found in the subcortical region and the lowest in the frontal cortex which is not in agreement with our findings. The major reason for this discrepancy is the difference in study protocol and in techniques used for acquiring and processing of data. Firstly, in the study from Peremans et al. (2001), 10 regions were evaluated, the piriform lobe and the brainstem were not included and the subcortical region (including the hippocampal, thalamic and striatal area) was globally evaluated. The region map of our study is more detailed (19 regions) and was created

based on MR instead of CT images which make it difficult to compare the absolute regional perfusion indices of both studies. Secondly, in the first study (Peremans et al., 2001) different hardware was used and a different processing protocol was applied on the data. Thirdly, sedation was given prior to tracer injection in the study of Peremans et al (2001). The effect of sedation with medetomidine on the regional cerebral blood flow in dogs was recently demonstrated by Waelbers et al. (2011) and therefore in more recent canine SPECT studies, the tracer is injected in the awake dog before the administration of any sedative or anesthetic. The important timing of injection of the tracer is also emphasized in human SPECT studies (Catafau, 2001).

A significant tracer uptake asymmetry towards the left brain hemisphere was detected in our study. Also regional asymmetries were detected in the temporal, frontal and parietal cortex towards the left. It is important to realize that general and regional brain asymmetries exist in normal dogs because the identification of rCBF abnormalities in brain SPECT is often based on the detection of interhemispheric asymmetries (Aubert-Broche et al., 2003; Vermeire et al., 2009b). Asymmetries in regional uptake in the normal dog were not described previously (Peremans et al., 2001; Waelbers et al., 2011). It is possible that they have not been detected due to the resolution limits and consequently higher prevalence of partial volume effects of the conventional SPECT system. Different human SPECT studies in healthy volunteers also found left-right asymmetries in hemispheric perfusion (Catafau et al., 1996; Krausz et al., 1998; Lobaugh et al., 2000; Van Laere et al., 2001). In contrast to our findings, the perfusion in the right hemisphere in humans is often higher than in the left. In human subjects, these hemispheric asymmetries in perfusion have been explained by volumetric differences between the right and left hemisphere (Catafau et al., 1996), but other hypotheses such as functional differences could also contribute. These hypotheses need to be further investigated in dogs.

A significant rostro-caudal gradient was found in our adult dogs with the higher PI in the occipital compared to the frontal cortex. This finding is in agreement with previous human and canine studies (Koyama et al., 1997; Peremans et al., 2001; Van Laere et al., 2001) and recently a similar negative rostro-caudal gradient was detected in a µ-SPECT study in normal adult cats (Waelbers et al., 2013). The perfusion in the global cortical and subcortical region was comparable with a Co-SCo index of nearly 100% (99.4%, Table 2). This index has not often been used in previous studies, but could be interesting to

apply in the future as a first screening tool to detect changes in cortical or subcortical perfusion in clinical or experimental studies.

This study has some limitations. It is known that anaesthesia influences brain perfusion (Waelbers et al., 2011; Waelbers et al., 2012a) and could therefore be considered a confounding factor. However, the tracer was injected prior to sedation or anaesthesia, therefore allowing an undisturbed distribution. This means that anaesthesia effects will not have influenced our results. Furthermore, because the lipophilic tracer crosses the blood-brain barrier rapidly and is transformed into a hydrophilic form and as a consequence, trapped within the neuron, the µ-SPECT image is a fixed image within 5 min after the tracer injection (Walovitch et al., 1994). As was shown previously (Waelbers et al., 2012b), a stable image, representing the rCBF at the moment of tracer injection, is obtained in dogs as long as the acquisition starts between 15 and 40 min after tracer administration, which was respected in this study (injection – acquisition interval of 35 min).

For this study a homogenous group of young adult male Beagle dogs was used. This has the consequence and limitation that breed, gender and age influences on the normal regional perfusion pattern in the dog cannot be evaluated in this study. Peremans et al. (2001) did not find an influence of age and gender on the conventional SPECT images of dogs, but only 1 older dog was included. Consequently, the effects of aging on conventional SPECT images were investigated in dogs older than 8 years and significant differences in regional perfusion were detected (Peremans et al., 2002). So, future canine µ-SPECT studies should investigate this as well.

Conclusion

Performing high-resolution µ-SPECT of the brain in dogs is feasible and a resolution of 2.3 mm can be obtained. The perfusion pattern in adult male dogs has the highest and lowest uptake in the parietal and the piriform cortex, respectively. Furthermore, a normal hemispheric asymmetry towards the left and a negative rostro-caudal gradient is present in the normal Beagle dog. Knowledge of this normal canine rCBF pattern, including normal asymmetries, is important before evaluating the results of ^{99m}Tc-ECD µ-SPECT in clinical and experimental studies in dogs.

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PART II:

THE MECHANISM OF ACTION OF VAGUS NERVE STIMULATION IN THE HEALTHY BEAGLE DOG

CHAPTER 3

VAGUS NERVE STIMULATION IN DOGS: SURGICAL IMPLANTATION TECHNIQUE, COMPLICATIONS, LONG-TERM FOLLOW-UP AND PRACTICAL CONSIDERATIONS

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Sources and manufacturers

[a] Cyberonics, Inc., Houston Texas, USA

[b] Dolorex, MSD AH, Brussels, Belgium

[c] Propovet, Abbott Laboratories, Berkshire, UK

[d] Isoflo, Abbott Laboratories, Berkshire, UK

[e] Rimadyl, Zoetis Belgium, Louvain-la-Neuve, Belgium

[f] Zoetis Belgium, Louvain-la-Neuve, Belgium

[g] Vetergesic, Alstoe Limited, York, UK

[h] Clavubactin, Le Vet BV, Oudewater, the Netherlands

Summary

The aims of this study were to describe the implantation procedure of a vagus nerve stimulation (VNS) device in dogs and to report complications experienced during short- and long-term follow-up.

A VNS Therapy® System was implanted in the left cervical region of anesthetized healthy Beagle dogs (n = 10). During and one day after implantation, electrocardiography (ECG) and impedance testing of the system were performed. The dogs were monitored daily and the impedance of the system was determined regularly. The VNS devices were surgically removed 3 years after implantation.

The implantation procedure succeeded in all dogs without intra-operative complications. The ECG monitoring and impedance test were within normal limits during and one day after surgery. Postoperative seroma formation was common. One dog developed an irreversible Horner's syndrome leading to removal of the device. Another dog developed a trauma-induced damage of the lead requiring surgical revision. The device could be safely removed in all dogs, however the electrodes were left in place to avoid damage to the nerve. At removal, the anchor tether was dislodged in 40% of dogs and the lead was twisted in 50% of dogs.

The implantation of a VNS Therapy system is safe and feasible in dogs, although, seroma formation, twisting of the lead and dislodgement of the anchor tether were common. Practical considerations to improve the technique are suggested. A regular evaluation of the lead impedance is important, as altered values can indicate serious complications.

Introduction

Vagus nerve stimulation (VNS) is an intermittent stimulation of the cervical vagus nerve using a commercially available implantable device (VNS Therapy® System[a]). This device consists of a pulse generator, helical electrodes that are coiled around the vagus nerve and a connecting lead (Landy et al., 1993). In human medicine, VNS is an established adjunctive treatment for refractory epilepsy. In people whose seizures are unresponsive to medical and surgical therapy, VNS can reduce seizure frequency and increase quality of life (Vale et al., 2011). Currently, VNS is also approved for the treatment of refractory depression and under the investigation for other disorders like migraine, Alzheimer's disease, eating disorders, neuropsychiatric disorders and chronic heart failure (George et al., 2002; Beekwilder and Beems, 2010; Schwartz, 2011).

Vagus nerve stimulation is not yet an established treatment in dogs, but can become increasingly important in the treatment of refractory epilepsy. Refractory epilepsy is an important problem in small animal practice as it occurs in up to 30% of dogs with idiopathic epilepsy (Farnbach, 1984; Lane and Bunch). Since many dogs with poorly controlled seizures do not survive, the search for alternative treatment options is warranted (Martlé et al., 2013). A key advantage of VNS in dogs is the independency of seizure focus localization. Zabara (1992) demonstrated an acute abortive effect of electrical stimulation of the cervical vagus nerve on chemically induced seizure activity in experimental dogs. VNS also seemed to reduce the seizure frequency in some dogs with idiopathic refractory epilepsy using a similar implantable device as in humans (Muñana et al., 2002).

The surgical implantation technique is a minimally invasive procedure that has been well described in humans (Reid, 1990; Landy et al., 1993; Schachter and Saper, 1998). The left vagus nerve is usually chosen for the treatment of epilepsy, as the goal is to influence the brain and to avoid side effects on the heart. There seems to be a higher risk of bradycardia with right-sided VNS (Cohn, 1912; Ardell and Randall, 1986; Kamath et al., 1992). The surgical technique in dogs is quite comparable as in humans and has been described briefly before (Muñana et al., 2002).

In humans, VNS therapy is usually well tolerated and has a low incidence of side effects (Ramsay et al., 1994). Three categories of complications have been recognized: (1)

surgical complications, (2) hardware or technical complications and (3) stimulation related complications. Here, we will focus on the first two categories. The most frequently reported surgery related complications in humans are vocal cord paralysis and infection. Intra-operative bradycardia or asystole (during impedance test of the system), cervical hypoesthesia, traumatic damage to surrounding blood vessels and keloid development have been reported less frequently (Kahlow and Olivecrona, 2013). With devices implanted in a moving subject, technical or hardware complications are inevitable. The most common appears to be a fracture of the lead needing surgical revision (Kahlow and Olivecrona, 2013), but the incidence has diminished considerably in humans with the development of newer leads with improved fatigue tolerance (Landy et al., 1993). The implantation of a similar device in 10 dogs with refractory epilepsy appeared to be safe and well tolerated. During the intraoperative impedance test of the device in dogs, transient bradycardia or even asystole appeared quite common (30%). Post-operative side effects were minor and often transient and were monitored during 6 months after implantation. Horner's syndrome, seroma formation and migration of the pulse generator have been described (Muñana et al., 2002).

This study aims to provide a detailed description of a slightly modified surgical implantation procedure of a VNS Therapy System in dogs in the left cervical region and to describe surgical and technical complications that have been encountered during the postoperative and long-term follow-up period of 3 years. This information will be useful for the implantation of other hardware devices (e.g. pacemakers) in the cervical region of dogs as well.

Materials & Methods

Animals

Ten male castrated, healthy Beagle dogs, aged between 1.5 and 2 years, weighing 14 to 19 kg were included in this study. This study was part of a larger research project investigating the mechanism of action of VNS in dogs and approval of the local Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/020) was obtained. All guidelines for animal welfare, imposed by the Ethical Committee, were respected. The dogs had no history of neurological or other diseases.

The implantable device

A VNS Therapy® System[a] was surgically implanted in the left cervical region of all dogs. The system consists of a pulse generator (Demipulse model 103), 3 helices and a connection lead (Model 304, 3.0 mm) in between (Figure 1). The pulse generator, which looks similar to a cardiac pacemaker, is housed in a titanium case of 45 x 32 x 6.9 mm, weighing around 16 g. The helices are 3 spiral coils, of which the most proximal 2 supply the electrical stimulation (anode and cathode electrode), while the most distal one is the anchor or tethering coil. The function of this anchor tether is to avoid traction on the nerve with movement of the neck (Fahy, 2010). A programming wand (Model 201) and software are used to communicate with and program the device.

Anesthetic protocol

After premedication with 0.3 mg/kg butorphanol[b] given intravenously (IV), general anesthesia was induced with propofol[c] (2-6 mg/kg IV to effect) and maintained with isoflurane[d] in oxygen. Before surgery carprofen[e] (4 mg/kg IV) and antibiotics (Clamoxyl, 10 mg/kg IV and Synulox, 12.5 mg/kg SC)[f] were administered to each dog and an intra-arterial 22G catheter was placed aseptically in a dorsal pedal artery. During the surgical procedure, there was a continuous monitoring of end tidal CO_2 concentration (ET CO_2 %), inspiratory oxygen fraction (FiO_2), peripheral hemoglobin saturation (SpO_2%) respiratory rate (RR) and pulse rate (PR). Also, regular arterial blood pressure measurements were obtained every 10 minutes and continuous ECG monitoring was performed.

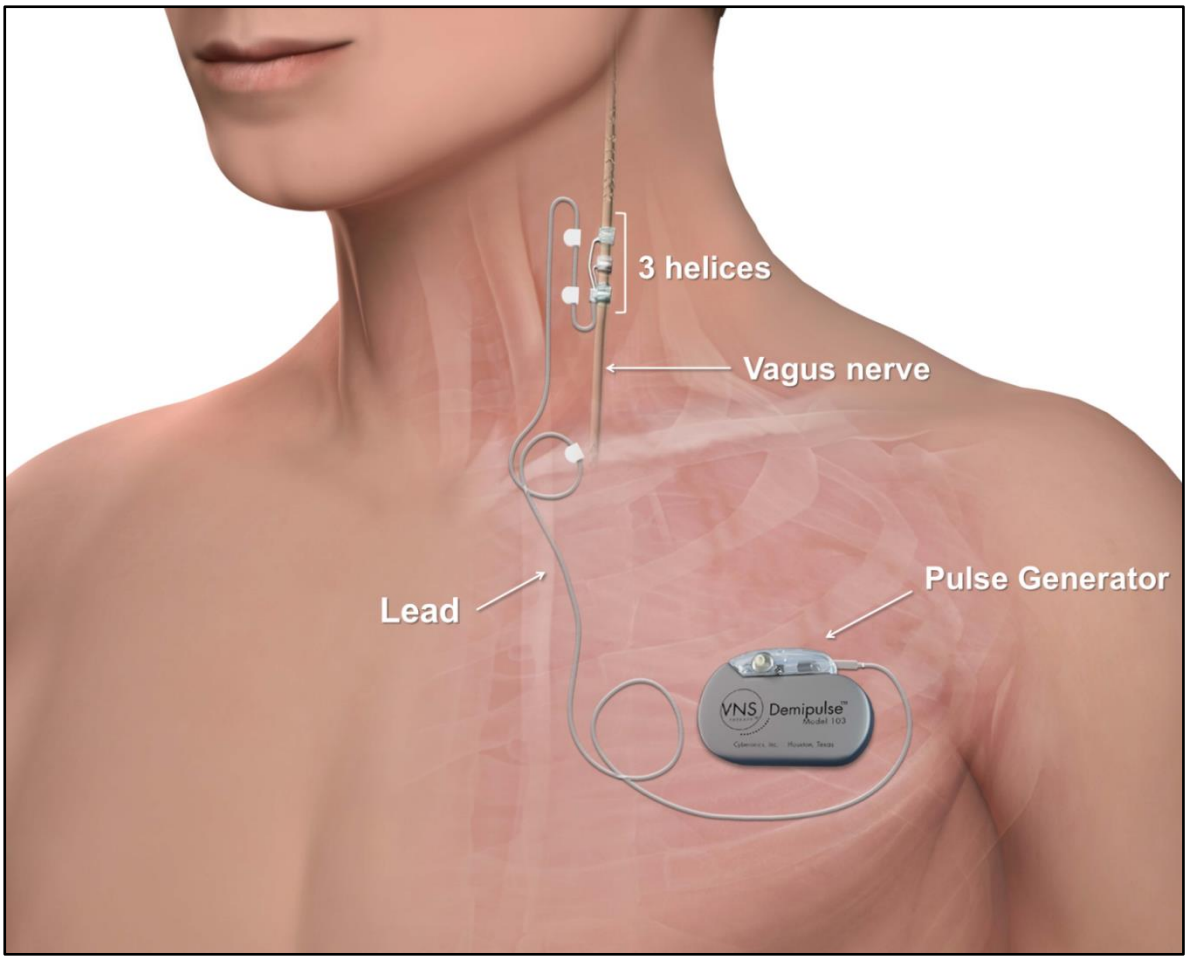

Figure 1: A VNS Therapy® System implanted in a man

(with permission of Cyberonics, Inc.)

Surgical implantation procedure

The dogs were placed in right lateral recumbence with the head and neck rotated 45° clockwise. The left thoracic limb was mildly retracted caudally. The skin was surgically prepared over the entire left cervical region, extending past the dorsal and ventral midlines. Two incisions in the cervical region were made: a dorsal incision for placement of the pulse generator and a ventral incision for placement of the helical electrodes around the left vagosympathetic trunk. The position of both incisions is demonstrated in Figure 2. The ventral linear skin incision of about 4 cm length was made approximately 0.5 cm dorsal and parallel to the jugular furrow. The subcutaneous tissues were incised and blunt dissection between the brachiocephalic and omotransversarius muscle was performed until the carotid sheath was identified. The carotid sheath was opened and the 3

containing structures (common carotid artery, vagosympathetic trunk and internal jugular vein) were identified.

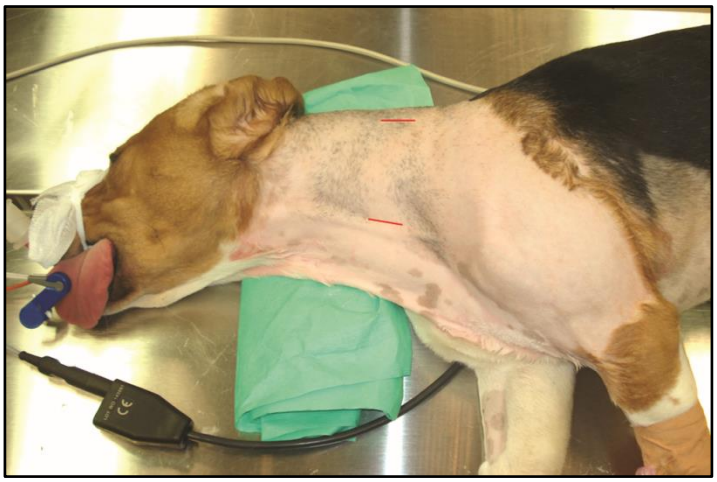

Figure 2: Localization of the ventral and dorsal incision

The vagosympathetic trunk was freed from the surrounding connective tissue and isolated with 2 vessel loops to ease manipulation (Figure 3). The 2 helical electrodes and anchor tether were coiled around the nerve beginning with the anchor tether, next placing the positive electrode (anode) and then placing the negative electrode (cathode) most cranially towards the head of the dog (Figure 4). Attention was paid to manipulate only the helical sutures and not the helices themselves to avoid damage of the electrodes. To provide adequate slack and neck movement, 3 strain relief loops were formed in the lead and the lead was fixated to the underlying fascia at 3 points between the ventral and dorsal incision site using silicone tie-downs and non-absorbable sutures. Then, a dorsal 4 cm linear skin incision was made about 2 cm from the dorsal midline with the caudal extent of the incision just cranial to the left scapula. A pocket underneath the fascia, just large enough for the pulse generator to easily fit in, was created by blunt dissection. A shunt-passing tool[a] was used to create a tunnel and guide the lead from the ventral incision to the pocket. The tunnel and lead passed underneath the brachiocephalic muscle. The lead was connected to the pulse generator using a hex screwdriver[a] and at that time the impedance of the system was tested to confirm the integrity of the system. The residual part of the lead was wound and placed next to the pulse generator in the subfascial pocket. The pulse generator was fixated with 1 or 2 non-absorbable polyfilament sutures to the underlying tissue. Then, the wounds were closed routinely.

