FACULTY OF MEDICINE AND HEALTH SCIENCES

Ghent University

Faculty of Medicine and Health Sciences

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The human dog

A TRANSLATIONAL NEUROBIOLOGICAL BRAIN MODEL ON THE MOLECULAR EFFECTS OF THE NON-INVASIVE BRAIN STIMULATION TECHNIQUE ACCELERATED HIGH FREQUENCY REPETITIVE TRANSCRANIAL MAGNETIC STIMULATION (HF RTMS)

Word count: 61533

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A dissertation submitted to Ghent University in partial fulfillment of the requirements for the degree of “Doctor in health sciences”
Academic year: 2018 - 2019

GHENT UNIVERSITY

Ik zou deze thesis willen opdragen aan mijn oma Irma Carchon die tijdens mijn doctoraatsstudie is overleden. Oma, op deze manier kan je er toch een beetje bij zijn.

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List of abbreviations

[^{11}C]DASB	11Carbon 3-Amino-4-(2-Dimethylaminomethylphenylsulfanyl)-Benzonitrile
[^{99m}Tc]HMPAO	99mTechnetium d,1 Hexamethylpropylene Amine Oxime
5-HIAA	5-Hydroxy-3-Indolacetic Acid
5-HT	Serotonin
5-HTP	5-Hydroxytryptophan
99Mo	99Molybdenum
99mTc –ECD	99mTechnetium Ethyl Cysteinate Dimer
ACC	Anterior Cingulate Cortex
aHF-rTMS	Accelerated High Frequency Repetitive Transcranial Magnetic Stimulation
APA	American Psychiatric Association
BI	Binding Potential
C-BARQ	Canine Behavioural Assessment And Research Questionnaire
CANMAT	Canadian Network for Mood and Anxiety Treatments
CI	Confidence Interval
CMT	Cortical Motor Threshold
COMT	Catechol-O-Methyl-Transferase
CORT	Corticosterone
CRI	Continuous Rate Infusion
CS	Conditioning Stimulus
CSF	Cerebrospinal Fluid
cTBS	Continuous Theta Burst Stimulation
CUMS	Chronic Unpredictable Mild Stress
DA	Dopamine
DAT	Dopamine Transporter
DBS	Deep Brain Stimulation
DLPFC	Dorsolateral Prefrontal Cortex
DLPMC	Dorsolateral Premotor Cortex
DOPAC	3,4 Dihydroxyphenylacetic Acid
ECT	Electro Convulsive Therapy
EEG	Electroencephalography
EMG	Electromyography
EST	Electroshock Therapy
fMRI	Functional Magnetic Resonance Imaging
HF-rTMS	High Frequency Repetitive Transcranial Magnetic Stimulation
HPA	Hypothalamic-Pituitary-Adrena
HVA	Homovanillic Acid
IM	Intramuscular
iTBS	Intermitted Theta Burst Stimulation
IV	Intravenous
LF-rTMS	Low Frequency Repetitive Transcranial Magnetic Stimulation

LH	Learned Helplessness
LTD	Long Term Depression
LTP	Long term Potentiation
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
MDD	Major Depressive Disorder
MEP	Motor Evoked Potential
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MT	Motor Threshold
NAD	Noradrenaline/Norepinephrine
NREM	Non-Rapid Eye Movement
PET	Positron Emission Tomography
PI	Perfusion Index
rCBF	Regional Cerebral Blood Flow
ROI	Region Of Interest
rTMS	Repetitive Transcranial Magnetic Stimulation
SD	Standard Deviation
SERT	Serotonin Transporter
sgACC	Subgenual Anterior Cingulate Cortex
SNRI	Selective Norepinephrine Re-uptake Inhibitor
SPECT	Single-Photon Emission Computed Tomography
SSRI	Selective Serotonin Re-uptake Inhibitor
TBS	Theta Burst Stimulation
TCA	Tricyclic Antidepressants
tDCS	Transcranial Direct Current Stimulation
TMS	Transcranial Magnetic Stimulation
TS	Test Stimulus
VOI	Volume Of Interest
WFSBP	World Federation of Societies of Biological Psychiatry

Chapter 1: General introduction

Transcranial magnetic stimulation

Transcranial magnetic stimulation (TMS) is a neurostimulation technique that has been extensively used for brain mapping [1, 2], and as a diagnostic and therapeutic tool in medical and veterinary neurology [3, 4]. It was set forward as a rapid, easy to use, pain free and non-invasive technique to induce electrical depolarisations in desired cortical brain regions [5]. This technique is based on Faraday's law of induction stating that by varying a magnetic field in time in a conducting environment an electric current is generated[3, 4, 6]. A varying magnetic field applied over a person's head, passes the scalp and skull almost unaltered and thereby inducing electrical currents in the underlying neurons[7, 8]. Thus TMS can be considered as "electrodeless electric stimulation of the brain via electromagnetic induction" [9]. Depending on the brain region and the frequency of the magnetic field, the induced electrical current may either stimulate neural activity, or inactivate it [10], this aligned with the releases of monoamines [11]. Not only targeted cortical areas can be affected but also, depending on the intensity of stimulation, distant interconnected brain regions [12].

Currently, in human applications, TMS over the left dorsolateral prefrontal cortex (DLPFC) has been FDA approved for the treatment of major depressive disorder (MDD). This target localization is based on earlier neuroimaging studies that indicated a reduced neuronal activity (blood flow and glucose metabolism) in the left DLPFC and raised activity in the right DLPFC in this mental illness [13-15]. This neuronal asymmetry has also been found in dogs suffering from anxiety disorders, indicating similar neuropathological mechanisms for canine psychopathology [16, 17]. Therefore, psychopathologies in dogs may act as a possible natural animal model for depression. Due to the neuropathological similarities between humans and dogs - and that canine anxiety disorders are treated with antidepressants[18, 19]. - dogs with anxiety disorders could portray a similar neurophysiological and behavioural response towards novel antidepressants such as TMS. Nonetheless, induced anxiety in rodents is still the current animal model for unravelling the neuropathology of depression and its treatment modalities, which may be considered as a suboptimal animal model for human psychopathology. To test rTMS treatment paradigms the canine model may be better suited.

The principle

Transcranial magnetic stimulation relies on a time varying magnetic field. A magnetic field can be generated by a permanent magnet or by an electrical current moving through a conductor, as discovered by Oersted. A magnetic field can be defined as magnetic flux density expressed in Ampere per meter or as magnetic field strength expressed in Tesla. As discovered by Faraday, varying a magnetic field over a stationary conductive circuit leads to the induction of an electrical current. The direction of the induced electrical current will be in such a way that it opposes the magnetic field by which it was generated (Lenz's Law). By consequence, a secondary magnetic field is created that opposes the original magnetic field.

A modern TMS stimulator consists of a stimulating coil and a charge-decharge system [9, 20]. The coil itself consists of well-insulated copper circular wire enclosed in a plastic cover. The dimensions and the shape of the coil play a crucial role in the TMS treatment since they determine the strength, focality and the dimensions of the induced electrical current [21-23]. In accordance with the inducing magnetic field, the induced current decreases rapidly with distance from the coil. This only allows for the induction of electrical current in superficial layers of the cortex (up to 2 cm deep). The charge-decharge system holds a charging system, one or more capacitors and an energy recover circuitry. The charging system generates the current to provoke the magnetic field whereas the capacitors allow for the electrical pulses to be rapidly generated, stored and discharged. After discharge, the current flows back to capacitor through an energy recovery unit that enables a quick recharge. An electronic semiconductor (thyristor) is placed between the charge-decharge system and the coil, which allows for a rapid transfer of the large currents from the capacitor to the coil. When the capacitor(s) is/are fully charged, the thyristor starts discharging the capacitor by passing currents true the coil. Based on the desired shape of the induced current, a specialized pulse-shape circuitry can be used to generate monophasic or biphasic pulses.

The pulse

Monophasic and biphasic pulses are the most widely used pulse shapes in TMS [24]. Monophasic pulses are created when the inducing current only flows in one direction whereas biphasic

pulses originate after the current has circulated through the coil in both directions. In contrast to biphasic pulses, the current provoking a monophasic pulse is completely lost and transferred into heat after discharge. In contrast, biphasic pulses allow a significant portion of the energy to be re-stored in the capacitors allowing a quick recharge of the capacitors. A third pulse form is the polyphasic pulse, which is created by oscillation of the inducing current. Thereby multiple biphasic pulses are sequentially created with decreasing energy and amplitudes.

The intensity or strength of the pulse depends on the voltage of the inducing current, which is expressed as percentage of the maximum machine output or as a percentage of a person's motor threshold. The cortical motor threshold (CMT) is general defined as the minimal TMS intensity required to provoke at least five out of 10 electromyographic (EMG) responses - of at least 50 μV - in a contralateral fully relaxed muscle (resting MT) [25]. If the person is performing a voluntary constant active contraction of that muscle, the active MT is assessed (EMG response of at least 200 μV).

TMS paradigms

Pulses can be administered to the target cortical region following different paradigms: single pulses, paired pulses, repetitive TMS (rTMS), and accelerated rTMS.

Single pulse TMS consists of applying a single pulse (either monophasic or biphasic) to a specific cortical region and is used in clinical and experimental settings. Applying a single pulse over the primary motor cortex can be used to assess the CMT, input/output curves or the cortical silence period [25-27]. Single pulses can also be applied over the primary visual cortex to determine the phosphene threshold [28].

Applying paired pulses constitutes of delivering two consecutive pulses, a conditional stimulus (CS) followed by a test stimulus (TS), with an interpulse interval ranging from 1 to 200 milliseconds to a cortical target [24, 29, 30]. The first pulse (CS) can modify the response of the second pulse (TS). The modification of the response depends upon the intensity of both pulses and the interval between them [30-35]. The target of both pulses may differ from each other [36].

rTMS is defined as sequentially applying mono- or biphasic pulses over a target cortical region [37]. It is able to produce long lasting effects on the target cortical region and remote regions

(cortical and subcortical), thereby differing from single and paired pulse TMS [38]. rTMS is used as a potential treatment modality for psychiatric and neurological conditions [39, 40]. A rTMS protocol is defined by its frequency, intensity, duration, train length, intertrain interval and the total number of pulses [26, 41-44]. When pulses are applied at a rate of 1 pulse per second (1Hz), the rTMS protocol is considered low frequency (LF-rTMS). On the other hand, when the pulse frequency is equal or higher than 5Hz, the rTMS protocol is defined as high frequency rTMS (HF-rTMS) [10, 45-47]. Generally, HF-rTMS is thought to provoke cortical excitability presumably through the mechanism of long term potentiation (LTP) whereas LF-rTMS rather reduces cortical excitability through long term depression (LTD), as noticed in the rodent hippocampus [10, 26, 39, 42, 44-46, 48]. It must be kept in mind that the train length, the intertrain interval, and the total amount of pulses can alter the facilitatory or inhibitory effect of a given rTMS paradigm [49, 50]. Even more, the individual's baseline cortical excitability influences the effect of an rTMS paradigm as well [47, 51]. For safety concerns, a classic active rTMS treatment for depression includes 5 daily high frequency sessions for a period of 3 to 6 weeks (on average 20 to 30 sessions). These sessions involve 20 to 75 HF-rTMS trains, lasting from 2 up to 10 seconds, with an intertrain interval of 20 to 30 seconds. Usually the sessions are given at an intensity of 100% to 120% of the patient's MT. The intertrain interval ranges from 20 to 30 seconds [52]. This daily administration can become a limiting factor for patients whom have to travel to the treatment facility. Even more, its slow clinical response makes its unsuitable for patients in need of a rapid treatment efficacy [53]. Recently studies have safely and successfully applied an accelerated HF-rTMS protocol, which condenses the classic treatment duration to several days [12, 53-58]. Accelerating a rTMS protocol has the advantage of not only a rapid improvement of the depressive symptoms (after 2 days) without provoking severe side effects, it significantly reduces the duration of treatment [56, 57, 59]. Importantly, a response rate similar to classic rTMS paradigms is achieved[56].

Target localisation

Localisation of the TMS target can be quite challenging in the prefrontal 'silent' cortical areas [60]. This is less challenging when targeting cortical regions that provoke a behavioural response such as the primary motor cortex or the visual cortex. Stimulation of these areas results in either a muscle

contraction or phosphenes respectively. In contrast, targeting brain regions that do not result in a behavioural response after stimulation, such as the dorsolateral prefrontal cortex frontal (DLPFC), are harder to locate. Even more, accurate localisation of a cortical area is hardened by interindividual variability in its anatomical or functional location. Three methods are routine used to locate a TMS target: the standard function guided method, the 10-20 international EEG system and an optically tracked neuronavigation system.

Non-stereotactic neuronavigation

The standard function guided method locates the DLPFC based on location of the primary motor cortex [61, 62]. First, a cap is placed over the patient's head, which holds marked anatomical landmarks to allow repositioning of the cap. Secondly, the primary motor cortex is identified by monitoring the response of the contralateral abductor pollicis brevis muscle. Finally, the DLPFC is localised by moving the coil 5 cm rostrally in the parasagittal plane of the region that provoked the most prominent motor response. Although this method allows for an easy to perform coil placement, it is only capable of correctly locating the DLPFC in 30% of the cases [63]. This, because it does not allow for individual anatomical variation, more specifically the distance between the primary motor cortex and the DLPFC [63, 64]. Other sources of error introduced by this method are: the repositioning of the cap between stimulation sessions and interobserver variability [64].

Another, more reliable, commonly used non-stereotactic neuronavigation technique is the 10-20 international electroencephalogram (EEG) system [65]. This system correlates external skull locations to underlying cortical regions by specific placement of EEG electrodes. Based on four anatomical landmarks (nasion, inion, left pre-auricular point and right pre-auricular point), the electrodes are placed in steps of 10% and 20% onto the skull. Thereby, this system takes into the individual skull variability. Nonetheless, this system does not consider the shape of the patient's head, the position of the target or the individual cortical morphology. Despite the high reliability of this technique, different cortical regions may be targeted in different patients when targeting the same electrode [65].

Both non-stereotactic neuronavigation techniques yield similar accuracies [66]. Nonetheless, they are less precise than stereotactic neuronavigation [64-68]. In comparison to stereotactic

neuronavigation, the standard function guided method should only be used in situations that do not require high spatial precision whereas the 10-20 EEG system could be used as a low-cost alternative.

Stereotactic neuronavigation

Stereotactic neuronavigation is system which is used in neurosurgery and neuropsychiatry to provide a three-dimensional orientation of the neurological structures enclosed in the skull and vertebral column. Stereotactic neuronavigation can be either frame based or frame-less. Frame based neuronavigation allows for navigation, along a mechanical frame, in the dorsal, transversal and sagittal plane. Frameless neuronavigation uses external fiducial markers (natural occurring or artificially added) and infrared cameras instead of a mechanical frame [67, 69-71]. Prior to stereotactic neuronavigation, a structural scan of the patient's head is required. Using a common reference frame, anatomical landmarks and the target are simultaneously registered on the patient's head and its scan. Thereby enabling real-time guided placement of the TMS coil over the chosen target.

While placing a TMS coil over the target, frameless neuronavigation provides a within-session repeatability of 1.7mm and a between session deviation of 2.5mm [71]. These deviations are comparable to the allowed measurement error of frameless neuronavigation for successful intracranial biopsies [72-77]. Nonetheless, the accuracy of frameless stereotactic neuronavigation is influenced machine made errors and man made errors [74]. Machine made errors include software combability, slice thickness, voxel dimension and the properties of the magnetic field of the MRI [72, 74, 78, 79]. Man made errors on the other hand originate from misapplication and insufficient experience or training of the operator [71, 80].

Cortical motor threshold

The appropriate TMS treatment intensity is set in function of the cortical excitability or the cortical motor threshold (CMT). An accurate and reliable determination of the CMT is of essence to reduce the risk of over- and under-stimulation of the subject. Over-stimulation could lead to severe adverse effects [81] whereas under-stimulation could cause treatment failure [82].

The CMT is in general defined as the minimal TMS intensity required to provoke at least five out of 10 electromyographic (EMG) responses - of at least 50 μV - in a contralateral fully relaxed muscle [25]. Rossini's paradigm to determine the CMT first locates the spot, which provokes the clearest

muscle contraction. Over this hot spot, a descending staircase method is applied to obtain the CMT. The drawback of this relative frequency method is being time consuming due to the use of relatively high number of pulses [83, 84]. Alternatively, although less accurate, the CMT can be determined by replacing the EMG response with observation of movements of the thumb or fingers [85]. Despite its drawbacks, the visual assessment of the CMT is currently the method of choice in clinical and experimental settings. An option to reduce the time and the number of pulses, would be to use only 6 instead of 10 trials. Nonetheless, for clinical and research purposes, it is advised to use at least 10 out of the 20 trials to obtain reproducible results [86]. Another alternative would be to use the Mills and Nithi paradigm, which uses two thresholds to estimate the CMT: the lower and the upper motor threshold. The arithmetic mean of both thresholds defines the CMT [87]. Despite the use of fewer stimuli to determine the CMT, it is as time consuming as the Rossini paradigm. A computational method to determine the CMT is the adaptive method. This method uses an S-shaped function to find the relationship between a set intensity and its probability of eliciting a motor evoked potential. Thereby an intensity is predicted which provokes at least 5 out of 10 motor evoked potentials. The adaptive method uses even less stimuli than the two-threshold method by Mills and Nithi. Drawbacks of this method are complexity and a high cost to be used clinically. This method may require additional software and hardware that needs to be purchased, this in comparison to the visual Rossini method. Even more, besides a reduction of stimuli holds the adaptive method no other advantage. All the above-mentioned methods may be used in research and clinical settings if EMG is being used [81, 88].

Even though the motor cortex excitability fluctuates across individuals, it remains stable within the individual over time [89, 90]. Nonetheless, an inaccurate CMT can still be registered due to physiological, technical and pharmacological reasons [88, 91]. Intrinsic fluctuations of the cortical and spinal neurons' excitability can account for the physiological differences in the CMT, whereas technical imprecisions are not only due to the measurement method but also the use of different coil types, coil-cortex distance, the TMS treatment itself and arousal level [25, 92-94]. Because the created magnetic field declines exponentially with the distance from the coil, increasing the coil-cortex distance increases the CMT [92, 93, 95]. It is obvious that every factor that increases the coil-cortex distance could potentially influence the CMT [96]. For example, aging may cause atrophy of the grey

matter, which provokes a significantly higher coil-cortex distance, and on its turn this could account for a higher CMT [92, 93].

It is yet unclear whether a repetitive TMS (rTMS) treatment influences the CMT, but the literature suggests no influence on the CMT [90]. Drugs, such as carbamazepine and phenytoin, augment the CMT [97-99]. Not only benzodiazepines influence the motor threshold, anaesthetics such as etomidate, propofol, thiopental, and isoflurane, may also supress motor evoked potentials (MEPs) induced by TMS [100, 101].

Transcranial magnetic stimulation and its role in depression

Major depressive disorder

Major depressive disorder (MDD) or depression is a common heritable psychiatric disease. It is defined by a consistent depressed mood, diminished interest in pleasure (anhedonia), a reduction in movement, feelings of guilt, the inability to think and recurrent thoughts of death. This disabling disease has a comorbidity rate ranging from 40 to 50% with anxiety [102, 103]. Worldwide 120 million people are affected, and it has a lifetime prevalence of 10 to 25% for women and 5 to 12% for men [104-106]. First-line treatments of depression consist out of psychotherapy or psychopharmaceuticals or a combination of both. When depression is treated with antidepressants, the remission rate is 30 to 40% [107].

The monoamine hypothesis of depression

Monoamines are neurotransmitters that consists of an amino group connected to an aromatic ring. This group is one of the four neurotransmitter groups. The other three groups are the amino acids, acetylcholine and neuropeptides. The group of monoamines holds two catecholamines (dopamine (DA) and noradrenaline (NAD)) and an indolamine (5-hydroxytriptamine (5-HT) or serotonin). The catecholamines are formed from the amino acid tyrosine, which undergoes hydroxylation and decarboxylation to become DA [108]. After synthetisation, DA is transported into vesicles. Upon neuronal excitation, the vesicles are emptied into the synaptic cleft and DA binds to its pre- or postsynaptic receptor. The dopaminergic transmission is terminated by a re-uptake or degradation of DA [109-111]. After re-uptake into the presynaptic neuron, mediated by the dopamine transporter (DAT), DA is stored in vesicles or, when accumulated freely in the cytosol, broken down by monoamine oxidase (MAO). The extracellular DA is also taken up by the glial cells surrounding the synaptic cleft, where it is degraded by MAO and catechol-O-methyl-transferase (COMT) [109]. The 2 main breakdown products of DA are 3,4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) [112-114]. Serotonin on the other hand, is synthesized from the essential amino acid L-tryptophan. Tryptophan is hydroxylated and decarboxylated to form sequentially 5-hydroxytryptophan (5-HTP) and serotonin (5-HT). Like DA, 5-HT is stored in vesicles prior to neuronal activation. After its

release, the serotonergic neuronal transmission is ended by removal of 5-HT from the synaptic cleft, mediated by the serotonin transporter (SERT). Serotonin that is not stored in vesicles is degraded by MAO and a dehydrogenase to form 5-hydroxy-3-indolacetic acid (5-HIAA)[115, 116]. The breakdown products of both DA (HVA and DOPAC) and 5-HT (5-HIAA) are diffused from the neuronal or glial cells into the extracellular space towards the cerebrospinal fluid (CSF) [117]. The concentration of the metabolites of DA and 5-HT resemble the turnover rate of the parent molecules. In addition, DOPAC, HVA and 5-HIAA have been put forward as biomarker for diseases in the central nervous system[118]. Although inconsistent results, a recent meta-analysis found a significant decrease in CSF HVA in depressed patients compared to healthy controls. Thus, HVA in the CSF could be a valid biomarker for depression [119].

The first findings supporting the link between monoamines and depression were presented by Shore and co-workers [120]. They found that reserpine, a treatment for high blood pressure that causes depression as a side effect, provoked a 5-HT depletion. Even more, this reserpine induced depression was reversible by administering 3,4-dihydroxyphenylalanine (L-DOPA), the precursor of DA and NAD [121]. Subsequently the drug iproniazid, used to treat tuberculosis, was found to have a positive influence on a person's mood. Later, it was proven that this drug exerted its anti-depressive action by inhibiting the MOA enzyme [122, 123]. Axelrod and his team discovered that imipramine, a tricyclic antidepressant, acted upon the reuptake mechanism of NAD [124, 125]. A similar action on re-uptake mechanism of action was found for 5-HT, by which it became clear that imipramine inhibits the re-uptake of NAD and 5-HT [126]. These findings led to the monoamine hypothesis of depression, which states that depression is caused by a deficiency of 5-HT, DA and NAD [119, 127-132]. As such, most psychopharmaceutical treatment modalities (e.g. selective serotonin reuptake inhibitors (SSRI)) for depression are based on normalizing monoaminergic neurotransmission.

The role of the serotonin transporter in depression

Alterations in the brain serotonin transmission play a key role in the development of depression [133]. Evidence supporting this statement comes from studies indicating a reduction in serotonin (5-HT) and its metabolites in depressed patients, a depressive relapse after tryptophan

depletion, a reduction of the 5-HT uptake binding sites in depression and the effectiveness of selective serotonin reuptake inhibitors (SSRIs) [134, 135].

SSRIs, the current first-line pharmacologically treatment modality for depression, exert their function by elevating the 5-HT level through blocking of the serotonin transporter (SERT) [136]. This transporter is a transmembrane protein that actively transports 5-HT from the synaptic cleft into the presynaptic neuron, thereby ending the 5-HT neurotransmission. By administering SSRIs, the availability of the SERT decreases. This causes a decreased 5-HT re-uptake speed, which is accompanied by an improvement of depression [137].

The density of the SERT plays, besides its availability, a crucial role in depression. Brain regions with the highest density of SERT are the thalamus, the hypothalamus, the amygdalae, the raphe nuclei, the nucleus caudatus and putamen [137-139]. Of note here, regions with high SERT density are constant between different species, such as rodents [140], pigs [141], cats [142], non-human primates [143] and dogs [144]. However, post mortem and imaging studies SERT density studies in depressed and healthy individuals revealed contradictory results. The SERT density in depressed patients can either be decreased [138, 145-153], increased [154], or be similar [155-157] as in non-depressed patients. Nonetheless, the chronic use of SSRIs mediates a decrease in the SERT density (down regulation), which can account for the long-term effects of the SSRIs [158-160].

First-line treatment modalities

Psychotherapy, psychopharmaceuticals and brain stimulation techniques have been established as effective treatment modalities for depression [161]. Despite the presence of a wide array of psychopharmaceuticals and psychotherapy, a large amount of people in need of treatment does not receive any care. This treatment gap for depression is, although varying by region, on average 54% [162].

Psychotherapy includes cognitive behavioural therapy, interpersonal psychotherapy, problem-solving therapy, behavioural activation and psychodynamic therapy. Cognitive behavioural therapy is focussed on reducing stress and inducing adaptive coping by correcting the patient's wrong beliefs and maladaptive information processing strategies [163]. This treatment combines behavioural and cognitive techniques [164]. If only behavioural techniques are used than it is called behavioural activation.

Interpersonal psychotherapy is a time limited (12-16 weeks) treatment that focuses on the patient's current interpersonal relationships [165]. Thereby, this strategy intervenes with symptom formation and the social dysfunction associated with depression [166]. Problem solving therapy teaches the patient how to solve life problems step-by-step [167, 168]. Psychodynamic therapy or psychoanalytic therapy aims at making the patient's conscious mind aware of its unconscious thoughts and feelings that have affected its thinking and behaviour. Psychotherapy can either be administered individually, in-group or as guided self-help and have proven to be as effective as psychopharmaceuticals. Nonetheless, on average half of the patients receiving psychotherapy stop their treatment prematurely [169]. Combining psychotherapy and psychopharmaceuticals are more effective than both stand-alone therapies [170-173].

The wide range of psychopharmaceuticals or antidepressants consists out of selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), selective norepinephrine reuptake inhibitors (SNRIs) and monoamine oxidase inhibitors (MAOIs). The action mechanism of these antidepressants is predominantly based on the inhibition SERT (SSRIs and TCAs), the norepinephrine reuptake transporter (TCAs, SNRIs) or the enzymatic breakdown (MAOIs) [174, 175]. The choice of the administered antidepressant depends upon the severity, chronicity and resistance to psychotherapy [176, 177]. After an initial trial treatment of 8-12 weeks, partial remission can be achieved. Once established, the dose of the antidepressant can be augmented and/or combined with other antidepressant or a switch to another antidepressant is tried in order to obtain full remission. Nonetheless, it is only after several weeks of treatment that the antidepressant effect is noticeable. This effect is likely the result of downstream effects that affect signal transduction and gene expression.

If psychotherapy and antidepressant do not lead to remission, then neuromodulation techniques may be administered. Neuromodulation techniques can be invasive or non-invasive. Electroconvulsive therapy (ECT (electroshock therapy (EST) in rodent)), an invasive neuromodulation technique, consists out of applying electrical shock directly to the patients scull thereby causing convulsion. This while the patient is under general anaesthesia. Non-invasive alternatives for ECT are transcranial direct current stimulation (tDCS) and transcranial magnetic stimulation (TMS) [178, 179].

TMS as treatment modality for depression

Functional imaging studies indicated that MDD is characterized by reduction in blood flow and glucose metabolism of the left prefrontal cortex, anterior cingulate cortex and caudate nucleus [14, 15, 180-182]. Even more, an imbalance between the left and right frontal cortex is present. A relatively hypoactive left frontal cortex is associated with a relatively hyperactive right frontal cortex [183, 184]. This imbalance reflects itself in the rTMS treatment protocols for MDD. Patients with MDD have benefitted by HF-rTMS over the left frontal cortex and LF-rTMS over the right frontal cortex, thereby with the assumption of respectively increasing or decreasing neuronal activity [53, 185-188]. Currently, only HF-rTMS treatment of the frontal cortex is FDA approved and accepted treatment for MDD by the American Psychiatric Association[1] (APA), the Canadian Network for Mood and Anxiety Treatments[2] (CANMAT), the World Federation of Societies of Biological Psychiatry [3] (WFSBP). More specifically, this approval was based on a large multicentre sham-controlled randomized study, which indicated superiority of an active HF-rTMS protocol over the sham condition. The applied HF-rTMS treatment was set at an intensity of 120% of the MT and 10 pulses per second. The intertrain interval was set was set at 26 seconds and a train length of 4 seconds. A treatment session lasted 37.5 minutes during which 3000 pulses were given in total. Five daily sessions were applied over the left frontal cortex during 4 to 6 weeks [189, 190]. In Europe, this technique has received its CE mark approval assigned to class IIa and IIb [191]. Recently, theta burst stimulation (TBS) for the treatment of MDD has also received its FDA approval. TBS is a specialized form of rTMS that, through its novel stimulation pattern, mimics neuronal oscillation patterns corresponding to effective cognitive processing. High frequency burst (50 Hz) are given either continuously (cTBS) or intermitted (iTBS). cTBS exerts an inhibitory effect whereas iTBS provokes an excitatory effect [192-194]. In comparison to the FDA approved classic HF-rTMS treatment for MDD, iTBS can achieve a comparable clinical effect after a

[1] https://www.psychiatry.org/

[2] http://www.canmat.org/

[3] https://www.wfsbp.org/home/

stimulation period of 3 minutes versus 37,5 minutes for the rTMS protocol. The iTBS protocol delivered pulses at a frequency of 50Hz during 2 seconds at a frequency of 5Hz. Per session 800 pulses were given. Five sessions were given every week (one per day) for 4 weeks. This implies that by the use iTBS the number of treated patients per day can be increased without compromising the clinical response [195].

There is evidence that therapeutic effect of TMS could be mediated by an enhancement of monoaminergic neurotransmission [11, 196, 197]. Functional imaging in healthy and depressed humans showed a transient increase of striatal dopamine after a single rTMS session over the left frontal cortex [198, 199]. Furthermore, single rTMS stimulation over the motor cortex can evoke central and peripheral release of dopamine [200, 201]. Low frequency rTMS over the motor cortex induces a decrease in HVA in the CSF of people with Parkinson's disease [202]. In anesthetized macaque monkeys, a single HF-rTMS treatment releases striatal dopamine [203]. In the rodent rTMS model, micro dialysis revealed a dopamine increase in the dorsal hippocampus, the shell of the nucleus accumbens and the dorsal striatum after acute HF-rTMS [11, 196, 197, 204, 205]. Acute HF-rTMS provokes a significant increase of the DA turnover rate in the frontal cortex and decrease in the striatum and the hippocampus [11]. Besides DA, acute HF-rTMS increases 5-HT and 5-HIAA in the rat's hippocampus, however the global 5-HT catabolic turnover rate was not affected [11]. Moreover, acute HF-rTMS over the left frontal cortex induced changes in the tryptophan/5-HT metabolism in the limbic system of healthy people [206]. On the other hand, Kanno found in rats that acute HF-rTMS inhibited an induced release of 5-HT [207]. Nonetheless, there is also evidence that acute rTMS might have no significant effect on the 5-HT level in rats [208]. Sibon et al (2007)[206] found, in comparison to stimulation of the left occipital cortex, that the tryptophan/5-HT metabolism was significantly decreased in the left parahippocampal gyrus and the right insula whereas an increase was found in the right cingulate gyrus and cuneus. These findings indicate that an acute HF-rTMS treatment can modulate the dopaminergic and serotonergic system, which could be noticeable in the brain and reflected periphery[201, 209].

Repetitive HF-TMS may exert its action through the SERT either through a downregulation of the SERT mRNA, an internalization of the SERT protein or indirectly by a release of 5-HT [11, 159, 210]. This results in an increase concentration of 5-HT in the synaptic cleft. Thereby, the mechanism of

action of rTMS may be similar to that of chronic SSRIs usage as hypothesized by Best [211]. This implies that when rTMS would be applied in subjects with a low vesicular (intracellular) 5-HT concentration (depression or anxiety), which is accompanied by a lower firing rate of the raphe nuclei, a normalization of response to bursts of the cells of the raphe nuclei would occur. This elevation in extracellular 5-HT in the raphe nuclei decreases the tonic firing rate of those cells (mediated by the 5-HT_{1A} autoreceptor) [212]. Consequentially, a decrease in 5-HT release in terminal regions of the raphe nuclei is provoked. It must be kept in mind that all the above changes not only comply with changes induced by psychotropic interventions but by neuromodulation modulation techniques as well. Shen (2003) found an immediate and long-term decreased SERT mRNA expression in the raphe nuclei after acute and chronic ECT [213]. Although they were not able to reproduce their findings, they also found an increased SERT in the frontal cortex as Hayakawa et al. (1995) previously reported [214, 215].

Transcranial magnetic stimulation and animal models for depression

Most of the rTMS studies on the monoaminergic system and the SERT were performed in rodents. Extrapolating this data to humans remain difficult due to several hurdles [216]. The first hurdle is the state of consciousness. Although rTMS can be conducted in awake humans and animals, anaesthesia/sedation may be needed in animal models [217]. The latter is preferred to stimulation while awake or under mechanical restraint due to technical (e.g. movement) and ethical considerations. Despite the fact that anaesthetics (e.g. dexmedetomidine, isoflurane, midazolam, ketamine) depress the neural activity [218-221], neural effects of rTMS have been shown in anesthetized rats. However, Gersner found different effects on neuroplasticity markers (BDNF, GluR1) between anesthetized and awake rats after rTMS [222]. Finally, conscious animals might react to the acoustic and tactile stimuli provoked by the TMS, which could cause a loss of focality and efficacy [223]. Focality is of the essence in order to mirror human TMS studies. A human figure-of-eight coil can easily affect 100-200 mm^2 of the underlying cortical area [224] and stimulate as focal as 0.5 cm^3 [22] whereas the global adult rat brain comprise on average only 1.5 cm^3 [223]. This implies that while focal stimulation is achieved in humans, whole brain stimulations are executed in the rat [217]. A solution to achieve focal stimulation in rats is the use of smaller, more focal coils. Notwithstanding, a reduction in coil size involves limiting factors such as coil overheating and a drop in efficiency [224]. As important as focality is the accuracy of the coil placement. Localization of the target region in rats is frequently done by means of stereotactic frames, which supplies a larger level of accuracy than non-stereotactic frameless neuronavigation systems. Nonetheless, time, safety and cost can be reduced when choosing frameless over frame-based systems [225]. Recently cats, dogs and monkeys have been subjected to (r)TMS [203, 226-231]. The use of these animals as model would allow accurate and focal stimulations in awake animals, mirroring human (r)TMS research. Although it has been possible to stimulate cats and monkeys awake [226, 229], anaesthesia or sedation may still be preferred [221]. Based on the phylogenetic closeness, the monkey would be the preferred animal model in preclinical rTMS research. Nevertheless, high costs and ethical considerations coincide with the use of monkeys, which limits the use of this species in

preclinical research [223]. Besides monkeys, dogs have proven their ability to be a valid natural animal model for several psychiatric conditions thereby presenting themselves as a novel TMS animal model [16, 229, 232-236]. In order to unravel the neurobiological effects of TMS, functional molecular brain imaging techniques such as positron emission tomography (PET) and single photon emitted computed tomography (SPECT) are used. The use of these techniques is limited in humans due to the involvement of radiation and related radioprotection issues, which forms less of a problem in animals. Subsequently, dogs would be a logical choice to perform longitudinal TMS studies in combination with brain imaging techniques.

Animal models for depression

Current theories concerning its neurobiological mechanism are mostly based on animal models [237, 238]. An animal model is considered relevant for human pathology when it fulfils three criteria: face validity, construct validity and predictive or pharmacological validity [238-240]. As put forward by Willner (1984)[240] "Face validity is assessed by whether antidepressant effects are only present on, or are potentiated by, chronic administration (1), and whether the model resembles depression in a number of respects (2), which are specific to depression (3), and do actually coexist in a specific sub-group of depressions (4); also, the model should not show features which are not seen clinically (5)". "Construct validity correspond to the fact that 'both the behaviour in the model (1) and the features of depression being modelled (2) can be unambiguously interpreted, and are homologous (3), and whether the feature being modelled stands in an established empirical (4) and theoretical (5) relationship to depression". "Predictive validity relies on five sub-criteria: 'whether a model correctly identifies (1) antidepressant treatments of pharmacologically diverse types (2), without making errors of omission (3) or commission (4), and whether potency in the model correlates with clinical potency (5)" [240]. Additionally, pathological validity can be evaluated. Pathological validity is only a potential criterion that validates animal models based on their similarities in post-mortem pathological or serological changes in depressed humans [238, 241].

Current animal models for depression are based on the aetiology of depression and can be achieved by exposure to acute or chronic stress and a disruption of the hypothalamic-pituitary-adrenal (HPA) axis.

The rodent animal models

a. Chronic Unpredictable Mild Stress (CUMS)

The CUMS model was developed to maximise the unpredictable nature of stressors and their time of delivery [242]. Initially, rats were subjected to 21 days of severe unpredictable stressors such as shock, food deprivation, water deprivation, tail pinch, dipping in cold water, change of cage mate. This model was, in rats, able to reduce their basal activity, eliminated their response to acute stress, induce anhedonia and increase their corticosterone level. Thereby the CUMS model was able to simulate signs of depression in rats. Even more, rats treated with an antidepressant restored their ability to react to acute stress [242-245]. Later, Willner et al. (1987) modified this model to reduce the severity of the stressors, thereby resembling stressors from the daily life [244].

When compared to the symptoms of depression indicated by the DSM-V, the CUMS model can provoke all symptoms of depression, including anxiety, in rats. Only symptoms inherent to humans such as the presence of a depressed mood or recurrent thoughts of death were not noticed in rats [246, 247]. Even more, the CUMS model shows neurobiological changes identical to those in depression [248]. Thereby confirming the construct and face validity of the CUMS model. The provoked symptoms can be reversed by antidepressants and neuromodulation techniques such as rTMS and electroshock therapy (EST) [249-252]. On the other hand, they cannot be reversed by pharmaceuticals that do not treat depression [246]. It has to be noted that the success of the CUMS model to provoke depression in rats depends on individual differences in susceptibility to stress, the severity of the stressors, and good laboratory practice [247, 253].

b. Corticosterone model

Stress activates the HPA axis, which results into an increase of serum glucocorticoids. These corticoids allow the body to respond to a stressor and a return to baseline conditions [254]. A persistent

overactive HPA axis is thought to mediate symptoms of depression. On the other hand, daily 3 weeks injections of corticosterone (CORT, a corticoid hormone) induces, dose dependently, depression-like behaviour and anxiety in rats [255-260]. Thereby confirming its face validity. Even more, administration of a glucocorticoid receptor antagonist such as mifepristone reduces depression in humans as well as depressive-like symptoms in corticosterone treated rats [261, 262]. Regarding construct validity, Su et al (2014) [263] discovered alterations of the glucose metabolism associated with depression: in the insula, limbic system, basal ganglia, thalamus and cerebellum. The CORT model in rats also induced these findings [264]. Van Laeken and his team (2018) were able to induce a significant decrease in glucose metabolism of the striatum and insular cortex while a higher metabolism was noticed in the cerebellum and the midbrain. The changes in the 5-HT_{1A} and 5-HT_{2A} receptor are parallel in humans suffering from MDD and rats treated with the CORT model [265-269].

c. Learned helplessness (LH)

Exposing an animal to an uncontrollable and inescapable stressor may lead this animal to the state of learned helplessness. The animal in the state of learned helplessness will fail to escape when exposed to the same but escapable stressor. Although this animal model suffers from a low reliability, it shares etiological and behavioural symptoms (face validity) with clinical depression in humans [270-272]. Animals in a state of learned helplessness express a decreased motor activity, anorexia, anhedonia, weight loss, and sleep disturbance [237, 273-278]. Even more the LH model has a good predictive validity since animals with LH respond well when treated with antidepressants such as selective serotonin re-uptake inhibitors (SSRIs), tricyclic antidepressants and monoamine oxidase inhibitors [279-281]. Even more, depressive symptoms in rats induced by the LH model are ameliorated by TMS and ECT [282-284]. Finally, animals that have developed LH express changes in their catecholamines when exposed to a stressor. Even more the effects of LH can be reversed by administering monoaminergic antidepressants [285-289]

The canine animal model

Besides rodents, the dog has been used as an animal model for depression. In fact, Seligman used dogs in his LH experiment [290]. During his experiment 150 dogs were subjected to electrical

stimulation of the paws while in an inescapable room. One hundred of these dogs failed to attempt to evade the shock while placed in an escapable room. Thereby, these authors concluded that LH in dogs was a fitting animal model for depression. However, it has to be kept in mind that not all dogs will fail to show signs of discomfort (LH) when exposed to stressors, this due to their temperament or by previous learning [291]. In order to respond to stressors, they can also choose to fight, flight, flee, or fidget [291, 292]. These behaviours may become more intense each time the animal tries to avoid the stressors and may therefore result in aggressive, fleeing, freezing or fiddling behaviour that is out of proportion thus leading to anxiety related conditions. Canine anxiety related conditions include aggression, separation anxiety, generalized anxiety, fears, phobias, and obsessive-compulsive disorders [293, 294].

Functional brain imaging studies with positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have identified similar alterations, between humans and dogs, in the neurotransmitter system and neuronal functions for impulsive aggression, compulsive disorders and other anxiety disorders [16, 232, 234, 295-297]. Anxious dogs show a decreased 5-HT_{2A} receptor binding index in the frontal, temporal and occipital cortex [234]. This cortical decreased binding index is also present in depressed untreated humans. Even more, anxious dogs display, as depressed humans, an asymmetry in frontal lobe neuronal activity. Improvement of dogs suffering from anxiety related conditions is seen when given psychopharmaceuticals or behavioural therapy or a combination of both, as in depressed human [18, 19, 293, 294, 298-301]. On the other hand, there are only few studies that calculated the optimal dosage regime for human psychopharmaceuticals in dogs based on functional brain imaging [302-305]. This could account for the initial failures of psychopharmaceutical treatment of canine anxiety related conditions. More specifically, due to the extrapolation of the administered dose based on human dose by body weight. Therefore, caution is needed when extrapolating drug dose regimens from humans to dogs[306-308]. Other treatment modalities such as electroconvulsive therapy are also able to improve behavioural changes in dogs suffering from LH [309], implying that dogs with anxiety related conditions would respond, neurophysiological and behaviourally, similar to humans and consequentially be used a natural occurring bidirectional translational animal model for TMS in depressed humans. Since no aHF-rTMS research has been performed in healthy dogs, the feasibility of

applying an aHF-rTMS protocol has to be assessed in healthy dogs. More specifically, it has to be investigated whether the same machinery, such as the coil and stereotactic localiser, and protocols can be safely used in dogs. Secondary, the applied aHF-rTMS protocols in healthy dogs should provoke similar neurobiological changes (e.g. on the regional cerebral perfusion, SERT availability and monoaminergic system) as in humans.

Functional brain imaging modalities

Disorders of the brain can be investigated structurally and functionally. The most commonly used functional brain imaging techniques include functional magnetic resonance imaging (fMRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT). fMRI is an imaging modality that evaluates functional connectivity in the brain either in rest or while performing a task. This technique is based on the blood oxygenation level dependent signal, which arises from the different magnetic properties of oxy and deoxyhaemoglobin in the blood [310]. PET and SPECT on the other hand are based on detection of gamma rays emitted by the patient after the injection of radionuclide [311, 312]. Radionuclides linked to a chemical substance, thereby forming a radiopharmaceutical, can be used to assess the brain glucose metabolism, blood flow and receptors.

SPECT

SPECT studies are based upon the spontaneous decay of a radionuclide resulting in the production of a single gamma ray with an energy ranging from 80 to 250 keV. After being administered to the subject, gamma rays traveling in a specific direction are selected by passing through a collimator. Gamma rays traveling in a direction different from the orientation of the collimator are blocked. When passing through the collimator successfully, the gamma ray reaches a scintillation crystal (a scintillator) upon which it produces multiple optical-wavelength photons. The number of produced photons is in proportion to the energy of the provoking gamma ray. Finally, each photon released by the scintillator is collected by photon multiplier tubes. These produce on their turn multiple electrons resulting in a detectable electrical current. Spatial resolution is dependent on several factors e.g. the radionuclide used, distance of the patient to the collimator, type of collimator…. For clinically used camera set-ups, spatial resolution is in general lower than that of PET.

The most frequently used isotopes for SPECT studies include ^{99m}Tc, ^{123}I and ^{111}In with a half-life of respectively 6 hours, 13.3 hours and 2.8 days. ^{99m}Technetium (^{99m}Tc) can be acquired from a ^{99}Molybdenum generator and emits gamma rays with energy of 140 keV. After elution, ^{99m}Tc can be directly added to exametazime (d,1 hexamethylpropylene amine oxime) forming the lipophilic radiopharmaceutical [^{99m}Tc]HMPAO. After intravenous administration, [^{99m}Tc]HMPAO passes

through the blood brain barrier and diffuses into the neurons. There, it is transformed and trapped as a hydrophilic secondary complex. Since this entrapped is in proportion to cerebral blood flow, [^{99m}Tc]HMPAO allows for the evaluation of the neuronal activity.

PET

Positron emission tomography is based on the spontaneous decay of a neutron-deficient radioisotope. During this process, a core proton is turned into a neutron, with the emission of a neutrino and a positron. The positron ((β^+) particle) has a mass identical to an electron but has an opposite charge. After traveling for a short distance (range, order of millimetres), the positron annihilates with an electron to produce 2 high-energy photons (each 511 keV). The two photons travel in a straight line in opposite directions (180°) and can be detected outside the patient using a gamma detector. When two gamma rays are detected simultaneously ("in coincidence"), it is assumed that they originated from the same decay/annihilation event. Only those photons arriving simultaneously at the opposing crystal surface will be accepted by the system as true events and as such will be allowed to contribute to the image. Due to this characteristic, electronic collimation is used instead of a physical collimator as in SPECT imaging. PET cameras are equipped with several rings of electronically interconnected gamma-detectors, which enable monitoring of the gamma-ray detection time.

The most extensively used radioisotopes for PET are ^{11}C, ^{15}O and ^{18}F. The first 2 radionuclides have a short half-life (20.3 minutes and 2.04 minutes respectively) and an on-site cyclotron is needed for production. The longer half-life of ^{18}F (109.8 minutes) allows transportation from commercially exploited cyclotrons [311]. In order to allow imaging of SERT the following PET radiopharmaceuticals or radiotracers can be used: 5-[^{18}F]fluoro-6-nitroquipazine, [^{11}C]McN5652, [^{11}C]MADAM, 4-[^{18}F]ADAM and [^{11}C]DASB. The latter has been set as the golden standard for SERT imaging due its high affinity, selectivity, high specific to nonspecific binding ratio, reversible high brain uptake and binding equilibrium within a reasonable time frame [313].

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Aims

The main objective of this doctoral thesis is twofold. First, the applicability and safety of an aHF-rTMS protocol over the left frontal cortex will be assessed in healthy dogs. Second, in order to get more insights into the immediate and long-term neurobiological effects of two different aHF-rTMS paradigms, an acute (single day) and chronic (four consecutive days) will be applied in healthy dogs and combined with SPECT (rCBF) and PET (SERT availability) assessed before and after stimulation.

Our general hypothesis is that aHF-RTMS over the left frontal cortex in dogs, using a human figure-of-eight coil would provoke similar functional changes as in humans. The effects provoked by 4-day accelerated HF-rTMS would impact stronger modulation of neuronal functions and the serotonergic system, a system consistently involved in the pathogenesis and treatment of mood and anxiety disorders both in man and dogs.

First, the feasibility of an aHF-rTMS in dogs will be tested by means of applying a single day aHF-rTMS protocol (5 stimulations on one day) with a regular human figure-of-eight coil placed over the left frontal cortex. Simultaneously, the accuracy of a human frameless neuronavigation system and motor threshold paradigm will be assessed in dogs. The immediate and long-term effects of aHF-rTMS on the brain will be assessed with [^{99m}Tc]HMPAO SPECT scans. It was hypothesized that the active aHF-rTMS protocol would induce changes in rCBF at the stimulation site and prove its superiority over a sham aHF-rTMS protocol.

Second, the effect of sedation and anaesthesia on the regional cerebral perfusion during an aHF-rTMS protocol will be assessed. Two aHF-rTMS protocols (single and a 4 day protocol) differing in the amount of sessions will be compared using [^{99m}Tc]HMPAO SPECT scans. We assumed that the anaesthesia would decrease the aHF-rTMS induced effects and that there would a larger rCBF increase when more aHF-rTMS sessions are applied.

Finally, the immediate and long term effects of both protocols on the SERT system will be evaluated using [^{11}C]DASB PET scans. Simultaneously, CSF and serum taps will be performed to find changes in central and peripheral 5-HT and DA and their metabolites. An increase in 5-HT and/or DA turn-over and/or their metabolites are expected to be present after the accelerated 4 days of stimulation

and supposedly significantly stronger when compared to single day or sham aHF-rTMS protocol. It is hypothesized that these changes would be reflected in the serum.

Chapter 2: The feasibility of aHF-rTMS in dogs.

Adapted from Changes in canine cerebral perfusion after accelerated HF-rTMS: a proof of concept study by Dockx et al (2018) [1]

Changes in canine cerebral perfusion after accelerated HF-rTMS: a proof of concept study.

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Abstract

In humans, researchers have proposed repetitive transcranial magnetic stimulation (rTMS) as treatment for several neuropsychiatric disorders. Other than in rodents, little research has been conducted in other species, such as dogs. Given the pathophysiological similarities between humans and dogs, the canine brain might react in a similar way to the effects of rTMS. However, the neurobiological effects of rTMS in dogs have not yet been investigated.

Therefore we designed a proof of concept study to evaluate the effect of rTMS on cerebral perfusion, measured with single-photon emission computed tomography (SPECT).

We performed an accelerated high frequency (aHF)-rTMS (20 Hz) protocol over the canine left frontal cortex. To accurately target this area, eight dogs underwent a 3 Tesla magnetic resonance imaging (MRI) scan before stimulation. Each dog's left frontal cortex was subjected to five consecutive aHF-rTMS sessions with a human figure-of-eight coil (the intensity set at 110% of the motor threshold). One week prior to and one day after the stimulations, dogs underwent a ^{99m}Tc-HMPAO (d,1 hexamethylpropylene amine oxime) SPECT scan and perfusion indices (PI) were obtained by semi-quantification.

aHF-rTMS resulted in a significantly increased PI in the left frontal cortex and the subcortical region. No significant differences were noted for the other regions.

As has been observed in humans, aHF-rTMS applied to the left frontal cortex alters regional perfusion in dogs.

Keywords: Canine; High frequency repetitive transcranial magnetic stimulation; Regional cerebral blood flow; Frontal cortex

Introduction

In 1985, Barker et al. proposed repetitive transcranial magnetic stimulation (rTMS) as a non-invasive method of inducing electrical depolarisations in targeted cortical brain regions [2]. This electromagnetic technique is based on Faraday's law of induction, which states that by varying an electromagnetic field in time, an electric field is generated in a conducting environment [3, 4]. When an electromagnetic coil is applied over the skull, a repetitive, changing, electromagnetic field induces secondary electrical currents in the neocortex. Depending on the brain region and the frequency of the magnetic field, the induced secondary electrical current can either stimulate neural activity, or inactivate it [5-7], associated with release of monoamines [8]. Additionally, depending on the intensity of stimulation, distant interconnected brain regions, and not just the targeted cortical areas, can be affected [9]. An rTMS treatment for major depressive disorder usually comprises 5 sessions (1 per day) per week over a period of 3 to 6 weeks. On the other hand, the same rTMS treatment can be given over a shorter time period (accelerated rTMS), resulting in similar, or even superior, effects on neuronal activity without causing severe side effects [10-12]

Repetitive TMS is currently being used to treat psychiatric and neurological disorders such major depressive disorder, epilepsy and anxiety in humans [13].

Additionally, rTMS is able to not only induce behavioural changes in rats, but also induces changes in monoamines, amino acids and blood derived neurotrophic factor as well [14-18]. However, extrapolating rTMS results from rodents to humans is not straightforward. On the other hand, dogs have proven to be a valid natural animal model for some of human neuropsychiatric disorders. Several studies involving positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have demonstrated similar alterations in neurotransmitter systems and neuronal function in humans [19-22] and dogs [23-25] suffering from impulsive aggression, anxiety disorders and compulsive disorders. Furthermore, both humans with depression and dogs with anxiety disorders demonstrate involvement of the frontal cortex. In humans, two networks have been implicated in depressive behaviour, one including the dorsolateral prefrontal cortex and the anterior cingulate cortex and the other consisting of the medial ventral frontal cortex and some subcortical regions [26]. In

depressed human patients, High Frequency repetitive Transcranial Magnetic Stimulation (HF-rTMS) of the left dorsolateral prefrontal cortex produced an increase in neuronal activity (increased regional cerebral blood flow [rCBF] or metabolism) and clinical improvement [27, 28]. Martlé et al. (2009) found interictal hypoperfusion of the subcortical region (including the thalamus) in epileptic dogs, which, as in humans, plays an important role in the initiation and propagation of seizures of epileptic disorders.

Currently, rTMS treatment of epilepsy is in its infancy in humans and recommendations regarding its use are noted as level C: 'possible efficacy for local rTMS over the left frontal cortex for epilepsy' [13]. Due to these neurobiological similarities of neuropsychiatric disease in dogs and man, rTMS might also benefit dogs with similar disorders.

Therefore, we sought to evaluate the effects on neuronal activity (measured with perfusion SPECT) after accelerated HF-rTMS (aHF-rTMS; 5 sessions in a single day) over the left frontal cortical area in normal dogs. Given the excitatory nature of HF-rTMS, we hypothesized that regional cerebral blood flow in the left frontal cortex would increase after aHF-rTMS.

Materials and methods

Animals

Eight healthy dogs (6 beagles, 2 mix breed foxhounds; 4 males, 4 females; aged between 4 and 8 years) that had never been treated with psychotropic medication and with no background of neurologic or behavioural diseases were included. Each dog received a clinical examination before and after the study. The Ghent University Ethical Committee approved this study and all guidelines for animal welfare, imposed by the Ethical Committee, were respected (EC 2014/85).