Figure 5 shows the schematic representation of a VNS device implanted in a dog. Within 48 hours after surgery a second impedance test was performed in the awake dogs while ECG was monitored to detect any change in heart rate or rhythm. A postoperative healing period of 1 month was respected before activation of the system.

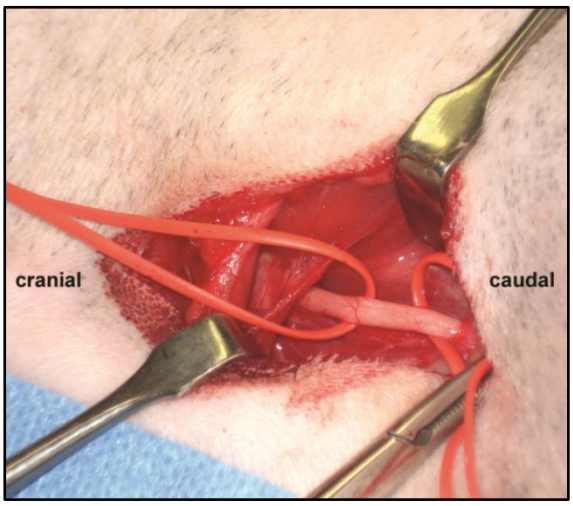

Figure 3: The left vagosympathetic trunk isolated with 2 vessel loops at the ventral incision site

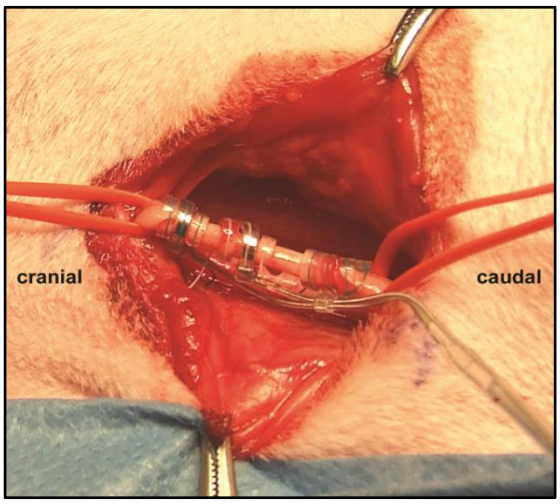

Figure 4: The 3 helices placed around the left vagosympathetic trunk at the ventral incision site

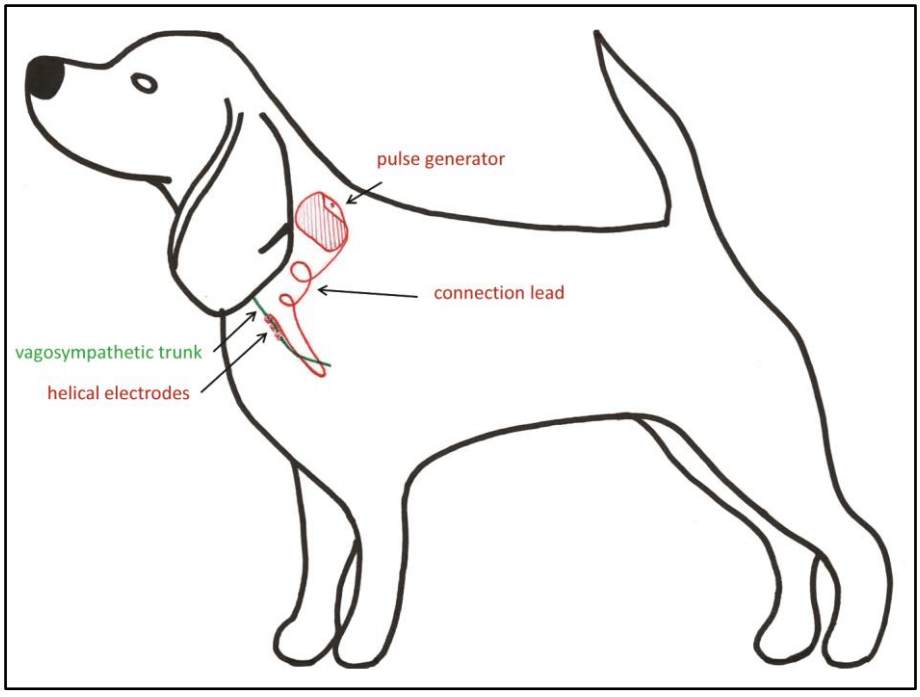

Figure 5: Schematic representation of a VNS device implanted in the left cervical region of a dog

Post-operative care and follow-up

For pain relief, buprenorphine[g] 0.01 mg/kg was administered intramuscularly (IM) every 6 hours the first 24 hours after surgery. Carprofen[e] (2 mg/kg BID) and amoxicillin-clavulanic acid[h] (12.5 mg/kg BID) were continued orally for 5 days. The first week after surgery, the dogs remained in the hospitalization area for close monitoring. A general examination together with a control of pupil size and wound healing was performed on a daily basis the first week. Subsequently, the dogs and their wounds were inspected on a daily basis and a thorough general examination was performed every week.

Impedance evaluation

The lead impedance of the VNS Therapy System was evaluated regularly using the System Diagnostics section of the software[a]. During this test, the pulse generator delivers a short pulse (130 µs) at 0.25 mA. Likewise, the programming software will report the lead impedance. Normal lead impedance is defined as a value between 600 and 7000 Ohms (Cyberonics Physician's manual, 2001). As mentioned before this impedance test was performed during surgery and within the first 48 hours after surgery. Subsequently, the impedance was evaluated every week the first 2 months after surgery, every 2 weeks the following 6 months and every 2 months until 1.5 year after surgery. Additional evaluations of the lead impedance were performed when abnormalities or complications were suspected.

Removal of the devices

The VNS device was surgically removed 3 years after the implantation. The helices were left in place, to avoid the potential for nerve injury during their removal. The lead was cut just below the anchor tether and was completely removed together with the silicone tie-downs and pulse generator. The pocket of the pulse generator was excised. Both wounds were closed routinely.

Results

Surgical implantation and anesthetic monitoring

The implantation of the VNS Therapy System succeeded in all dogs and no intraoperative abnormalities or complications were noticed. During the whole surgical procedure and especially during the impedance test of the device no signs of bradycardia or asystole were detected with ECG. The other anesthetic parameters (ET CO_2%, FiO_2, SpO_2%, RR and arterial blood pressure) remained stable as well throughout the procedure. ECG monitoring, performed within 48 hours after the implantation during the second impedance test, did not reveal changes in heart rate or rhythm.

Minor transient postoperative complications

Seroma formation was common in our study. Three dogs developed a seroma at the ventral incision site, 3 dogs developed a seroma at the dorsal incision site and one dog developed both. Some seromas required draining and placement of a mild pressure bandage. In most dogs, a seroma developed approximately 10 days after implantation and resolved within 2 to 3 weeks (mean duration of the seroma (± SD) was 12 (± 5) days). None of the dogs developed signs of infection.

One dog developed hoarseness the first day after the implantation which resolved spontaneously within 2 days.

Complications requiring device removal or revision surgery: 2 cases

Case 1: Horner's syndrome

One dog developed a left-sided Horner's syndrome 22 days after surgery (Figure 6). This particular dog had also developed a seroma at the ventral and dorsal incision site. At the ventral incision site, the seroma was present from day 9 to 11 after surgery. The seroma at the dorsal incision site appeared 12 days after surgery. Several drainages combined with placement of a bandage were performed and the seroma resolved 32 days after the implantation. The Horner's syndrome was still present at that time. General physical and neurological examination revealed no other abnormalities. The lead impedance, which was within normal limits before, suddenly had changed around the time of the onset of the Horner's syndrome (from 1140 to 532 Ohms). This led to the suspicion that the

system was not functioning properly and that there was a problem at the level of the vagosympathetic trunk causing the irreversible Horner's syndrome.

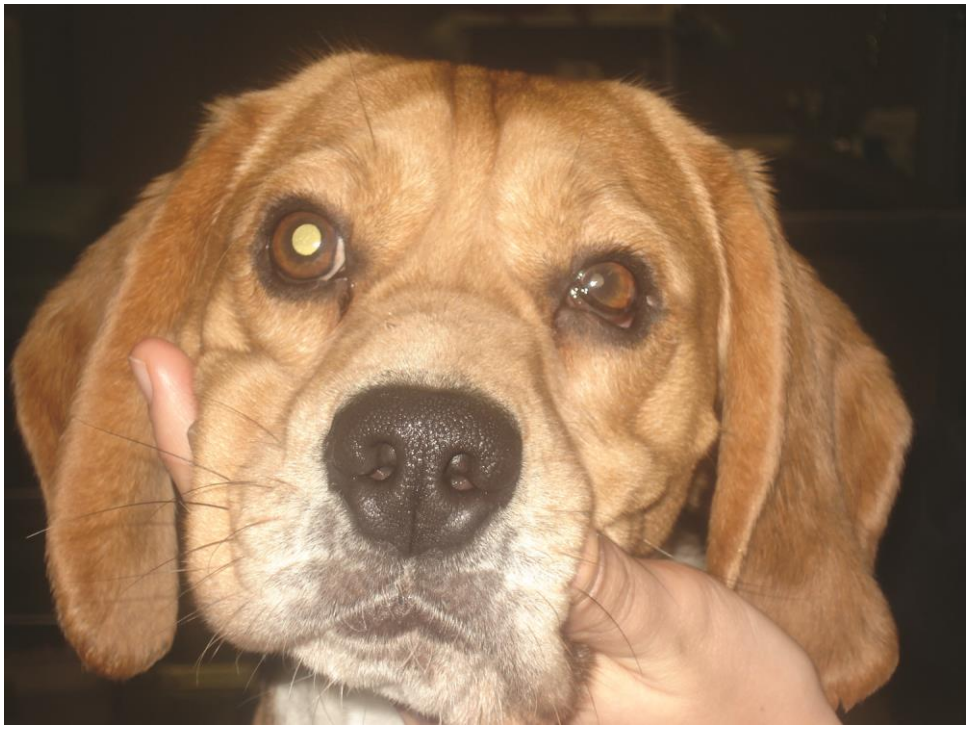

Figure 6: Left-sided Horner's syndrome (case 1)

Radiography (RX) and ultrasound (US) of the left cervical region were performed. Both remained inconclusive about the cause of the Horner's syndrome. However, on US the left-sided vagosympathetic trunk was focally thickened (0.22 mm Ø) compared to the right-sided nerve (0.11 mm Ø).

Because the Horner's syndrome persisted, surgical exploration was imperative and was performed 5 months after initial implantation. The surgical approach was similar to the initial implantation. After identifying the left vagosympathetic trunk, it was seen that the anchor tether (distal helix) was no longer wound around the nerve, but it was encapsulated by a migrated silicone tie-down and both were compressing the vagosympathetic trunk (Figure 7). After removal of the helices the nerve had a red and swollen appearance and this dog was excluded from further VNS research. Thus, the lead and pulse generator were removed. When the pulse generator was removed, the lead underneath had a twisted, knotted appearance. The Horner's syndrome has improved over time but a mild anisocoria remains present 3 years after removal of the device.

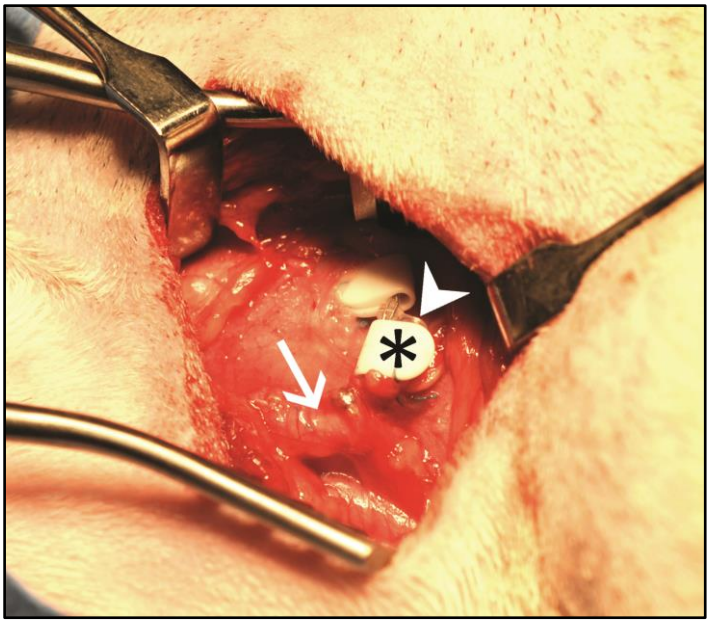

Figure 7: Surgical exploration of case 1: the anchor tether (arrowhead) is encapsulated in a migrated silicone tie-down (asterisk) and both are compressing the vagosympathetic trunk (arrow)

Case 2: Traumatic lead damage

Another dog suddenly developed a seroma at the dorsal incision site 5 months after the implantation and this was probably induced by trauma (e.g. dog fight, dog play, scratching, rubbing). Together with the seroma development, the impedance was unmeasurably low (< 200 Ohms). Before, the impedance was stable around 1200 Ohms. RX of the cervical region showed an irregular, twisted appearance of the lead (Figure 8). Although the seroma resolved within a few days, the impedance value still remained low, so surgical exploration was performed the next week. After opening the dorsal incision site, the lead showed a twisted, knotted appearance (Figure 9) surrounded by fibrous tissue. At the ventral incision site, the lead between both incisions had an abnormal yellow to brown color reflecting damage of its silicone sheath (Figure 10). The 3 helices were nicely in place around the vagosympathetic trunk and encapsulated in fibrous tissue. To avoid damaging the nerve by excessive manipulation, the lead was cut just below the anchor tether and a new set of electrodes was placed cranial to the old ones. The new lead was then fixated similarly as before and connected to the same pulse generator. The system was tested intra-operatively and a normal lead impedance was obtained.

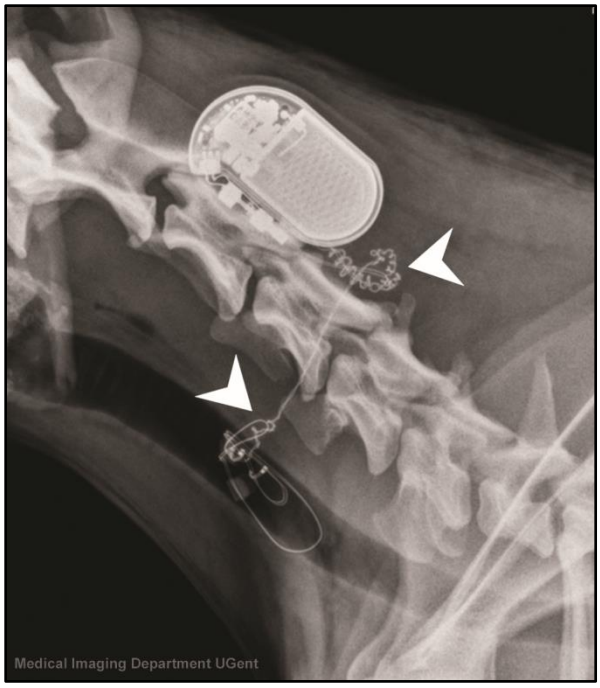

Figure 8: Lateral RX of the cervical region (case 2): the lead has a twisted, irregular appearance (arrowheads)

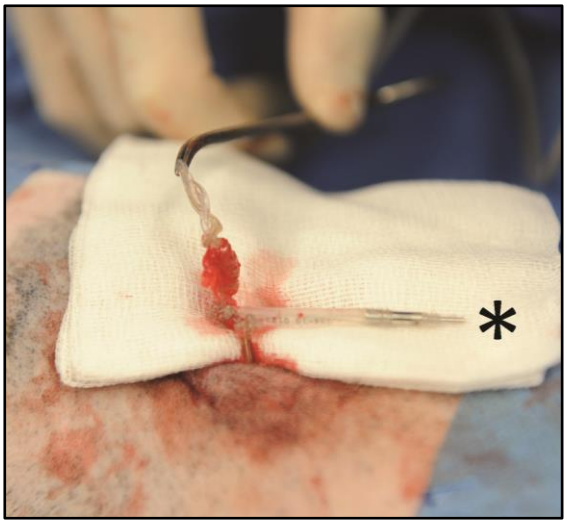

Figure 9: The lead at the level of the pulse generator (asterisk) has a twisted and knotted appearance

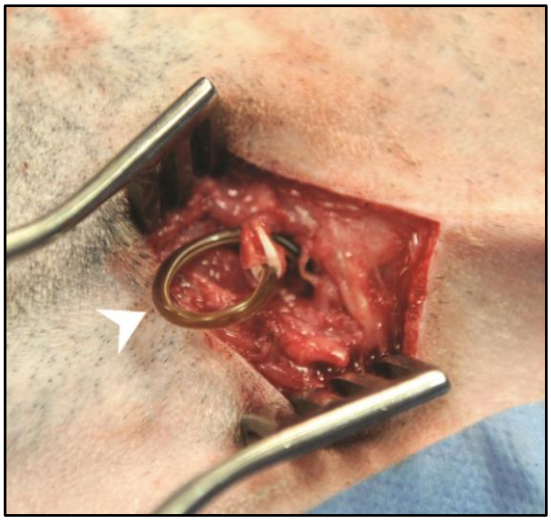

Figure 10: The lead between the dorsal and ventral incision site has an abnormal color (arrowhead)

Lead impedances

During the intraoperative device test, the impedance values were within normal limits in all dogs (mean ± SD: 972 ± 170 Ohms). Also, during the second device test within 48 hours after surgery no abnormal impedance values were detected (mean ± SD: 790 ± 62 Ohms). Until 1.5 year after implantation, the impedances remained normal in all dogs except for the 2 cases described above.

Removal of the devices

In 8 dogs the device was removed after 3 years at the end of the study, in 1 dog it was removed 5 months after implantation due to the Horner's syndrome (case 1) and in another dog the device was removed after 1.5 years due to the development of primary hypothyroidism.

In 5 dogs (50%), the lead had a twisted appearance, most commonly at the level of the pulse generator. Traumatic damage of the silicone sheath was detected in 3 of these 5 dogs. At the level of the vagosympathetic trunk the distal helix (= anchor tether) was detached from the nerve in 4 dogs (40%).

Discussion

The implantation of a VNS Therapy system in the left cervical region is a minimally invasive, feasible technique in dogs. No intraoperative complications occurred, but transient seroma formation was common during the early postoperative period. Twisting and damage of the lead and movement of the anchor tether was also frequently noticed. Two complications requiring surgical revision or removal of the device were associated with sudden changes in the impedance value.

In humans, complications related to the surgical implantation of a VNS Therapy System are uncommon and usually minor and transient (George et al., 2000; Kotagal, 2011). Postoperative side effects related to the surgical implantation of a device in the cervical region seem to be more common in dogs than humans. This is not surprising as dogs do not adjust their natural behavior after surgery and they do not understand what they have been implanted with and why.