Magnetic resonance imaging

All eight dogs underwent magnetic resonance imaging (MRI). Images were collected on a Siemens 3 Tesla Magnetom Trio Tim system (Siemens Medical Systems) using the phased-array spine coil and a phased-array body matrix coil. The dogs were pre-medicated in a quiet room with dexmedetomidine (Dexdomitor, Orion Corporation) at 375µg/m^2 by intramuscular injection. General anaesthesia was induced IV with propofol at 2-3 mg/kg intravenously (Propovet Multidose, Abbott

Laboratories) through a cephalic vein catheter. Anaesthesia was maintained with isoflurane (Isoflo, Abbott Laboratories) in oxygen given to effect. Animals were placed head first in sternal recumbence in the scanner bore, with the front legs extended caudally.

A structural scan was acquired using a T1-weighted 3D MPRAGE sequence with 176 sagittal slices. Following sequence parameters were used: TR = 2250 ms, TE = 4,18 ms, TI = 900 ms, parallel acquisition method = GRAPPA with acceleration factor = 2, matrix size = 256 × 256, sagittal, FOV = 220 mm, flip angle = 9°, voxel size = 0.9 × 0.86 × 0.86 mm^3.

The left frontal cortex was identified using an anatomical atlas (Dua-Sharma, Sharma et al. 1970) and located in each dog on the acquired MRI images. The centre of the left frontal cortex was defined as the left cortical region located in the upper fourth part between the rostral segment of the lateral rhinal sulcus and the prorean sulcus; halfway the distance from the caudal rim of the olfactory lobe and the presylvian sulcus.

Neuronavigation

Prior to neuronavigation, the dogs were sedated with dexmedetomidine at 375µg/m^2 by intramuscular injection. Neuronavigation is used to localize structures in a closed space (e.g., the skull). Neuronavigation (Brainsight, Rogue-resolutions Ltd) consists of a computer coupled to an infrared camera and a subject tracker (equipped with infrared reflective beads), fiducial markers as reference for the position of the structure(s) in space and a free handheld stereotactic pointing device. The sedated dogs were placed in sternal recumbence, with the subject tracker attached to the neck. The 3 Tesla MRI data were used to provide spatial information and skin reconstructions. On this reconstruction, three to four fiducial markers (protuberentia occipitalis externa, top of the nose and lateral or medial corner of the eye) were identified with the stereotactic pointing device. The target's centre, the left frontal cortex, was identified on the MRI data set and the neuronavigation software then determined the external position of the target with the help of the handheld pointer combined with the spatial information of the fiducial markers.

The coordinates of the target site were individually measured on the skull (x-axis = distance between the top of the nose and the protuberentia occipitalis externa; y-axis= perpendicular on x-axis) using measuring tape (for a full description see Dockx et al., 2017)[30].

Experimental procedures

The dogs underwent operant conditioning (positive reinforcement) to become accustomed to the stimulation room, the researcher, the placing of and the sound of a sham coil. General anaesthesia was necessary during the stimulation sessions. The dogs were sedated with dexmedetomidine (Dexdomitor, Orion Corporation), dosed at 375 $\mu g/m^2$ by intramuscular injection. When deemed necessary an additional dose of 183 $\mu g/m^2$ was administered. The stimulation intensity was set at 110% of the motor threshold. The motor threshold was determined as the intensity that provoked 5 out of 10 visible muscle contractions in the right upper front limb.

An accelerated High Frequency (aHF-rTMS) protocol was chosen. This stimulation protocol, executed over the frontal cortex in humans, is FDA approved and accepted as a treatment for treatment-resistant major depressive disorder by the APA, the CANMAT and the, WFSBP [13, 31].

The left frontal cortex of each dog was subjected to 5 consecutive stimulation sessions in one day with a figure-of-eight coil (Magstim Company Limited) at 20 Hz (Fig. 1). The sessions consisted of 40 trains of 1.9 s duration, separated by a 12 s intertrain interval (1560 pulses per session). The time interval between sessions was 10 to 15 min. This protocol was identical to the daily aHF-rTMS performed in major depressive disorder patients [32, 33].

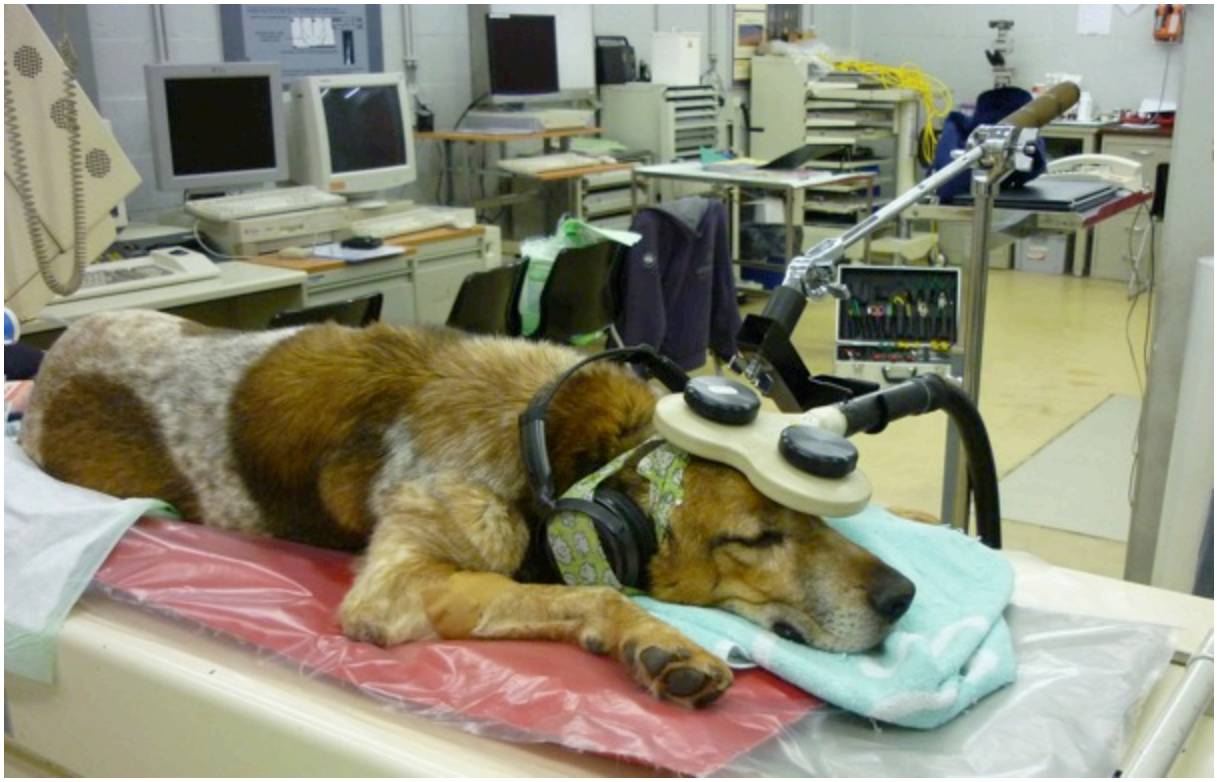

Fig. 1: A mixed-breed sedated foxhound undergoing high frequency repetitive transcranial magnetic stimulation (HF-rTMS). The centre of coil was positioned over the left frontal cortex perpendicular to the scull. Old headphones and earplugs where used to reduce any possible negative effects on the subjects hearing and sedation status.

Behavioural assessment

Each dog received a clinical examination and behavioural assessment before and after the study, which was scored using a validated behavioural questionnaire (canine behavioural assessment and research questionnaire; C-BARQ) [34]. This questionnaire contains everyday canine behavioural situations, each given a score from one to five by the faculty animal caretaker. The dogs were evaluated for the following behaviours: anxiety, fear and aggression, trainability, excitability, separation-related behaviour, attachment, attention seeking and chasing behaviour. The final score for each type of behaviour was obtained by calculating the mean of each scored behavioural situation reflecting on that type of behaviour.

SPECT Tracer

$^{99m}TcO_4^-$ was eluted from a ^{99}Mo generator (less than 24 h before each SPECT acquisition) and directly added to exametazime (d,1 hexamethylpropylene amine oxime (HMPAO); Ceretec, GE

Healthcare). A dose of 31,2 MBq (± 4,5 MBq) ^{99m}Tc-HMPAO per kilogram bodyweight was administered intravenously, through the cephalic catheter, to the sedated dogs approximately 15-20 min before the induction of general anaesthesia. This lipophilic tracer initially diffuses into the brain cells, where it is transformed and trapped as a hydrophilic secondary complex [35, 36].

SPECT scanning procedure

One week prior to the aHF-rTMS, each dog received a blank HMPAO-SPECT scan using sedation and anaesthesia protocols as described above. The SPECT scan was acquired 30-35 min after tracer injection. All dogs were scanned in ventral recumbence with a triple headed gamma camera (Triad, Trionix), equipped with low energy ultrahigh-resolution parallel hole collimators (tomographic resolution FWHM=9 mm). Data were acquired over a circular 360° rotation, for 20 min in step-and-shoot mode (120 steps, 10 s per step, 3° steps) on a 128×128 matrix. Peak energy was set at 140 keV and a symmetrical window width of 20%. Images were then processed using iterative reconstruction and a Butterworth filter (cut-off 1.6 cycli/cm, order 5) was applied. Pixel size was 1.72 mm. Twenty-four h after the final aHF-rTMS session all dogs underwent another SPECT scan.

Image analysis

Perfusion images from each dog were automatically registered to a template, generated from 14 beagle dogs (9 male, 5 female, mean age 50 months ± 20), using BRASS software (brain registration and automated SPECT semiquantification, Nuclear diagnostics) [37]. This template, based automated registration method, eliminates subjective operator-dependent region definition and the automatic registration facilitates the fitting procedure, necessary to compensate for intra-individual differences in anatomical brain size and shape. On this template a region map was generated, including 11 separate manually drawn volumes of interest (VOI), positioned (Fig. 2) over the frontal, temporal, parietal and occipital lobes of both hemispheres and over the cerebellum and the subcortical structures. To calculate the rCBF ratios (perfusion index), regional radioactivity was normalized to the radioactivity of the entire brain.

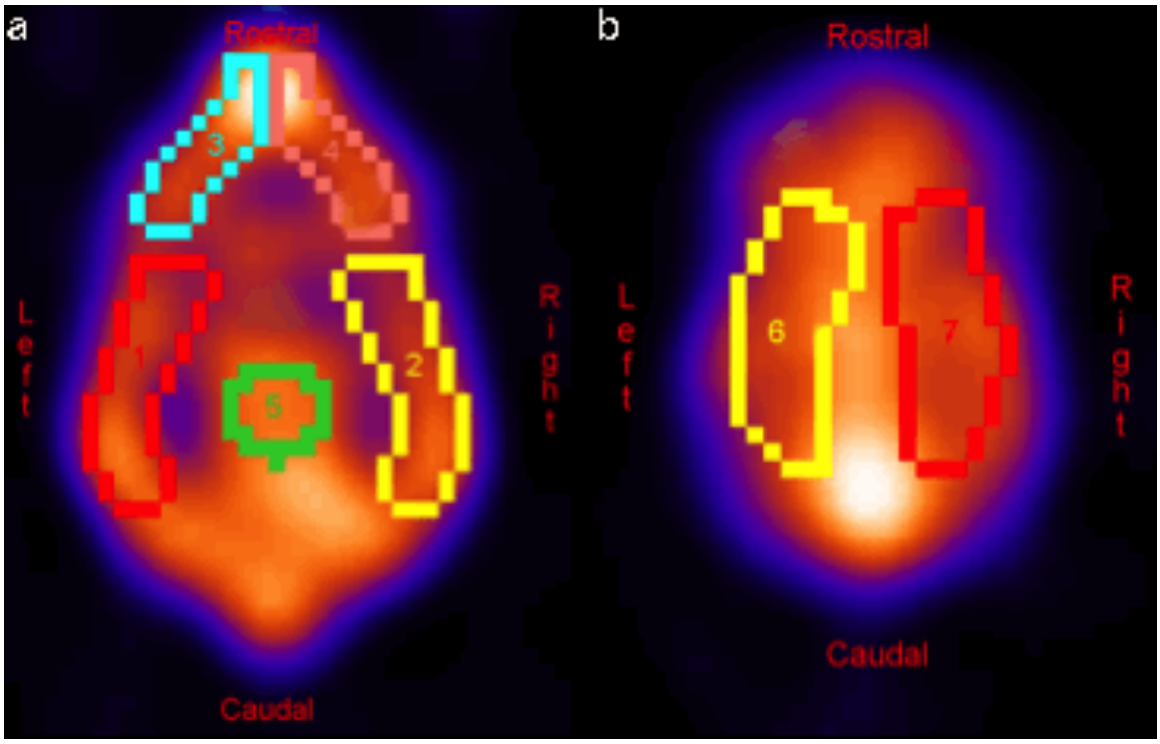

Fig. 2: 99mTc-HMPAO single-photon emission computed tomography (SPECT) images post stimulation. (a) mid-dorsal slice (b) high-dorsal slice; 1 left temporal; 2 right temporal; 3 left frontal; 4 right frontal; 5 subcortical; 6 left parietal; 7 right parietal.

Statistical analysis

To our knowledge, no literature concerning the effects of TMS on the rCBF in dogs exists. Therefore we chose a proof-of-concept design containing a one-group pre-test/post-test setup, with an absence of a control group. Two limitations should be kept in mind: a small sample size and the study design without control.

Rstudio 1.0.136 (R: a language and environment for statistical computing; R core team; R foundation for statistical computing, 2016) with packages MASS (version 7.3-45) and Sommer (version 3.0) was used for all statistical analyses.

The dataset contained the PI of 14 manually drawn VOI's (left temporal, right temporal, cerebellum, subcortical, olfactory lobe, left frontal, right frontal, left occipital, right occipital, left parietal, right parietal, left hemisphere, right hemisphere and total brain) at 2 different time points (baseline and 24 h after the last session) after aHF-rTMS stimulation under sedation. A multivariate

linear mixed model with heterogeneous variances (unstructured) was set up. The outcome variables included the PI of the 14 VOI's whereas the fixed factors (breed, age and sex) and random factors (time and animal) were set as predictor variables. A random intercept was included into the model. Model building was done by Log-likelihood ratio tests (Chi-squared) for nested models, after which interaction terms were taken into account. The Welsh-Satterthwaite equation was used to calculate the degrees of freedoms. The type-I error was set at 0.05.

Results

The fixed factors (sex, age and breed) were excluded from the final model by the log-likelihood tests. Time was the only predictor of PI in the final model. The PI of the left frontal cortex and subcortical region was increased by 0.05 (95%CI [0.008; 0.081], $P = 0.01$) and 0.15 (95%CI [0.089;0.207], $P < 0.001$) in all subjects (Fig. 3). The PI of the other brain regions remained unchanged (Table 1). The total brain, left and right cortical hemisphere tracer uptake after stimulation did not differ from the baseline tracer uptake. No behavioural changes were noted (Table 2).

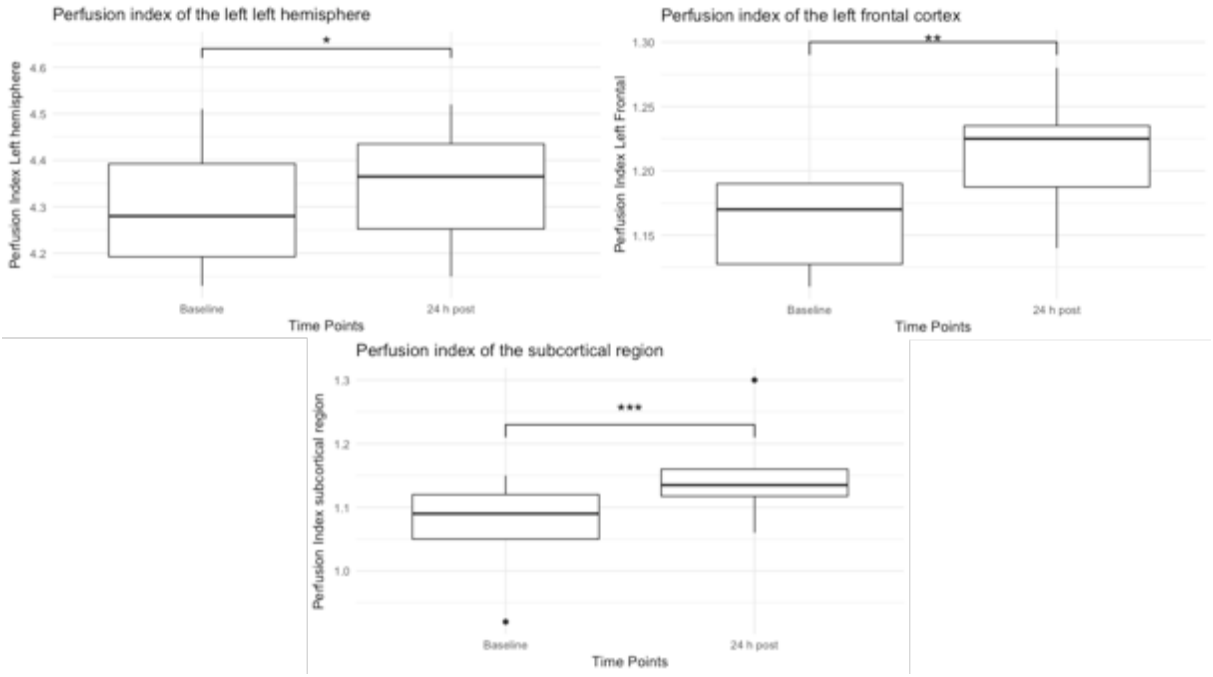

Fig. 3: Boxplot of the perfusion indices of the left frontal cortex and subcortical areas. *p<0.01; **p<0.001

Region	Estimate	SD	95% CI		p-value
			Lower	Upper	
Left temporal	-0.017	0.045	-0.044	0.010	0.21
Right temporal	-0.021	0.036	-0.059	0.017	0.27
Cerebellum	-0.022	0.061	-0.062	0.018	0.27
Subcortical	0.147	0.091	0.089	0.207	<0.001**
Bulbus olfactorius	0.022	0.136	-0.077	0.120	0.66
Left frontal	0.045	0.051	0.009	0.081	0.01*
Right frontal	0.003	0.065	-0.040	0.045	0.9011
Left occipital	-0.055	0.125	-0.138	0.029	0.20
Right occipital	0.016	0.099	-0.047	0.079	0.61
Left parietal	0.021	0.061	-0.025	0.067	0.36
Right parietal	0.020	0.057	-0.029	0.068	0.42
Total left hemisphere	0.063	0.151	-0.038	0.164	0.22
Total right hemisphere	-0.061	0.082	-0.131	0.009	0.08
Total	-0.034	0.114	-0.115	0.047	0.41

Table 1: Measured difference in perfusion indices for selected brain regions in healthy dogs before and after repetitive transcranial magnetic stimulation. Total left hemisphere = left temporal + left frontal + left occipital + left parietal; Total right hemisphere = right temporal + right frontal + right occipital + right parietal; Total = sum all eleven regions *p<0.01, **p<0.0001.

	Dog number							
	1	2	3	4	5	6	7	8
Traning and obedience	2.00	2.25	1.88	2.25	2.13	1.25	1.88	1.75
Owner-directed aggression	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stranger-directed aggression	0.29	0.00	0.00	0.29	0.29	0.29	0.00	0.00
Dog-directed aggression/fear	0.25	0.00	0.00	0.00	0.25	0.25	0.13	0.00
Familiar dog aggression	0.25	0.25	0.25	0.00	0.75	0.33	0.25	0.00
Chasing	Na	Na	Na	Na	Na	Na	Na	Na
Stranger-directed fear	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67
Nonsocial fear	0.00	0.20	0.20	0.60	0.50	0.25	0.50	0.50
Separation-related problems	0.75	0.88	0.63	0.75	0.75	0.75	0.50	0.38
Touch sensitivity	0.33	0.00	0.00	0.25	0.25	0.00	0.33	0.67
Excitability	2.25	1.75	2.00	1.50	1.50	1.25	1.00	0.75
Attachment/attention-seeking	1.80	2.60	1.60	1.60	1.75	1.20	1.40	1.60
Energy	3.00	2.00	2.00	2.00	2.00	2.50	2.00	1.50

Table 2: Behavioural scoring prior to the high frequency repetitive transcranial magnetic stimulation (HF-rTMS) session.

Discussion

Our findings indicate that an accelerated HF-rTMS protocol increases the regional cerebral blood flow at the stimulation site and the subcortical region in healthy dogs. This increased rCBF is detectable 24 h post stimulation without notable side effects (neurological, behavioural).

The remote stimulation of the subcortical area by local stimulation at the left frontal cortex might be the result of white matter connections between the different cortical and subcortical areas. Similar findings are reported in humans when the left dorsolateral prefrontal cortex is stimulated with HF-rTMS [9, 38-40]. High frequency stimulation over the left dorsolateral prefrontal cortex provokes an increase in rCBF at the stimulation site and in the subcortical area, including structures belonging to the limbic system. These changes in rCBF are thought to be one of the mechanisms behind its beneficial action in depression, a disease accompanied by left frontal cortex and limbic dysfunction [5, 38, 41]. We were not able to identify changes in the separate structures of the subcortical regions because of the low image resolution. The lack of changes in other brain regions could be the result of the chosen stimulation parameters/protocols [42], individual response to rTMS [43], the time interval between stimulation and the successive brain scan, the spatial resolution of SPECT, the quantitative data analysis technique or a combination of these factors. Of note here is that for every subject we targeted the left frontal cortex using individual brain anatomy based on neuronavigation, limiting the risk that other areas of the brain were stimulated.

A limitation of the study, besides the sample size and study design, is that a human figure-of-eight coil was used to stimulate the dogs' frontal cortices. A beagle's head is considerably smaller than a human head. Furthermore, the canine frontal lobe occupies approximately half the volume (as a percentage of the hemisphere) of the human analogue [44, 45]. However, the distribution of the magnetic field, created by the coil, is not influenced by the size and the shape of the skull [46]. In addition, the magnetic field size created by the used coil type, at a depth of 1.5 cm, covers a cortical area of less then 5 cm^2 when stimulating at 110% motor threshold [47]. The smaller size of the frontal cortex did not hamper the precision of stimulation, as no neighbouring cortical areas were stimulated. Therefore it can be assumed that a human figure-of-eight coil can be used to focally stimulate the canine frontal

cortex. Another confounder is the sedation with dexmedetomidine used prior to tracer injection. However, our research group demonstrated that dexmedetomidine caused no alterations in the frontal cortex region but decreased perfusion in the subcortical region, using ^{99m}Tc-ethyl cysteinate dimer (^{99m}Tc –ECD) [48]. It is well known that different tracers can result in differences in perfusion indices but in a comparative study with ^{99m}Tc -ECD and ^{9m}Tc -HMPAO, HMPAO had a lower perfusion index than ECD [36]. Both findings accentuate the findings of increased PI in the subcortical area after aHF-rTMS. Sedation and anaesthesia can also influence the outcome of an rTMS intervention because of the suppressive effect on neuronal activity. Since they are inevitable for rTMS applications, the influence of different forms of sedation and anaesthetics on perfusion alterations by rTMS should be evaluated in future studies. Strict adherence to one specific anaesthetic protocol is also mandatory. Sedation and anaesthesia can also influence the outcome an rTMS paradigm since they depress the neuronal activity. Since they are inevitable for rTMS applications, the influence of different forms of sedation and anaesthetics on rTMS should be researched.

A SPECT study performed in dogs with anxiety disorders demonstrated decreased perfusion of the left frontal cortex, when compared to dogs without anxiety disorders [23]. In that study, researchers found an average of 0.04 (SEM: 0.01) decrease in the left frontal PI. This decrease is of the same magnitude as the increased perfusion of the left frontal cortex, between baseline and post stimulation, that we observed in the current study. In human patients, perfusion alterations caused by high or low frequency TMS coincided with mood improvement [49-51]. Although speculative at this point, this might suggest that dogs with an anxiety disorder could benefit from (accelerated) HF-rTMS over the left frontal cortex. HF-rTMS could also be used for other neurological disorders such as epilepsy. Martle et al (2009) demonstrated that neuronal function during the interictal state was abnormal in epileptic dogs compared to normal dogs. Remote effects of rTMS on subcortical areas that we observed might be relevant for use of rTMS in canine epilepsy. Further research is needed to substantiate this assumption.

Conclusions

Despite the lack of a control group, this study showed a significant rCBF increase at stimulation site after HF-rTMS and in the subcortical region, whereas no changes where noted in behaviour or clinical examination. This proof-of-concept study suggests that HF-rTMS is a safe, non-invasive and pain free method of increasing rCBF at stimulation site and in remote areas in dogs. This study paves the way for investigations of neuronal effects in larger placebo-controlled populations, using SPECT or other functional imaging techniques such as positron emission tomography and functional MRI in order to refine treatment parameters and efficacy, and finally, for rTMS as a treatment modality for canine neurological disorders.

Acknowledgements

This research was funded by Ghent university special research fund (BOF 01J05715) and was supported by the Ghent university multidisciplinary research partnership 'The integrative neuroscience of behavioural control'. We thank the division of veterinary nuclear medicine (department of medical imaging and small animal orthopaedics, university of Ghent) to provide access to their infrastructure.

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Chapter 3: Non-stereotactic neuronavigation in dogs

Adapted from *Accurate external localization of the left frontal cortex in dogs by using pointer based frameless neuronavigation* by Dockx et al (2017[1])

Accurate external localization of the left frontal cortex in dogs by using pointer based frameless neuronavigation

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Abstract

In humans, non-stereotactic frameless neuronavigation systems are used as a topographical tool for non-invasive brain stimulation methods such as Transcranial Magnetic Stimulation (TMS). TMS studies in dogs may provide treatment modalities for several neuropsychological disorders in dogs. Nevertheless, an accurate non-invasive localization of a stimulation target has not yet been performed in this species.

This study was primarily put forward to externally locate the left frontal cortex in 18 healthy dogs by means of a human non-stereotactic neuronavigation system. Secondly, the accuracy of the external localization was assessed.

18 healthy dogs, drawn at random from the research colony present at the faculty of Veterinary Medicine (Ghent University), were used. Two sets of coordinates (X, Y, Z and X", Y", Z") were compared on each dog their tomographical dataset.

The non-stereotactic neuronavigation system was able to externally locate the frontal cortex in dogs with accuracy comparable with human studies.

This result indicates that a non-stereotactic neuronavigation system can accurately externally locate the left frontal cortex and paves the way to use guided non-invasive brain stimulation methods as an alternative treatment procedure for neurological and behavioral disorders in dogs. This technique could, in analogy with human guided non-invasive brain stimulation, provide a better treatment outcome for dogs suffering from anxiety disorders when compared to its non-guided alternative.

Introduction

Neuronavigation systems provide a three dimensional orientation of the neurological structures contained in the skull and vertebral column. They can either be frame or frameless based and have currently their main application in canine neurosurgery. In this field, frame based neuronavigation uses a mechanical frame, mounted on the skull. This allows navigation along the dorsal, transversal and sagittal plane of the brain [2]. Frameless neuronavigation on the other hand does not require a mounted frame to locate a brain region. It uses external fiducial markers (natural occurring or artificially added) and infrared cameras. Both commercially available neuronavigation systems, including a veterinary-specific frameless neuronavigation system (Brainsight stereotactic vet system, Rogue Research, Montreal, Quebec), have been used for stereotactic neurologic procedures such as biopsy or injections in the canine brain [3-8].