Bradycardia or asystole during intraoperative device testing has been reported in dogs and humans (Asconape et al., 1999; Muñana et al., 2002). Cardiac effects of VNS are not unexpected because of the vagus nerve's efferent influence on the heart. Therefore, the VNS electrodes are usually implanted around the left vagus nerve for the treatment of epilepsy, because the left vagus nerve has less cardiac input than the right in humans and dogs (Cohn, 1912; Ardell and Randall, 1986; Kamath et al., 1992). Additionally, the electrode contacts are placed in an orientation (negative pole rostral) with the aim of primarily activating afferent cervical vagal fibers, however co-activation of the efferent fibers cannot be fully prevented (Banzett et al., 1999). A higher incidence of cardiac side effects during surgery in dogs than in humans was reported previously (Muñana et al., 2002). This might seem illogical on the one hand, since in dogs the electrodes are wrapped around the vagosympathetic trunk, as both nerves are fused in the cervical region (Mizeres, 1955). Consequently, additional sympathetic stimulation and influence on the heart cannot be excluded. On the other hand, anatomical differences of the cardiac branches of the vagus nerve are most likely responsible for this higher incidence of bradycardia in dogs (Muñana et al., 2002). In humans, care is taken to place the VNS electrodes distal to the superior and inferior cervical cardiac branches of the vagus nerve (Reid, 1990). In dogs, it is impossible to spare the cardiac branches from stimulation,

because these branches leave the nerve more distally in the thoracic cavity (Muñana et al., 2002). No intraoperative ECG changes were detected in our study, but the impedance test was performed at a lower output current (0.25 mA) compared to the previous canine VNS study (1 mA) (Muñana et al., 2002). Also, in our study the anesthetic protocol was carefully chosen to minimize cardiorespiratory depression (Lamont et al., 2007).

Vocal cord palsy affects about 1 to 6% of people after implantation and is usually transient (Kahlow and Olivecrona, 2013). It is probably related to intraoperative manipulation of the vagus nerve or disruption of the blood supply in the area (Fernando and Lord, 1994; Vonck et al., 2004). Transient hoarseness was obvious in 1 dog in our study, but may have been underestimated as these Beagle dogs do not bark or whine on command.

The surgical implantation procedure of a commercially available VNS device in dogs has been described previously (Muñana et al., 2002); however, small adaptations were made in our study. In our dogs, a smaller type of pulse generator was used and both incisions were smaller. At the ventral incision site, the carotid sheath was approached by blunt dissection between the brachiocephalic and omotransversarius muscle, avoiding incision through muscle tissue. The pulse generator was not placed in a subcutaneous pocket, but underneath the fascia, to create an extra protective layer and a tighter pocket. For similar reasons the lead was tunneled below the brachiocephalic muscle instead of subcutaneously. Despite these efforts, a higher incidence of seromas was present (70% compared to 20% (Muñana et al., 2002)). Our study was performed in experimental Beagle dogs, whereas Muñana et al. (2002) implanted the device in client-owned dogs with refractory epilepsy. Although efforts have been made to monitor the Beagle dogs closely during the postoperative period, they will have been less supervised than a client-owned dog. This might have contributed to the higher seroma development in our study.

Possible improvements of the surgical technique in the future could be to place implantable devices in a more stable anatomical position for example more dorsally in the cervical region or even caudal to the interscapular region. The placement of the pulse generator underneath the cervical musculature can also be considered, but communication with the programming wand could be altered and surgical revisions or removals will be more difficult. Postoperatively, we recommend to place a preventive mildly compressive cervical bandage (Domenech et al., 2005). Still, placing an adequately compressive

bandage in the canine cervical region for several days remains a true challenge. Also, since excessive movement of the neck could have contributed to the seroma formation, serious restriction of movement during the first 2 weeks after surgical implantation can be useful.

One dog in our study developed a left-sided Horner's syndrome indicating damage to the left sympathetic nerve. Horner's syndrome has been reported as rare complication in humans undergoing surgical implantation of the device and is most likely due to transient dysfunction of third-order sympathetic fibers within the carotid sheath (Kim et al., 2001). This complication was noticed in 20% of dogs with refractory epilepsy, 24 hours after implantation and it spontaneously resolved within 2 to 4 weeks (Muñana et al., 2002). The anatomical differences between the human and canine cervical vagus nerve most likely account for the higher incidence of Horner's syndrome in dogs. In dogs, the cervical vagus nerve is fused with the sympathetic nerve to form the vagosympathetic trunk (Mizeres, 1955), whereas in humans, the cervical portion of the vagus nerve is adjacent to, but completely separated from the sympathetic nerve. In our dog, Horner's syndrome occurred 22 days after surgery which indicates that causes directly related to the surgical implantation, such as a transient compromise of blood supply to the sympathetic nerve, local edema or direct mechanical damage resulting from manipulation of the nerve, seemed unlikely. Surgical exploration 5 months after the initial implantation revealed a dislodgement of the distal anchor tether and migration of the silicone tie-down both compressing the nerve. This was the only dog that developed a seroma at both incision sites. It is unclear whether there is an association between both complications, but the seroma at the dorsal incision site could have caused unwanted movement and twisting of the pulse generator or lead underneath and subsequently traction on the lead distally at the level of the nerve. The lead at the level of the pulse generator certainly had a twisted appearance.

The lead of the dog suffering an external trauma 5 months after the surgery had a similar twisted and knotted appearance at the level of the pulse generator. This seems comparable to the "pacemaker Twiddler syndrome" described both in humans and dogs, which is characterized by accidental or deliberate rotation of an implanted pulse generator leading to winding, dislodgement or even fracture of the lead (Bayliss et al., 1968; Young and Bailey, 2002; De Monte et al., 2013). This hypothesis assumes that the sutures fixating the pulse generator in the pocket got loosened. Twiddling in animals may be caused by

scratching with a paw, but local muscular action during normal activities seems more likely to contribute (Been and Darke, 1988). It seems reasonable that a twisted lead will be more prone to traumatic damage due to increased fatigue of the wire. Remarkably, when the devices were removed 3 years after implantation, the lead had an abnormally twisted appearance in 3 other dogs as well. Since we used human leads, we hypothesized that these might have been too long. The leads need to be sufficient in length to avoid traction on the nerve and to allow strain-relief loops, but in each dog, a few additional windings had to be placed together with the pulse generator in the subfascial pocket. The use of customized leads in the future, adapted to the size of the dog, is advisable. Additionally, improved fixation techniques for the pulse generator as well as for the lead are warranted.

Impedance measurements were regularly performed in this study using the System Diagnostics test of the software. This test evaluates the lead impedance of the VNS Therapy System, as well as the pulse generator's ability to deliver the programmed stimulation (Cyberonics Physician's manual, 2001). It is an essential test during and after surgery to assure the integrity of the system. Sudden changes in impedance values can indicate complications as observed in the 2 dogs requiring surgical exploration.

Conclusion

The implantation of a VNS Therapy system in the left cervical region is a minimally invasive, feasible technique in dogs. Intra-operative complications did not arise. Postoperatively, several complications occurred such as seroma formation, twisting and traumatic damage of the lead and movement of the distal helix. Hence, all efforts should be made to prevent seroma formation as this transient complication can also contribute to movement and damage of the system. Regular impedance testing is important as altered impedance values can indicate serious complications requiring surgical revision of the system.

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CHAPTER 4

REGIONAL BRAIN PERFUSION CHANGES DURING STANDARD AND MICROBURST VAGUS NERVE STIMULATION IN DOGS

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Sources and manufacturers

[a] Cyberonics, Houston, TX, USA

[b] Neurolite, Lamepro, Raamdonksveer, The Netherlands

[c] Dolorex, MSD AH, Brussels, Belgium

[d] Propovet, Abbott Laboratories, Queensborough, UK

[e] Isoflo, Abbott Laboratories, Queensborough, UK

[g] Triad, Trionix, Twinsburg, OH, USA

[h] HiSPECT, Bioscan, Paris, France

Summary

Vagus nerve stimulation (VNS) is an effective adjunctive treatment for refractory epilepsy in humans, but its mechanism of action (MOA) and optimal stimulation parameters are still unknown. Functional neuroimaging studies could provide better insight into the brain structures involved in the activity of VNS, but have not yet been described in dogs. The aim of this study was to investigate the effect of acute VNS on the regional cerebral blood flow (rCBF) in dogs using micro-SPECT (µ-SPECT). Additionally, a novel stimulation paradigm (microburst VNS) was used and compared with standard VNS.

A VNS Therapy® System was implanted in ten Beagle dogs. µ-SPECT was performed after sham, standard and microburst VNS in a randomized, cross-over study. Nineteen volumes of interest (VOIs) were semi-quantitatively analyzed and perfusion indices (PIs) were calculated. Furthermore, a rostro-caudal gradient (R-C), an asymmetry index (AI) and a cortical-subcortical index (Co-SCo) were determined. The SPECT results after standard and microburst VNS were compared pairwise with sham stimulation.

Acute standard VNS did not cause significant rCBF alterations. Acute microburst VNS caused a significant hypoperfusion in the left frontal lobe ($P = 0.023$) and in the right parietal lobe ($P = 0.035$). Both stimulation paradigms did not cause changes in R-C, AI nor Co-SCo.

Microburst VNS seems more potent than standard VNS to modulate the rCBF in the dog. Our results promote further research towards the antiepileptic effect of microburst VNS in dogs and humans.

Introduction

Vagus nerve stimulation (VNS) is a well-established adjunctive treatment in human patients with medically or surgically refractory epilepsy (Ben-Menachem, 2002; Boon et al., 2002). Several studies, including two large double-blinded randomized clinical trials have shown effectiveness of VNS, which even increases over time, in different forms of human epilepsy (Ben-Menachem et al., 1994; DeGiorgio et al., 2000; Groves and Brown, 2005).

Neither the exact antiepileptic MOA nor the optimal stimulation parameters for VNS are known (Chae et al., 2003; Alexander and McNamara, 2012), despite a lot of research in humans with refractory epilepsy (Boon et al., 2002) and in experimental animal models (Zanchetti et al., 1952; Chase et al., 1967; Lockard et al., 1990; Woodbury and Woodbury, 1990; Zabara, 1992; Naritoku et al., 1995; Takaya et al., 1996; Krahl et al., 1998; Fernandez-Guardiola et al., 1999; Walker et al., 1999; Krahl et al., 2001; Dedeurwaerdere et al., 2005a; Alexander and McNamara, 2012). A better understanding of the exact MOA is important, because it could lead to the identification of predictive factors for responsiveness and to optimization of stimulation parameters. Functional brain imaging studies have the potential to reveal better insight into the brain structures involved in the activity of VNS. The main conclusion of human Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT) and functional Magnetic Resonance Imaging (fMRI) studies is that VNS causes immediate and long-term changes in anatomical structures belonging to the afferent vagus nerve pathways, although there is some disagreement between different studies (Boon et al., 2002; Chae et al., 2003). A lot of confounding factors may be responsible for this discrepancy (Dedeurwaerdere et al., 2005b). As our study is performed in healthy dogs, the epilepsy-related factors are avoided and the true effect of VNS on the rCBF is assessed. In addition, we believe that it is useful to expand the research into the MOA of VNS from rodents towards canines as quite some similarities exist between human and canine epilepsy (Chandler, 2006). Furthermore, the canine and human brain size and function share more similarities than the rodent and human brain. Until now, only a few studies examining the antiepileptic effect or MOA of VNS in dogs have been performed (Zabara, 1992; Speciale and Stahlbrodt, 1999; Muñana et al., 2002; Castoro et al., 2011; Yoo et al., 2013).

There is a continuous search for more effective stimulation paradigms and in this study, a recently developed experimental stimulation paradigm, called microburst VNS, is evaluated and compared with standard VNS. The rationale of the microburst stimulation is that multiple stimulations within a short time period could induce a more robust synaptic response at central vagal targets (Ito and Craig, 2005, 2008; Alexander and McNamara, 2012).

The aim of our study was to evaluate the effect of acute VNS on the regional brain perfusion in healthy dogs using µ-SPECT. Furthermore, a recently developed experimental stimulation paradigm, microburst VNS, was compared with standard VNS.

Materials & Methods

Animal preparation

This study was approved by the local Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/020). All guidelines for animal welfare, imposed by the Ethical Committee, were respected. To minimize gender, breed and age influences, ten male castrated healthy Beagle dogs, aged between 1.5 and 2 years, weighing between 14 and 19 kg were included in this study. The dogs had no history of neurological or other diseases and were trained to being handled for imaging procedures. Low-field MRI of the brain was performed in all dogs to exclude intracranial abnormalities.

All animals were implanted with a VNS Therapy® System[a], consisting of a pulse generator (Demipulse™ Model 103) and a lead with helical electrodes (single-pin bipolar Lead, 3.0 mm size). A previously described surgical technique (Zabara, 1992; Muñana et al., 2002) was used. The 3 helical electrodes were coiled around the isolated vagosympathetic trunk. During surgery, the end tidal CO_2 concentration, inspiratory oxygen fraction, peripheral hemoglobin saturation (SpO_2%) and pulse rate were continuously monitored. At the same time, the heart rhythm was analyzed by visual assessment of an electrocardiogram (ECG). The lead impedance was measured to ensure the integrity of the stimulation electrode and the functionality of the stimulation. A second device test was performed within 48 hours after surgery and at that time an additional ECG was evaluated during 10 min to monitor for any changes in heart rate or rhythm. A healing period of one month was respected during which no VNS was performed.

Stimulation parameters

For this study, acute VNS was defined as 55 min of intermittent active stimulation. To achieve adequate duty cycles during the 55 min of stimulation it was chosen to give rapid-cycling stimulation, meaning an on-time of stimulation of 7 seconds and an off-time of 18 seconds. Two types of active stimulation (standard and microburst VNS) and a sham stimulation were given to each dog. The microburst VNS is a novel stimulation paradigm where stimulation is delivered in small bursts instead of single pulses as in standard VNS (Figures 1 and 2). Stimulation parameters used for standard VNS included

a pulse width of 500 µs and an inter-pulse interval of 33 ms (30 Hz). Stimulation parameters for microburst VNS included a pulse width of 500 µs, an inter-pulse interval of 3.3 ms (300 Hz), 3 pulses/burst and an inter-burst interval of 0.4 s. The stimulation frequency of 300 Hz was chosen based on neurophysiological studies which used similar stimulation parameters. In these studies, high frequency stimulation was used to enhance synaptic transfer and produce vagal-evoked responses in the thalamus (Ito and Craig, 2005, 2008). The optimal stimulation output current was individually determined one month after the implantation during a ramping-up procedure (with increasing steps of 0.125 mA) and was defined as the highest current for which no cough was elicited, similar as in a previous canine VNS study (Muñana et al., 2002). This output current was checked on a weekly basis and adapted if necessary. For the sham stimulation the animals underwent the same procedures as with active VNS except that the output current was set at 0 mA.

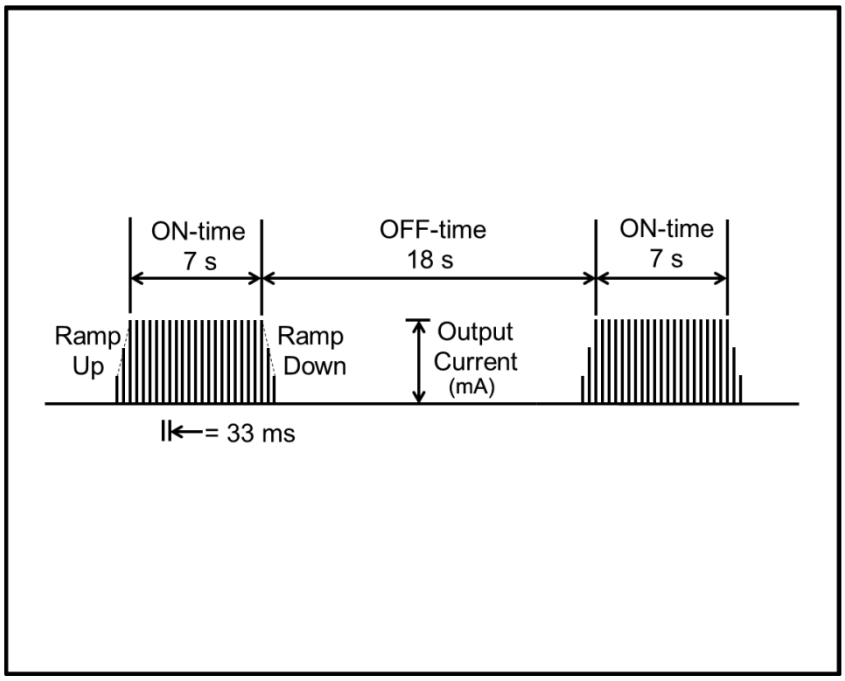

Figure 1: ON/OFF Standard VNS: Graphical representation of the waveform for intermittent standard stimulation (s = seconds; ms = milliseconds; mA = milliampere)

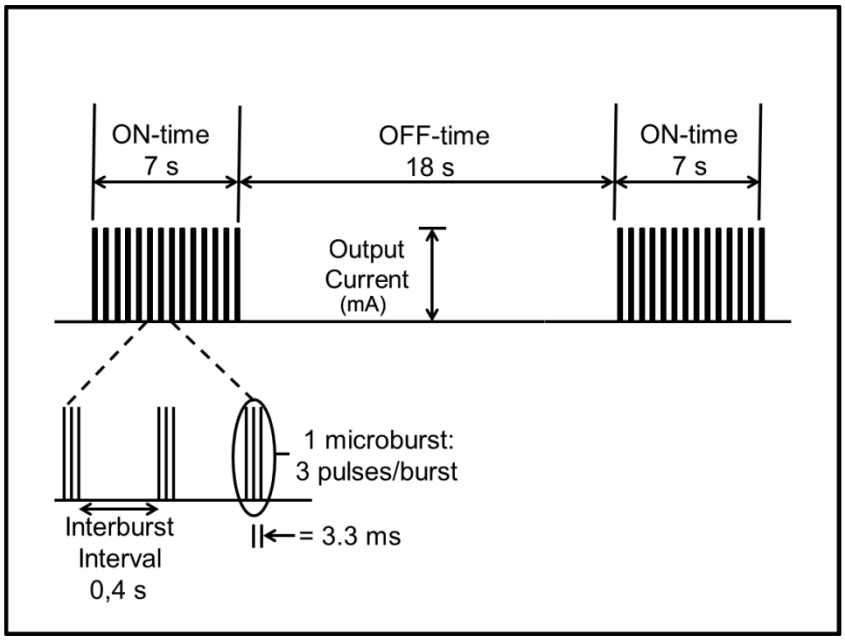

Figure 2. ON/OFF Microburst VNS: Graphical representation of the waveform for intermittent microburst stimulation (s = seconds; ms = milliseconds; mA = milliampere)

Study design

In a randomized, single-blinded cross-over study, Technetium-99m-Ethyl Cysteinate Dimer (^{99m}Tc-ECD) µ-SPECT was performed three times in each dog after sham stimulation, standard and microburst VNS with a mean wash-out period of 20 days (range: 7 - 34 days) between different paradigms. VNS was started 45 min before injection of the tracer and was continued for 10 more minutes after injection. During VNS the dogs were continuously under direct supervision to monitor possible side effects.

Tracer

After 45 min of VNS, ^{99m}Tc-ECD[b] (injected activity: range 742-794 MBq, mean ± SD: 765 ± 13 MBq) was injected IV in the awake dog.

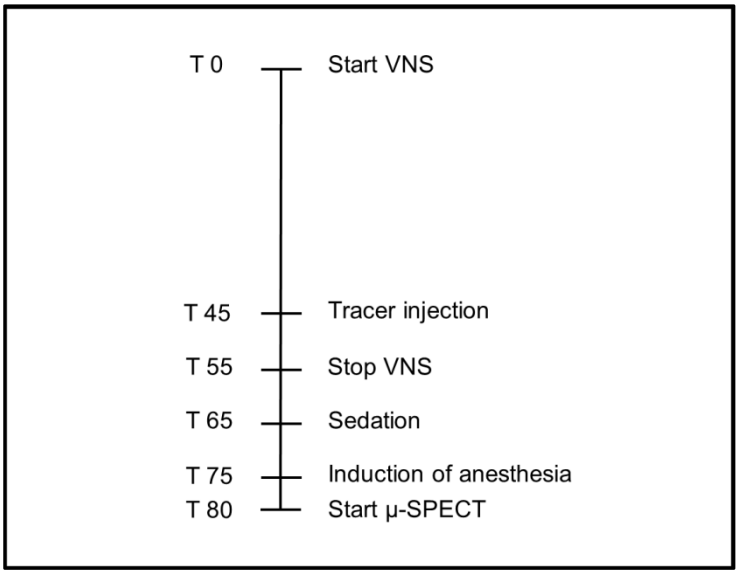

Figure 3: Timeline of the different proceedings during an acute VNS experiment (T x = x minutes)

Anesthetic protocol

Before the start of the experiment a catheter was placed in the cephalic vein and the dogs were allowed to relax for five minutes. The dogs were sedated with butorphanol[c] and general anesthesia was induced with intravenous propofol[d] to effect and maintained, after endotracheal intubation, with isoflurane[e] in oxygen. The timing of sedation, induction and start of the acquisition with respect to the 55 min of VNS is illustrated in Figure 3. There was a time span of 35 min between tracer injection and the start of the acquisition in all dogs, which fits within the ideal time span of 15 to 40 min to obtain optimal SPECT data in the dog (Waelbers et al., 2012). Dogs were allowed to breathe spontaneously during acquisition and heart rate and peripheral hemoglobin saturation (SpO_2%) were monitored using a pulse oximeter. During the acquisition SpO_2 remained above 92% in all subjects.

µ-SPECT imaging

All dogs were positioned in ventral recumbence. Micro-SPECT was performed using a standard triple-head gamma camera[f], equipped with three multi-pinhole collimators[h] (6 multi-focused holes, 3 mm Ø). The radius of rotation (ROR) was set at 21.5 cm. The resolution of the system was 2.3 mm (Dobbeleir and Peremans, 2006). Data were acquired in step-and-shoot mode (10 steps, 36° angular step, 120 s/step) and the total acquisition time was 20 min for each dog. Camera and table positioning were recorded to ensure optimal intra-individual comparison.

Processing protocol and data analysis

Images were reconstructed using a dedicated ordered subset-expectation maximization (OSEM) algorithm (nine iterations, five subsets) (Scivis) and a Butterworth filter was applied (order 5, cut-off frequency 2.5 cycles/cm). The individual dog's perfusion images were automatically registered to a previously created template, generated from the same 10 dogs before implantation of the VNS system using BRASS software (Brain Registration and Automated SPECT Semi-quantification, Nuclear diagnostics). Using this automated registration method, a compensation for size and shape differences was applied. The regional brain activity was quantified based on a predefined region map consisting of 19 volumes of interest (VOIs): left and right frontal, temporal, parietal, piriform and occipital cortical regions (LF, RF, LT, RT, LP, RP, LPi, RPi, LO, RO); left and right thalamus (LTh, RTh), hippocampus (LHi, RHi) and corpus striatum (LCs, RCs);

the cerebellum (CER), the olfactory bulbs (BO) and the brainstem (BS) (Martlé et al., 2013). A routinely used semi-quantification procedure (BRASS, Hermes, Nuclear Diagnostics) was employed to calculate the counts and voxels of every VOI. Perfusion indices (PIs), representing the regional perfusion in every VOI, were calculated by normalizing the counts/voxel of each VOI to the counts/voxel of the whole brain (all VOIs). Additionally, a rostro-caudal gradient, an asymmetry index and a cortical-subcortical index were calculated for each µ-SPECT study, as previously defined (Martlé et al., 2013).

The optimal stimulation currents used for standard and microburst VNS were statistically compared using a paired T-test. Statistical analysis was based on a mixed model with period and condition as fixed effects and dog as random effect. The two VNS conditions (standard and microburst) were compared pairwise with sham using Dunnett's multiple comparisons technique. For these comparisons with control adjusted *P*-values are reported. Global level of significance was set at $P < 0.05$ and data are presented as mean ± standard deviation (SD).

Results

The MRI of the brain was normal in all dogs. During surgical implantation of the VNS device no changes in heart rate and rhythm on ECG or other complications were noted. Testing of the lead impedance of the device was normal during surgery (mean: 972 ± 170 Ohms, normal range: 200-7000 Ohms) and within 48 hours after surgery (mean: 790 ± 62 Ohms) in all dogs. Also, no ECG changes were noticed at that time.

One dog had to be excluded from the study because of the development of an irreversible Horner's syndrome 22 days after the surgery. So eventually, nine dogs participated in the study. Only mild, transient post-operative complications such as seroma formation at the site of surgery (7 dogs) and hoarseness (1 dog) were noticed. All dogs tolerated the VNS very well and no side effects were detected during stimulation.

The stimulation parameters that were used for the VNS were fixed except for the stimulation output current which was individually determined. The optimal output current used for standard VNS ranged between 0.375 and 1 mA (mean ± SD: 0.639 ± 0.190 mA) and for microburst VNS a range between 0.250 and 1 mA (mean ± SD: 0.625 ± 0.220 mA) was used. No significant difference was found between the optimal output currents used in the two different conditions ($P = 0.68$).

Table 1 represents the PIs of the different VOIs for the three stimulation conditions. Acute standard VNS did not cause significant regional brain perfusion alterations. Acute microburst VNS caused a significant hypoperfusion in the left frontal lobe ($P = 0.023$) and in the right parietal lobe ($P = 0.035$). No significant effect of standard or microburst VNS was found on the R-C gradient, AI and Co-SCo index (Table 2).

Table 1: ^{99m}Tc-ECD Perfusion Indices after sham (Sh), standard (St) and microburst (M) VNS in 9 dogs (represented as mean ± SD)

VOI	Sh	St	M
BS	0.61 ± 0.11	0.61 ± 0.11	0.62 ± 0.11
BO	0.74 ± 0.10	0.75 ± 0.08	0.76 ± 0.09
CER	1.00 ± 0.03	1.01 ± 0.06	1.01 ± 0.03
LPi	0.67 ± 0.09	0.65 ± 0.07	0.69 ± 0.07
RPi	0.62 ± 0.08	0.63 ± 0.05	0.63 ± 0.06
LT	1.08 ± 0.03	1.07 ± 0.03	1.07 ± 0.03
RT	1.02 ± 0.04	1.03 ± 0.04	1.02 ± 0.03
LF	1.08 ± 0.04	1.07 ± 0.06	1.06 ± 0.04*
RF	0.98 ± 0.04	1.00 ± 0.03	0.99 ± 0.03
LO	1.20 ± 0.07	1.19 ± 0.06	1.19 ± 0.06
RO	1.20 ± 0.07	1.20 ± 0.05	1.19 ± 0.06
LP	1.25 ± 0.03	1.25 ± 0.03	1.24 ± 0.03
RP	1.23 ± 0.05	1.22 ± 0.02	1.21 ± 0.04*
LHi	0.97 ± 0.06	1.00 ± 0.06	0.97 ± 0.04
RHi	0.95 ± 0.07	0.97 ± 0.06	0.93 ± 0.04
LTh	1.15 ± 0.05	1.14 ± 0.05	1.13 ± 0.04
RTh	1.07 ± 0.05	1.06 ± 0.03	1.07 ± 0.04
LCs	1.13 ± 0.05	1.13 ± 0.04	1.15 ± 0.05
RCs	1.10 ± 0.03	1.12 ± 0.05	1.11 ± 0.06

VOI = volume of interest, BS = brainstem, BO = olfactory bulbs, CER = cerebellum, LPi = left piriform cortex, RPi = right piriform cortex, LT = left temporal cortex, RT = right temporal cortex, LF = left frontal cortex, RF = right frontal cortex, LO = left occipital cortex, RO = right occipital cortex, LP = left parietal cortex, RP = right parietal cortex, LHi = left hippocampus, RHi = right hippocampus, LTh = left thalamus, RTh = right thalamus, LCs = left corpus striatum, RCs = right corpus striatum

* significant difference with sham control, adjusted *P*-value lower than 0.05

Table 2: Rostro-caudal gradients (R-C), asymmetry indices (AI) and cortical-subcortical indices (Co-SCo) after sham (Sh), standard (St) and microburst (M) VNS in 9 dogs (represented as mean ± SD)

	Sh	St	M	*P* St vs. Sh	*P* M vs. Sh
R-C	-7.72 ± 2.53	-7.07 ± 1.90	-7.10 ± 1.97	0.69	0.72
AI	-5.30 ± 2.58	-3.90 ± 2.60	-4.43 ± 3.39	0.35	0.64
Co-SCo	99.40 ± 4.55	98.74 ± 3.67	99.14 ± 3.53	0.73	0.95

Discussion

This ^{99m}Tc-ECD µ-SPECT study investigated the effect of acute standard and microburst VNS on the regional brain perfusion in dogs. A hypoperfusion in the left frontal and right parietal cortex was found after microburst VNS whereas acute standard VNS did not elicit changes in rCBF.

Interestingly, microburst VNS, which could be a promising new stimulation paradigm, seems to have more potency than standard VNS to induce SPECT changes in the brain of normal dogs. The reason to develop and use microburst stimulation is mainly based on the phenomenon of paired-pulse facilitation, meaning that two stimulations of a presynaptic terminal within a short period of time result in a larger evoked synaptic response to the second stimulation (Zucker and Regehr, 2002). In primate studies to map evoked potentials in the thalamus, paired-pulse stimulations of the vagus nerve at 300 Hz frequency produced multi-unit discharges at central vagal targets, particularly within the parafascicular and basal ventromedial nucleus of the thalamus (Ito and Craig, 2005, 2008). Further, in a study to map vagal-evoked single-unit activity in the monkey thalamus, it was reported that the probability of producing an evoked response increased as the number of high frequency stimulation pulses was increased from single, paired to triple stimuli (Hallowitz and MacLean, 1977). Therefore, it can be expected that short bursts of stimulation of the vagus nerve ("microbursts") are more potent to influence central effectors of VNS and could possibly be associated with a stronger antiepileptic effect than standard VNS. A recent study in the rat kindling model suggested that microburst stimulation may indeed be an improved stimulation paradigm (Alexander and McNamara, 2012). Based on our results, further research to investigate the antiepileptic effect of microburst VNS in dogs with spontaneous epilepsy or in canine seizure models is warranted.

Unexpectedly, no significant rCBF alterations were induced using standard VNS parameters. Possibly, the duration of the stimulation was too short, although SPECT or PET changes have been detected even after a shorter period of VNS using standard parameters in rats and humans (Garnett et al., 1992; Henry et al., 1998; Ring et al., 2000; Van Laere et al., 2002, Barnes et al., 2003; Dedeurwaerdere et al., 2005b; Vonck et al., 2008). Still, it would be interesting to assess the chronic effects of VNS on the rCBF in dogs. Although the actual effect of VNS in healthy subjects could be evaluated in this

study, the possible different reaction to VNS between an epileptic and a healthy brain must be taken into account as well. Finally, it also has to be acknowledged that the power of this study could have been too low to show effects of standard VNS.

Several SPECT and PET studies of the brain have already been performed in humans with epilepsy and their general conclusion is that VNS causes changes in anatomic structures that are part of the afferent vagus nerve pathways (Boon et al., 2002; Chae et al., 2003). More specifically, changes in the thalamus and limbic structures are the most consistent findings (Garnett et al., 1992; Ko et al., 1996; Henry et al., 1998, 1999; Ring et al., 2000; Vonck et al., 2000; Van Laere et al., 2002; Barnes et al., 2003; Vonck et al., 2008). However, a lot of disagreement exists regarding other activated structures and the type of change (hypo- or hyperperfusion). Surprisingly, neither standard nor microburst VNS caused significant changes in the thalamic or hippocampal region in dogs. These discrepancies could be due to different confounding factors (Dedeurwaerdere et al., 2005b). An important one is that previous studies in human epileptic patients could not always exclude the effect of seizures or previous resective surgery on SPECT or PET results, with the thalamus and limbic system often involved in seizure activity as well (Henry et al., 1990; Yune et al., 1998; Boon et al., 2002). Also in dogs with idiopathic epilepsy, interictal SPECT changes have been demonstrated, especially in the subcortical, thalamic region (Martlé et al., 2009). Therefore, we decided to evaluate the effect of VNS in healthy dogs that did not suffer from epilepsy or other neurological diseases. A pilot study investigating PET changes induced by VNS in healthy rats described significant changes mainly in the hippocampus of the rats, but the thalamus was not involved (Dedeurwaerdere et al., 2005b). A lot of other factors can be responsible for the variation across different studies such as species differences, differences in methodology and study design in general. In contrast with humans, where the electrodes for VNS are positioned around the vagus nerve, in dogs these electrodes are placed around the vagosympathetic trunk as a whole as in the cervical region both nerves are fused. It cannot be excluded that this additional sympathetic stimulation influenced the results of our study. More specifically, VNS related (stimulation parameters and duration of stimulation) and imaging related factors (imaging modality, type of tracer, image acquisition and analysis) are difficult to compare between different studies as well. Unfortunately, there is not a single consistent way of analyzing functional brain images and very different approaches have been used to process VNS – SPECT/PET data. Some studies used VOI analysis

(Henry et al., 1998, 1999; Ring et al., 2000; Vonck et al., 2000; Van Laere et al. 2002; Vonck et al., 2008), as in our study, but others were based on statistical parametric mapping (SPM) (Garnett et al., 1992; Barnes et al., 2003) or voxel-based analysis (Ko et al., 1996). Finally, previous functional brain imaging studies in humans did not use microburst VNS as a stimulation paradigm, which makes a comparison difficult.

In our study, a significant hypoperfusion was found after acute microburst VNS in two cortical regions (the left frontal and right parietal lobe). Several human studies, investigating the effect of acute VNS on the rCBF in people with epilepsy or depression, detected similar involvement of the frontal and parietal lobes (Henry et al., 1998; Barnes et al., 2003; Conway et al., 2006). VNS induced PET changes in the parietal lobes were also detected by Ko et al. (1998, 1999) and a fMRI study performed during VNS in 4 human patients with intractable partial seizures described an activation of the frontal and parietal structures bilaterally (Sucholeiki et al., 2002). It cannot be concluded from our study if these cortical rCBF changes are involved in the antiepileptic MOA of VNS, but it seems reasonable that suppression of the regional perfusion and so indirectly of the neuronal activity (Roy and Sherrington, 1890) in certain cortical regions could have a seizure suppressing effect.

Conclusion

Acute microburst VNS suppresses the regional perfusion in the frontal and parietal cortex in normal dogs. These findings support further research into the antiepileptic effect of this stimulation paradigm both in humans and dogs.

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CHAPTER 5

THE EFFECT OF VAGUS NERVE STIMULATION ON CSF MONOAMINES AND THE PTZ SEIZURE THRESHOLD IN DOGS

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Sources and manufacturers

[a] Cyberonics, Inc., Houston Texas, USA

[b] Dolorex, MSD AH, Brussels, Belgium

[c] Propovet, Abbott Laboratories, Queensborough, UK

[d] LC Packings/Dionex, The Netherlands

[e] Antec, the Netherlands

[f] Bioanalytical Systems, West Lafayette, USA

[g] Clarity software version 3.0.2, Data Apex, The Czech Republic

[h] Graseby 3400, Smiths Medical, Belgium

[i] Valium, NV Roche, Belgium

[j] Phenoleptil, Le Vet BV, Oudewater, the Netherlands

Summary

Vagus nerve stimulation (VNS) is an established treatment for refractory epilepsy in humans, but the precise mechanism of action (MOA), predictive responsive factors and the optimal stimulation parameters remain to be elucidated.

We aimed to investigate the effect of two VNS paradigms on cerebrospinal fluid (CSF) neurotransmitter levels and on the seizure threshold in the canine pentylenetetrazole (PTZ) model.

Eight Beagle dogs, implanted with a VNS Therapy® System, participated in a cross-over study. Levels of serotonin (5HT), norepinephrine (NE) and dopamine (DA) were quantified in the CSF after one hour of sham, standard and microburst VNS with a wash-out period of 1 month. One week later, the PTZ seizure threshold was determined. As a positive control, the PTZ seizure threshold was determined after a single oral dose of phenobarbital (PB).

Standard and microburst VNS caused a significant increase of NE levels in the CSF (P = 0.03 and P = 0.02 respectively). No significant changes in 5HT or DA levels were detected. Standard and microburst VNS did not cause significant changes in the PTZ seizure threshold compared to sham. PB caused an increase in the PTZ threshold compared to baseline ($P < 0.001$).

VNS induces an increase of NE in the canine brain, which supports previous findings indicating that the MOA of VNS is mediated by the locus coeruleus-norepinephrine (LC/NE) system. Importantly, this study demonstrates that this increase in NE is measurable in the CSF. One hour of VNS did not affect seizure threshold in the canine PTZ model. Therefore, the role of NE in the antiepileptic effect of VNS in dogs remains to be elucidated.

Introduction

Vagus nerve stimulation (VNS) often leads to a considerable reduction in seizure frequency in patients with refractory epilepsy (Ben-Menachem, 2002). However, approximately one third of implanted patients does not respond to even long-term treatment with VNS (DeGiorgio et al., 2000). Since the antiepileptic mechanism of action (MOA) of VNS is not fully understood, no rational hypotheses can be derived to help identify predictive factors for clinical response. Also, the currently applied stimulation parameters are not evidence-based. Further elucidation of the MOA could lead to an improvement of VNS efficacy by identifying early biomarkers for response or biomarkers for optimized stimulation paradigms.

Studies in rats have demonstrated neurotransmitter changes induced by VNS in specific brain structures using microdialysis (Hassert, et al., 2004; Roosevelt et al., 2006; Follesa et al., 2007; Raedt et al., 2011; Manta et al., 2013) and the most consistent finding was a local increase in extracellular norepinephrine (NE) levels of the hippocampus (Roosevelt et al., 2006; Raedt et al., 2011; Manta et al., 2013), amygdala (Hassert et al., 2004) or prefrontal cortex (Follesa et al., 2007; Manta et al., 2013). Other studies measured neuronal firing rates in the LC, a nucleus in the brainstem that sends noradrenergic input to many levels of the central nervous system, to investigate the effect of VNS on the noradrenergic system (Dorr and Debonnel, 2006; Manta et al., 2009). Importantly, it was shown in rats that the LC/NE system is not only activated by VNS, but that its activation and concurrent increased NE levels correlate with the antiepileptic effects of VNS (Krahl et al., 1998; Raedt et al., 2011). Although these studies have revealed important clues about the role of NE in the antiepileptic MOA of VNS, they currently cannot be translated into a clinically applicable strategy in patients to screen for optimal VNS candidates or optimal stimulation parameters. In this context, it is useful to investigate whether VNS induces neurotransmitter changes in the CSF and whether these changes can be correlated with an antiepileptic effect. To examine the relevance of certain neurotransmitter changes as biomarkers, it is important that the antiepileptic effect of VNS is simultaneously evaluated in the same group of animals.