Neuronavigation is, besides in neurosurgery, used in human neuropsychiatry as a tool for non-invasive brain stimulation methods such as Transcranial Magnetic Stimulation (TMS)[9]. TMS has become a major player in the treatment modalities of neuropsychiatric conditions like depression and anxiety [10]. In order to accurately and repeatedly stimulate the targeted neocortical regions, coil navigation methods such as MRI based – non-stereotactic frameless neuronavigation systems are essential [11]. Moreover, a good localization of the target region provides a better treatment outcome [12].

In dogs with behavioral disorders, a deficiency in both neuronal function as well as the serotonergic system have been reported in the frontocortical region in general and the left in particular [13-16]. Guided non-invasive brain stimulation methods may therefore have a place in future treatments of drug resistant neuropsychiatric disorders in the canine species necessitating, similar to men, an accurate localization of the target region. A more accurate localization of the target region might, in analogy with humans, provide a better treatment outcome [12].

It is clear that feasibility and accuracy studies should precede guided non-invasive brain stimulation methods in dogs. However, to date, no studies have been conducted using a human non-stereotactic frameless neuronavigation system in dogs for non-invasive procedures such as TMS. Hence, the main purpose of this study was to test the feasibility of this type of neuronavigation system

to externally locate the left frontal cortex. Additionally the accuracy of the external localization was determined by means of comparing the coordinates of the set target and its external location.

Material and method

Animals

Eighteen dogs (2 fox-hounds and 16 beagles; 9 neutered males; 8 neutered females and 1 intact female: aged between 8 months and 8 years old) were included in this study. The dogs were owned by the Ghent University department of Small Animal and the Ghent University department of Veterinary medical imaging and small animal orthopaedics. The Ghent University Ethical Committee approved this study and all guidelines for animal welfare, imposed by the Ethical Committee, were respected (EC 2015_38).

Neuronavigation protocol (Fig. 1)

In order to obtain a tomographical dataset, all dogs underwent magnetic resonance imaging (MRI). A Siemens 3T Magnetom Trio Tim system (Siemens Medical Systems, Erlangen, Germany), using the phased-array spine coil and a phased-array body matrix coil, was used to collect the data set. Following the placement of an intravenous cephalic catheter, the dogs were pre-medicated intramuscularly (IM) with dexmedetomidine (375 μg/m^2 body surface, Dexdomitor®, Orion Corporation, Espoo, Finland). Propofol (Propovet Multidose®, Abbott Laboratories, Berkshire, UK, 1-2 mg/kg given to effect) was administered IV to induce general anaesthesia. Anaesthesia was maintained with isoflurane (Isoflo®, Abbott Laboratories, Berkshire, UK) in oxygen using a rebreathing system. The dogs were placed head first and sternally in the scanner bore. After completion of the MRI acquisition, the dogs were allowed to recover.

During this recovery phase, the dogs were placed in sternal recumbence, with the head positioned in a self-made mould (Fig. 2). The subject tracker was attached to the neck region in such a way that the infrared camera could detect the marker balls. If needed, the dogs were given dexmedetomidine (0,5-1 μg/kg; IV) to complete the following neuronavigation protocol.

First, the tomographical data were loaded into the neuronavigation system (Brainsight, Rogue-resolutions Ltd, Cardiff, UK). Using the software, a skin reconstruction was made. Next, three to four fiducial markers (landmarks) were created and identified on the skin reconstructions: the top of the nose, the external occipital protuberance and one or both medial corners of the eyes. In some dogs, the MRI coil caused a moderate to severe ventro-abaxial displacement of the skin, which hindered the identification of one of the medial corners of the eyes. In these cases the medial corner(s) were identified based on the MRI images instead of the skin reconstructions. Subsequently, the center of the left frontal cortex was set as target and was manually identified on the tomographical dataset. The center of the left frontal cortex was determined as the cortical region situated in the upper fourth part between the prorean sulcus and the rostral part of the lateral rhinal sulcus; halfway the distance from the caudal rim of the olfactory lobe and the presylvian sulcus. The target's external position was located by holding a pointer – connected to three reflecting balls - perpendicular over the target region as indicated by the neuronavigation software. This external position was marked on the fur using a permanent marker. The position of the mark was then confirmed by once again locating the target's external position with the pointer. At last, the position of the mark was registered in a Cartesian coordinate system. The Y-axis ran from the external occipital protuberance to the top of the nose, whereas the X-axis was placed perpendicular to the Y-axis containing the center of the externally placed mark.

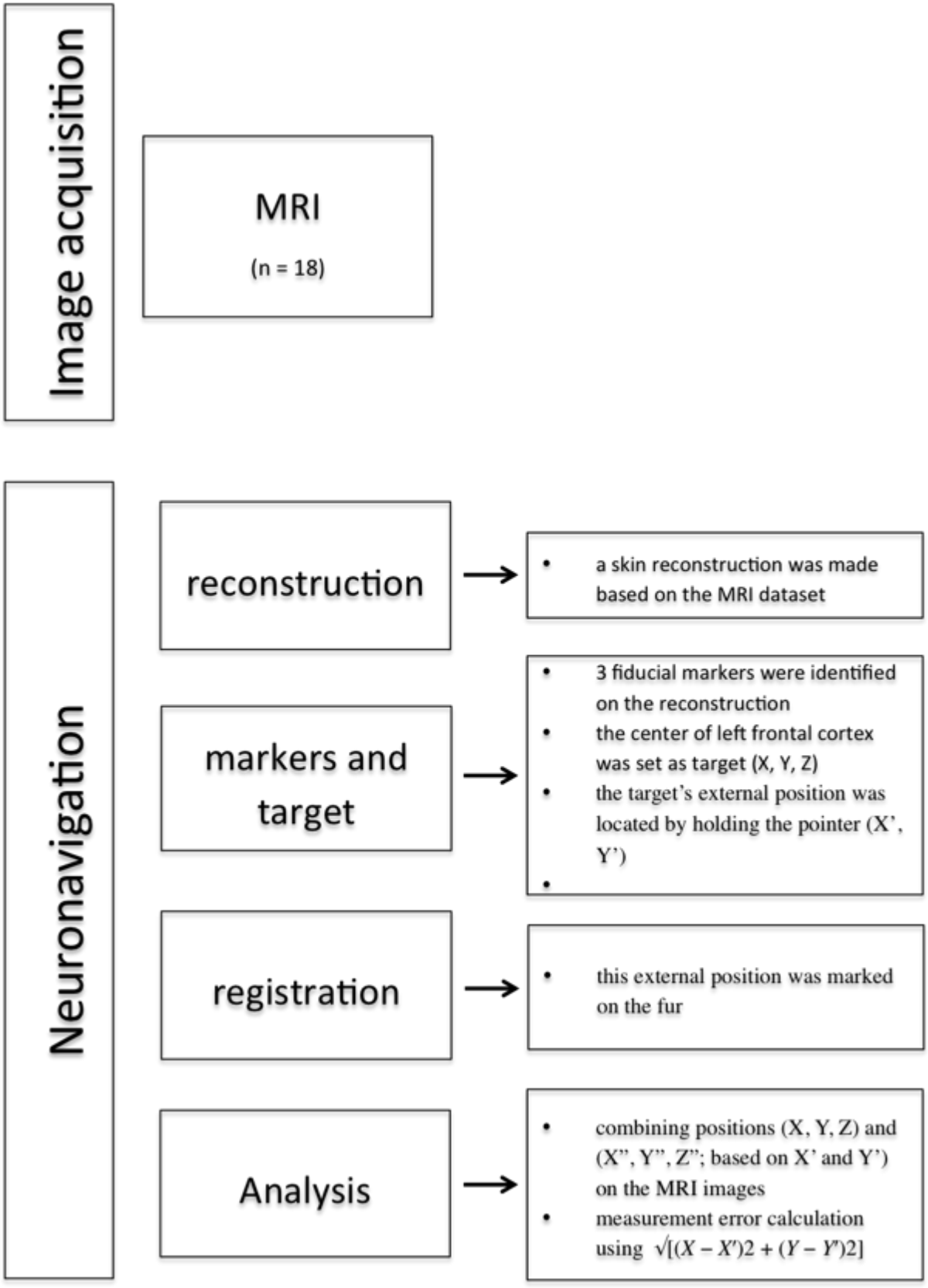

Fig. 1: Overview methodology of the study.

Fig. 2: A beagle placed in sternal recumbence, with the head fixed in a self-made mould. The subject tracker is attached to the mould and the dog in the neck region. The infrared camera was placed in front of the dog.

Accuracy assessment

In this cross-sectional study, the accuracy of a human frameless neuronavigation system was assessed by comparing two sets of coordinates. The first set of coordinates (X, Y, Z) was obtained by identifying the center of the target region on the tomographical dataset. The Cartesian coordinate system, used to measure the target's external position, provided the second set (X', Y'). This second set was plotted onto the tomographical dataset, creating a third set of coordinates (X", Y", Z"). The average difference, measured in centimeters, between the two sets of coordinates was calculated for each dog on the dorsal plane of the skull (Fig. 3). This provided the latero-lateral deviation (X – X") and the rostro-caudal deviation (Y – Y"). The measurement error was calculated for each dog using the Pythagorean theorem $\sqrt{[(X - X')^2 + (Y - Y')^2]}$. The dorso-ventral deviation (Z – Z") was not incorporated in this theorem. It was hypothesized that the median measurement error could not differ from zero. Osirix 6.5.2 was used to calculate the differences between the two sets of coordinates.

Stimulation depth

In addition the depth (dorso-ventral distance) was assessed to evaluate the feasibility of TMS in dogs. Osirix 6.5.2 was used to calculate the depth between the two sets of coordinates.

Statistical analysis

SPSS 23 (The Statistical Package for Social Science SPSS Inc, USA) was used to compute all the performed tests. Normality was tested by executing the Shapiro-Wilk test (significance set at $p < 0.05$). Outliers were identified and removed from the dataset. To assess the measurement error, a Wilcoxon one-sample signed rank test was used with a hypothesized median of 0.00 cm. In addition, a Wilcoxon signed rank test was put forward to check for differences between each rostro-caudal distance, latero-lateral distance and depth measured on its two different planes.

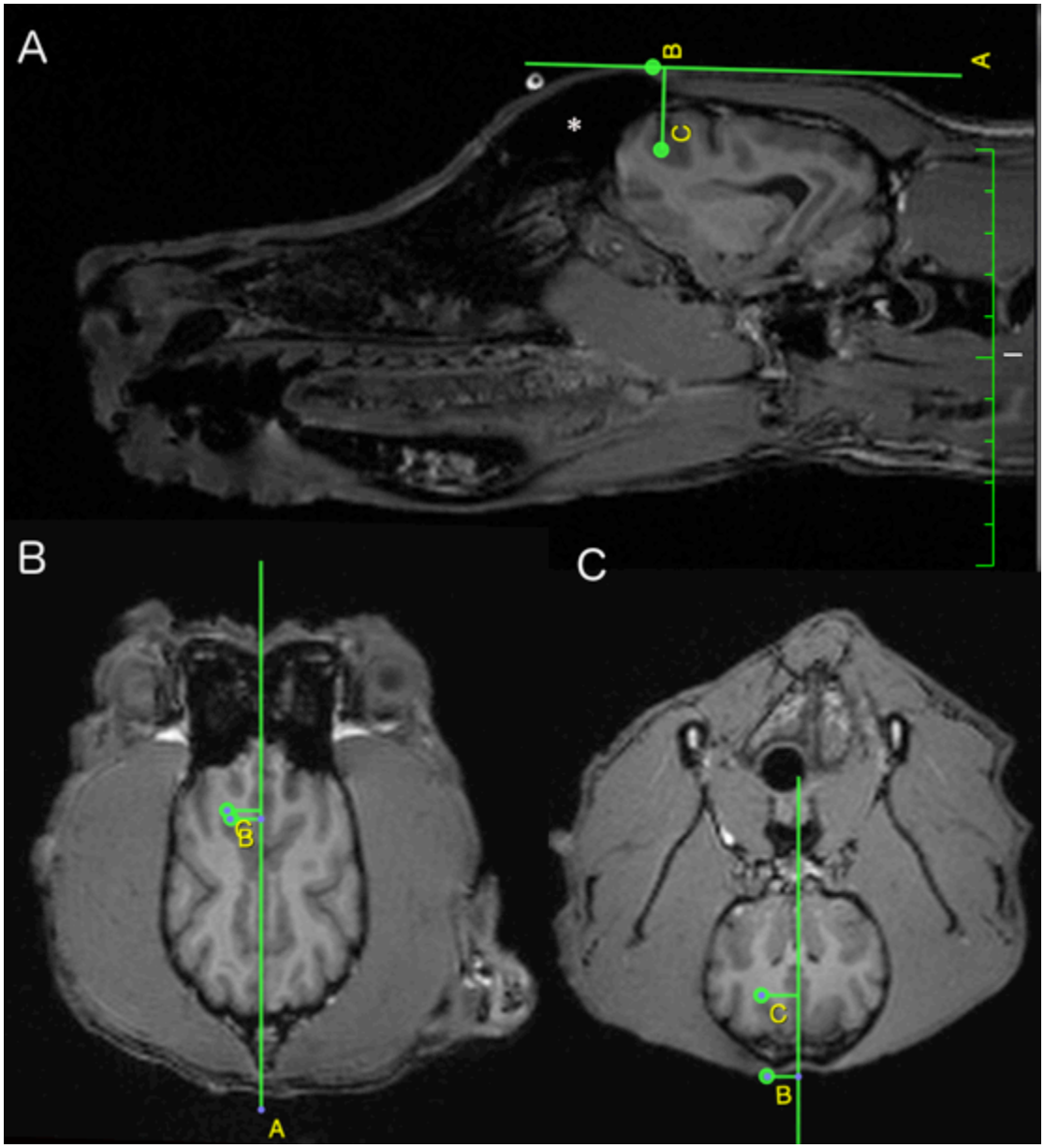

Fig. 3: Sagittal (A), dorsal (B) and transversal (C) view of the canine brain at the level of the left frontal cortex. On all views is C the center of the left frontal cortex (set as target for the neuronavigation), whereas B is the target's external position on the skull. The sagittal view indicates the depth (C-B) and the rostro-caudal deviation (C minus B). The dorsal view shows the latero-lateral deviation (C minus B) and again the rostro-caudal deviation (B-C). The transversal view provides the depth (B-C) and again the latero-lateral deviation (C minus B). (*Left part of the frontal sinus)

Results

The Shapiro-Wilk test revealed data that were not normally distributed. Dog number 2, 7 and 12 were identified as outliers and pairwise removed from the dataset. The Wilcoxon one-sample signed rank test showed a statistically significant difference ($p < 0.001$) between the hypothesized median measurement error (0.00 cm) and the actual measurement error between the left frontal cortex and its external location on the skull. The actual median measurement error was 0.26 cm. The average measurement error constrains 0.36 cm (sd = 0.22 cm, ranged from 0.14 cm to 0.712 cm, 95%CI [-0.06; 0.79]).

Table 1 shows the mean differences between the two sets of coordinates on each view. No significant differences were found between each rostro-caudal distance, latero-lateral distance and depth measured on its two different planes.

Between the left frontal cortex and its external location on the skull there is an average deviation in latero-lateral distance of 0.17, sd = 0.14cm. The rostro-caudal difference between both is 0.33, sd = 0.19 cm. The stimulation depth was on average 1.78 cm, sd 0.37 cm.

Plane	Distance	Mean	SD	SE	Min	Max
DORSAL	LATERO - LATERAL	0.17	0.13	0.03	0.05	0.53
	ROSTRO - CAUDAL	0.31	0.21	0.05	0.08	0.68
SAGITTAL	DEPTH	1.76	0.40	0.09	1.30	3,1
	ROSTRO - CAUDAL	0.35	0.18	0.05	0.11	0.66
TRANSVERSAL	DEPTH	1.79	0.35	0.08	1.33	2.90
	LATERO - LATERAL	0.16	0.15	0.04	0.03	0.55

Table 1: The mean differences (in cm) between the two sets of coordinates for each dog on the dorsal, sagittal and transversal plane of the skull.

Discussion

An external location was found for the set target in each dog by means of the used neuronavigation method, which could allow non-invasive brain stimulation methods such as guided rTMS. These techniques could therefore be used in future treatments of neuropsychiatric disorders in dogs.

Two dogs were identified in the dataset as outliers. Movements of the dog or disturbance of the digital reference frame were most likely to create these outliers. It was hypothesized that the median measurement error would not differ significantly from zero (hypothesized median measurement error).

In contrast to what was expected, the median measurement error differed significantly from zero. Hereby, it could be concluded that this type of neuronavigation system is not accurate in dogs and thereby not feasible to use in this species. However, a measurement error of less than 0.3 cm is accepted for the use of a neuronavigation system to obtain intracranial biopsies [17-23]. In analogue, this study was able to localize the left frontal cortex external position in dogs with a median measurement error of 0.26 cm (average 0.36 cm). Thus, it appears that this study was able to determine the left frontal cortex's external position in dogs. In comparison, the Brainsight stereotactic vet system was able to place Deep Brain Stimulation (DBS) electrodes with an accuracy of 0.46 cm (sd = 0.15 cm) in normal dogs [24] and contains an upper bound needle placement error limit of 0.331 cm [25]. This implies that a human frameless non-stereotactic neuronavigation system can be as accurate as a neuronavigation system specifically developed for stereotactic veterinary use. However, the dog's frontal lobe is considerably smaller in volume when compared to its human counterpart [26]. This may require a higher level of accuracy when externally locating the frontal cortex in dogs.

On average, the depth of the center of the frontal cortex in dogs constrains 1.78 cm (range from 1.30 cm to 3,10 cm). In comparison, the center of the frontal cortex in humans has a depth ranging from 2.00 cm to 3.00 cm. Despite the obvious discrepancy between a human and beagle head, a comparable target depth for the frontal cortex is present. This similar average target depth could be explained by the presence of a large frontal sinus in dogs, as depicted in Fig. 3. The frontal sinus could

comprise a distinguished amount of the distance between the scalp and the center of the target, provoking an increase in scalp-cortex distance. In its turn, an enlargement of the scalp-cortex region can elicit differences in the magnetic field strength created - during a TMS protocol - in the underlying cortical region. Furthermore, at depths up to 1.5 cm, the magnetic field strength - created by classic TMS figure-of-eight coil at an intensity of 120% motor threshold - remains above the threshold of neuronal stimulation [27]. This implies that the presence of a large frontal sinus might limit the possibility to transcranially stimulate distant brain regions.

When assessing the measurement error, this study only included the latero-lateral (X – X") deviation and the rostro-caudal (Y – Y") deviation. The dorso-ventral (Z – Z") deviation was not included. This was because the scope of this study was to superficially determine the external localization of the frontal cortex. Adding the dorso-ventral deviation to the theorem would greatly negatively influence the outcome. When performing accuracy studies of frameless neuronavigation systems, it must be kept in mind that its accuracy is influenced by user dependent (measuring errors, handler) and mechanical errors (technical limitations and imaging procedure), which makes it implausible to achieve the predefined hypothetical difference of zero [20, 28, 29]. A second limitation was created while obtaining the tomographical dataset. The used coils caused, during the MRI acquisition, a moderate to severe ventro-abaxial displacement of the skin. This displacement was noticeable on the skin reconstruction, which hindered the identification of the fiducial markers. In addition, the chosen fiducial markers were closely located to one other, which might negatively influence the accuracy of the external localization[29]. On the other hand, artificial fiducial markers, which should be attached to the head, could negatively influence the accuracy of the neuronavigation system [3].

Conclusions

This study was able to externally locate the left frontal cortex in dogs using a human commercially available neuronavigation system with a high level of accuracy. This could allow non-invasive brain stimulation methods such as guided rTMS to be used in future treatments of neuropsychiatric disorders in dogs. Error calculations, to eliminate the machine error, combined with

artificial fiducial markers could augment the found accuracy of non-stereotactic frameless neuronavigation in dogs.

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Chapter 4: Cortical motor threshold determination in dogs

Derived from Cortical motor threshold determination in dogs, by Dockx, R. et al. (Under review by the journal of Research in veterinary science)

Cortical motor threshold determination in dogs

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Abstract:

In humans, determining the cortical motor threshold (CMT) is the first critical step in successfully applying a transcranial magnetic stimulation (TMS) treatment. Stimulus intensity, safety and efficacy of a TMS treatment are dependent of the correct assessment of the CMT. The CMT is on its turn inherent to the individual and is easily influenced. Given that this TMS technique is also applied on dogs, as in humans, an accurate and reliable technique for the measurement of the CMT should be available.

Using a visual descending staircase paradigm (Rossini paradigm), the CMT repeatability was assessed and compared to the electromyographic (EMG) variant. Subsequently, the CMT was measured under sedation and general anaesthesia (isoflurane). Finally, the coil-cortex distance was associated with the CMT, weight, age and gender.

Over a period of one year, the CMT remained constant. A higher CMT was measured when using EMG (*P*-value <0.001) and under general anaesthesia (*P*-value = 0.005). A Gender difference was noticed for the CMT and coil-cortex distance. Males have larger CMT and coil cortex distance than females (*P*-value = 0.007 and *P*-value = 0.008). Within both genders, the CMT was positively and linearly associated (*P*-value < 0.05) with the weight and age of the animals. Only within the female subjects, a positive linear association was found between the CMT and the coil-cortex distance (*P*-value = 0.02).

This visual paradigm can be reliably used over time and during a TMS treatment. Therefore, this paradigm study implies that this method can be used under clinical and experimental settings. It has to be kept in mind that when using EMG or assessment of CMT under general anaesthesia, a higher CMT is to be expected. As in humans, every parameter that influences the coil-cortex distance may influence the CMT.

Keywords: Dog; Cortical motor threshold; Transcranial magnetic stimulation; Electromyography; Anaesthesia; Coil-cortex distance

Introduction

Transcranial magnetic stimulation (TMS) is a non-invasive neuromodulation technique used in human clinical and experimental settings. Based on the principle of electromagnetic induction this technique is able to induce an electrical current in a targeted neocortical brain region. The appropriate intensity of these induced currents is set in function of the cortical excitability or the resting motor threshold (CMT). An accurate and reliable determination of the CMT is of essence to reduce the risk of over- and under-stimulation of the subject. Over-stimulation could lead to severe adverse effects [1] whereas under-stimulation could cause treatment failure [2].

In humans, the CMT is in general defined as the minimal TMS intensity required to provoke at least five out of 10 electromyographic (EMG) responses - of at least 50 μV - in a contralateral fully relaxed muscle [3]. Rossini's paradigm to determine the CMT first locates the spot, which provokes the clearest muscle contraction. Over this hot spot, a descending staircase method is applied to obtain the CMT. The drawback of this relative frequency method is being time consuming due to the use of relatively high number of pulses [4, 5]. Alternatively, although less accurate, the CMT can be determined by replacing the EMG response with observation of movements of the thumb or fingers [6]. Despite its drawbacks, the visual assessment of the CMT is currently the method of choice in clinical and experimental settings. An option to reduce the time and the number of pulses, would be to use only 6 instead of 10 trials. Nonetheless, for clinical and research purposes, it is advised to use at least 10 out of the 20 trials to obtain reproducible results [7]. Another alternative would be to use the Mills and Nithi paradigm, which uses two thresholds to estimate the CMT: the lower and the upper motor threshold. The arithmetic mean of both thresholds defines the CMT. Despite the use of fewer stimuli to determine the CMT, it is as time consuming as the Rossini paradigm. A computational method to determine the CMT is the adaptive method. This method uses an S-shaped function to find the relationship between a set intensity and its probability of eliciting a motor evoked potential. Thereby an intensity is predicted which provokes at least 5 out of 10 motor evoked potentials. The adaptive method uses even less stimuli than the two-threshold method by Mills and Nithi. Drawbacks of this method are complexity and a high cost to be used clinically. This method may require additional

software and hardware that needs to be purchased, this in comparison to the visual Rossini method. Even more, besides a reduction of stimuli holds the adaptive method no other advantage. All the above-mentioned methods may be used in research and clinical settings provided that EMG is being used [1, 8].

Despite the fact that the motor cortex excitability fluctuates across individuals, it remains stable within the individual over time [9, 10]. Nonetheless, an inaccurate CMT can still be registered due to physiological, technical and pharmacological reasons [8, 11]. Intrinsic fluctuations of the cortical and spinal neurons' excitability can account for the physiological differences in the CMT, whereas technical imprecisions are not only due to the measurement method but also the use of different coil types, coil-cortex distance, the TMS treatment itself and arousal level [3, 12-14]. Due to the fact that the created magnetic field declines exponentially with the distance from the coil, increasing the coil-cortex distance increases the CMT [12, 13, 15]. It is obvious that every factor that increases the coil-cortex distance could potentially influence the CMT [16]. For example, aging causes atrophy of the grey matter, which provokes a significantly higher coil-cortex distance, which on it turn might account for a higher CMT [12, 13].

It is yet unclear whether a repetitive TMS (rTMS) treatment influences the CMT, but the literature suggests no influence on the CMT [10]. Drugs, such as carbamazepine and phenytoin augment the CMT [17-19]. Not only benzodiazepines influence the motor threshold, anaesthetics such as etomidate, propofol, thiopental, and isoflurane may supress motor evoked potentials (MEPs) induced by TMS [20, 21].

Dogs show similar natural behavioural problems with a neurobiological base that resembles the neurobiology of some of the human psychiatric disorders (anxiety, impulsive aggression, OCD) [22-24]. Our research group was recently able to accurately apply neuronavigation and accelerated high frequency repetitive TMS (aHF-rTMS) in dogs with measurable brain perfusion alterations in the left frontal cortical area and the subcortical regions [25-28]. Since the used coil, the coil-cortex distance, the TMS treatment itself, the arousal level, age and drugs easily influence the CMT, a better knowledge concerning the canine CMT in is necessary, especially in view of the clinical application of rTMS in dogs. In order to reduce the risk of possible side effects and a poor rTMS treatment response in dogs, a

reliable CMT measurement is of the essence. Therefore, the first goal of this study was to assess and evaluate the reliability and repeatability of the visual Rossini paradigm. Secondly, the visually determined CMT was compared to CMT measured with EMG to evaluate whether any difference in the measured CMT was noticeable. Thirdly, the CMT measured under sedation and general anaesthesia was evaluated with the hypothesis that general anaesthesia would cause a higher CMT. Finally, it was hypothesized that an increased coil-cortex distance would increase the CMT. Also the effect of aging, gender, and weight was explored in this context.

Materials and methods

Ethics

The guidelines for animal welfare, imposed by the ethical committee were respected. This study (EC 2015_140; EC 2016_070) was approved by The Ghent University Ethical Committee (2/03/2016, 08/11/2016). The dogs were socially-housed according to the Belgian legislation and received environmental enrichment.