At this moment, the optimal VNS parameters remain unknown and the standard stimulation parameters used for VNS are rather empirical (Fanselow, 2012). There is a

continuous search for more effective stimulation paradigms and in this study, a recently developed experimental stimulation paradigm, called microburst VNS, is evaluated and compared to standard VNS. The rationale of the microburst stimulation is that multiple stimulations within a short time period could induce a more robust synaptic response at central vagal targets (Ito and Craig, 2005, 2008; Alexander and McNamara, 2012). The antiepileptic capability of microburst VNS in humans and dogs has not yet been described.

The aim of this study was (1) to evaluate neurotransmitter changes in the CSF of dogs after one hour of standard and microburst VNS and (2) to assess whether these stimulation paradigms induce changes in the PTZ seizure threshold in the same dogs.

Materials & Methods

Animals

Eight healthy male castrated Beagle dogs, aged between 3 and 3.5 years old, weighing 13 to 18 kg were included in this study. The dogs had no history of neurological or other diseases. They had a VNS Therapy® System[a] implanted in the left cervical region under general anesthesia. This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2011-160). All guidelines for animal welfare, imposed by the Ethical Committee, were respected. The dogs were habituated to the VNS activation/deactivation procedure and to the examination room.

VNS stimulation parameters

For this study, acute VNS was defined as 1 hour of intermittent active stimulation. To achieve adequate duty cycles during this short period of stimulation it was chosen to give rapid-cycling stimulation, meaning an on-time of stimulation of 7 seconds and an off-time of 18 seconds. Two types of active stimulation (standard and microburst VNS) and a sham stimulation were given to each dog. The microburst VNS is an experimental stimulation paradigm where electrical stimuli are delivered in small bursts instead of single pulses as in standard VNS. Stimulation parameters used for the standard VNS included a pulse width of 500 µs and a frequency of 30 Hz. Stimulation parameters used for microburst VNS included a pulse width of 500 µs, a frequency of 300 Hz, 3 pulses/burst and an inter-burst interval of 0.4 s. The stimulation output current was individually determined during a ramping-up procedure (with increments of 0.125 mA) and was defined as the highest current for which no cough was elicited, similar as in a previous canine VNS study (Muñana et al., 2002). This output current was checked on a weekly basis and adapted when necessary. For the sham stimulation the animals underwent the same procedures as with active VNS stimulation except that the output current was set at 0 mA.

Experimental design (Figure 1)

In a first cross-over study, three cisternal CSF samples were obtained from each dog following three different acute VNS paradigms (one hour of sham, standard or microburst VNS). One week after each CSF tap, a PTZ experiment was performed following the same acute stimulation paradigm. The order of stimulation paradigms was randomized in

each dog and blinded for the persons evaluating the PTZ experiments. A wash-out period of 1 month was respected between consecutive CSF taps and between successive PTZ experiments. The VNS was administered to awake and freely moving dogs.

In a second cross-over experiment and as a positive control of our canine PTZ model we determined the PTZ threshold after a single oral dose of PB (20 mg/kg) and compared it to the baseline PTZ threshold in each dog.

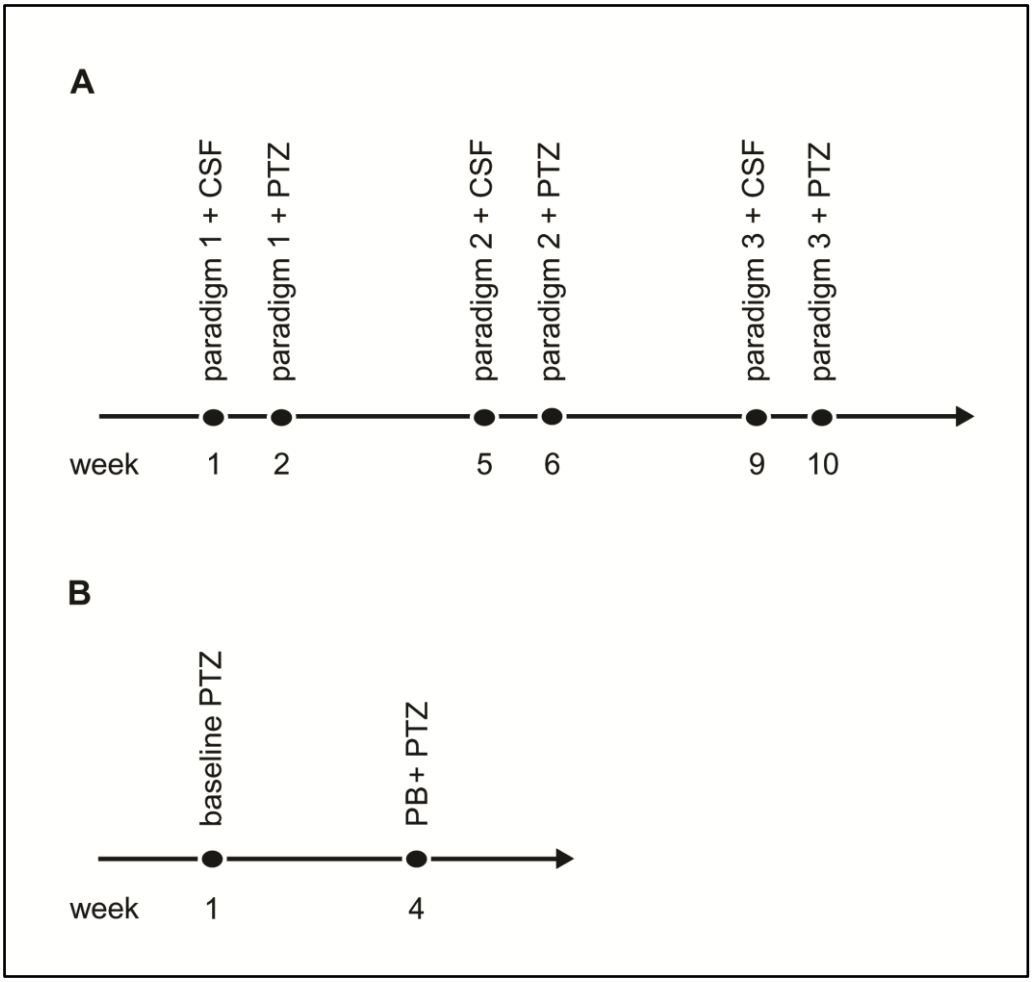

Figure 1: Timeline to demonstrate the experimental design of the VNS cross-over study (A) and the phenobarbital (PB) cross-over study (B). The order of the 3 different stimulation paradigms (sham, standard and microburst VNS) was randomized in each dog. Also, the order of baseline or post-PB PTZ threshold determination was randomized in each dog

VNS – CSF experiment

Before the start of the experiment a catheter was placed in the cephalic vein and the dogs were allowed to relax for five minutes. Then, the VNS Therapy System was activated (standard or microburst VNS) or pseudo-activated (sham stimulation) for 60 minutes. During this time the dogs were awake, able to move around in the examination room and under direct supervision. After one hour, the VNS was stopped and the dogs were sedated with butorphanol[b] (0.3 mg/kg IV). Ten minutes later general anesthesia was induced with intravenous propofol[c] (2-6 mg/kg to effect) and a CSF tap was performed at the cerebellomedullary cistern with the dog positioned in right lateral recumbence.

CSF analysis

An antioxidant solution (50 µl) containing 100 mM acetic acid, 3.3 mM L-cysteine, 0.27 mM disodium edetate and 12.5 µM ascorbic acid was added to the CSF (200 µl) to preserve the monoamines. All samples were stored at – 80°C until liquid chromatography (LChr) analysis.

The LChr system consisted of a temperature-controlled (15°C) FAMOS autosampler[d] with a sample loop injection volume of 10 µL, a Dionex DEGASYS DG-1210 degasser, a 307 Gilson piston pump and a DECADE II electrochemical detector with a µ-VT03 flow cell (0.7 mm glassy carbon working electrode, 25 µm spacer)[e]. The detection potential was set at + 450 mV versus a Ag/AgCl reference electrode. The mobile phase was a mixture of 89% V/V aqueous buffer solution (100 mM sodium acetate trihydrate, 20 mM citric acid monohydrate, 2 mM sodium decanesulfonate, 0.5 mM disodium edentate, pH 5.5) and 11% V/V acetonitrile. The flow rate of the mobile phase was set at 60 µL/min. The stationary phase consisted of a microbore UniJet C8 column (100 x 1.0 mm, 5 µm)[f] on which separation of the monoamines was performed at 35°C. Data acquisition was carried out by specific software[g].

VNS – PTZ experiment

One week later and similarly as for the CSF experiments, a catheter was placed in the cephalic vein and the dogs were allowed to relax for five minutes before the start of the experiment. Then, the VNS Therapy System was activated (standard or microburst VNS) or pseudo-activated (sham stimulation). During VNS, the dogs were awake, able to move

around in the examination room and under direct supervision. After 55 minutes of stimulation, the dogs were placed on a table and an IV line for PTZ infusion was connected to the catheter. After 60 minutes of VNS, an infusion of 3% PTZ in 0.9% NaCl was started and delivered at a constant rate of 3 ml/min using a syringe pump[h], following a previously described protocol (Löscher et al., 2004). At the moment of the first whole body myoclonic twitch, the infusion was immediately stopped and a diazepam[i] bolus (1 mg/kg IV) was administered to avoid generalization or repetition of the myoclonic twitch. However, when generalization of the seizure occurred, it was controlled with a bolus of propofol (2-3 mg/kg IV). VNS continued during the PTZ infusion and was stopped after the administration of the diazepam bolus. The dogs were monitored for additional seizures the first 24 hours after each PTZ experiment.

Phenobarbital – PTZ experiment

An additional cross-over positive control experiment was performed in which the PTZ threshold was determined after a single oral dose of PB[j] and compared with the baseline PTZ threshold (without PB). In each dog, both PTZ threshold determinations were performed in a randomized order with a wash-out period of three weeks to ensure sufficient clearance of PB. PB was administered orally (20 mg/kg) and 3 hours post dosage administration (= approximate t_{max}) a blood sample for serum concentration monitoring was taken and the PTZ infusion was started. At the moment of the first whole body myoclonic twitch the PTZ infusion was stopped and a diazepam bolus (0.2 mg/kg IV) was administered.

PTZ threshold determination

For each PTZ experiment, the PTZ threshold was defined as the amount of PTZ (in mg/kg) required to provoke the first whole body myoclonic twitch. The exact moment of the first whole body myoclonic twitch after the start of the infusion was determined by post-experimental video analysis by two blinded observers. As a fixed concentration and infusion rate of PTZ was used, the threshold dose of PTZ could then be calculated.

Statistical analysis

The output currents used for standard and microburst VNS were statistically compared using a paired T-test. The different stimulation paradigms were compared with respect to CSF and PTZ results using a mixed model with period and stimulation paradigm as fixed

effects and dog as random effect and were tested at the 5% significance level. CSF and PTZ results for the two VNS conditions (standard and microburst) were compared pairwise with sham using Dunnett's multiple comparisons technique and adjusted *P*-values for these multiple comparisons are reported.

Results

No obvious side effects of the VNS were noticed. In one dog, muscle tremors or spasms of the left front limb were noticed during the on-time of stimulation. The output current used for standard VNS ranged between 0.250 and 1.250 mA (mean ± SD: 0.516 ± 0.289 mA) and for microburst VNS a range between 0.250 and 1.125 mA (mean ± SD: 0.500 ± 0.250 mA) was used. No significant difference was found between the output currents used in the two different conditions (P = 0.35).

The results for the CSF neurotransmitter levels are presented in Table 1. Both standard and microburst VNS caused a significant increase of NE levels in the CSF (p = 0.03 and p = 0.02, respectively) (Figure 2A). This increase was noticed in six dogs (75%) and a mean increase in NE of 45% and 44% was achieved after standard and microburst VNS respectively. In the dog where the muscle tremors of the left thoracic limb were noticed during VNS, NE levels were not increased. No significant alterations in DA or 5HT levels were detected in the CSF after acute standard or microburst VNS.

Table 1: Mean (± SD) CSF monoamine concentrations (in nM) after the 3 different stimulation paradigms (n=8)

Monoamine	Sham	Standard	Microburst
DA	0.143 (± 0.078)	0.190 (± 0.135)	0.222 (± 0.170)
NE	0.366 (± 0.096)	0.499 (± 0.125)*	0.481 (± 0.148)*
5HT	0.744 (± 0.398)	0.947 (± 0.972)	0.604 (± 0.155)

SD = standard deviation, nM = nanomolair, DA = dopamine, NE = norepinephrine, 5HT = serotonin; * significant difference with sham control, adjusted P-value lower than 0.05

In the PTZ experiment, the appearance of the first clinically observable whole body myoclonic twitch was similar within each dog. The PTZ thresholds were not significantly different after acute standard or microburst VNS compared to sham (Figure 2B). The PTZ threshold after a single oral dose of PB was significantly increased compared to the baseline PTZ threshold (P < 0.001) with a mean increase of 63% (Figure 3). The serum

concentration of PB was situated within the therapeutic range (20–45 µg/ml, (Farnbach, 1984)) in all dogs. Secondary generalization of the myoclonic twitch was noticed during 5 of the 24 VNS – PTZ experiments in 3 dogs and during 4 of the 16 PB – PTZ experiments in 3 dogs. The 3 dogs experiencing a generalized seizure were not the same animals each time except for one dog whose whole body myoclonic twitch evolved into a generalized tonic-clonic seizure in each PTZ experiment. No additional seizures were seen during the 24 h monitoring period after each experiment.

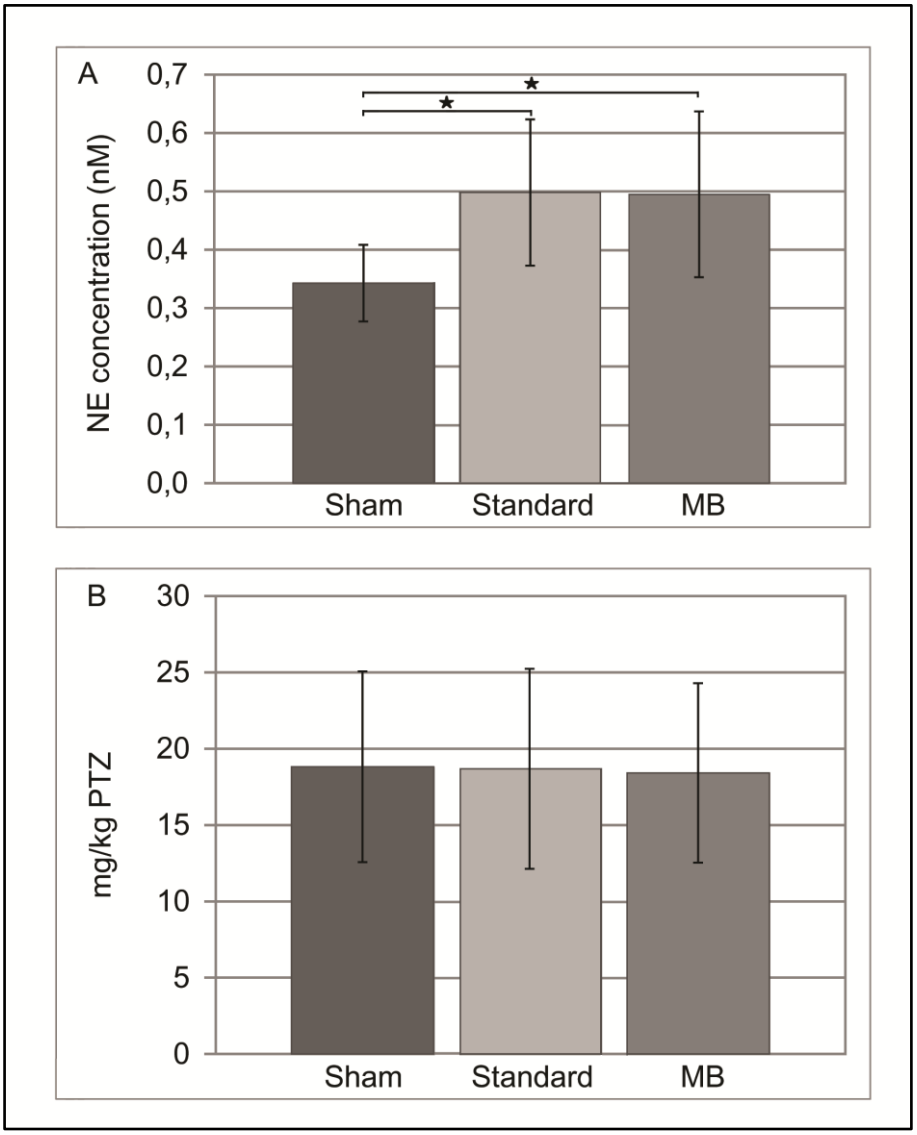

Figure 2: Graphical representation of the CSF NE levels (nM) (mean ± SD) (A) and the PTZ seizure thresholds (mean ± SD) (B) after acute sham, standard and microburst VNS

★Significant difference with sham control ($P < 0.05$)

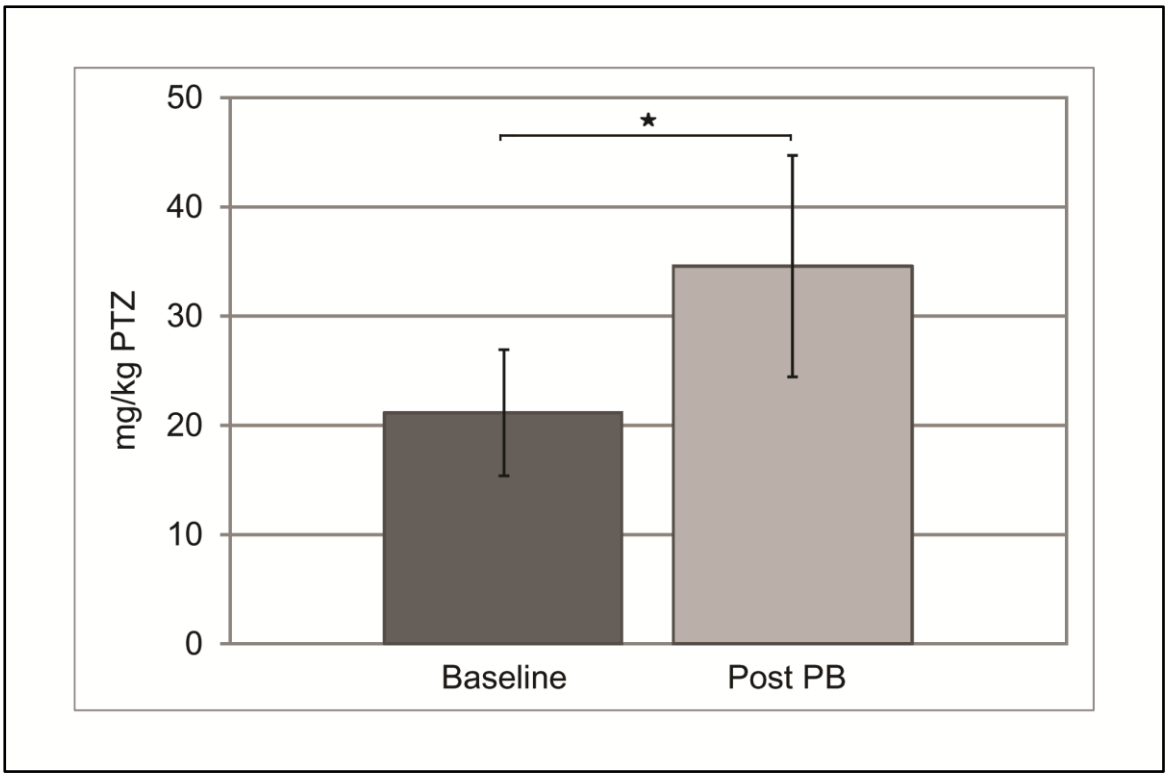

Figure 3: Graphical representation of the PTZ seizure threshold (mean ± SD) after PB administration (post PB) compared to the baseline threshold.