Long-term repeatability of the CMT

Six healthy Beagle dogs underwent three, six months separated, assessments of the CMT under general anaesthesia. The dogs were premedicated with butorphanol IV (0.2 mg/kg, Dolorex, Intervet), after which midazolam (0.2 mg/kg, Dormicum, Roche) and propofol (Propovet Multidose, Abbott Laboratories, 1-2 mg/kg given to effect) were given to induce general anaesthesia. General anaesthesia was maintained with isoflurane (Isoflo, Abbott Laboratories) in oxygen using a rebreathing system. The dogs were placed in ventral recumbence, ECG electrodes were attached, cotton wool was placed in the external ear canal, an infrared light was placed on the dogs to keep them warm, the left frontal cortex was identified on the skull and the TMS machine (Magstim 200:Novametrix Medical systems LTD, Dyfed, United kingdom) was switched on and tested. The CMT of the left motor cortex was than measured 10-15 min after the induction of the general anaesthesia. The spot for determining the CMT was defined as the area, which provoked the clearest muscular contraction of the right upper front limb. After localisation of this hot spot, a machine output

was found which induced 100% of muscular twitches, after which the output was decreased in steps of 5%, 2% and 1% until at least five of 10 consecutive pulses induced a visible muscular twitch (descending staircase method) (Rossini et al., 1994). The CMT is expressed as percentage of maximal machine output.

Influence of an accelerated HF-rTMS treatment on the CMT

Twenty-two healthy neutered dogs were randomly divided into two groups. Both were subjected to four consecutive days of five sessions of an accelerated HF-rTMS protocol either sham or active. The figure-of-eight coil (air-cooled, mean diameter of 90mm; Magstim Company Limited, Wales, UK) was placed, respectively, perpendicular (sham) and parallel (active) onto the skull. The protocol was applied over the left frontal cortex with the figure-of-eight coil, and consisted out of 40 trains of 1.9 s duration, separated by a 12 s intertrain interval (1560 pulses per session). After 10-15 min the next session was applied. This protocol was an exact copy of one day of accelerated HF-rTMS performed in depressed patients [29, 30]. The anaesthesia, positioning, preparation and the CMT measurement of the animals were similar as expounded in the previous section.

Visual and EMG assessment of the CMT under sedation

Seven healthy dogs underwent a visual and EMG assessment of the CMT under sedation. The CMT was determined in the same way as previously explained. Positioning and preparation was similar as in previous sections described. A cephalic catheter was placed, through which they received a dexmedetomidine (Dexdomitor, Orion Corporation) bolus of 3 µg/kg (IV). This bolus was followed by a dexmedetomidine continuous rate infusion (CRI) at 1-3 µg/kg/h. During this CRI, the CMT was assessed visually and with an EMG (Natus synergy ultrapro S100, Natus neurology incorporated) simultaneously. Visually, the CMT was determined as the machine output that could provoke five out of the 10 visual muscle twitches in the contralateral front limb. Electromyographically, the CMT was defined as the machine output that could induce at least five out of 10 measurable motor evoked potentials (filter settings 20Hz-2kHz; gain 50µV) [31]. The visual and EMG measurements were blinded from each other. The monopolar recording electrode (disposable monopolar needles 37 mm, Teca corporation), Teflon-coated except for the tip, was placed in the centre of the musculus extensor carpi

radialis (cranial to the lateral humeral epicondyle). The reference electrode was placed subcutaneously at the level of the carpus. The ground electrode (disposable subdermal needle 12mm, Acertys healthcare), a blank subdermal needle electrode, was placed subcutaneously at the level of the olecranon [32, 33]. In order to determine the CMT, the same descending staircase method was used as described in the previous sections of the materials and methods.

Influence of sedation and general anaesthesia on the CMT

Eight healthy Beagle dogs were subjected twice - six months in time separated - to an assessment of the CMT under sedation and under anaesthesia. At first, the CMT was assessed under intramuscular (IM) sedation with dexmedetomidine (Dexdomitor, Orion Corporation) at 375 $\mu g/m^2$ body surface. When necessary an additional dose of 183 $\mu g/m^2$ was injected IM. Six months later, the dogs underwent the same protocol but under anaesthesia. General anaesthesia, positioning, preparation and the CMT assessment were identical as described in third section of the materials and methods.

Relationship between the coil-cortex distance and motor threshold

In 22 Beagle dogs, the distance between the centre of the left frontal cortex and the coil was calculated on the sagittal and transversal plane in Osirix 6.5.2 (Pixmeo Sarl, Bernex, Switserland). The centre of the left frontal cortex was identified on the MRI images as the cortical region located in the upper fourth part between the prorean sulcus and the rostral part of the lateral rhinal sulcus, more specifically in the middle of the distance between the caudal rim of the olfactory lobe and the presylvian sulcus. The coil-cortex distance was defined as the distance between the centre of the left frontal cortex and the outer rim of the skull, this perpendicular onto the skull. It was chosen to perform all the CMT assessments under anaesthesia. General anaesthesia, positioning, preparation and CMT assessment was performed as described in the previous section.

Statistical analysis

Rstudio 1.1.383 (R: A Language and Environment for Statistical Computing; R Core Team; R Foundation for Statistical Computing, Vienna, Austria, 2016) with packages nlme (version 3.1-131),

lawstat (version 3.2), ggplot (version 2.2.1) and sommer (version 3.0) were used to compute all analyses.

Long-term repeatability of the CMT

Three times the CMT was assessed visually in six anesthetized dogs. Onto this data, a simple repeated measures main effects model was fitted containing the CMT as outcome variable and time as predictor variable. The subjects modulated as a random effect to account for any correlations between repeated measurements. In addition, a random slope was added to the model to account for the changes in variance over time. The first time point was set as reference level. The alpha was set at 0.05, two-tailed. The assumptions of normality of the error terms, linearity of the regression function, homoscedasticity were checked by using plots and statistical tests (Shapiro-Wilk normality test and Levene's test of equality of variances).

Influence of an accelerated HF-rTMS treatment on the CMT

This dataset contains the CMT of 21 dogs, measured visually, under general anaesthesia, once a day prior to a four-day active or sham HF-rTMS treatment. Onto this data, a simple main effects model was fitted containing the CMT as outcome variable and treatment group and time as predictor variables. The model was comparable to the one set up in the previous section. A time by treatment interaction was included into the model. Time point 1 of the sham group was set as reference level. The alpha was set at 0.05, two-tailed. The assumptions were assessed as described above.

Visual and EMG assessment of the CMT under sedation

This dataset holds two variables: the motor threshold and group. The variable group indicates whether the CMT was measured electromyographically or visually. A paired two-sample T-test was opted. A QQ-plot was made to investigate the plausibility of normality. In addition, an F-test for unequal variances was performed.

Influence of sedation and general anaesthesia on the CMT

This dataset holds two variables: the motor threshold and group. The variable group indicates whether the CMT was measured under sedation or anaesthesia. A paired two-sample T-test was opted. As above, normality and homoscedasticity were checked.

Relationship between the coil-cortex distance and motor threshold

A dataset containing the continuous variables CMT, coil-cortex distance, age (months) and weight (kg) and the categorical variable gender was created. First, a full multivariate linear mixed model was created with the predictor values coil-cortex and gender and outcome variables CMT, age and weight. Secondly, another multivariate linear mixed model was set with predictor values CMT and gender and outcome variables age, coil-cortex distance and weight. Each time, the subjects were modulated as a random effect, an unstructured variance/covariance matrix structure was chosen and interaction between the predictor variables was considered. The alpha was set at 0.05, two-tailed. The assumptions of normality of the error terms, linearity of the regression function, homoscedasticity were checked by using plots.

Results

Long term repeatability of the CMT

No significant main effect of time was found, indicating that CMT assessment remains constant over time (Fig. 1).

Influence of an accelerated HF-rTMS treatment on the CMT

The CMT did not significantly change over time within each treatment group (Table 1).

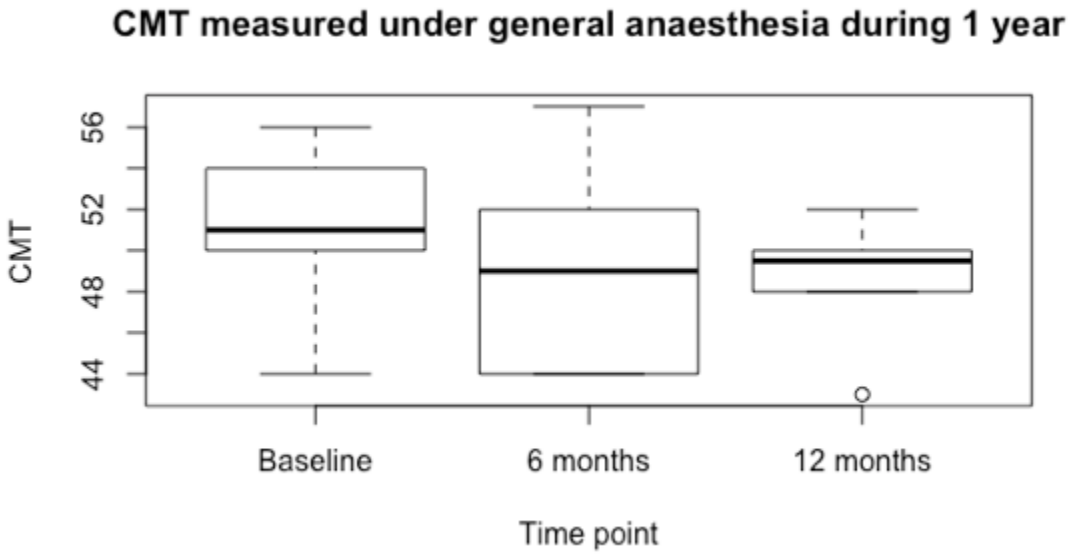

Fig. 1: Boxplot of the cortical motor threshold (CMT) measured during one year (every six months) of six healthy beagle dogs.

Treatment	Time	Mean	sd[a]	Min	Max
Active	1	53.50	10.02	41.00	75.00
(n = 16)	2	53.56	11.28	35.00	75.00
	3	53.44	11.38	35.00	75.00
	4	52.25	10.89	35.00	74.00
Sham	1	49.33	8.51	34.00	60.00
(n = 8)	2	50.44	7.60	37.00	62.00
	3	49.89	8.19	37.00	61.00
	4	50.44	8.59	34.00	61.00

Table 1: CMT measured each day during a four day active and sham treatment. [a] sd, Standard deviation

Visual and EMG assessment of the CMT under sedation

A discrepant average CMT was noticeable when measured electromyographically or visually: sample mean 92.86% (sd = 9.06) of the CMT measured electromyographically and 52.86% (sd = 12.40) when assessed visually (Fig. 2). The F-test for unequal variances revealed equal variance between both groups (*P*-value = 0.46). No deviations of normality were detected. The paired T-test with equal variances indicated a significant difference (*P*-value < 0.001) in CMT between both groups of 40.00% (95%CI [29.19; 50.81]).

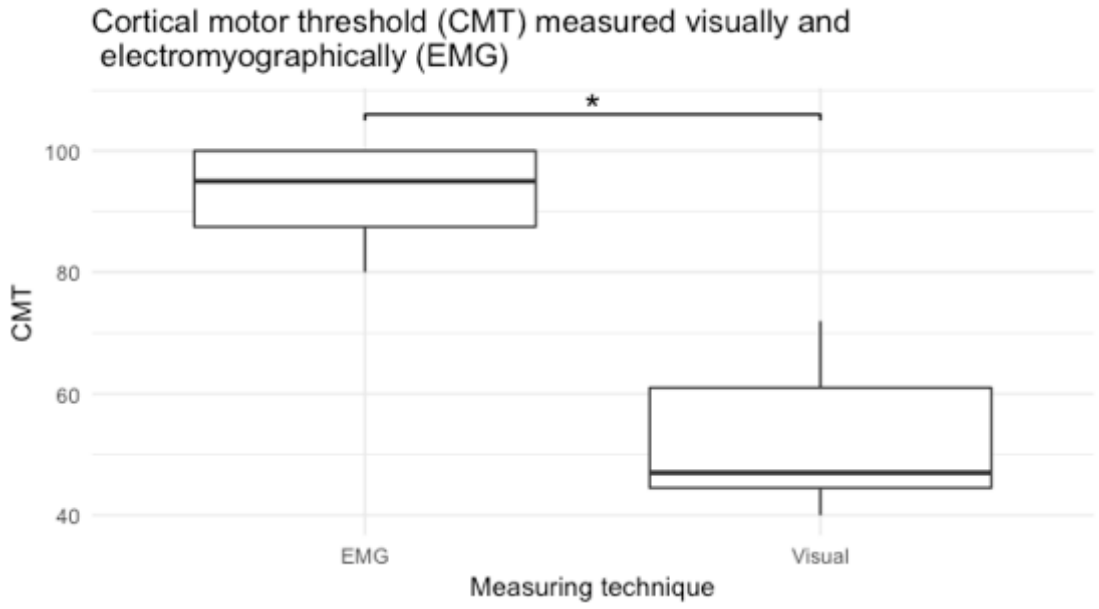

Fig. 2: Boxplot of the cortical motor threshold (CMT) measured visually and electromyographically. (* *P*-value <0.001)

Influence of sedation and general anaesthesia on the CMT

Overall, the CMT tended to be lower when measured visually under sedation than under general anaesthesia: sample mean 48.88% (sd = 3.76) for the CMT measured under sedation and 60.88% (sd = 8.79) under general anaesthesia (Fig. 3). The *F*-test for unequal variances revealed a significant (*P*-value = 0.04) unequal variance between both groups. No deviations of normality were detected. The paired *T*-test with unequal variances indicated a significant difference (*P*-value = 0.005) in CMT between both groups of 12.00% (95%CI [5.08; 18.92]).

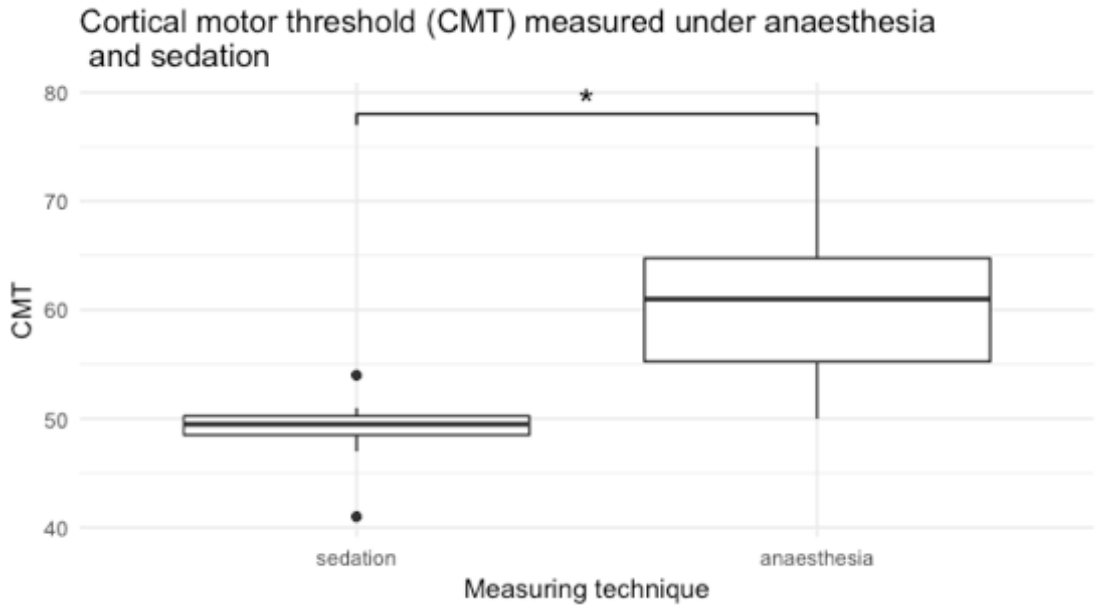

Fig. 3: Boxplot of the cortical motor threshold (CMT) measured under anaesthesia and sedation. (* *P*-value <0.01)

Relationship between the coil-cortex distance and motor threshold

Both models showed a significant interaction between both predictors (Table 2, 3, 4). Therefore, both models were refitted onto the dataset for each gender. Hence, four multivariate models were made. Male beagle dogs had, in comparison to female dogs, a significantly larger CMT and a coil-cortex distance (respectively 11.81% of the machine output (95%CI [3.53; 20.09])., *P*-value = 0.007) and 0.52 cm (95%CI [0.21; 0.83])., , *P*-value = 0.002)). In addition, the age of the male gender

group was significantly (*P*-value = 0.008) higher than the female group. The included male beagles were on average 36.16 months older (95%CI [10.23; 62.09]) than the female dogs.

Within the male gender group, a significant positive linear association was found between: weight and CMT (*P*-value = <0.001), age and CMT (*P*-value = 0.02) and between weight and coil-cortex distance (*P*-value = 0.004). For the female gender a significant positive linear association was found between: weight and CMT (*P*-value = 0.001), age and CMT (*P*-value = 0.04), weight and coil-cortex distance (*P*-value = 0.004) and between the coil-cortex distance and the CMT (*P*-value = 0.02).

Gender	Factor	Mean	sd[a]	Min	Max
Male	Age (months)	66.93	29.42	15.00	98.00
(n = 14)	Weight (Kg)	14.98	6.16	7.00	28.00
	Coil-cortex (cm)	1.79	0.38	1.26	2.60
	CMT[b] (% machine output)	54.86	7.16	41.00	70.00
Female	Age (months)	48.00	25.41	13.00	95.00
(n = 8)	Weight (Kg)	11.30	1.34	9.80	13.50
	Coil-cortex (cm)	1.54	0.23	1.19	1.82
	CMT[b] (% machine output)	50.62	11.89	35.00	75.00

Table 2: Descriptive statistics of the relationship between the coil-cortex distance and motor threshold. [a] sd, Standard deviation; [b] CMT, Cortical motor threshold

	Weight	Age	Coil-cortex distance
Intercept	0.025*	0.100	<0.001***
Male	0.026*	0.296	<0.001***
CMT	0.853	0.001**	<0.001***
Male:CMT	0.005**	0.293	<0.001***

Table 3: Output main effect and interaction test for the predictors gender and CMT. ****P*-value <0.05; *****P*-value <0.01; ******P*-value <0.001

	Weight	Age	CMT
Intercept	0.805	0.007**	<0.001***
Male	0.302	0.026*	0.004**
Coil –cortex distance	0.362	0.028*	0.001**
Male:Coil-cortex distance	0.269	0.009**	0.004**

Table 4: Output main effect and interaction test for the predictors gender and coil-cortex distance. ****P*-value <0.05; *****P*-value <0.01; ******P*-value <0.001

Discussion

Long term repeatability of the CMT and influence of an aHF-rTMS protocol

No CMT changes over time or an influence of the applied aHF-rTMS protocol on the CMT were noticed during this study. Thereby confirming current CMT reliability research [9, 10, 34].

Visual and EMG assessment of the CMT under sedation

A discrepancy was found between the CMT measured visually and with EMG. A 40% higher machine output (95%CI [29.19; 50.81]) was on average needed to provoke at least five out of 10 positive responses with EMG. This finding is in direct contrast to the findings in human studies where a lower or equal CMT is measured by means of EMG [14, 31, 35]. The reason for the discrepancy may be explained by the applied methodology. The CMT in this study was assessed using the same methodology as the canine motor evoked potential studies, where the recording electrodes are placed in the musculus tibialis cranialis and the musculus extensor carpi radialis [36, 37]. These studies used machine outputs of 80%, 90% or 100% and were able to detect, as in this study, an EMG response. Thereby, this study confirms that it is possible to provoke a MEP, measured with EMG, in dexmedetomidine-sedated dogs. Nonetheless, when lowering the stimulation intensity, no EMG responses were observed whereas visually muscular contractions were still noticeable. Nonetheless, these contractions were only visible in the proximal part of the front limb (Fig. 4). This could be explained by the fact that a relatively large coil, when compared to humans, could have produced electromagnetic fields over a larger area of the motor cortex. Thereby muscles with lower thresholds are activated, thus causing the visual method to provoke a lower CMT. Even more, in dogs, retraction of front limb has a larger cortical representation than flexion of the digits or extension of the carpus [38]. This strengthens the findings of this study that the visual CMT determination in sedated dogs leads to a lower CMT. Studies investigating the effect of spatial placement of the electrodes on the CMT, combined with the visual paradigm, should be conducted. These studies would provide more information whether the measured discrepancy is caused by the used methodology or by stimulating larger portions of the motor cortex. These studies could help determining a safe method for determining supra-threshold rTMS intensities.

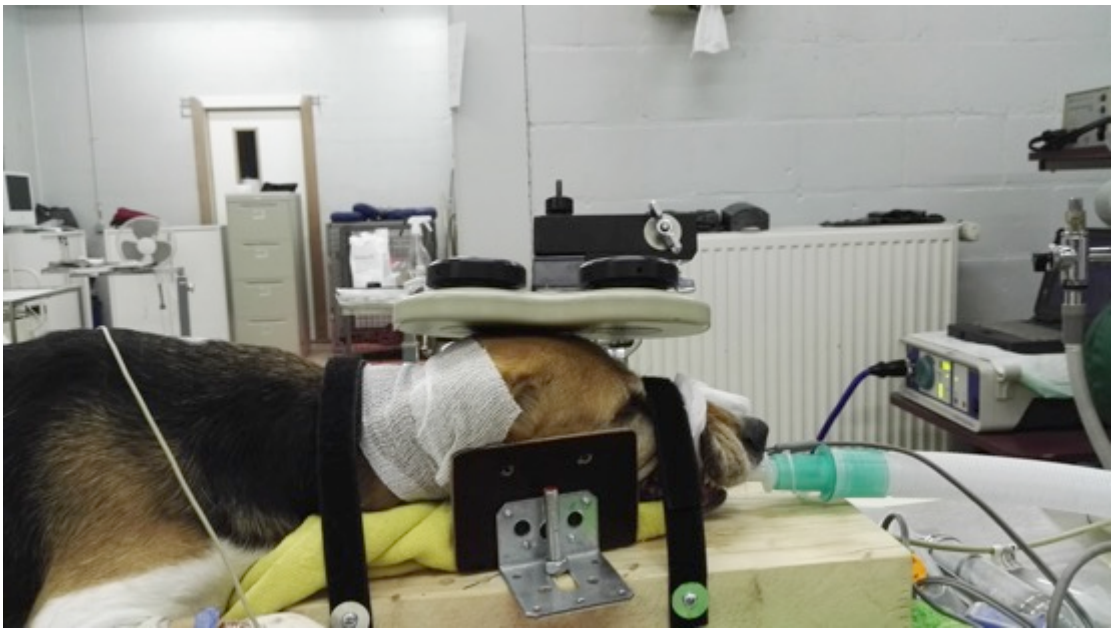

Fig. 4: An anaesthetized dog (isoflurane) being subjected to an active accelerated high frequency repetitive transcranial magnetic stimulation protocol (aHF-rTMS)

Influence of sedation and general anaesthesia on the CMT

Drugs can alter the intracortical excitability, albeit to a different degree. Here, sedation with dexdetomidine gave a CMT that was 12% (95%CI [5.08; 18.92]) of the total machine output lower than when measured under general propofol/isoflurane anaesthesia. Anaesthetic agents such as propofol, etomidate, methohexital and thiopental are known to abolish MEP in 86%, 43%, 47% and 80% of the test subjects respectively [20, 21]. Under both anaesthetic protocols the CMT was visually measurable, thereby confirming its measurability under dexmedetomidine [20, 39-43]. Since dexmedetomidine barely influences the MEP [39], this in contrast to isoflurane, a different CMT was expected. Consequently, a significantly higher CMT was found under isoflurane. Besides the effects of isoflurane, other agents used in our protocol might influence CMT measurements. High doses of midazolam allow MEP measurements, whereas propofol has a dose-dependent MEP response and in only 14% of the cases the MEP is maintained after complete induction [20, 36]. Nonetheless, a visible CMT was measured 10-15 min after propofol induction during this study. Propofol has a rapid hypnotic onset (60–90 s) and, when given as a bolus, a short duration of action (10 min) [44]. It is

noteworthy to recall that the CMT was only assessed in this study when general anaesthesia was maintained by isoflurane, 10-15 min after the induction. Despite the fact that after these 10-15 min the depressive action of propofol is minimal, the inhaled isoflurane would suppress the MEP [20] or at least decrease its amplitude [43]. Nonetheless, a visual unilateral muscular contraction in the right front limb was visible. A plausible explanation for the presence of a visible MEP could be that the depressive effect of the propofol bolus has worn off and that the isoflurane dosage, at that time, was too low to completely abolish a visual muscle contraction. In order to test the assumption that the CMT is measurable under a low dosage of isoflurane an experiment should be set up that uses low doses isoflurane to induce and maintain general anaesthesia, this with and without the use of propofol. Another setting, albeit difficult to accomplish, is to assess the CMT in conscious animals.

Relationship between the coil-cortex distance and motor threshold

The magnetic field produced by a TMS coil declines exponentially with the distance to the coil [45]. This implies that any factor influencing the coil-cortex distance could potentially affect the delivered intensity of the magnetic stimulation [13]. In this study, the male dogs had on average a 0.52 cm larger coil-cortex distance than the female dogs. This could be explained by the canine sexual dimorphism. Male dogs tend to be larger than female dogs due to the inhibition of major gene networks [46]. This dimorphism is also present in the cephalic index (CI). This index, characterized by the ratio of the head width divided by the length, is larger in male dogs in some breeds [47, 48]. With a larger head, the coil-cortex distance increases and a higher CMT is expected. This was confirmed since male dogs had an 11.81% of the total machine output higher CMT than the female dogs. No sexual dimorphism concerning the skull thickness is present in humans. However, sexual dimorphism was found in the diploe. Men tend to have a larger diploe the women [49]. No association between gender and the CMT is present in humans [50]. However, in humans the changing ovarian steroid level modulates the cortical excitability. Therefore, a variability in the MEP amplitude may arise caused by the various stages of menstrual cycle [51, 52]. Hormonal cycle of the used female dogs was controlled since all animals were neutered. Although a larger coil-cortex distance and CMT was found in the

male group, only in the female group a significant positive association was found between the coil-cortex distance and the CMT.

In accordance with the results presented by Kozel et al. (2000)[13], this study showed a significant association between the age of the subjects and the coil-cortex distance. Even more, a comparable association is present between a dog's age and its CMT [53-55]. On the other hand, Mills and Nithi found no significant association in humans between an individual's age and its CMT. Atrophy of the grey matter of the aging brain occurs in general and regional volume reduction has been reported including the frontal lobe [56, 57]. Peculiar is that a discrepancy in regional cortical aging atrophy was found between males and females. Males have a significantly higher age related loss in grey matter in the frontal lobe [56] whereas females have higher age related grey matter atrophy in the temporal lobe. This discrepancy, taken together with the sexual morphological dimorphism and the fact the tested male group was on average significantly older, could explain why in this study only a significant association was found in the female group between the coil-cortex distance and the CMT. To find conclusive evidence that a significant association between the coil-cortex distance and the CMT is also present in male dogs, the experiment should be repeated but with a more heterogeneous male group for the factor age, similar to the female group.

Finally, the coil-cortex distance and CMT were positively associated with the weight of the dogs. This indicates that that dogs that weigh more would have a larger coil-cortex distance and a higher CMT. Only within the female sample, a significant association was found between the CMT and the coil distance to cortex. Since all the dogs had a body condition score between four and five out of nine and the fact that weight is significantly correlated with the height of a dog [58], it could be assumed that the factor weight resembles more the size of the dog rather than merely its weight. This would imply that a larger dog would have a larger coil to cortex distance and CMT. To determine whether the constitution of the dog influences the coil-cortex distance and thereby the CMT, the dogs' weight should be measured along with their height and skull dimensions.