*Significant difference with sham control ($P < 0.05$)

Discussion

The first objective of our study was to assess whether acute VNS results in neurotransmitter changes measurable in the CSF of a dog model. Dogs were used in this study, since the use of this animal model for translational research may have certain advantages. First of all, the collection of CSF in dogs is a standard procedure with few complications when performed by an experienced veterinary neurologist (Cook and DeNicola, 1988). Although CSF can be collected in rodents as well, it leads more often to blood contamination, neurological complications and only a small amount of fluid can be collected compared to dogs (Pegg et al., 2010). Secondly, the prevalence and semiology of naturally occurring canine epilepsy are comparable to human epilepsy (Chandler, 2006) which renders translational research for new epilepsy treatments in a canine model justified. Also from a veterinary point of view, research towards non-pharmacological treatments for refractory canine epilepsy is needed (Martlé et al., 2014, Muñana et al., 2013). Finally, the canine PTZ model, in which a myoclonic twitch is induced in healthy dogs by intravenous infusion of the chemical convulsant pentylenetetrazole has been well described previously (Löscher et al., 2004, 2013) and the same protocol was used in this study.

In this study, a significant increase of NE was detected in the canine CSF after one hour of VNS. Also in rats VNS activates the LC/NE system (Hassert, et al., 2004; Dorr and Debonnel, 2006; Roosevelt et al., 2006; Follesa et al., 2007; Manta et al., 2009; Raedt et al., 2011; Manta et al., 2013). Activation of the LC/NE system has a crucial role in the seizure-suppressing effect of VNS (Krahl et al., 1998; Raedt et al., 2011). In a rat model for limbic seizures, VNS-induced increase of hippocampal NE levels is a biomarker for the antiepileptic efficacy of VNS (Raedt et al., 2011). Despite this valuable information, these studies used microdialysis or extracellular unitary recordings in specific brain structures. However, these invasive techniques are not practical to use in patients as a screening tool for responsiveness to VNS. Collection of CSF on the other hand is a less invasive and standard procedure which could be performed in potential VNS candidates after a short period of, for example, external stimulation of the vagus nerve (Stefan et al., 2012). Until now, only little is known about the influence of VNS on CSF neurotransmitters. Two studies evaluated the effect of chronic VNS on certain CSF metabolites in human epileptic patients (Hammond et al., 1992; Ben-Menachem et al.,

1995). However, NE levels were not measured in these studies. To our knowledge, NE levels in the CSF were only investigated in depressed patients before and after two months of VNS and no significant alteration was demonstrated (Carpenter et al., 2004). As our study demonstrates that VNS induces an increase of NE detectable in the CSF of a dog model, it supports also further research towards non-invasive biomarkers of NE increase in humans. Recently, it was shown that VNS induces an increase of the P300 amplitude, a non-invasive marker of LC/NE activity in humans and that this increase was correlated with a positive response to therapy (De Taeye et al., 2014).

A second important aim of this study was to assess the antiepileptic effects of acute VNS in the same canine subjects. However, no significant increases of the PTZ seizure threshold after one hour of standard or microburst VNS could be demonstrated in this canine PTZ model. Our model was validated by a positive control test with PB, a clinically established antiepileptic drug in dogs (Farnbach, 1984). A significant increase of the PTZ threshold of 63% was found in our dogs after a single oral dose of PB (20 mg/kg) compared to baseline, which is in correspondence with the increase of 83% detected by Löscher et al. (2013).

One could argue that VNS is possibly not able to exert anticonvulsive effects against PTZ-induced seizures. Nevertheless, Zabara (1992) has shown an acute abortive effect of VNS on PTZ induced convulsions in two dogs and in the rat PTZ model a positive effect of VNS on seizure severity has been demonstrated by several research groups (Takaya et al., 1996; Krahl et al., 2001, 2003; Zhang et al., 2008; Sahin et al., 2009).

In our study, the prophylactic anticonvulsive property of one hour of VNS was evaluated; this timing was based on a rat PTZ study where one hour of VNS was sufficient to induce antiepileptic effects (Takaya et al., 1996). To increase the number of pulses delivered to the nerve during this short period of stimulation, we decided to use a rapid-cycling stimulation protocol instead of the 5 minutes off – 30 seconds on intermittent stimulation most often used in a clinical setting. Despite the use of this stimulation protocol, one hour of VNS may have been too short to lead to an antiepileptic effect in dogs, certainly because it is known from human studies that the effectiveness of VNS improves over time (Morris and Mueller, 1999; Vonck et al., 1999; Ben-Menachem, 2002; Boon et al., 2002).

It would be interesting to evaluate the antiepileptic effects of chronic VNS and to see whether correlations exist with acute changes in NE CSF levels within the same subjects.

It is also possible that VNS could be less effective in dogs than in humans. Studies investigating the antiepileptic effect of VNS in dogs are limited and performed on a small number of dogs (Zabara, 1992; Speciale and Stahlbrodt, 1999; Muñana et al., 2002), however, they have shown some antiepileptic effectiveness. Zabara (1992) demonstrated an acute abortive effect on chemically induced seizures in dogs. Furthermore, some dogs with spontaneous refractory epilepsy, implanted with a similar VNS device as in humans, seemed to respond to VNS (Muñana et al., 2002).

In our study, it was demonstrated that microburst VNS, a recently developed experimental stimulation paradigm, is as potent as standard VNS to induce an increase in NE levels in the canine CSF. The rationale of microburst stimulation is mainly based on the phenomenon of paired-pulse facilitation, meaning that two stimulations of a presynaptic terminal within a short period of time result in a larger evoked synaptic response to the second stimulation (Zucker and Regehr, 2002). Hence, it has been shown that multiple stimulations of the vagus nerve within a short period of time evoked a larger response at central vagal targets (Ito and Craig, 2005, 2008). Therefore, it can be expected that short bursts of stimulation of the vagus nerve ("microbursts") are more potent in affecting central mediators of VNS and could possibly be associated with a stronger antiepileptic effect compared to standard VNS. A recent study in the rat kindling model suggested that microburst stimulation may indeed represent an improved stimulation paradigm (Alexander and McNamara, 2012). In our study, the effects of microburst and standard VNS on CSF neurotransmitter levels and PTZ thresholds are comparable.

Conclusion

To the author's knowledge, this is the first study describing a significant increase in NE in the canine CSF after two different VNS paradigms, which further supports that the LC-NE system is involved in the MOA of VNS. However, no immediate antiepileptic effect of acute VNS could be demonstrated in this canine PTZ model. Considering the important protective role of NE in seizures, further research on this VNS induced NE increase in the CSF is recommended, both in dogs and humans.

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CHAPTER 6

EVALUATION OF HEART RATE VARIABILITY IN DOGS DURING STANDARD AND MICROBURST VAGUS NERVE STIMULATION

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Sources and manufacturers

[a] Holtersoft Ultima Software version 2.5.4 (Novacor SA)

Summary

Vagus nerve stimulation (VNS) is an established treatment for epilepsy and depression in human patients. Both in humans and dogs, the optimal stimulation parameters remain unknown. Delivering afferent bursts of stimulation may be promising to increase efficacy, but requires evaluation of potential effects on the heart due to unavoidable efferent stimulation. The present study investigated heart rate variability in healthy Beagle dogs treated with 1 hour of sham, standard or microburst left-sided VNS in a cross-over design. No significant differences were found between the stimulation paradigms for any of the cardiac parameters. Short-term left-sided VNS, including a novel bursting pattern (microburst VNS), had no effect on the heart rhythm in ambulatory healthy dogs. Studies in a larger number of animals with long-term VNS are recommended.

Introduction

VNS is an effective treatment for human epilepsy and depression with the vagus nerve providing a unique entrance to the brain (Beekwilder and Beems, 2010). The optimal stimulation parameters for VNS are unknown (Ben-Menachem, 2012). There is a continuous search for more effective stimulation paradigms and in this study, the influence on the heart of a recently developed experimental stimulation paradigm, called microburst VNS, is evaluated and compared to standard VNS in dogs.

This study is part of a research project investigating the effects of VNS on the canine brain. The primary aim of this study was to screen for possible cardiac side effects of acute VNS. The initial goal was not to affect the heart, so therefore the VNS system was implanted on the left side with the electrode contacts placed in an orientation that primarily aims at activating afferent cervical vagal fibers, required for central nervous system effects in treating disorders like epilepsy.

Materials & Methods

Nine Beagle dogs, aged between 1.5 and 2 years, implanted with a VNS Therapy® System in the left cervical region participated in this randomized, sham-controlled, cross-over study. Holter monitoring was performed during 55 minutes of sham, standard and microburst VNS in each dog. Stimulation parameters used for both VNS paradigms are summarized in Table 1. The optimal stimulation output current was individually determined during a ramping-up procedure and was set subthreshold for coughing. To achieve maximum stimulation on-time during the 55 minutes of VNS, the rapid duty cycle of the VNS therapy system (7s on and 18s off time) was used. In the sham stimulation condition, the animals underwent similar procedures except that the output current was set at 0 mA.

Table 1: The stimulation parameters used in this study for intermittent standard and microburst VNS

Standard VNS		**Microburst VNS**	
Signal frequency (Hz)	30	Signal frequency (Hz)	300
Pulse duration (μs)	500	Pulse duration (μs)	500
Signal on-time (s)	7	Number of pulses/burst	3
Signal off-time (s)	18	Interburst interval (s)	0.4
		Burst on-time (s)	7
		Burst off-time (s)	18

The Holter data were processed using specific software[a]. Since the dogs were handled in the beginning and at the end of the VNS session, the initial and final 10 minutes were not used for statistical analysis to avoid interference of stress or manipulation. So, for each experiment 35 minutes, corresponding to 84 duty cycles of VNS, were analyzed. The following parameters were determined: number of QRS, mean NN (mean normal to normal interval), SDNN (standard deviation of all normal to normal intervals), SDANN (standard deviation of the averages of NN intervals), rMSSD (square root of the mean of the sum of the squares of differences between adjacent NN intervals), pNN50 (numbers of pairs of adjacent NN intervals differing by more than 50 ms in the entire recording divided by the total number of all NN intervals), SDNNIDX (the mean of the standard

deviations of NN intervals of all 5 min segments in the entire recording), VLF Power (very low frequency power, < 0.04 Hz), LF Power (low frequency power, 0.04 – 0.15 Hz), HF Power (high frequency power, 0.15 – 0.4 Hz). The low frequency/high frequency ratio (LF/HF) was used to quantify the sympathovagal balance as an indicator of autonomic modulation. A mixed model analysis was performed and Bonferroni correction was applied.

Results

The Holter parameters during the 3 different stimulation paradigms are represented as a mean ± SD in Table 2. No significant differences were detected for any of the parameters between sham, standard or microburst VNS.

Table 2: Results of the different Holter parameters (represented as mean ± SD) during the 3 stimulation paradigms (n=9). Also, the *P*-values of the pairwise comparison with sham stimulation are reported.

Holter parameter	Sham	Standard	Microburst	*P* (St vs Sh)	*P* (MB vs Sh)
# QRS	3801 ± 632	3717 ± 472	3450 ± 461	1.00	0.18
meanNN (ms)	567 ± 79	574 ± 77	619 ± 87	1.00	0.20
SDNN (ms)	130 ± 42	151 ± 56	176 ± 71	1.00	0.11
SDANN (ms)	41 ± 22	60 ± 38	75 ± 40	0.88	0.06
SDNNIDX (ms)	119 ± 73	119 ± 29	159 ± 114	1.00	1.00
rMSSD (ms)	92 ± 54	109 ± 57	132 ± 57	1.00	0.17
pNN50 (%)	35 ± 19	38 ± 16	45 ± 15	1.00	0.22
VLF Power (ms^2)	6956 ± 4264	14336 ± 11976	15904 ± 11391	0.31	0.12
LF Power (ms^2)	4323 ± 4509	5413 ± 5271	9291 ± 14064	1.00	1.00
HF Power (ms^2)	4420 ± 3728	8629 ± 7926	8563 ± 6030	0.27	0.29
LF/HF	1.1 ± 0.6	0.9 ± 0.5	1.0 ± 0.8	1.00	1.00

St vs Sh: standard versus sham pairwise comparison; MB vs Sh: microburst versus sham pairwise comparison; # QRS: number of QRS; meanNN: mean NN interval; SDNN: standard deviation of all normal to normal intervals; SDANN: standard deviation of the averages of NN intervals; SDNNIDX: the mean of the standard deviations of NN intervals of all 5 min segments in the entire recording; rMSSD: square root of the mean of the sum of the squares of differences between adjacent NN intervals; pNN50: numbers of pairs of adjacent NN intervals differing by more than 50 ms in the entire recording divided by the total number of all NN intervals; VLF Power: very low frequency power; LF Power: low frequency power; HF Power: high frequency power; LF/HF: low frequency/high frequency ratio; ms: milliseconds

Discussion

Acute left-sided standard and microburst VNS did not affect the heart rate variability in this group of healthy awake dogs. Given the important relationship of the vagus nerve to cardiac function, the potential occurrence of cardiac arrhythmias was a serious initial concern when using VNS to treat human refractory epilepsy (Asconape et al., 1999). Despite this initial concern, clinically relevant cardiac side effects are rarely noticed during left-sided VNS in humans. To avoid cardiac complications the left vagus nerve is usually chosen as it has less cardiac influence than the right nerve in humans and dogs (Kahlow and Olivecrona, 2013; Kamath et al., 1992; Munana et al., 2002; Spuck et al., 2008). This can be explained by an asymmetrical vagal innervation of the heart: the right vagus nerve is more associated with the cardiac atria and innervates the sinoatrial node, whereas the left vagus nerve is more associated with the ventricles and innervates the atrioventricular node (Groves and Brown, 2005). Also, VNS therapy electrode contacts are placed in an orientation (negative pole rostral) that primarily activates afferent cervical vagal fibres. Furthermore, electrodes are placed at a location distally from the branching off of efferent cardiac vagal branches in humans in order to avoid stimulation of these fibres during VNS. However, it is impossible to completely avoid activation of efferent fibres, vagal cardiac branching may differ individually and bradycardia and asystole have been described anecdotally, mainly during the intraoperative testing of the device where anaesthesia appeared to play a major role. (Asconape et al., 1999; Schwartz, 2011).

Important anatomical differences exist between the human and canine cervical vagus nerve which, in theory, could also lead to differences in the cardiac influence of VNS. In dogs, the vagus nerve is fused with the sympathetic nerve in the cervical region, so the electrodes are wrapped around the vagosympathetic trunk. Furthermore, the cardiac branches leave the vagus nerve more distally in the thoracic region in dogs, so it is impossible to spare these branches when the electrodes are placed around the nerve (Muñana et al., 2002). One study reported intraoperative bradycardia in 30% of dogs (Muñana et al., 2002) which seemed quite high, but no cardiac side effects were reported in the non-anaesthetized dogs which is in agreement with our findings. A comparison of different stimulation protocols was not performed in this previous study.

The influence of microburst stimulation, a recently developed stimulation paradigm, on the heart has not been investigated previously. The rationale of the microburst stimulation is that multiple stimulations within a short time period could induce a more robust synaptic response at central vagal targets (Alexander and McNamara, 2012; Ito and Craig, 2005, 2008). So, the possibility exists that this could also lead to a greater effect on the heart compared to standard VNS. No clinically relevant cardiac effects were found in our study that should be considered as a pilot trial with a relatively low number of dogs and short-term stimulation.

Conclusion

Short-term left-sided standard and microburst VNS had no effect on the heart rhythm of healthy Beagle dogs, although larger and long-term VNS studies are warranted.

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General Discussion

A major evolution occurred in both the diagnosis and therapy of human epilepsy during the past decades. The introduction of functional imaging modalities such as Single Photon Emission Computed Tomography (SPECT) and new therapeutic options such as vagus nerve stimulation (VNS) were important milestones. Despite this, the diagnostic accuracy of and therapeutic options for canine epilepsy remain limited. A better understanding of the pathophysiology of canine epilepsy could further improve the classification of this common disorder, and lead to the optimization of existing and the development of new therapeutic options. Furthermore, investigating canine epilepsy is not only useful from a veterinary point of view, but the epileptic dog can also serve as a translational animal model for human epilepsy (Löscher, 1997).

This research project tries to address both diagnostic and therapeutic limitations of canine epilepsy. Therefore, it consisted of two parts addressing different research questions. In the **first part**, we investigated whether Single Photon Emission Computed Tomography (SPECT) could be a feasible technique to detect cerebral perfusion alterations in dogs with idiopathic epilepsy and if these alterations could be related to the origin of seizures. Furthermore, an attempt was made to improve the resolution of brain SPECT in dogs. In the **second part**, the effect of vagus nerve stimulation (VNS) on the canine brain was investigated in healthy Beagle dogs. Functional brain imaging, neurochemical and seizure-suppressing effects of VNS were examined in a canine model. Furthermore, a new burst type of stimulation (microburst) was compared with standard VNS.

1. SPECT and canine idiopathic epilepsy

In human medicine, functional brain imaging techniques can detect epileptogenic foci in morphologically unremarkable areas, and they are less invasive than intracranial electroencephalography (EEG) evaluation. Furthermore, these techniques can improve the understanding of the pathophysiology of epilepsy, as their unique ability to image *in vivo* changes in the brain has provided valuable insights into the mechanisms of epileptogenesis and seizure propagation in humans (la Fougère et al., 2009). Elucidating the neurobiology of canine epilepsy is essential to obtain a species specific, detailed classification system and an expansion of treatment options.

Following this rationale, regional brain perfusion changes in dogs with idiopathic epilepsy were investigated in the **first chapter** of this research project. We concluded that regional brain perfusion in the subcortical region was suppressed interictally in dogs with idiopathic epilepsy (n = 12). However, it was impossible to visualize more detailed structures within this subcortical volume of interest (VOI) due to resolution limits of conventional SPECT. A considerable part of this subcortical area consists of the thalamus, a structure that may play an important role in the pathophysiology of canine idiopathic epilepsy. The thalamus can be involved in the origin of seizures, according to the centrencephalic theory, which proposes that most generalized seizure activity originates in deep structures of the thalamus and brainstem and is projected to the cortex thereafter (Russo, 1981). Also in humans, the thalamus has a primary role in the initiation and propagation of seizures in several types of epilepsy (Spiegel and Wycis, 1950; Williams, 1953; Henry et al., 1990; Prevett et al., 1995; Yune et al., 1998). The hypoperfusion in the thalamic region found in the dogs of our study could have been a consequence of seizures through the corticothalamic diaschisis phenomenon, meaning that the subcortical hypoperfusion results from an interrupted afferent axonal supply from the epileptogenic cortex (Yune et al., 1998).

Interestingly, the thalamus is also involved in the mechanism of action (MOA) of different neurostimulation treatments for human refractory epilepsy. It is an important target of deep brain stimulation in humans where electrical stimulation of the anterior nucleus of the thalamus has beneficial seizure suppressing effects (Fisher, 2013). Furthermore, the thalamus is involved in the antiepileptic MOA of VNS in humans (Henry et al., 1999; Vonck et al., 2008). In the **fourth chapter** of this PhD thesis, rCBF in the thalamus was not altered by a short period of VNS in healthy Beagle dogs, so the role of the thalamus in the MOA of VNS in dogs remains uncertain.

In humans, interictal SPECT can be useful for localization of the epileptogenic focus (Yune et al., 1998), but is often part of a multimodality work-up. The epileptogenic focus usually appears as an area of hypoperfusion on interictal SPECT, as an area of hyperperfusion on ictal SPECT and as an area of hypometabolization on interictal PET, but the sensitivity between these modalities differs. Ictal SPECT clearly has a higher sensitivity than interictal SPECT (Devous et al., 1998; Spanaki et al., 1999; Weil et al., 2001) and the sensitivity of interictal PET is higher than that of interictal SPECT (Spencer, 1994). As part of a multimodality work-up, it would have been useful to

investigate ictal SPECT changes in dogs with idiopathic epilepsy. However, ictal SPECT studies are difficult to perform as they require the injection of the tracer immediately at the start of a seizure (Menzel et al., 1997; Van Paesschen et al., 2007). As seizures in dogs tend to generalize rapidly (Berendt and Gram, 1999), a true image from the start of the seizure would be difficult to obtain. Furthermore, a specialized monitoring unit with nuclear tracer available onsite is often difficult to achieve in veterinary medicine.

In the **second chapter** we demonstrated that micro-SPECT (µ-SPECT) is a feasible technique to investigate the regional brain perfusion of healthy Beagle dogs and that a substantial improvement of resolution could be obtained using this technique compared to conventional SPECT (2.3 mm instead of 8 mm) (Peremans et al., 2001). Also, a new detailed VOI map based on Magnetic Resonance Imaging (MRI) was created and allowed the evaluation of regional cerebral blood flow (rCBF) in 19 different regions, including important subcortical structures. Interestingly, a general hemispherical and regional asymmetry in brain perfusion towards the left was demonstrated in the temporal, parietal and frontal cortex of our healthy Beagle dogs. These changes have not been demonstrated previously in adult dogs using conventional SPECT (Peremans et al., 2001; Waelbers et al., 2011). Asymmetries in hemispheric perfusion have also been detected in healthy people using conventional SPECT and in cats using µ-SPECT (Catafau et al., 1996; Krausz et al., 1998; Lobaugh et al., 2000; Van Laere et al., 2001; Waelbers et al., 2013). In humans, they have been attributed to volumetric and possibly also functional differences (Catafau et al., 1996). It is important for future µ-SPECT studies to acknowledge the fact that asymmetries in the canine brain can be normal, because in cerebral diseases (e.g. epilepsy), the identification of rCBF abnormalities has been based on the detection of interhemispheric asymmetries (Catafau, 2001; Aubert-Broche et al., 2003; Vermeire et al., 2009a; McArthur et al., 2011).

Methodological considerations and future perspectives

The number of dogs with idiopathic epilepsy investigated in the **first chapter** was rather small, especially since their demographics and clinical variables were heterogeneous. Most dogs received antiepileptic drugs (AEDs) at the moment of the SPECT study, which is inevitably a possible confounding factor in many human SPECT studies as well.

Unfortunately, the exact influence of different AEDs on brain perfusion and metabolism remains largely unknown. Phenobarbital (PB) and phenytoin can cause diffuse cerebral hypoperfusion in humans (Jibiki et al., 1993). Therefore, in our study, a semiquantitative analysis of the SPECT data was performed by normalizing the regional brain perfusion to the total counts of the brain. Hence, the possible confounding effects of AEDs on global brain perfusion should be minimized. There is a need for further studies to evaluate the influence of AEDs on regional brain perfusion in dogs and humans.

Concerning the multi-modality work-up, it would have been useful to include interictal EEG in our dogs with idiopathic epilepsy (**chapter 1**), as EEG can investigate the epileptic nature of detected SPECT abnormalities. Recently, interictal scalp EEG was used to investigate the epileptic nature of PET abnormalities in epileptic Lagotto Romagnolo and Finnish Spitz dogs (Jokinen et al., 2013; Viitmaa et al., 2014). However, the use of scalp EEG in dogs is associated with serious limitations such as muscle artifacts and the need for sedation (Russo, 1981). Furthermore, interictal scalp EEG seems to have a low sensitivity to detect seizure activity in dogs with epilepsy and is not considered a useful screening method (Brauer et al., 2012; Pakozdy et al., 2012). Intracranial EEG recording remains the gold standard in humans for localization of the epileptogenic focus, but requires the surgical implantation of electrodes. Recently, some progress has been made by investigating the use of wireless implantable EEG devices in dogs, but further research will be needed (Davis et al., 2011). For the aforementioned reasons and due to the lack of technical equipment and expertise, EEG was not performed in the present PhD thesis.

SPECT was used in this research project as it offers numerous advantages. It is less expensive and more widely available than PET. Furthermore, ^{99m}Tc-ECD SPECT has the unique “frozen image” capacity (Peremans et al., 2001), which is certainly of value in veterinary medicine as it allows the tracer to be injected in the awake animal while the acquisition can be postponed until general anesthesia is induced (Waelbers et al., 2010). Furthermore, this capacity creates the opportunity to obtain SPECT images from the ictal period, as the start of the acquisition can be delayed until the seizure has been controlled (Ichise et al., 1997; la Fougère et al., 2009). Despite the benefits of SPECT, spatial resolution limits - mainly due to attenuation, scatter and partial volume effects - remain a major concern (Catafau, 2001). Consequently, not all registered photons are with certainty derived from the actual VOI, which can lead to an under- or overestimation of

rCBF in certain regions. The limited spatial resolution (7-8 mm) of the conventional SPECT system used in the **first chapter** precluded a detailed assessment of the suppressed rCBF in the subcortical region. The use of a µ-SPECT system with multi-pinhole collimators in **chapter 2 and 4** led to a clear improvement of spatial resolution (2.3 mm), and this technique offers future possibilities to investigate the canine brain into detail. The currently available µ-SPECT system is feasible to use in Beagle dogs, but the field of view is limited (Peremans et al., 2005b), which hampers its use in the evaluation of the brain of larger dog breeds. Since many dogs with idiopathic epilepsy are large breed dogs, µ-SPECT might not be the optimal technique to use. Recently, new "resolution recovery" software (e.g. Hybrid, Hermes, NUD, Sweden) has become available to process conventional SPECT data. This software corrects for attenuation based on CT images, for scatter based on Monte Carlo methods and for collimator response. In this way, resolution may improve from 8 to 5 mm according to the producers (Waelbers, 2012a; LeBlanc and Peremans, 2014). The detailed VOI map created in **chapter 2** can also be used with this software. Therefore, future studies evaluating rCBF in epileptic dogs, can use this software for data analysis.

The µ-SPECT study was performed in a homogenous group of healthy young adult male Beagle dogs, which precludes the evaluation of gender and age differences on the rCBF in dogs. Age and gender differences in brain perfusion exist in healthy people (Van Laere et al., 2001). Significant rCBF differences have also been detected in dogs older than 8 years with conventional SPECT (Peremans et al., 2002). Future studies investigating age and gender differences with µ-SPECT are warranted.

As immobilization of the animal is essential, general anesthesia is required to perform brain SPECT in veterinary medicine. Certain anesthetics definitely influence ^{99m}Tc-ECD SPECT images in dogs (Waelbers et al., 2011; Waelbers et al., 2012b). However, in the SPECT studies of this PhD thesis (**chapter 1, 2 and 4**), influence of anesthesia was avoided by injecting the tracer prior to sedation or anesthesia, hence allowing an undisturbed tracer distribution and creating a "frozen image" in the awake dog. In humans, this fixed image is obtained within a couple of minutes after tracer injection (Walovitch et al., 1994). In dogs a stable image, representing rCBF at the moment of tracer injection, is obtained as long as the acquisition starts between 15 and 40 min after tracer administration (Waelbers et al., 2012c), which was respected in the present work.

Evaluation of SPECT images was consistently performed by semiquantification **(chapter 1, 2 and 4)**, which is more objective than visual analysis. Automated registration of the perfusion data to a template, combined with an automatically applied VOI map, results in a more reliable analysis compared to manual registration and visual interpretation (Slomka et al., 1997). On the other hand, visual analysis, in comparison with semiquantitative analysis, had a higher sensitivity in the detection of areas of hypometabolism with PET in epileptic dogs (Jokinen et al., 2013). Therefore, it might still be rewarding to compare both in further studies.

In the 3 SPECT studies of this thesis (**chapter 1, 2 and 4**), brain perfusion was evaluated using the tracer ^{99m}Tc-ECD, which revealed interesting information as rCBF is strongly related to regional brain metabolism and neuronal activity (Leonard et al., 1986; Warwick, 2004). However, in addition to the evaluation of rCBF, SPECT can also be used to evaluate certain neurotransmitter systems and their receptors using specific tracers (LeBlanc and Peremans, 2014). Several receptor ligands have been used to investigate the neurochemical basis of epilepsy in humans with PET or SPECT (la Fougère et al., 2009). Benzodiazepine/GABA receptor, serotonin receptor and dopamine receptor brain imaging have been investigated in humans with epilepsy, but results remain controversial (la Fougère et al., 2009). Neurotransmitter systems in epileptic dogs have not yet been investigated with SPECT, although similar research has been performed in dogs with behavioral disorders at our SPECT department (Peremans et al., 2003, 2005a, 2006; Vermeire et al., 2009b, 2011, 2012). Neuroreceptor brain imaging studies in dogs with idiopathic epilepsy might aid in the localization of epileptogenic foci and in the understanding of the neurobiology of epilepsy. Also, this type of functional brain imaging could be applied to investigate the effects of VNS on certain neurotransmitter systems in dogs.

2. The mechanism of action of vagus nerve stimulation in the Beagle dog

Since almost two decades, VNS is an established treatment for refractory epilepsy in humans (Ben-Menachem, 2002). However, approximately one third of treated patients do not respond even to long-term treatment (DeGiorgio et al., 2000). Since the antiepileptic MOA of VNS is not fully understood, no rational hypotheses have been derived to help identify predictive factors for clinical response. Also, the currently applied stimulation parameters are not evidence-based. Further elucidation of the MOA could lead to an improvement of VNS efficacy by identifying early biomarkers of response to treatment or biomarkers for optimized stimulation paradigms.

The second part of this PhD thesis was dedicated to the investigation of the influence of VNS on the canine brain. The use of this animal model for translational research has certain advantages. First of all, naturally occurring canine epilepsy is comparable to human epilepsy, particularly regarding its prevalence and semiology (Chandler, 2006). This renders translational research in a canine model justified. Secondly, the canine brain shares more similarities with the human brain regarding its size and function than with the rodent brain. The collection of cerebrospinal fluid (CSF) in dogs is a routine procedure with only few complications. Also, a larger sample than in rodents can be collected (Cook and DeNicola, 1988; Pegg et al., 2010). Finally, canine seizure models, such as the PTZ model, have been well described previously (Löscher et al., 2004; Löscher et al., 2013) and a similar protocol was used in **chapter 5.**

Functional brain imaging is able to identify brain structures altered by VNS in humans and rodents (Chae et al., 2003), but this was not yet investigated in dogs. Micro-SPECT, a feasible technique to assess regional brain perfusion in Beagle dogs (see **chapter 2**), was used in **chapter 4** to investigate rCBF changes induced by a short period of standard and microburst VNS in healthy Beagle dogs. A hypoperfusion in the left frontal and right parietal cortex was induced by microburst VNS, whereas standard VNS did not elicit changes in canine rCBF. Several human PET or SPECT studies, investigating the effect of acute VNS on the rCBF in people with epilepsy or depression, detected a similar involvement of the frontal and parietal lobes (Henry et al., 1998; Ko et al., 1999; Barnes et al., 2003; Conway et al., 2006). Moreover, functional MRI performed during VNS in a

small group of human patients with intractable focal seizures described an activation of the frontal and parietal structures (Sucholeiki et al., 2002). From our study, it cannot be concluded that the cortical rCBF alterations are involved in the antiepileptic MOA of VNS, but it seems reasonable that suppression of rCBF, coupled indirectly to neuronal activity (Roy and Sherrington, 1890), in certain cortical regions could be associated with a seizure suppressing effect. Epileptiform activity has also been detected in the frontal lobe of dogs with idiopathic epilepsy using EEG (Brauer et al., 2012). Furthermore, structural lesions in the frontal lobe in dogs are often associated with seizures (Schwartz et al., 2011).

On the neurochemical level, a significant increase of NE was detected in the canine CSF after one hour of VNS (**chapter 5**). The influence of VNS on the LC/NE system has been previously demonstrated in rodents (Hassert et al., 2004; Dorr and Debonnel, 2006; Roosevelt et al., 2006; Follesa et al., 2007; Manta et al., 2009; Raedt et al., 2011; Manta et al., 2013). Moreover, it was found that activation of the LC, associated with a focal NE increase, was correlated to the antiepileptic efficacy of VNS (Krahl et al., 1998; Raedt et al., 2011). These studies used invasive techniques (e.g. microdialysis), which are not practical to use in patients as a screening tool for responsiveness to VNS. On the other hand, collection of CSF is a minimally invasive, well known procedure. Importantly, our study (**chapter 5**) is the first to demonstrate an increase of NE, induced by VNS, measurable in the CSF. This finding supports also further research into non-invasive biomarkers of NE increase, such as the P300 amplitude, whose increase has been correlated with a positive antiepileptic effect of VNS in humans (De Taeye et al., 2014).

An important aim of the second part of this PhD thesis was to compare the influence of a novel and promising stimulation paradigm (microburst VNS) with standard VNS. The rationale of microburst stimulation is primarily based on the phenomenon of paired-pulse facilitation, meaning that two stimulations of a presynaptic terminal within a short period of time result in a larger evoked synaptic response to the second stimulation (Zucker and Regehr, 2002). Hence, it has been shown in primates that multiple stimulations of the vagus nerve within a short period of time evoked a larger response at central vagal targets (Hallowitz and MacLean, 1977; Ito and Craig, 2005, 2008). Therefore, it can be expected that short bursts of stimulation ("microbursts") of the vagus nerve are more potent in affecting central mediators of VNS and could possibly be associated with a stronger antiepileptic effect compared to standard VNS. In the rat kindling model, it was suggested

recently that microburst VNS may indeed represent an improved stimulation paradigm (Alexander and McNamara, 2012). Interestingly, we demonstrated that microburst VNS seems to have more potency than standard VNS to induce µ-SPECT changes in the brain of healthy dogs (**chapter 4**), which reflects the higher potency of affecting central targets. On the neurochemical level, microburst VNS is thought to be as potent as standard VNS to induce an increase of NE levels in the canine CSF (**chapter 5**). Despite these promising effects of microburst VNS on the canine brain, no antiepileptic effect could be demonstrated in the canine PTZ model (**chapter 5**). Still, further research is warranted to investigate the antiepileptic effect of microburst VNS, since the effectiveness of VNS improves over time in humans (Morris and Mueller, 1999; Vonck et al., 1999) and only the effects of one hour of stimulation in one particular canine seizure model were investigated in the present work.

Also from a veterinary point of view, research towards non-pharmacological treatments for refractory canine epilepsy is needed (Muñana, 2013), as currently, many dogs with refractory epilepsy are eventually euthanized or die due to uncontrollable seizures (Arrol et al., 2012; Monteiro et al., 2012). VNS has the advantage of being independent of the localization of the epileptogenic focus and of owner compliance. Earlier studies investigating the antiepileptic effect of VNS in dogs seemed promising, but the number of studies are limited. An experimental study in dogs demonstrated that chemically induced seizures could be aborted by stimulation of the cervical vagus nerve (Zabara, 1992). Furthermore, ocular compression, which is an indirect way of stimulating the vagus nerve, has been shown to be beneficial for controlling seizures in a few dogs (Speciale and Stahlbrodt, 1999). Only one placebo controlled, double-blinded cross-over study evaluated the safety and efficacy of VNS in 10 dogs with refractory epilepsy using a similar implantable device as in humans (Muñana et al., 2002). No significant difference in seizure frequency, duration and severity was detected between the treatment and control period of 13 weeks, but when the final 4 weeks of both periods were compared, a significant decrease in seizure frequency was found during the treatment period. In our study, no antiepileptic effect of a short period of VNS could be demonstrated in the canine PTZ model **(chapter 5)**.

VNS, as a tool to treat refractory epilepsy, aims to mainly activate the afferent fibers of the vagus nerve, but co-activation of the efferent fibers is inevitable (Banzett et al., 1999).

Given the influence of the efferent fibers of the vagus nerve on the heart, the potential occurrence of cardiac side effects was a serious initial concern when VNS was introduced as a treatment for refractory epilepsy in humans (Asconape et al., 1999). Despite this concern, clinically relevant cardiac side effects of VNS are rarely noticed in humans. Bradycardia and asystole have occasionally been reported, mainly during intraoperative testing of the device, which is probably due to the simultaneous influence of anesthesia and VNS (Asconape et al., 1999; Schwartz, 2011). To avoid cardiac complications, the left vagus nerve is usually chosen as it has less cardiac influence than the right nerve, both in humans and dogs (Kamath et al., 1992; Muñana et al., 2002; Spuck et al., 2008; Kahlow and Olivecrona, 2013). Furthermore, the helices are placed distally from the vagal cervical cardiac branches in humans to avoid stimulation of these fibers during VNS. This is impossible in dogs due to anatomical differences and therefore, dogs might be more sensitive to cardiac effects of VNS than humans (Muñana et al., 2002). For this reason and because the cardiac side effects of microburst VNS had not yet been evaluated, the influence of standard and microburst VNS on heart rate variability was examined in healthy Beagle dogs using Holter monitoring (**chapter 6**). No effects of neither standard nor microburst VNS on the different Holter parameters were noticed, but long-term studies in a larger number of animals are recommended before definitive conclusions can be drawn.

VNS is usually well tolerated in humans with a low incidence of side effects (Ramsay et al., 1994). No obvious side effects of the stimulation were noticed in our Beagle dogs, but it is important to acknowledge that only short periods of stimulation were delivered. Effects of chronic VNS cannot be evaluated in the present work. Furthermore, the occurrence of paresthesia and hoarseness, which are common, but often transient stimulation-related side effects of VNS in humans (Ben-Menachem, 2002), is difficult to evaluate in dogs. Surgical complications did not occur, but postoperative or technical problems were frequently encountered in our Beagle dogs and needed surgical exploration in 2 dogs. These complications are reported in **chapter 3** and suggestions have been made to refine the surgical technique.

Methodological considerations and future perspectives

The studies in the second part of this PhD thesis were performed in healthy Beagle dogs. We considered it useful to get experienced with the VNS Therapy® system and to assess the antiepileptic effect in a canine model, before implanting these VNS devices in canine epileptic patients. Also, by investigating regional brain perfusion and CSF changes induced by VNS in healthy dogs, epilepsy related confounding factors have been excluded both on the neuroimaging and neurochemical level. However, it may be possible that the healthy and epileptic brain respond differently to VNS. Therefore, the MOA of VNS should be investigated in dogs with spontaneous epilepsy as well.