Conclusion

The results of this study justify the use of the visual Rossini paradigm in canine experimental and clinical settings.

Acknowledgements

This research was funded by Ghent university special research fund (BOF 01J05715) and was supported by the Ghent university multidisciplinary research partnership 'The integrative neuroscience of behavioural control'. We thank the division of veterinary nuclear medicine (department of medical imaging and small animal orthopaedics, university of Ghent) to provide access to their infrastructure.

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Chapter 5: Influences of anaesthesia and number of sessions on aHF-rTMS

Adapted from Anaesthesia, not number of sessions, influences the magnitude and duration of an aHF-rTMS in dogs by Dockx et al. (2017) [1]

Anaesthesia, not number of sessions, influences the magnitude and duration of an aHF-rTMS in dogs

Running title: Anaesthesia and number of HF-rTMS session in dogs

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Abstract

Currently, the rat has been a useful animal model in brain stimulation research. Nevertheless, extrapolating results from rodent repetitive Transcranial Magnetic Stimulation (rTMS) research to humans contains several hurdles. This suggests the desperate need for a large animal model in translational rTMS research. The dog would be a valid choice, not only due to the fact that humans and dogs share a neurophysiological background, but a similar neuropathological background as well.

In order to evaluate the feasibility of the canine rTMS animal model, this study aimed to evaluate the neurophysiological response in dogs on a, clinically used, accelerated high frequency (aHF) rTMS protocol. This aHF-rTMS (20 Hz) protocol was performed under anaesthesia or sedation and either 20 sessions or 5 sessions were given to each dog.

21 healthy dogs were randomly subjected to one of the four aHF-rTMS protocols (1 sham and 3 active protocols). For each dog, the perfusion indices (PI), of a [^{99m}Tc]HMPAO scan at 4 time points, for the left frontal cortex (stimulation target) were calculated for each protocol.

Concerning sham stimulation, the average PI remained at the baseline level. The main result was the presence of a direct transitory increase in rCBF at the stimulation site, both under anaesthesia and sedation. Nevertheless, the measured increase in rCBF was higher but shorter duration under sedation. The magnitude of this increase was not influenced by number of sessions. No changes in rCBF were found in remote brain regions.

This study shows that, despite the influence of anaesthesia and sedation, comparable and clinically relevant effects on the rCBF can be obtained in dogs. Since less methodological hurdles have to be overcome and comparable results can be obtained, it would be acceptable to put the dog forward as an alternative translational rTMS animal model.

Introduction

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique that relies on Faraday's law of induction. An electric pulse is generated and is sent through a dense winding of conducting material, the stimulating coil, creating a magnetic field perpendicular to the pulse flow. When applied alternated over the head, electrical currents - with an opposite direction to the pulse flow - are induced in the superficial cortical areas [2, 3]. Repetitive TMS is used clinically, to treat major depression, anxiety disorders, Alzheimer disease, chronic pain, stroke, tinnitus, etc. Only a level A recommendation is given for the beneficial effects of High Frequency rTMS (HF-rTMS) in major depressive disorder (MDD) and neuropathic pain [3, 4]. The stimulation in humans is applied while being conscious, this in contrast to (r)TMS in animals, where anaesthesia or sedation is required. The latter is applied for ethical and technical considerations (e.g. movement).

Despite the beneficial effects of HF-rTMS, there is still a desperate need for animal models in the field of rTMS. The current animal models are frequently used to (1) assess the basic mechanisms of rTMS, (2) evaluate the neurobiological changes induced by the magnetic field, (3) appraise its influence on neuropsychiatric disorders, and (4) examine the influence of the different stimulation parameters [2, 5]. Repetitive TMS research in rats has attributed a lot in unravelling its mechanism. This research has not only shown changes in monoamines [6-9], amino acids [10, 11] and Blood Derived Neurotrophic Factor (BDNF) [12, 13], but in behaviour and neuropsychiatric rat models as well [9, 14, 15]. Nonetheless, the rat model does not comply with human studies completely.

In order to mirror human TMS studies, accurate and focal stimulation in animals is essential. A human figure-of-eight coil can easily affect 100-200 mm^2 of the underlying cortical area [16] and stimulate as focal as 0.5 cm^3 [17] whereas the adult rat brain comprise on average only 1.5 cm^3. [5]. This implies that while focal stimulation is achieved in humans, whole brain stimulations are executed in the rat [18]. A solution to achieve focal stimulation in rats is the use of smaller, more focal coils. Notwithstanding, a reduction in coil size involves limiting factors such as coil overheating and a drop in efficiency [16]. As important as focality is the accuracy of the coil placement. Localization of the target region in rats is frequently done by means of stereotactic frames, which supplies a larger level

of accuracy than non-stereotactic frameless neuronavigation systems. Nonetheless, time, safety and cost can be reduced when choosing frameless over frame-based systems [19]. It is clear that, when extrapolating rTMS research from rodents to humans, some hurdles must be overcome [5].

Although rTMS can be conducted in awake humans and animals, anaesthesia/sedation may be needed in animal models [18]. Stimulation under sedation and anaesthesia is preferred to stimulation while awake/mechanically restraint. While conscious, dogs might react on the acoustic and tactile stimuli provoked by the TMS, which could cause a loss of focality and efficacy. Despite the fact that anaesthesia (e.g. dexmedetomidine, isoflurane, midazolam, ketamine) depresses the neural activity [20-23], neural effects of rTMS have been shown in anesthetized rats. However, Gersner et al. (2011) found different effects on neuroplasticity markers (BDNF, GluR1) between anesthetized and awake rats after rTMS.

Recently cats, dogs and monkeys have been subjected to (r)TMS [24-31]. The use of these animals as model would allow accurate and focal stimulations in awake animals, mirroring human (r)TMS research. Although it has been possible to stimulate cats and monkeys awake [25, 27], anaesthesia or sedation may still be preferred [20]. Based on the phylogenetic closeness, the monkey would be the preferred animal model in preclinical rTMS research. Nevertheless, high costs and ethical considerations coincide with the use of monkeys, which limits the use of this species in preclinical research [5]. Besides monkeys, dogs have proven their ability to be a valid natural animal model for several psychiatric conditions [27, 30, 32-36]. While conscious, animals might react to the acoustic and tactile stimuli provoked by the TMS, which could cause a loss of focality and efficacy [5]. Therefore, this study aims to evaluate and compare the short and long-term effects, by means of changes in regional cerebral perfusion (Perfusion Index, PI), provoked by a navigated, accelerated, High Frequency (20 Hz) rTMS (aHF-rTMS) protocol over the left frontal cortex [37] in 21 healthy dogs. Two stimulation conditions are focussed on: the number of sessions (20 sessions vs 5 sessions) and the consciousness state (anaesthesia vs sedation). It was hypothesized that the PI, at the stimulation site would differ significantly ($\alpha = 0.05$) for each stimulation condition. [99mTc]HMPAO SPECT (Single Photon Emission Computed Tomography; d, 1, hexamethylpropylene amine oxime) scans were used

to semi-quantify the perfusion indices (PI) of the cortical, cerebellar, subcortical areas and the olfactory bulb [38, 39].

Materials and methods

Animals

Twenty-one healthy neutered dogs (2 fox-hounds and 15 beagles; 13 males and 4 neutered females; aged between 3 and 8 years old) were incorporated in this study. For practical reasons, four out of 21 dogs were randomly selected for reuse. Only after a three-month washout period (equals 6 months after the last stimulation session) and a return to baseline perfusion index (measured by SPECT) the dogs were reused and considered as a new test subject. Hence, 21 (17 used and 4 reused) dogs entered the study. This study includes, mere for statistical comparison, own data extracted from Dockx et al (2015). The guidelines for animal welfare, imposed by the ethical committee were respected. This study (EC 2015_38) was approved by The Ghent University Ethical Committee. The dogs were provided by the department of veterinary medical imaging and small animal orthopedics and the department small animals of the faculty of veterinary medicine. The dogs were permanently housed in groups (newly built housing since this year in the new farm of small pets) of 8 on an internal surface of 15 m^2 with permanent access to an outside area of 15 m^2. The floor coverings in the inner part consisted of wood shavings. Frequently, toys such as Kongs® were given to the animals and were twice a day released onto an enclosed play area. In addition, the dogs were regularly walked by students of the faculty of veterinary medicine. After the HMPAO scans, the dogs stayed 1 night at the Nuclear Veterinary Department, where they were accommodated in a separate 3.5 x 3.7 meter cage. No animals were sacrifised at the end of this study. A sample size calculation was performed based on a prediction linear mixed model with a delta (predicated difference) equal to 0.05 and a power of 0.80. This provided a sample size of 7.48 animals per group. Therefore, a sample size of 8 animals per group was chosen.

Neuronavigation protocol

In order to perform the neuronavigation, a tomographical dataset was required. Each dog underwent a magnetic resonance imaging (MRI) scan. This acquisition was performed by a Siemens 3T Magnetom Trio Tim system (Siemens Medical Systems, Erlangen, Germany). A phased-array spine coil and a phased-array body matrix coil were used to obtain the data set. A T1-weighted 3D MPRAGE sequence with 176 sagittal slices was acquired. The following sequence parameters were used: TR = 2250 ms, TE = 4,18 ms, TI = 900 ms, parallel acquisition method = GRAPPA with acceleration factor = 2, matrix size = 256 ~ 256, sagittal, FOV = 220 mm, flip angle = 9°, voxel size = 0.9 ~ 0.86 ~ 0.86 mm3.

After the placement of an intravenous cephalic catheter, the dogs were intramuscularly (IM) premedicated with dexmedetomidine 375 μg/m^2 body surface, Dexdomitor®, Orion Corporation, Espoo, Finland). General anaesthesia was achieved by an intravenous (IV) propofol injection (Propovet Multidose®, Abbott Laboratories, Berkshire, UK, 1-2 mg/kg given to effect) and maintained with isoflurane (Isoflo®, Abbott Laboratories, Berkshire, UK) in oxygen through a rebreathing system.

During the MRI acquisition the dogs were sternally positioned, head first in the scanner bore. The neuronavigation was performed while the dogs were recovering from general anaesthesia. When needed, the dogs were given dexmedetomidine (0,5-1 μg/kg to effect; IV) to finish the neuronavigation. The dogs' heads were fixated in a self-made mould and the subject tracker was attached to the neck region.

After the data were loaded into the software (Brainsight, Rogue-resolutions Ltd, Cardiff, UK) a skin reconstruction was made, on which three to four fiducial markers (landmarks) were set and identified. The left frontal cortex was targeted by manually identifying its centre on the MRI data set. The target's external position was located by holding a pointer – connected to three reflecting balls - perpendicular over the target region as indicated by the neuronavigation software (for a full description we refer to Dockx et al. (2017, accepted for publication in PeerJ).

The stimulation protocol

The 21 dogs were randomly divided into 3 unequal groups: group 1 (n=5), group 2 (n=8) and group 3 (n=8). Five neutered male beagle of 6.6 years (sd = 2.01) and 8 neutered beagles (2 females, 6 males) of 5.75 years old (sd = 2.14) were included in group 1 respectively group 2. Group 3 comprised 2 male neutered foxhounds and 6 (3 females, 3 males) beagle dogs (all neutered) with an average age of 6.25 years (sd = 0.98).

The data extracted from Dockx et al. (2015)[40] was derived from 8 neutered dogs, group 4. This group consisted of 6 beagles and 2 mix breed foxhounds (4 males, 4 females; aged between 4 and 8 years).

By using positive reinforcement techniques, all dogs were accustomed to the researchers, the stimulating room and the sound and placing of a sham coil. This was done several months before the start of the stimulation experiment. For cardiovascular reasons, it was chosen to perform all stimulation protocols in groups 1, 2 and 3 under general anaesthesia. Premedication consisted of butorphanol IV (0.2 mg/kg; Dolorex®; Intervet Belgium NV). After onset of sedation anaesthesia was induced intravenously by administering midazolam (0.2 mg/kg; Dormicum®; Roche Nederland B.V.) immediately followed by propofol (Propovet Multidose®, Abbott Laboratories, Berkshire, UK, 1-2 mg/kg given to effect). General anaesthesia was maintained with isoflurane (Isoflo®, Abbott Laboratories, Berkshire, UK) in oxygen using a rebreathing system. Group 4 underwent the stimulation protocol under IM sedation with dexmedetomidine at 375 $\mu g/m^2$ body surface. When deemed necessary an additional dose of 183 $\mu g/m^2$ was injected intramuscularly.

Immediately following the induction of anaesthesia/sedation, the motor threshold of the left motor cortex was determined. A cortical motor threshold (CMT) of 100% was defined as the set machine output (Magstim Company Limited, Wales, UK) that could provoke 5 out of 10 visible muscle contractions in the right upper front limb. After the assessment of the CMT, the external localisation of the centre of the left frontal cortex was located based on the previously measured X,Y positions and marked with a permanent marker on the fur. The centre of a standard figure-of-eight coil was placed perpendicular over the mark with the handle pointing abaxial. For the sham group, the coil was placed in a 90-degree angle with one wing making contact with the skull. HF-rTMS protocol (20Hz, 110%

CMT) was applied to the left frontal cortex. The animals received, based on the group they were divided into, 20 sham sessions under general anaesthesia (group 1; 5 daily sessions during 4 days), 5 active sessions under anaesthesia or sedation on 1 day (group 2 and group 4 respectively) or 20 active sessions under general anaesthesia (group 3; 5 daily sessions during 4 days). Each session contained 40 trains of 1.9 seconds each. The trains were separated by a 12 second intertrain interval (in total 1560 pulses were given per session). The time interval between sessions was 10 to 15 minutes. This protocol was an exact copy of an accelerated HF-rTMS treatment protocol performed in MDD patients at our medial university hospital [43, 44]. The depth of the anaesthesia during each stage of the study was monitored by an anaesthesiologist. The anesthetic depth was clinically monitored (ventral position of the eye and absence of the eyelid-reflex). When deemed necessary, the isoflurane dose was adjusted to maintain the same depth.

Under sedation, each rTMS session was preceded and followed by checking the sedation depth. Only when the dogs were not responsive to external stimuli, the next session could be applied.

Tracer

Less than 24 hours prior to each SPECT scan a ^{99}Mo generator was eluted. Approximately 1,85 GBq $^{99m}TcO_4$ was added to the exametazime (d,1 hexamethylpropylene amine oxime (HMPAO); Ceretec®, GE Healthcare LTD, UK).

SPECT scanning procedure

Prior to the stimulation sessions, each dog received a baseline [^{99m}Tc]HMPAO-SPECT scan. After the last stimulation session, the dogs received 3 additional HMPAO-SPECT scans: 24 hours, 1 month and 3 months post stimulation. In order to perform these scans, an intravenous cephalic catheter was placed, and the dogs were IM pre-medicated with dexmedetomidine (375µg/m2 body surface). When sedated, the dogs were given 348,54 MBq (sd 26,64 MBq)99mTc-HMPAO IV. Induction of anaesthesia was achieved 15 -20 minutes after the tracer injection by administering propofol IV (1-2 mg/kg body weight to effect). Again, general anesthesia was maintained with isoflurane in oxygen through a rebreathing system. Respiratory and electrocardiographic monitoring was used during the entire duration of each scan. Equipped with low energy ultrahigh-resolution parallel hole collimators

(tomographic resolution FWHM=9 mm), a triple head gamma camera (Triad, Trionix, Twinsburg, OH, USA) was used 30-35 minutes after the tracer injection to acquire the data. The camera collected data over a circular 360° rotation in a step-and-shoot mode during 20 minutes (120 steps, 10 sec per step, 3° steps) on a 128~128 matrix. Afterwards, the data were iteratively reconstructed and a Butterworth filter (cut-off 1.4 cycli/cm, order 5) was added.

Image analysis

A template containing 11 fixed, different brain regions (both frontal, temporal, parietal and occipital lobes, the cerebellum, olfactory bulb and the subcortical area) was, using BRASS software (Brain Registration and Automated SPECT Semiquantification, Nuclear diagnostics, Sweden), fitted onto each SPECT scan. This template, composed from 14 dogs (9 male, 5 female, mean age 50 months ± 20), eliminates the operator dependent demarcation of the volumes of interest (VOI). Hereby facilitating the fitting procedure that is necessary to compensate for inter-individual differences in brain size and shape. The regional Cerebral Blood Flow (rCBF; perfusion index (PI)) was automatically calculated for each individual dog by normalizing the regional radioactivity to the radioactivity of the entire brain. The left frontal cortex was of major interest but since the PIs of the other 10 regions were automatically calculated, these data were also included in the analysis.

Statistical analysis

Rstudio 1.0.136 (R: A Language and Environment for Statistical Computing; R Core Team; R Foundation for Statistical Computing, Vienna, Austria, 2016, https://www.R-project.org/) with package nlme version 3.1-131 was used to compute all analyses.

At first, a simple main effects model was fitted with PI as outcome variable and time point and treatment group set as fixed effects. Forward stepwise regressions model building was used (α_{in} = 0.1, α_{out} = 0.15). During the model building process, multicollinearity was taken into account. After identifying the main effects, the interactions between the different variables were assessed ($\alpha_{in} = \alpha_{out}$ = 0.05).

For the first dataset, PI of the left frontal cortex was determined at 4 time points (baseline, 24 hours post, 1 month and 3 months) after stimulation under general anaesthesia. The primary objective

was to compare PI at each time point between and within the treatment groups: 20 sessions sham stimulation as reference, and 5 vs. 20 sessions of active stimulation. A linear mixed model was fitted on this response variable with treatment group and time points as fixed-effect factors and subjects as random effect to account for correlations between repeated measurements. A random slope was included in the model to account for a change in variance over time. The presence of a time point by treatment interaction was also considered in the model. Gender and age were considered as fixed effect. Post hoc, this model was fitted onto the PI of the remaining 10 VOI in order to detect any distant effects of the stimulation protocol.

For comparing aHF-rTMS stimulation under general anaesthesia and sedation, a previous dataset (Dockx et al., 2015) was used, where PI of the left frontal cortex was determined by SPECT scan at the same 4 time points after 5 active sessions under sedation (group 4). The model is the same as the previous one, except that the treatment groups are now stimulation under sedation and anaesthesia (always 5 active sessions).

The significance level of was set at 0.05, two-tailed. The assumptions of normality of the error terms, linearity of the regression function, homoscedasticity and independence of the error term were checked based on diagnostics plots combined with statistical tests (Bartlett test of homogeneity of variances and the Shapiro-Wilk normality test).

Results

The set model assumptions, with the exception of independence due to the repeated measures, were met based on the diagnostics plots. The Bartlett test of homogeneity and the Shapiro-Wilk normality test revealed a p-value larger than the pre-set significance level of 0.05, two-tailed. No outliers were detected. The variables “gender” and “age” did not have a significant influence on the outcome variable and were thus removed from the model.

<u>The influence of the number of sessions on the short- and long-term effects of an aHF-rTMS protocol under anaesthesia.</u>

The fitted model was written as $E(Yt|T_1,T_2) = \beta_0 + \beta_1 t_1 + \beta_2 t_2 + \beta_3 t_3 + \beta_4 T_1 + \beta_5 T_2 + \beta_6 t_1 T_1 + \beta_7 t_2 T_1 + \beta_8 t_3 T_1 + \beta_9 t_1 T_2 + \beta_{10} t_2 T_2 + \beta_{11} t_3 T_2$ with Yt (PI left frontal cortex) as response variable. The first

predictor value t denoted the different SPECT scan time points, t1 the first of three (k-1=4-1=3) dummies (=1 if time point = “24 hours post” or 0 otherwise), t2 the second dummy (=1 if time point = “1 month post” or 0 otherwise) and t3 (=1 if time point = “3 months post” or 0 otherwise). With T denoting the different stimulation conditions (treatment variable, categorical), T1 the first of 2 dummies (=1 if the stimulation protocol = “5 active sessions under anaesthesia” or 0 otherwise) and T2 (=1 if the stimulation protocol = “20 active sessions under anaesthesia” or 0 otherwise).

Based on table 1 there is a significant influence ($p < 0.05$) of the treatment group on the average PI of the left frontal cortex when compared to the baseline PI of the first group (20 sham sessions under anaesthesia), whereas time points showed no main effect. Based on the set model, contrasts were created within each treatment group and time point.

Within group 1 (20 sham sessions under anaesthesia) no significant differences in average PI were found between the different time points (Fig 1). Group 2 and 3 had an average PI at 24 hours post stimulation that differed significantly from the baseline PI as well as from the PI at 3 months after the last stimulation session (Table 2, Fig 1).

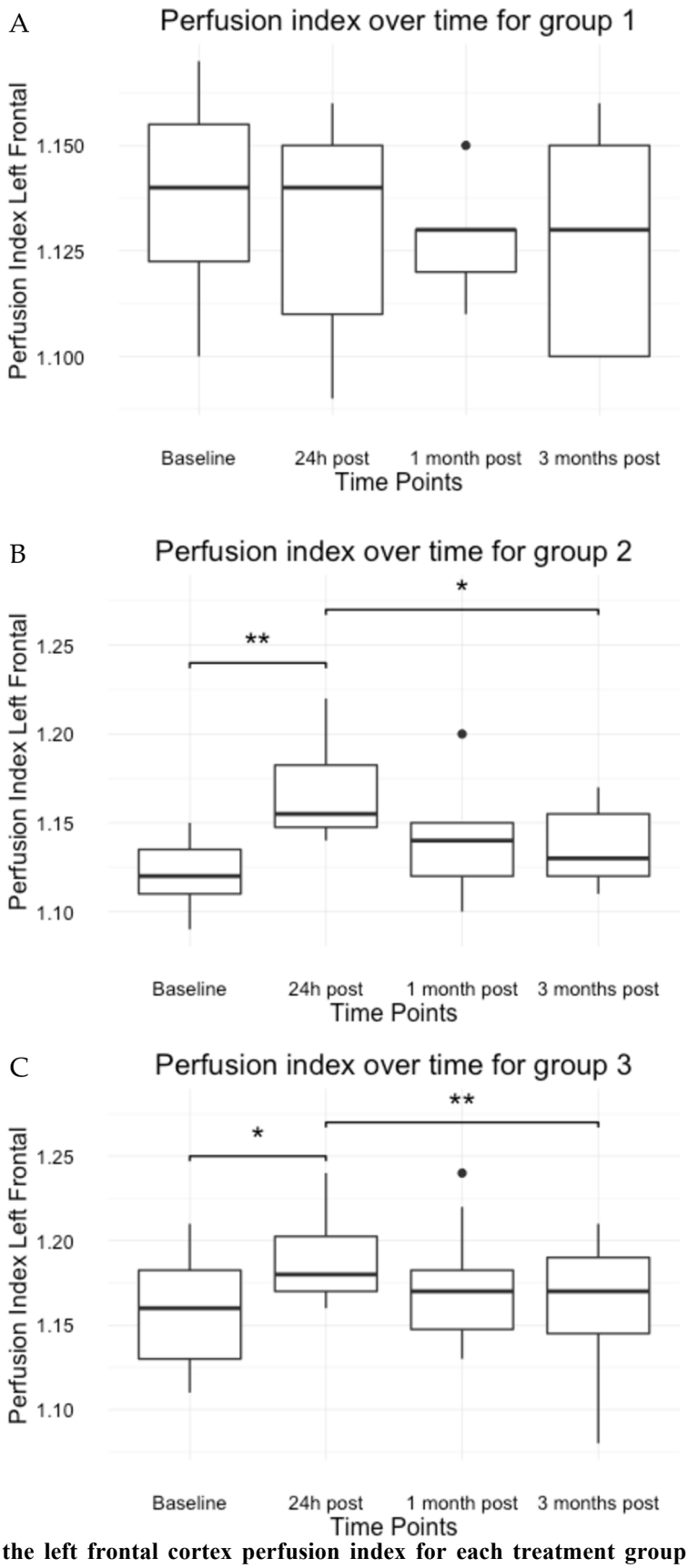

Fig. 1: Boxplots of the left frontal cortex perfusion index for each treatment group based on the first model. (A = group 1; B = group 2; C = group 3; * < 0.01; **<0.001).

Between the treatment groups, differences in PI were found 24h post and 1 month after the last stimulation session. At baseline no differences in average PI were found between the active stimulation protocols and the sham stimulation, whereas both active stimulation protocols differed significantly with the sham protocol at 24 hours post stimulation. At the given time point the average PI increased, compared to the reference level, 0.050 (95% CI -0.003; 0.102) and 0.039 (95% CI -0.016; 0.090) in group 2 respectively group 3. Between the two active stimulation protocols (1 day versus 5 days under anaesthesia), there was no significant difference between the average PI at the 24 hours' time. One month post stimulation there was no difference in average PI between group 1 and 2. Nonetheless a significant difference was found between group 1 and group 3 ($p = 0.014$) and between group 2 and group 3 ($p = 0.042$). Three months after the last stimulation session, no significant differences were found over all stimulation groups. As seen in S1, each dog can react differently on the stimulation. Nonetheless, an average transient increase for each active protocol is present (Fig 2).

Post hoc the statistical model showed no changes in average PI for the brain regions other than the left frontal cortex, compared to the reference level (S5). Post hoc the statistical model showed no changes in average PI for the brain regions other than the left frontal cortex, compared to the reference level (S5 Fig).

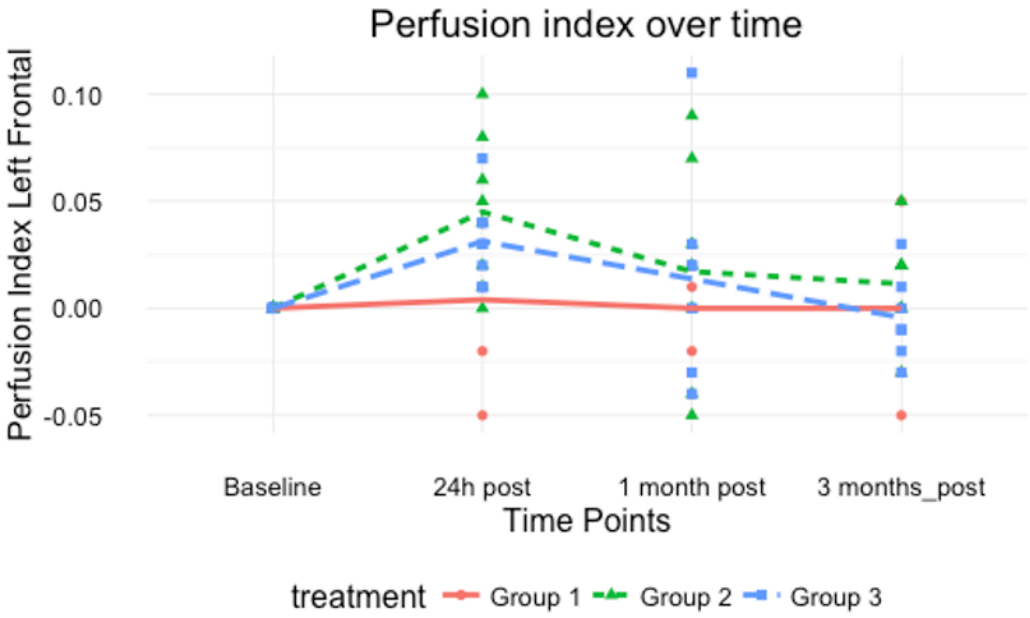

Fig 2. Difference in Perfusion Index of the left frontal cortex for the three treatment groups under anaesthesia.