Only the effects of a short period of VNS were assessed. To increase the number of pulses delivered to the nerve during this short period of stimulation, a rapid-cycling stimulation protocol (7s on – 18s off) was used instead of the classic intermittent stimulation protocol (30s on – 5min off) most often used in a clinical setting (Ben-Menachem, 2002). In humans, the effectiveness of VNS clearly improves over time (Morris and Mueller, 1999; Vonck et al., 1999). Also, the Holter monitoring, performed in **chapter 6**, was short, which precludes the evaluation of naturally occurring circadian variations. Therefore, a challenge would be to investigate the effects of chronic VNS on functional brain imaging, neurochemical, antiepileptic and cardiac parameters.

In the neurotransmitter study (**chapter 5**) we focused on the evaluation of monoamines based on the biomarker role of NE in rodents (Raedt et al., 2011). However, other neurotransmitters (e.g. glutamate, GABA) can be involved in the MOA of VNS as well. Future studies should expand the research towards other neurotransmitter systems. Also, the site of CSF collection (lumbar vs. cervical) could have an influence on neurotransmitter results, and this should be taken into account. In our Beagle dogs, CSF was obtained from the cerebellomedullary cistern, but in humans a lumbar puncture is usually the method of choice to collect CSF.

Neither standard nor microburst VNS increased the PTZ seizure threshold in our Beagle dogs, whereas the positive control test with PB showed a significant increase of this threshold (**chapter 5**). As mentioned before, it cannot be excluded that the period of stimulation was too short to induce a significant change in PTZ seizure threshold. The possibility that VNS is not able to exert any antiepileptic effect against PTZ-induced

seizures seems unlikely, because an acute abortive effect of VNS on PTZ induced convulsions in dogs has been demonstrated (Zabara, 1992). Furthermore, a positive effect of VNS on seizure severity has been shown in the rat PTZ model (Takaya et al., 1996; Krahl et al., 2001, 2003; Zhang et al., 2008; Sahin et al., 2009). It is also possible that VNS could be less effective in dogs than in humans, with potentially anatomical differences in the cervical vagus nerve between dogs and humans playing a role. Previous studies investigating the antiepileptic effect of VNS in dogs are limited and were performed only on a small number of dogs. However, antiepileptic efficacy was demonstrated in these studies (Zabara, 1992; Speciale and Stahlbrodt, 1999; Muñana et al., 2002). Consequently, it remains unknown whether the µ-SPECT (**chapter 4**) and CSF changes (**chapter 5**), induced by VNS, have an antiepileptic significance. Future studies using VNS in dogs should prioritize the evaluation of its antiepileptic effect, before further translational research is performed in this species.

The stimulation output current of VNS, used in the different chapters, was defined as the subcoughing threshold, according to Muñana et al. (2002). In analogy to that study, the strength of this current tolerated by our Beagle dogs was low compared to the current used in human clinical trials. Hence, it is possible that more significant effects of VNS could have been obtained if higher stimulation currents would have been used. However, the effectiveness of VNS has not been correlated to the degree of output current in humans (Muñana et al., 2002). Still, it could be warranted to compare the effects of different stimulation output currents on the canine brain.

Postoperative complications after implantation of a VNS Therapy system were relatively common in this small group of healthy Beagle dogs (**chapter 3**). We assume that these complications will not have influenced our study results significantly, as dogs with serious complications were excluded from the study or surgically implanted with a new VNS device. Also, despite the fact that one helix (anchor tether) was dislodged in 4 dogs, the two stimulation electrodes remained correctly implanted in all dogs.

Conclusions

Interictal conventional SPECT demonstrated a suppression of the subcortical brain perfusion, but was not able to reveal cortical rCBF alterations in dogs with idiopathic epilepsy. Whether this reflects an important role for the thalamus in the pathophysiology of canine idiopathic epilepsy is unclear. Future SPECT studies in dogs with idiopathic epilepsy should include larger patient numbers and could use the recently developed Hybrid software for data processing. Ideally, future studies should include ictal SPECT analysis.

Micro-SPECT of the brain is a feasible technique to evaluate regional brain perfusion in healthy Beagle dogs. Furthermore, an improved resolution compared to conventional SPECT is obtained allowing the assessment of rCBF in 19 different VOIs. It is important to acknowledge the fact that hemispheric and regional asymmetries are present in healthy dogs. Despite the great potential of this technique for experimental research, the currently available µ-SPECT system cannot be used in large breed dogs due to the limited field of view.

Microburst VNS appears to be more potent than standard VNS in the induction of regional brain perfusion changes in healthy Beagle dogs. A short period of microburst stimulation suppresses rCBF in the frontal and parietal cortex. Furthermore, this promising stimulation paradigm was as potent as standard VNS to induce a NE increase in the CSF of healthy Beagle dogs and was considered as safe as standard VNS when evaluating cardiovascular side effects. Unfortunately, no antiepileptic effect of either standard or microburst VNS could be demonstrated in our canine PTZ model. However, as only a short period of stimulation was evaluated, long-term VNS studies in dogs are required.

This is the first time that a VNS induced increase of NE, measurable in the CSF, is described. Considering the important protective role of NE in seizures and the biomarker role of NE for VNS efficacy in rodents, further research towards this CSF NE increase and its potential biomarker role are recommended both in dogs and humans.

VNS appeared to be a safe and well tolerated treatment in dogs. Ideally, this should be evaluated in a larger group of dogs receiving long-term VNS treatment. The surgical implantation of a VNS Therapy® System in dogs is minimally invasive, feasible and safe.

However, refinements of the surgical technique are advised to reduce post-operative complications.

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Summary

Epilepsy is one of the most common neurological disorders both in humans and dogs. The majority of dogs have idiopathic epilepsy and often they are managed successfully with antiepileptic drugs. However, up to 30% of dogs are refractory to treatment, which is comparable to the prevalence of refractory epilepsy in humans. A better understanding of the neurobiology of epilepsy could lead to the optimization and expansion of therapeutic options in dogs. This rationale was followed in the **first part of this PhD thesis (chapter 1 – 2)**, where the functional brain imaging technique SPECT was used to investigate the brain of dogs with idiopathic epilepsy.

The past 2 decades there has been an enormous expansion of treatment options for human epilepsy, in contrast to canine epilepsy. However, a lot of these treatments still need to be optimized. Research in animal models may contribute. The **second part of this PhD thesis (chapter 3 – 6)** investigated the mechanism of action (MOA) of Vagus Nerve Stimulation (VNS) in a canine model. VNS is an established effective add-on treatment for refractory epilepsy in humans, but the biomarkers for response and optimal stimulation parameters remain unknown. Also in this second part, a novel stimulation paradigm, microburst VNS, was compared with standard VNS.

As a **general introduction**, a brief review is given on the basic principles of canine epilepsy with a focus on classification, diagnostic work-up, standard treatment and pharmacoresistance. Additionally, the working mechanism of SPECT, a comparison between conventional and high-resolution micro-SPECT (µ-SPECT) and the use of SPECT in human epilepsy are described. Finally, VNS is explained and its effectiveness and safety in human epilepsy is summarized. Also, the limited available information on VNS in dogs is provided.

In the **first chapter,** conventional SPECT was used to investigate if regional brain perfusion alterations occurred during the interictal period of dogs with idiopathic epilepsy (*1st research aim*). The regional brain perfusion was evaluated in 11 regions and compared between the epileptic and control group. A significant hypoperfusion was demonstrated in the subcortical region of the epileptic dogs. No cortical or cerebellar perfusion changes were noticed. So, SPECT is a feasible technique to detect regional brain perfusion changes in dogs with idiopathic epilepsy and the subcortical area, more specifically the thalamus, may have an interesting role in the pathophysiology of canine idiopathic epilepsy.

The **second chapter** aimed at improving the limited resolution of the conventional SPECT system to evaluate canine brain perfusion. The feasibility of a high-resolution µ-SPECT system to evaluate regional brain perfusion was evaluated in healthy Beagle dogs (*2nd research aim*). Furthermore, a new, more detailed Volume of Interest (VOI) map was created based on Magnetic Resonance (MR) images. Functional imaging of the canine brain is possible using µ-SPECT, however, the currently available µ-SPECT system cannot be used in large breed dogs. Another aim of this study was to describe the normal regional brain perfusion pattern in adult healthy Beagle dogs. The highest and lowest regional brain perfusion was found in the parietal and piriform cortex respectively. Importantly, hemispherical and regional asymmetry in regional brain perfusion was demonstrated in the brain of healthy Beagle dogs.

In the **third chapter** of this PhD thesis, we provide a detailed description of the surgical implantation technique of a VNS Therapy® System in dogs (*3rd research aim*). Moreover, short- and long-term complications are reported. The implantation procedure succeeded safely in all dogs, but postoperative complications were common. Practical considerations to improve the surgical technique in the future are summarized. A regular impedance evaluation of the system seems important in dogs.

In the **fourth chapter**, the effect of a short period of standard and microburst VNS on the regional brain perfusion of healthy Beagle dogs was examined using µ-SPECT (*4th research aim*). Acute microburst VNS suppressed the perfusion in the frontal and parietal lobes, but acute standard VNS did not cause perfusion alterations. In conclusion, microburst VNS is more potent than standard VNS to modulate regional brain perfusion in dogs.

In the **fifth chapter**, the effect of a short period of standard and microburst VNS on monoamine levels in the cerebrospinal fluid (CSF) of healthy Beagle dogs was investigated (*5th research aim*). Furthermore, the antiepileptic effect of a short period of VNS was investigated, using the canine pentylenetetrazole (PTZ) model (*6th research aim*). Both standard and microburst VNS caused a significant increase of norepinephrine (NE) levels in the CSF. However, neither standard nor microburst VNS caused an increase of the PTZ seizure threshold. So, VNS induces an increase of NE in the canine brain that is measurable in the CSF. Nevertheless, this could not be linked to an

antiepileptic effect of VNS using the PTZ model in our dogs. Our results encourage further investigation of the biomarker role of NE in VNS efficacy.

The **last chapter** assessed cardiovascular safety of acute standard and microburst VNS in healthy Beagle dogs, using Holter monitoring (*7th research aim*). Heart rate variability parameters were compared and no significant changes in any of the Holter parameters were demonstrated. Short-term, left-sided standard and microburst VNS have no effect on heart rhythm in healthy dogs, although larger and long-term VNS studies are recommended.

Samenvatting

Epilepsie is één van de meest voorkomende neurologische aandoeningen bij de mens en de hond. Het grootste deel van de honden heeft idiopathische epilepsie en reageert goed op medicamenteuze behandeling. Toch is tot 30 % van de behandelde honden farmacoresistent, wat goed overeenkomt met de prevalentie van refractaire epilepsie bij de mens. Het beter begrijpen van de neurobiologische achtergrond van epilepsie zou kunnen leiden tot optimalisering en uitbreiding van de behandelingsmogelijkheden. Deze redenering werd gevolgd in het **eerste deel van deze doctoraatsthesis (hoofdstuk 1 - 2)** waar de functionele beeldvormingstechniek Single Photon Emission Computed Tomography (SPECT) gebruikt werd om de hersenen van de hond met idiopathische epilepsie te onderzoeken.

De laatste 2 decennia heeft er een enorme uitbreiding plaatsgevonden van de behandelingsmogelijkheden van epilepsie bij de mens, dit in tegenstelling tot de hond. Vele van deze behandelingen moeten echter nog geoptimaliseerd worden. Onderzoek door middel van diermodellen kan hierbij van nut zijn. In het **tweede deel van deze doctoraatsthesis** (**hoofdstuk 3 - 6**) werd het werkingsmechanisme van nervus vagus stimulatie (NVS) onderzocht bij de hond als diermodel. NVS is een effectieve behandeling voor refractaire epilepsie bij de mens, maar het is nog niet gekend welke factoren de respons op behandeling kunnen voorspellen en welke stimulatieparameters optimaal zijn. In dit tweede deel werd tevens een nieuw stimulatie type, microburst stimulatie genaamd, vergeleken met standaard NVS.

In de **algemene inleiding** wordt een kort overzicht gegeven van de basis principes van epilepsie bij de hond, waarbij de nadruk wordt gelegd op de classificatie, diagnostiek, standaard behandeling en farmacoresistentie. Tevens wordt het werkingsmechanisme van SPECT toegelicht, wordt conventionele met micro-SPECT (µ-SPECT) vergeleken en wordt de rol van SPECT in de diagnostiek van humane epilepsie beschreven. Tenslotte wordt er uitgelegd wat NVS is en wordt er een kort overzicht gegeven van de effectiviteit en veiligheid van NVS bij de mens met epilepsie aangevuld met de beknopte informatie die beschikbaar is over NVS bij de hond.

In het **eerste hoofdstuk** werd conventionele SPECT gebruikt om na te gaan of er veranderingen in hersendoorbloeding kunnen vastgesteld worden bij honden met idiopathische epilepsie tijdens de interictale fase (*1^{e} onderzoeksdoelstelling*). De regionale hersendoorbloeding werd beoordeeld in 11 regio's en de bevindingen van de

groep honden met epilepsie werden vergeleken met een controle groep. Een significant lagere doorbloeding werd aangetoond in de subcorticale regio van de honden met epilepsie. Er werden geen veranderingen gevonden in de corticale of cerebellaire regio's. SPECT is dus een nuttige techniek om veranderingen in hersendoorbloeding aan te tonen bij honden met idiopathische epilepsie en de subcorticale regio, meer specifiek de thalamus, vervult mogelijk een interessante rol in de pathofysiologie van epilepsie bij de hond.

Het doel van het **tweede hoofdstuk** was de beperkte resolutie van het conventionele SPECT systeem, gebruikt om de hersendoorbloeding bij de hond te beoordelen, te verbeteren. De mogelijkheid om de hersendoorbloeding te beoordelen met een µ-SPECT systeem met hogere resolutie werd nagegaan bij gezonde Beagles (*2e onderzoeksdoelstelling*). Bovendien werd een nieuwe, meer gedetailleerde kaart ontwikkeld waarop 19 verschillende hersengebieden werden afgelijnd, gebaseerd op Magnetische Resonantie. Uit deze studie blijkt dat µ-SPECT een geschikte techniek is om de regionale hersendoorbloeding bij de hond te beoordelen, maar dat het huidig beschikbare µ-SPECT systeem niet kan gebruikt worden bij grote hondenrassen. Een volgende doelstelling in dit hoofdstuk was om de regionale hersendoorbloeding bij de normale volwassen hond te beschrijven. De hoogste en laagste regionale hersendoorbloeding werd respectievelijk vastgesteld in de pariëtale en piriforme cortex. Tevens werd er een algemene en regionale asymmetrie in de hersendoorbloeding aangetoond in de hersenen van gezonde Beagles.

In het **derde hoofdstuk** van deze doctoraatsthesis wordt een gedetailleerde beschrijving gegeven van de chirurgische implantatie techniek van een vagale zenuwstimulator bij de hond (*3e onderzoeksdoelstelling*). Vervolgens worden ook de complicaties na chirurgie beschreven op korte en lange termijn. De chirurgische implantatie lukte goed en bleek veilig bij alle honden, maar postoperatieve complicaties kwamen regelmatig voor. Praktische aanbevelingen om de chirurgische techniek te verbeteren worden opgesomd. Zo is het bij de hond belangrijk om de impedantie van het geïmplanteerde systeem regelmatig te controleren.

In het **vierde hoofdstuk** werd door middel van µ-SPECT het effect van een korte periode van standaard en microburst NVS op de regionale hersendoorbloeding van Beagles nagegaan (*4e onderzoeksdoelstelling*). Acute microburst NVS onderdrukte de

hersendoorbloeding in de frontale en pariëtale hersenkwabben. Standaard NVS veroorzaakte geen veranderingen in hersendoorbloeding. Microburst NVS beïnvloedt de regionale hersendoorbloeding dus meer dan standaard NVS bij de hond.

In het **vijfde hoofdstuk** werd de invloed van een korte periode van standaard en microburst NVS onderzocht op het gehalte aan monoamines in het cerebrospinaal vocht (CSV) (*5e onderzoeksdoelstelling*). Daarnaast werd het anti-epileptisch effect van een korte periode van NVS nagegaan gebruik makend van het pentylenetetrazole (PTZ) model bij de hond (*6e onderzoeksdoelstelling*). Zowel standaard als microburst NVS veroorzaakte een stijging van het noradrenaline (NA) gehalte in het CSV. Geen van beide stimulatie types veroorzaakten echter een stijging van de PTZ aanvalsdrempel. NVS veroorzaakt dus een stijging van NA in de hersenen, welke meetbaar is in het CSV bij de hond. Dit kon echter niet gelinkt worden aan een anti-epileptisch effect van NVS gebruik makend van het PTZ model bij onze honden. Verder onderzoek naar een mogelijke rol van NA als biomarker voor de doeltreffendheid van NVS is dus aangewezen.

In **hoofdstuk 6** werden mogelijke cardiovasculaire neveneffecten van een korte periode van standaard en microburst NVS geëvalueerd door middel van Holter monitoring bij gezonde Beagles (*7e onderzoeksdoelstelling*). Parameters die de variabiliteit in hartslag weergeven werden vergeleken en voor geen enkele parameter werden significante verschillen aangetoond. Een korte periode van standaard of microburst stimulatie van de linker vagale zenuw veroorzaakt dus geen veranderingen in hartritme bij gezonde Beagles. Het is echter aangewezen dit te revalueren bij een groter aantal honden en gedurende een langere periode van NVS.

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Curriculum Vitae

Valentine Martlé werd geboren op 25 juli 1980 in Gent. Ze behaalde haar diploma secundair onderwijs in de richting Latijn – wiskunde aan het Sint-Bavohumaniora te Gent en startte hierna in 1998 met de studies Diergeneeskunde aan de Universiteit Gent. In 2004 behaalde ze het diploma van dierenarts met grote onderscheiding.

Aansluitend deed ze een roterend internship kleine huisdieren aan de Vakgroep Geneeskunde en Klinische Biologie van de Kleine Huisdieren in de Faculteit Diergeneeskunde van de Universiteit Gent te Merelbeke, waarvan ze in 2005 een getuigschrift behaalde. Vanaf oktober 2005 startte ze een specialisatie opleiding (residency) in de neurologie en neurochirurgie onder de supervisie van Prof. Luc Van Ham. Dit resulteerde in 2010 in het behalen van het Diploma van Europees Specialist in de Diergeneeskundige Neurologie (DiplECVN).

Geïnteresseerd door wetenschappelijk onderzoek, startte zij ondertussen op 1 oktober 2009 met dit doctoraatsonderzoek dat werd gefinancierd door een mandaat van het Bijzonder Onderzoeksfonds (BOF) van de Universiteit Gent. In het kader van dit onderzoek behaalde zij in 2009 het diploma van proefleider (FELASA categorie C). Tevens verkreeg zij begin 2014 het getuigschrift van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Buiten dit onderzoeksproject was zij ook deeltijds betrokken bij de supervisie en klinische opleiding van de residents neurologie. Zij was eveneens mede-promotor van een aantal scripties en masterproeven.

Valentine is auteur of mede-auteur van verschillende wetenschappelijke publicaties in nationale en internationale tijdschriften. Zij nam de voorbije jaren actief deel aan meerdere nationale en internationale congressen. In 2008 won zij de prijs (John Presthus award) voor beste orale presentatie op het congres van het Europees College van Diergeneeskundige Neurologie (ECVN) in Rhodos. Tevens was zij in 2012 lid van het lokaal organiserend comité en spreker op het ECVN congres in Gent.

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