The influence of depth of anaesthesia on the short and long term effects of a single day aHF-rTMS protocol.

Similar to the previous model, the results indicate differences in PI between and within each treatment group. The intercept was set at the baseline time point of the 5 active sessions stimulation protocol under anaesthesia. The fitted model was written as $E(Yt|T1) = \beta_0 + \beta_1 t_1 + \beta_2 t_2 + \beta_3 T_1 + \beta_4 t_1 T_1 + \beta_5 t_2 T_1$ with Yt (PI left frontal cortex) as response variable. The first predictor value t denoted the different SPECT scan time points, t_1 the first of three (k-1=3-1=2) dummies (=1 if time point = "24 hours post" or 0 otherwise) and t_2 the second dummy (=1 if time point = "3 month post" or 0 otherwise. With T denoting the stimulation protocol (treatment variable, categorical), T_1 the only dummy (=1 if the stimulation protocol = "5 active sessions under sedation" or 0 otherwise). The presence of a time point by stimulation protocol interaction was considered in the model. By default, the first level of the time point and stimulation protocol group was coded as the reference level (baseline and 5 active stimulation sessions under anaesthesia).

The two stimulation protocols differed from each other (*p*-value = 0.009). Table 3 indicates that the effect of the stimulation is again present at the stimulation site for both protocols but that the effect last longer under anaesthesia. Even more, a significant difference of 0.036 (95%CI 0.009;

0.064) was found between the average PI at time point 24h post whereas this difference was not present at time point 3 months post stimulation under sedation. The PI returned to baseline after 3 months under sedation whereas it did not under anaesthesia (Fig 3, Table 4). As seen in S2 each dog can react differently on the stimulation. Nonetheless, an average transient increase for each active protocol is present (Fig 4). Post hoc the statistical model showed no changes in average PI for the brain regions other than the left frontal cortex, compared to the reference level (S6).

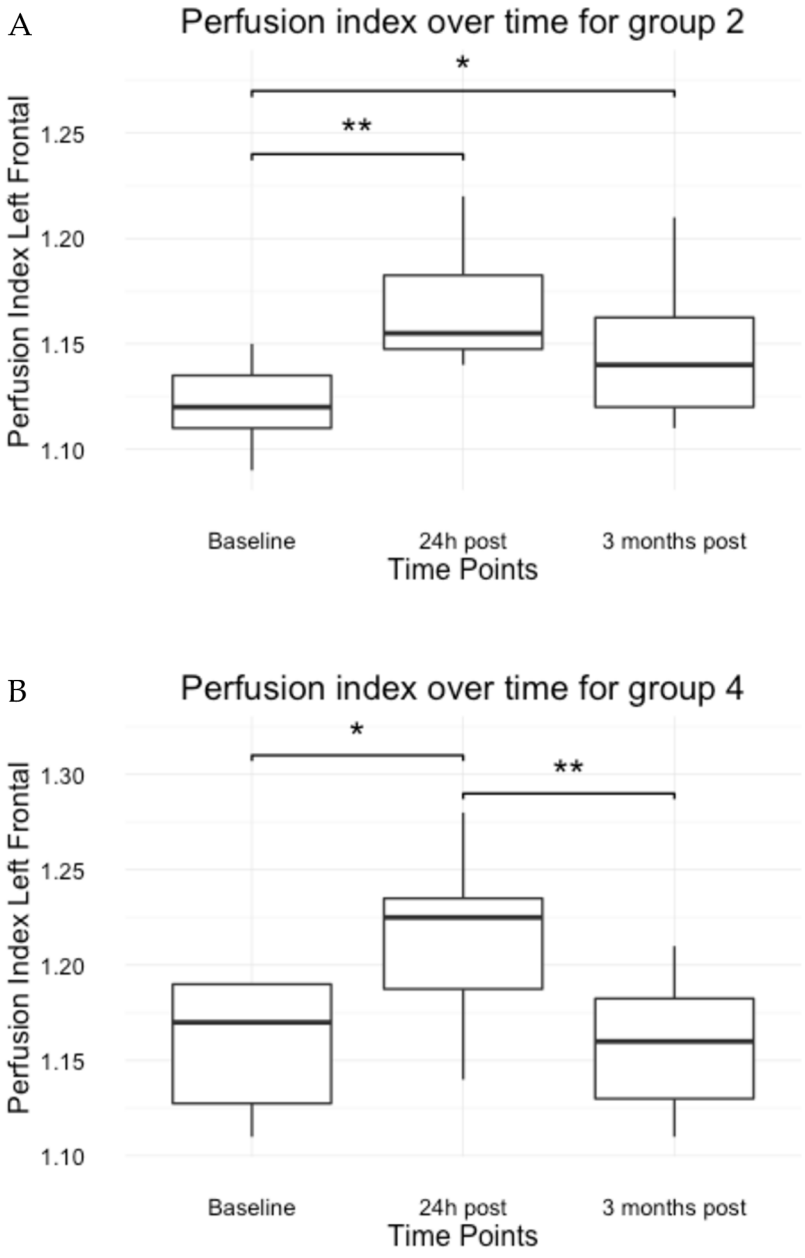

Fig. 3: Boxplots of the left frontal cortex perfusion index for each treatment group based on the second model. (A = group 2; B = group 4; * < 0.05; **<0.01)

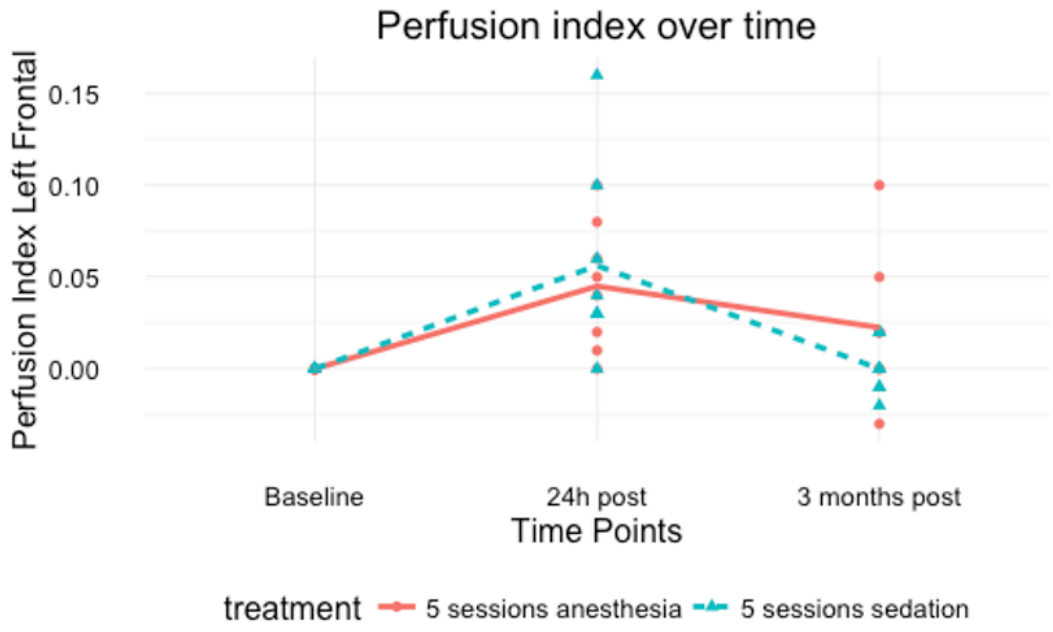

Fig. 4: Difference in Perfusion Index of the left frontal cortex for the two treatment groups that underwent 5 aHF-rTMS sessions.

	Model 1 output		
	numDF	*F-value*	*p-value*
(Intercept)	1	7130.60	<0.001**
Time point	3	0.11	0.95
Treatment group	2	3.58	0.03*
Interaction	6	1.34	0.25

(* < 0.05; **<0.001)

Table 1: Output type III main effect test for the stimulation protocols under anaesthesia.

	5 active sessions	20 active sessions
	p-value	*p-value*
Baseline - 24h post	<0.001**	0.009*
Baseline - 1 month post	0.267	0.320
Baseline - 3 months post	0.129	0.978
24h post - 1 month post	0.074	0.247
24h post - 3 months post	0.004*	0.001**
1 month post - 3 months post	0.913	0.288

(* < 0.01; **<0.001)

Table 2: Multiple comparison for each time point within the active stimulation protocols.

	Model 2 output		
	numDF	*F-value*	*p-value*
(Intercept)	1	12785.57	<0.001**
Time point	2	5.19	0.010*
Treatment group	1	6.67	0.013*
Interaction	2	1.82	0.175

(* < 0.05; **<0.001)

Table 3: Output type III main effect test for the 5 session stimulation protocols.

	5 active session anaesthesia	5 active sessions sedation
	p-value	*p-value*
Baseline - 24h post	0.007**	0.001**
Baseline - 3 months post	0.030*	1.000
24h post - 3 months post	0.649	0.001**

Table 4: Multiple comparison for each time point within the 5 active sessions stimulation protocols. (* < 0.05; **<0.01;).

Discussion

Accelerated HF-rTMS - delivered over the left frontal cortex - was applied one or four consecutive days in sedated or anesthetized dogs. In line with comparable research in depressed patients, sham stimulation did not show short- or long-term effects on cerebral perfusion [45-47]. Active aHF-rTMS resulted in a transitory increase in rCBF at the stimulation site, under anaesthesia as well as under sedation. Although the magnitude of this increase was not influenced by the number of sessions, the increase in rCBF was higher but more short-lived under sedation. Under anaesthesia, both active protocols (5 and 20 sessions aHF-rTMS) provoked increased neuronal activity in the left frontal cortex, which lasted until 1 month after stimulation. This is comparable with the work executed in human subjects, undergoing 10 consecutive daily HF-rTMS sessions (one session/day), that observed an increased neuronal activity lasting up to 2 weeks post stimulation [45, 46, 48]. Moreover, this study obtained a comparable increase in rCBF of the left frontal cortex (2-3%) as Catafau et al (2001)[46] reported in 7 depressed patients (medication-resistant). Although we did not assess behavioural measurements, this observed perfusion increase might initiate speculation on the potential role of rTMS in behaviour-disordered dogs, especially in anxiety disordered animals that have been reported to suffer from hypoperfusion in the left frontal cortex [34]. Similarly, increased rCBF in the left frontal region after HF-rTMS has been associated with clinical improvement in MDD patients [49].

In this study, we only detected increased rCBF after active aHF-rTMS limited to the stimulated area, and not more widespread in the structurally and functionally connected areas. A reason for the absence of remote effects could be the state in which the animals were stimulated. A study on human subject by Massimini et al. (2005) [50] found that during non-rapid eye movement sleep (NREM) the initial response at stimulation site did not propagate to distant regions. They explained this by a loss of cortical integration during NREM sleep, which also occurs during midazolam-induced loss of consciousness [50, 51]. Ferrarelli et al. (2010) [51] found an hd-EEG response with a short lasting high positive-negative wave under midazolam anaesthesia indicating a local and shorter TMS activity in contrast to the effect registered during wakefulness. Aside from the effects of midazolam, all volatile anaesthetics can affect neuroplasticity, reduce excitatory and augment inhibitory neuronal

transmission. In conclusion, it is therefore possible that the absence of remote activation is confounded by the combined use of midazolam and isoflurane.

Despite the presence of a (linear) dose-response relationship [52-54], the measured significant increase in rCBF after stimulation was identical for the 2 active protocols (5 session or 20 sessions) under anaesthesia. This implies that an increase in number of sessions does not seem to influence the magnitude of the rCBF increase. Nonetheless, this does not rule out the presence of a (linear) dose-response relationship. In this study, it was not possible to determine a response rate, which excludes the possibility to examine the presence of a dose-response relationship.

Although the descriptive analysis of this study revealed an individual response to the aHF-rTMS protocol (Fig 3 and 4), no main effect was found for the fixed variables age and gender. This is in line with studies performed in human subjects that found that gender and age were no significant predictor variables for the outcome of an rTMS treatment [49, 55-58]. Nonetheless, a better rTMS outcome has been found in younger people [59] and rats [60]. It is hypothesized that in older individuals the distance between the frontal cortex and the skull increases increase, limiting the rTMS response. Since aging also causes atrophy of the canine brain [61], it is plausible that the canine cortex-scalp distance increases as well, thus provoking an age-related response. In the current study, no age main effect was found, due to the selection of an age homogeneous population. Although a gender dependent rTMS response is not present, it appears that a high oestradiol to progesterone ratio may positively influence the outcome of SSRI's and rTMS treatment. [62]. This study used neutered female dogs, suggesting a lower oestradiol to progesterone ratio and therefor a lower rTMS response compared to intact female dogs.

When using a coil that provides a larger coil to brain size, the focality and efficacy might plummet [18]. Nonetheless, focality can be assumed in this study since only an increase in rCBF was found for the stimulation target and not in surrounding cortical regions. A focal increase in rCBF, might indicate that no whole brain stimulation took place notwithstanding the presence of a larger coil to brain size ration. The, in comparison, smaller canine brain could not have been able to capture the total flux generated by the coil, which might help to explain the absence of remote effects in this study.

Although this study has some major advantages such as including individual neuronavigation, some limitations must be kept in mind. An active coil, tilted 90 degrees was used as the sham condition. Despite the fact that an active coil, held this way, can provoke minor voltages in the underlying cortical tissue [63]. However, no changes in the rCBF were noticed in the control group. Only the one-day protocol under sedation was explored, leaving the question whether accelerated HF-rTMS under sedation allows propagation of the initial response at the stimulation site, unanswered. In the current study, 21 neutered healthy dogs were included. More information is needed regarding the effects of the gonadal status, age and natural brain disorders on the neuromodulation of the accelerated HF-rTMS protocols. Hereby, clearer insights into the canine rTMS model can be obtained and its use as a valid translational model for rTMS research. Nonetheless, it must be emphasised that the obtained results should be interpreted with caution. In order to exclude the effects of anaesthesia in the canine rTMS model; different anaesthetic/sedative protocols should be compared to conscious dogs while rTMS is applied. The final limitation is that SPECT has in comparison to PET a lower sensitivity.

Conslusions

To conclude, the results in this study suggest that accelerated HF-rTMS can provoke the neuronal activation in the stimulated cortical region in healthy anesthetized dogs. Because these findings - acquired with human rTMS apparatus - in healthy dogs strongly resemble SPECT findings in humans, is it reasonable to reserve a role for the dog as an alternative animal model for rTMS research in humans.

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Chapter 6: The ambiguous role of the serotonin transporter in the neurobiological mechanism of aHF-rTMS paradigms

Acute accelerated high frequency rTMS causes an immediate local and remote increase in the serotonin transporter binding index, measured with [^{11}C]DASB.

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Abstract:

Repetitive Transcranial magnetic stimulation (rTMS) has proven to be a useful tool for the treatment of depression. Nonetheless, its exact neurophysiological mechanism remains unclear. Since antidepressants - such as selective serotonin re-uptake inhibitors - act on the serotonin transporter (SERT), it is plausible that rTMS acts upon this transporter as well. Therefore, this study monitored the effect of a single day (5 sessions) and a 4-day accelerated (20 sessions) high-frequency rTMS (aHF-rTMS) treatment on the regional SERT availability in healthy beagle dogs.

22 dogs were randomly divided into 3 unequal groups: 5 active sessions (n=10), 20 active sessions (n=8), and 20 sham sessions (n=4). Each dog received an anatomical MRI, neuronavigation and a baseline [^{11}C]DASB PET scan, followed by an aHF-rTMS treatment over the left frontal cortex. 24 hours, 1 month and 3 months after the last stimulation session [^{11}C]DASB PET scans were acquired. The SERT binding index (BI) of 23 brain regions was calculated.

SERT BI changes over time were not significantly different in the 20 sessions sham group. Differences in SERT BI were noticed between both active protocols in several brain regions. However, acute (24 hours post) changes were found in the left frontal cortex, left hippocampus, pons and left thalamus. Whereas long term changes were noticed in the (pre)subgenual cortices, the left temporal cortex and right parietal cortex.

A similar mechanism of action was observed as treatment of depression with SSRIs and TMS. Moreover, this study stresses the importance of the number of sessions in the treatment of depression with TMS.

Keywords:SERT, [^{11}C]DASB, aHF-rTMS, SSRI

Introduction

Alterations in the brain serotonin transmission play a key role in the development of depression [1]. Evidence supporting this statement comes from studies indicating a reduction in serotonin (5-HT) and its metabolites in depressed patients, a depressive relapse after tryptophan depletion, a reduction of the 5-HT uptake binding sites in depression and the effectiveness of selective serotonin reuptake inhibitors (SSRIs) [2, 3].

SSRIs, the first-line pharmacologically treatment modality for depression, exert their function by elevating the 5-HT level through blocking of the serotonin transporter (SERT) [4]. This transporter is a transmembrane protein that actively transports 5-HT from the synaptic cleft into the presynaptic neuron, thereby ending the 5-HT neurotransmission. Consequentially, by administering SSRIs, a decreased 5-HT re-uptake speed is acquired which is accompanied by an improvement of depression [5]. The density of the SERT plays, besides its availability, a crucial role in depression. Brain regions with the highest density of SERT are the thalamus, hypothalamus, amygdala, raphe nuclei, nucleus caudatus and putamen [5-7]. Of note here, regions with high SERT density are constant between different species such as rodents [8], pigs [9], cats [10], non-human primates [11] and dogs [12]. These regions are also involved in the regulation of mood (depression), anxiety and fear. However, post mortem and imaging studies SERT density studies in depressed and healthy individuals revealed contradictory results. The SERT density in depressed patients can either be decreased [6, 13-21], increased [22] or similar [23-25] as in non-depressed patients. Nonetheless, the chronic use of SSRIs mediates a decrease in the SERT density (down regulation), which can account for the long-term effects of the SSRIs [26-28].

Brain modulation techniques such as electroconvulsive therapy (ECT) and repetitive transcranial magnetic stimulation (rTMS) can alter the density of the SERT [29, 30] as well. As SSRIs, ECT can either increase or decrease the SERT availability in the synaptic cleft [31-35] whereas for its alternative, TMS, only a decrease of the SERT transcription was found [30]. This decrease in SERT mRNA was found only in the cerebellum with brainstem of mice after whole brain TMS stimulation (10 days, 20 Hz, 2 s; 20 times/day; inter-stimulus interval 1 min). Ten days after cessation of this TMS

protocol, a normalization of the SERT mRNA was found. These findings indicated that chronic TMS would result in similar functional changes as SSRIs.

Although ECT is still considered to be more efficacious, TMS shows no significant differences in response rate [36-38]. Yet, a TMS parameter that needs to be optimized is the duration of the treatment. Initially, short duration treatments were given to patients whereas recently more sessions yield better clinical results [39-42]. Nowadays, in order to reduce the prolonging of a rTMS treatment, several sessions are given on a single day [43, 44]. However, the question remains whether to alter the number of sessions or the number of pulses when accelerating a rTMS treatment. Recent studies suggest that in order to achieve a better clinical outcome, a raise in the number of pulses should be pursued [45, 46]. This despite of the contradictory results presented by Kedzior et al. (2014) who found that fewer sessions provoked a better clinical outcome. Since few evidence of the modulatory effect of accelerated high frequency repetitive TMS (aHF-rTMS) on the SERT exist and the fact that different number of pulses mediate the effect of a TMS treatment, this study aimed to investigate the short- and long-term influence of 3 aHF-rTMS treatments over the left frontal cortex in healthy Beagle dogs using [^{11}C]DASB (3-amino-4-(2-dimethylaminomethylphenylsulfanyl)benzonitrile) PET scans. The 3 treatment groups were 5 active sessions (1 day), 20 active sessions (5 session during 4 consecutive days) and 20 sham sessions (5 sessions during 4 consecutive days). Our first hypothesis was that changes in the SERT binding indices would emerge in different brain regions, preferably in those with a high SERT density. Secondary, we aimed to see differences in SERT binding indices between the 2 active aHF-rTMS protocols.

Materials and methods

Ethics

The Ghent University Ethical Committee approved this study (approval number EC 2015/140; date of approval).

Animals

Twelve healthy Beagle dogs (5 female neutered, 1 female intact, 5 male neutered, 1 male intact) were used in this study. Ten of these dogs were reused, this only after a three-month wash out period (6 months after applying the last stimulation session) and a return to baseline SERT binding index (BI) measured by an [^{11}C]DASB PET scan. In this way, 22 dogs were included in this study. The dogs were owned by the department of veterinary medical imaging and small animal orthopaedics and the department small animals of the faculty of veterinary medicine. The dogs were permanently housed, at the Ghent university faculty of veterinary medicine, in groups of 8 on an internal surface of 15 m^2, with permanent access to an outside area of 15 m^2. The floor coverings in the inner part consisted of wood shavings. Frequently, toys such as Kongs were given to the animals and were twice a day released onto an enclosed play area. In addition, students of the faculty of veterinary medicine regularly walked the dogs.

Neuronavigation

The study aimed to apply an aHF-rTMS treatment over the left frontal cortex. Therefore, a frameless neuronavigation system was used to provide the external localisation of the left frontal cortex of each dog. First, a tomographical dataset (Siemens 3 Ts Magnetom Trio Tim) was acquired; after which neuronavigation was performed as described by Dockx et al. (2017)[48].

The stimulation protocol

The 22 dogs were ad random divided into 3 unequal groups. The first group consisted out of 10 dogs (5 neutered males and females). The second group held 8 dogs (3 neutered females, 1 intact female, 3 neutered males, 1 chemically neutered male). The last group consisted out of 4 animals (2 neutered males, 1 chemically neutered male, 1 neutered female). Several months prior to the stimulation, positive reinforcement was used to accustom all dogs to the researcher, the experimental room, the placing of the coil and the sound of the coil.

All stimulations were applied under general anaesthesia. Premedication consisted of butorphanol IV (0.2 mg/kg; Dolorex1; Intervet Belgium NV). After onset of sedation, anaesthesia was induced intravenously by administering midazolam (0.2 mg/kg; Dormicum; Roche Nederland B.V.)

immediately followed by propofol (Propovet Multidose, Abbott Laboratories, Berkshire, UK, 1±2 mg/kg given to effect). General anaesthesia was maintained with isoflurane (Isoflo, Abbott Laboratories, Berkshire, UK) in oxygen using a rebreathing system. Immediately following the induction of anaesthesia, the motor threshold of the left motor cortex was determined. A motor threshold (MT) of 100% was defined as the set machine output (Magstim Company Limited, Wales, UK) that could provoke 5 out of 10 visible muscle contractions in the right upper front limb. The MT assessment was performed as described in chapter five.

Group 1 received 5 daily sessions, whereas group 2 and 3 received 20, active or sham respectively, sessions (5 daily sessions during consecutive 4 days). Each session contained 40 trains of 1.9 seconds each. The trains were separated by a 12 second intertrain interval (in total 1560 pulses were given per session). The time interval between sessions was 10 to 15 minutes. This protocol (20Hz, 110% MT) was an exact copy of an accelerated HF-rTMS treatment protocol performed in MDD patients at our medical university hospital [49].

The anesthetic depth was clinically monitored (ventral position of the eye and absence of the eyelid-reflex). When deemed necessary, the isoflurane dose was adjusted to maintain the same depth.

Radiosynthesis

N-methylation of the precursor N-desmethyl-DASB (50 µg, ABX, Radeberg, Germany), with a [^{11}C]methyl triflate, was performed in order to synthesize the SERT ligand [^{11}C]DASB. Radiochemical purity of more than 99% was achieved [12].

Imaging procedure

All dogs received an intravenous cephalic catheter and were intramuscularly (IM) premedicated with dexmedetomidine 375 µg/m^2. The anaesthetic protocol was similar to that described in the study of Van Laeken et al, 2016[50]). In brief, general anaesthesia was achieved by intravenously injecting (IV) propofol (Propovet Multidose, Abbott Laboratories, Berkshire, UK, 1±2 mg/kg given to effect). Isoflurane (Isoflo1, Abbott Laboratories, Berkshire, UK) was used for maintenance. Prior to the [^{11}C]DASB PET scan, all dogs underwent, under general anaesthesia, an MRI on a Siemens 3 Ts Magnetom Trio Tim system (Siemens Medical Systems, Erlangen, Germany)

using a phased-array spine coil and a phased-array body matrix coil. A structural scan was acquired with the following sequence parameters: TR = 2250 ms, TE = 4,18 ms, TI = 900 ms, parallel acquisition method = GRAPPA with acceleration factor = 2, matrix size = 256 ~ 256, sagittal, FOV = 220mm, flip angle = 9Ê, voxel size = 0.9 ~ 0.86 ~ 0.86 mm^3.

All dogs underwent 4 [^{11}C]DASB PET scans: a baseline, 24 hours, 1 month and 3 months after the last aHF-rTMS treatment session was applied. An [^{11}C] DASB bolus was IV injected. Thirty minutes after the bolus injections, the dogs were placed in sternal recumbence, with the front limbs extended caudally. A CT scan was taken for attenuation correction. Forty minutes after the bolus injection, a 20 minutes static scan was performed with the PET camera (Gemini PET/CT, Philips, Eindhoven, The Netherlands)

PET analysis

Pmod (version 3.405, PMOD Technologies Ltd., Zurich, Switzerland) was used to analyze the PET data. The PET-CT data were fitted onto their corresponding MRI to provide anatomical information. The stereotactic atlas by Dua-sharma et al. (1970) [51] was used to delineate 24 regions (Fig. 1) of interest (ROI's): nucleus caudatus left, nucleus caudatus right, hippocampus left, hippocampus right, amygdala left, amygdala right, frontal cortex left, frontal cortex right, pons, medulla, midbrain, temporal cortex left, temporal cortex right, occipital cortex left, occipital cortex right, parietal cortex left, parietal cortex right, thalamus left, thalamus right, subgenual cortex, presubgenual cortex, anterior cingulate cortex, posterior cingulate cortex and cerebellum (excluding the vermis). A non-displaceable binding potential (BI) was calculated for each ROI at each time point with the cerebellum (excluding the vermis) as reference region.

Statistical analysis

Rstudio 1.1.456 (R: A Language and Environment for Statistical Computing; R Core Team; R Foundation for Statistical Computing, Vienna, Austria, 2016, https://www.R-project.org/) with packages MASS (version 7.3-50), doBy (version 4.6-2), sommer (version 3.0), stats (version 3.4.2) and emmeans (version 1.3.0) were used to compute all analyses.

Onto the SERT data, a multivariate linear mixed model was fitted. The BI of 23 ROI's (exclusion of the reference region cerebellum) were set as response variables. Treatment group and time points were set as fixed-effect factors. The presence of a time point by treatment interaction was also considered in the model. In addition, time and animal were set as random factors. A random intercept was included into the model. The Welsh–Satterthwaite equation was used to calculate the degrees of freedoms. The type I error was set at 0.05. Normality of the error terms, linearity of the regression function and homoscedasticity of the error terms were checked using diagnostic plots and statistical tests (Bartlett test of homogeneity of variances and the Shapiro-Wilk normality test).

A multivariate linear mixed model with heterogeneous unstructured variance was fitted onto the data. The model was written as $E(Yt|T1,T2) = \beta_0 + \beta_1 t_1 + \beta_2 t_2 + \beta_3 t_3 + \beta_4 T_1 + \beta_5 T_2 + \beta_6 t_1 T_1 + \beta_7 t_2 T_1 + \beta_8 t_3 T_1 + \beta_9 t_1 T_2 + \beta_{10} t_2 T_2 + \beta_{11} t_3 T_2$ with Yt as response variable. The BI of the 23 VOI's (continuous) was set as response value whereas time and treatment (both categorical) were set as predictor value. The factor time (continuous) and animal (categorical) were set as random factors. The predictor time (t) denotes the different timepoint with t_1 the first of three (k-1 = 4-1 = 3) dummies (= 1 if time point = "24 hours post" or 0 otherwise), t_2 the second dummy (= 1 if time point = "1 month post" or 0 otherwise), t_3 (= 1 if time point = "3 months post" or 0 otherwise. The treatment predictor (T) indicated the different treatment modalities with T_1 the first of two (k-1 = 3-1 = 2) dummies (= 1 if treatment = "20 sessions active" or 0 otherwise) and T2 (= 1 if treatment = "5 sessions active" or 0 otherwise). The reference level (for each region) was set as the PI at baseline in the control group (intercept).

Post hoc, linear contrasts were set up for each ROI for which the previous model indicated a significant time by treatment interaction.

Results

The model revealed (Table 1) for the 5 active sessions a marginal significant increase (estimate = 0.17; 95% CI [-0.01; 0.34], *P*-value = 0.06) in SERT BI of the left frontal cortex, 24 hours after the 5 sessions were administered. Three months after the 5 sessions were administered, a significant increase in SERT BI in the right parietal cortex (estimate = 0.06; 95% CI [0.03; 0.29], *P*-

value = 0.005) and left temporal cortex (estimate =0.21; 95% CI [0.07; 0.34], *P*-value = 0.005) was noticeable. The model did not reveal any significant time by treatment interactions for the remaining ROI's.

For the 20 active sessions group (Table 1), the model indicated a significant increase (estimate = 0.39; 95% CI [0.01; 0.78], *P*-value = 0.04) in the pons SERT BI 24 hours after the last sessions was given. At the same time, a marginally significant decrease of 0,33 in BI was found in the left hippocampus (95% CI; [-0.68; 0.01], *P*-value = 0.06). A significant decrease (estimate = -0.55; 95% CI [-1.06; -0.04], *P*-value = 0.04) was found 1 month later in the left thalamic region. This decrease in the left thalamic area was accompanied by a decrease in SERT BI in the presubgenual (estimate = -0.38; 95% CI [-0.68; -0.08], *P*-value = 0.01) and subgenual cortex (estimate = -0.69; 95% CI [-1.11; -0.27], *P*-value = 0.002). Similar to the 5 active session treatment, a significant increase in SERT BI was found in the right parietal cortex (estimate = 0.19; 95% CI [0.06; 0.318], *P*-value = 0.02). The model did not reveal any significant time by treatment interactions for the remaining ROI's.

The post hoc, linear contrasts were set up (Table 2 – 5), based on the multivariate mixed model, for the left frontal cortex, left temporal cortex, right parietal cortex, left hippocampus, left thalamus, presubgenual cortex and subgenual cortex.

The linear contrasts revealed within the 5 active sessions treatment an immediate increase (24 hours) in BI of the left frontal cortex, which normalized after 1 month. In addition, this treatment provoked a decrease in BI in the left thalamic area and subgenual cortex 3 months after stimulation (Table 2, 4). The left temporal cortex showed at 24 hours an increase in SERT BI whereas a decrease was seen after one month (Table 4). Nonetheless, these changes did not differ significantly when compared to the BI in the 20 sessions sham treatment (Table 3, 5).

Within the 20-session active treatment, a decrease in the left hippocampal BI was seen from 24 hours after the treatment until 3 months later. The pons showed an increase in BI at 24 hours post treatment. At 1 and 3 months, a decrease was also present in the left thalamic region (Table 2). The subgenual and genual cortex showed a decrease in SERT BI 1 month after stimulation. When compared to the 20 sessions sham treatment, only a significant increase and decrease was seen in the

pons (24 hours) and the left thalamic region (1 month) respectively (Table 3). This was accompanied by a decrease in the presubgenual and subgenual cortex (Table 5).

Between both active treatments, significant differences in the BI of the left frontal cortex, left hippocampus, left thalamic area, presubgenual cortex, subgenual cortex, left temporal cortex and right parietal cortex occurred for the different time points (Table 3 and 5). No differences in BI were noticeable between both active protocols in the pons (Table 3).

Discussion

The results of this study indicate that aHF-rTMS, single- or 4-days protocol, over the left frontal cortex can alter the SERT BI of different brain regions over time. The single day (5 sessions) aHF-rTMS protocol induced after 24 hours at stimulation site an increase in the SERT BI at the stimulation site. Nonetheless, this local effect was no longer present 24 hours after applying aHF-rTMS after 4 consecutive days (20 sessions). At the same time, alterations in the SERT BI were already present in the pons, the region containing the raphe nuclei, and the left hippocampus. It must be kept in mind that, since the treatment was applied for 4 consecutive days, there is a time lag of 72 hours between the 24 hours' time point of the 5 sessions protocol and the 20 sessions protocol. Four weeks after the 4 days protocol, the SERT BI of left thalamic region, the presubgenual cortex and genual cortex decreased significantly. Interestingly, between both active protocols, no differences in BI was found in the pons despite a significant time by treatment interaction was found for the 20 active sessions treatment.

Alteration in the SERT BI of the pons, the region containing the raphe nuclei, is in line with Ikeda et al. (2005) [30], who found a down regulation of SERT mRNA in the cerebellum with brainstem after chronic rTMS (20 days, 20Hz, 2s, 20 times/day, 1 min inter stimulus interval) in mice. This decrease could have been potentiated through the afferent connection between the left frontal cortex and the raphe nuclei. This hypothesis is strengthened by the fact that marginal significant alterations were noticed 24 hours after the 5 sessions protocol was applied. This decrease in the pons SERT BI could be the result of 3 pathways. First, since this is in accordance with Ikeda et al. (2005) [30], rTMS could induce a down regulation of the SERT mRNA. Secondly, the rTMS could have provoked and

internalization of the SERT protein [26]. Thirdly, rTMS may have provoked a release of serotonin in the cells of the raphe nuclei [52]. The result of all pathways is an increased concentration of 5-HT in the synaptic cleft. Thereby, the mechanism of action of rTMS may be similar to that of long-term SSRIs usage as hypothesized by Best et al. (2011) [53]. In essence, the re-uptake of 5-HT was decreased and the extracellular 5-HT in the raphe nucleus was thereby raised. This elevation in extracellular 5-HT in the raphe nuclei decreases the tonic firing rate of those cells (mediated by the 5-HT_{1A} autoreceptor) [54]. Consequentially, a decrease in 5-HT release in terminal regions of the raphe nuclei is provoked. Thereby, a reduction in 5-HT release would theoretically provoke a rise in SERT BI in the neocortex. In accordance, an increased SERT BI was found 3 months after the last stimulation was applied in the right parietal cortex for both active protocols. At that time point, significant alterations of the SERT BI were no longer noticeable in the brain region containing the raphe nuclei. This suggests that when rTMS would be applied in subjects with a low vesicular (intracellular) 5-HT concentration (depression or anxiety), which is accompanied by a lower firing rate of the raphe nuclei, a normalization of response to bursts of the cells of the raphe nuclei would occur. It has to be kept in mind that all of the above changes not only comply with changes induced by psychotropic interventions but by brain modulation techniques as well. Shen et al (2001)[35] found an immediate and long-term decreased SERT mRNA expression in the raphe nuclei after acute and chronic electroconvulsive stimulation. Although they were not able to reproduce their findings, they also found an increased SERT in the frontal cortex as Hayakawa et al. (1995) reported [31, 32].

Successful (non)pharmacological treatment of depression has been associated with a reduction of the hyperactivity of the subgenual anterior cingulate cortex (sgACC) [55]. Even more, studies evaluating the chronic use of SSRI on the SERT have hinted an association between a high pre-treatment SERT binding in the sgACC and a positive clinical response to the administered SSRIs [56, 57]. Recently, Baeken and his team found a significant correlation between clinical improvement, after HF-rTMS was applied over the left prefrontal cortex, and a reduction of the glucose metabolism of the sgACC, two weeks after the protocol was applied [49]. Furthermore, the current study assessed the effect of the HF-rTMS, used by Baeken, and found a decrease in SERT BI in the (pre)sgACC after four weeks. Thus, both studies indicate a delayed effect of the HF-rTMS, applied to the left frontal cortex,

on the sgACC. Although speculative, the reduction of the hyperactivity of the sgACC in depression, and its anti-depressive action, could be mediated by a decrease in availability of the SERT or by consequence, an increase in the extracellular 5-HT concentration.

Applying more rTMS treatments or more pulses per session yield better clinical results [58]. Moreover, this study found, besides alterations in BI in similar brain regions, differences in BI induced in different brain regions. More specifically, acute (24 hours post treatment) differences, present in the left frontal cortex, left hippocampus, left thalamus and presubgenual cortex, were noticeable. As hinted earlier, the differences at this time point could be caused by the time lag between the 24 hours' time point of each active treatment. This would imply that 72 h after applying the first 5 five sessions of a 20-session treatment a normalization of the SERT BI in the frontal cortex might already occur. Nonetheless, in the pons (region containing the raphe nuclei) no acute or chronic differences (1 month and 3 months post treatment) were found between both active protocols. In contrast, the active protocols showed significantly different BI at on month in the left thalamus, presubgenual cortex, subgenual cortex, left temporal cortex and right parietal cortex. This could imply that with a higher number of pulses, changes in the SERT BI would occur in a wider variety of brain regions. In addition, Dockx et al. (2017)[59] found that the immediate (24 hours post) increase in regional cerebral blood flow (rCBF) persisted longer (until 1 month) when 20 sessions of an aHF-rTMS protocol was applied. Therefore, the assumption that more sessions of an aHF-rTMS might result in other clinical results when compared to fewer sessions of the same aHF-rTMS protocol is strengthened.

Despite its insights into the modulation of the SERT by an aHF-rTMS protocol, some limitations have to be kept in mind. The aHF-rTMS protocol was only applied onto a small sample size of 12 dogs, which were reused. Nonetheless, significant effects on the SERT BI were noticed only within both active treatment groups. Table 3 and 5 indicate that no differences in baseline measurements were noticed between groups, thereby justifying the reuse of the dogs during the experiment with minimal risks of carry-over effects. During the sham treatment, an active coil was placed tilted, 90 degrees, over the left frontal cortex. An active coil placed in this manner can provoke minor voltages in the underlying cortical tissue [60]. Despite this fact, no changes in SERT BI were seen within the sham treatment group. All treatments were applied under general anaesthesia whereas

sedation provokes larger changes in rCBF after an aHF-rTMS protocol [59]. Therefore, the results obtained in this study should be re-evaluated under different anaesthetic protocols and during full consciousness.

Conclusion

Both active aHF-rTMS protocols were able to induce changes - either local and remote - in SERT BI measured with [^{11}C]DASB, thereby confirming our first hypothesis. In addition, administering more sessions of an aHF-rTMS protocol induces changes in SERT BI of brain regions other than when applying fewer sessions, thereby stressing the importance of the number of sessions of a TMS treatment.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This research was funded by Ghent university special research fund (BOF 01J05715) and was supported by the Ghent university multidisciplinary research partnership 'The integrative neuroscience of behavioural control'. We thank the division of veterinary nuclear medicine (department of medical imaging and small animal orthopaedics, university of Ghent) to provide access to their infrastructure.

Appendix: supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: …

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	Left hippocampus	Left frontal cortex	Pons	Left thalamus	Presubgenual cortex	Subgenual cortex	Left temporal cortex	Right parietal cortex
Intercept	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
24 hours	0.932	0.507	0.297	0.258	0.639	0.580	0.514	0.153
1 month	0.680	0.515	0.372	0.631	0.315	0.124	0.220	0.111
3 months	0.080	0.029*	0.058	0.209	0.064	0.765	0.004**	0.002**
20 sessions active	0.126	0.331	0.826	0.907	0.778	0.144	0.357	0.116
5 sessions active	0.252	0.344	0.067	0.462	0.532	0.033*	0.082	0.075
24 hours:20 sessions active	0.058	0.966	0.044*	0.930	0.352	0.175	0.348	0.374
1 month:20 sessions active	0.138	0.589	0.603	0.036*	0.015*	0.002**	0.607	0.933
3 months:20 sessions active	0.634	0.076	0.294	0.810	0.306	0.367	0.092	0.018*
24 hours:5 sessions active	0.494	0.060	0.488	0.301	0.817	0.879	0.120	0.073
1 month:5 sessions active	0.918	0.595	0.820	0.809	0.412	0.156	0.408	0.075
3 months:5 sessions active	0.276	0.373	0.570	0.495	0.362	0.316	0.005**	0.005**

Table 1: Output main effect and interaction test for the predictors time and treatment group. ****P*-value <0.05; *****P*-value <0.01

	20 sessions sham					5 sessions active					20 sessions active				
	Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
Left frontal cortex															
$T_1 - T_0$	-0.05	0.07	-0.19	0.09	0,51	0.12	0.04	0.04	0.20	0,01*	-0.04	0.05	-0,14	-0,28	0,34
$T_2 - T_0$	-0.04	0.07	-0.18	0.10	0,51	-0.09	0.04	-0.17	-0.01	0,05*	0.00	0.05	-0,10	-0,20	0,98
$T_3 - T_0$	-0.14	0.06	-0.26	-0.02	0,03*	-0.07	0.04	-0.15	0.01	0,08	-0.00	0.04	-0,08	-0,16	0,96
$T_2 - T_1$	0.00	0.08	-0.16	0.16	0,96	-0.20	0.05	-0.30	-0.10	0,0001***	0.05	0.05	-0,05	-0,10	0,38
$T_3 - T_1$	-0.09	0.07	-0.23	0.05	0,20	-0.19	0.05	-0.29	-0.09	0,0002***	0.04	0.05	-0,06	-0,12	0,37
$T_3 - T_2$	-0.10	0.07	-0.24	0.04	0,15	0.01	0.04	-0.07	0.09	0,77	-0.00	0.05	-0,10	-0,20	0,94
Left Hippocampus															
$T_1 - T_0$	-0.01	0.14	-0.29	0.27	0,93	0.10	0.09	-0.08	0.28	0,25	-0.35	0.09	-0,53	-1,07	0,001**
$T_2 - T_0$	-0.06	0.15	-0.36	0.24	0,68	-0.08	0.09	-0.26	0.10	0,40	-0.34	0.11	-0,56	-1,13	0,003**
$T_3 - T_0$	-0.22	0.12	-0.46	0.02	0,08	-0.06	0.08	-0.22	0.10	0,47	-0.30	0.09	-0,48	-0,97	0,002**
$T_2 - T_1$	-0.04	0.16	-0.36	0.28	0,76	-0.18	0.10	-0.38	0.02	0,07	0.01	0.11	-0,21	-0,43	0,91
$T_3 - T_1$	-0.21	0.14	-0.49	0.07	0,13	-0.16	0.09	-0.34	0.02	0,06	0.05	0.09	-0,13	-0,26	0,56
$T_3 - T_2$	-0.16	0.14	-0.44	0.12	0,24	0.02	0.09	-0.16	0.20	0,83	0.04	0.10	-0,16	-0,33	0,70
Pons															
$T_1 - T_0$	-0.17	0.16	-0.49	0.15	0,29	-0.04	0.10	-0.24	0.16	0,71	0.22	0.10	0,02	0,04	0,03*
$T_2 - T_0$	-0.13	0.14	-0.41	0.15	0,37	-0.09	0.09	-0.27	0.09	0,32	-0.04	0.11	-0,26	-0,53	0,74
$T_3 - T_0$	-0.25	0.13	-0.51	0.01	0,06	-0.16	0.09	-0.34	0.02	0,06	-0.08	0.09	-0,26	-0,53	0,37
$T_2 - T_1$	0.04	0.17	-0.30	0.38	0,82	-0.05	0.11	-0.27	0.17	0,62	-0.26	0.11	-0,48	-0,97	0,03*
$T_3 - T_1$	-0.09	0.16	-0.41	0.23	0,60	-0.13	0.10	-0.33	0.07	0,21	-0.31	0.10	-0,51	-1,03	0,004**
$T_3 - T_2$	-0.13	0.14	-0.41	0.15	0,38	-0.07	0.09	-0.25	0.11	0,43	-0.05	0.11	-0,27	-0,55	0,65
Left thalamus															
$T_1 - T_0$	-0.24	0.21	-0.66	0.18	0,26	0.01	0.13	-0.25	0.27	0,89	-0.22	0.14	-0,50	-1,01	0,12
$T_2 - T_0$	0.10	0.20	-0.30	0.50	0,63	0.04	0.13	-0.22	0.30	0,76	-0.45	0.15	-0,75	-1,52	0,004**
$T_3 - T_0$	-0.23	0.18	-0.59	0.13	0,21	-0.38	0.12	-0.62	-0.14	0,002**	-0.28	0.13	-0,54	-1,09	0,03**
$T_2 - T_1$	0.34	0.22	-0.10	0.78	0,13	0.02	0.13	-0.24	0.28	0,87	-0.23	0.15	-0,53	-1,07	0,12
$T_3 - T_1$	0.01	0.19	-0.37	0.39	0,96	-0.40	0.12	-0.64	-0.16	0,002**	-0.07	0.12	-0,31	-0,63	0,60
$T_3 - T_2$	-0.33	0.19	-0,71	0.05	0,09	-0.42	0.12	-0.66	-0.18	0,001	0.17	0.14	-0,11	-0,23	0,24

Table 2: Multiple comparison for each time point within each treatment group. (* < 0.05; **<0.01; *<0.001).**

	5 sessions active - 20 sessions sham					20 sessions active - 20 sessions sham					5 sessions active - 20 sessions active				
	Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
Left frontal cortex															
T_0	-0.06	0.06	-0.18	0.06	0.34	-0.07	0.07	-0.21	0.07	0.33	0.00	0.05	-0.10	0.10	0.94
T_1	0.10	0.07	-0.04	0.24	0.18	-0.06	0.08	-0.22	0.10	0.43	0.16	0.06	0.04	0.28	0.01*
T_2	-0.10	0.07	-0.24	0.04	0.15	-0.02	0.07	-0.16	0.12	0.80	0.19	0.14	-0.09	0.47	0.17
T_3	0.01	0.07	-0.13	0.15	0.92	0.08	0.07	-0.06	0.22	0.26	-0.07	0.05	-0.17	0.03	0.20
Left hippocampus															
T_0	0.17	0.15	-0.13	0.47	0.25	0.24	0.15	-0.06	0.54	0.13	-0.07	0.12	-0.31	0.17	0.57
T_1	0.29	0.16	-0.03	0.61	0.08	-0.10	0.16	-0.42	0.22	0.56	0.39	0.12	0.15	0.63	0.003**
T_2	0.16	0.16	-0.16	0.48	0.35	-0.03	0.17	-0.37	0.31	0.84	-0.09	0.06	-0.21	0.03	0.15
T_3	0.33	0.13	0.07	0.59	0.02*	0.16	0.14	-0.12	0.44	0.24	0.17	0.11	-0.05	0.39	0.14
Pons															
T_0	0.32	0.17	-0.02	0.66	0.07	0.04	0.18	-0.32	0.40	0.83	0.28	0.14	0.00	0.56	0.05*
T_1	0.45	0.20	0.05	0.85	0.03*	0.43	0.20	0.03	0.83	0.04*	0.02	0.15	-0.28	0.32	0.91
T_2	0.35	0.18	-0.01	0.71	0.06	0.13	0.19	-0.25	0.51	0.49	0.22	0.14	-0.06	0.50	0.14
T_3	0.41	0.17	0.07	0.75	0.02*	0.21	0.18	-0.15	0.57	0.24	0.20	0.14	-0.08	0.48	0.15
Left thalamus															
T_0	0.16	0.21	-0.26	0.58	0.46	0.03	0.22	-0.41	0.47	0.91	0.13	0.17	-0.21	0.47	0.45
T_1	0.41	0.23	-0.05	0.87	0.07	0.05	0.23	-0.41	0.51	0.83	0.37	0.17	0.03	0.71	0.04*
T_2	0.10	0.22	-0.34	0.54	0.66	-0.52	0.23	-0.98	-0.06	0.03*	0.62	0.18	0.26	0.98	0.001**
T_3	0.01	0.19	-0.37	0.39	0.96	-0.03	0.20	-0.43	0.37	0.89	0.04	0.16	-0.28	0.36	0.82

Table 3: Multiple comparison for each treatment group within each time point. (* < 0.05; **<0.01; *<0.001).**

	20 sessions sham					5 sessions active					20 sessions active				
	Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
Presubgenual cortex															
$T_1 - T_0$	0.06	0.12	-0,18	0,30	0,64	0.02	0.08	-0,14	0,18	0,75	-0.08	0.08	-0,24	0,08	0,32
$T_2 - T_0$	0.12	0.12	-0,12	0,36	0,31	0.00	0.07	-0,14	0,14	0,95	-0.26	0.09	-0,44	-0,08	0,01*
$T_3 - T_0$	-0.21	0.11	-0,43	0,01	0,06	-0.09	0.07	-0,23	0,05	0,22	-0.07	0.08	-0,23	0,09	0,37
$T_2 - T_1$	-0.27	0.11	-0,49	-0,05	0,02*	-0.02	0.08	-0,18	0,14	0,79	-0.18	0.08	-0,34	-0,02	0,04*
$T_3 - T_1$	0.06	0.12	-0,18	0,30	0,61	-0.12	0.07	-0,26	0,02	0,12	0.01	0.07	-0,13	0,15	0,90
$T_3 - T_2$	-0.34	0.11	-0,56	-0,12	0,00**	-0.10	0.07	-0,24	0,04	0,19	0.18	0.08	0,02	0,34	0,03*
Subgenual cortex															
$T_1 - T_0$	0.10	0.18	-0,27	0,47	0,58	0.13	0.11	-0,09	0,35	0,24	-0.20	0.12	-0,44	0,04	0,10
$T_2 - T_0$	0.26	0.17	-0,08	0,60	0,13	-0.02	0.11	-0,24	0,20	0,83	-0.42	0.12	-0,66	-0,18	0,001**
$T_3 - T_0$	-0.05	0.16	-0,37	0,27	0,77	-0.24	0.10	-0,44	-0,04	0,03	-0.22	0.11	-0,44	0,00	0,05
$T_2 - T_1$	0.16	0.18	-0,21	0,53	0,37	-0.16	0.11	-0,38	0,06	0,17	-0.23	0.12	-0,47	0,01	0,06
$T_3 - T_1$	-0.15	0.17	-0,49	0,19	0,39	-0.37	0.11	-0,59	-0,15	0,002**	-0.03	0.11	-0,25	0,19	0,80
$T_3 - T_2$	-0.31	0.16	-0,63	0,01	0,06	-0.22	0.10	-0,42	-0,02	0,04	0.20	0.12	-0,04	0,44	0,09
Left temporal cortex															
$T_1 - T_0$	-0.04	0.06	-0,16	0,08	0,51	0.08	0.04	0,00	0,16	0,06	0.03	0.04	-0,05	0,11	0,47
$T_2 - T_0$	-0.08	0.06	-0,20	0,04	0,22	-0.14	0.04	-0,22	-0,06	0,001**	-0.04	0.04	-0,12	0,04	0,42
$T_3 - T_0$	-0.17	0.06	-0,29	-0,05	0,004	0.03	0.04	-0,05	0,11	0,43	-0.06	0.04	-0,14	0,02	0,17
$T_2 - T_1$	-0.03	0.07	-0,17	0,11	0,60	-0.21	0.04	-0,29	-0,13	<0,001***	-0.07	0.04	-0,15	0,01	0,15
$T_3 - T_1$	-0.13	0.06	-0,25	-0,01	0,04*	-0.05	0.04	-0,13	0,03	0,25	-0.09	0.04	-0,17	-0,01	0,04*
$T_3 - T_2$	-0.10	0.06	-0,22	0,02	0,11	0.17	0.04	0,09	0,25	<0,001***	-0.02	0.04	-0,10	0,06	0,67
Right parietal cortex															
$T_1 - T_0$	-0.08	0.06	-0,20	0,04	0,15	0.04	0.04	-0,04	0,12	0,27	-0.02	0.04	-0,10	0,06	0,55
$T_2 - T_0$	-0.09	0.05	-0,19	0,01	0,11	0.03	0.03	-0,03	0,09	0,41	-0.09	0.04	-0,17	-0,01	0,02*
$T_3 - T_0$	-0.17	0.05	-0,27	-0,07	0,002**	0.01	0.03	-0,05	0,07	0,68	-0.01	0.04	-0,09	0,07	0,71
$T_2 - T_1$	-0.00	0.06	-0,12	0,12	0,97	-0.01	0.04	-0,09	0,07	0,73	-0.07	0.04	-0,15	0,01	0,09
$T_3 - T_1$	-0.09	0.06	-0,21	0,03	0,14	-0.03	0.04	-0,11	0,05	0,49	0.01	0.04	-0,07	0,09	0,82
$T_3 - T_2$	-0.09	0.06	-0,21	0,03	0,12	-0.01	0.04	-0,09	0,07	0,71	0.08	0.04	0,00	0,16	0,06

Table 4: Multiple comparison for each time point within each treatment group. (* < 0.05; **<0.01; *<0.001).**

	5 sessions active - 20 sessions sham					20 sessions active - 20 sessions sham					5 sessions active - 20 sessions active				
	Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
Presubgenual cortex															
T_0	0.08	0.13	-0.18	0.34	0.53	0.04	0.13	-0.22	0.30	0.78	0.04	0.10	-0.16	0.24	0.68
T_1	0.05	0.13	-0.21	0.31	0.71	-0.10	0.13	-0.36	0.16	0.45	0.15	0.10	-0.05	0.35	0.14
T_2	-0.04	0.13	-0.30	0.22	0.78	-0.34	0.13	-0.60	-0.08	0.01*	0.30	0.10	0.10	0.50	0.01*
T_3	0.20	0.12	-0.04	0.44	0.09	0.18	0.12	-0.06	0.42	0.14	0.02	0.09	-0.16	0.20	0.80
Subgenual cortex															
T_0	0.42	0.19	0.03	0.81	0.03*	0.29	0.19	-0.10	0.68	0.15	0.13	0.15	-0.17	0.43	0.41
T_1	0.45	0.20	0.04	0.86	0.03*	-0.01	0.20	-0.42	0.40	0.98	0.46	0.15	0.16	0.76	0.01*
T_2	0.13	0.19	-0.26	0.52	0.48	-0.40	0.19	-0.79	-0.01	0.05*	0.53	0.15	0.23	0.83	0.00**
T_3	0.22	0.18	-0.15	0.59	0.21	0.11	0.18	-0.26	0.48	0.53	0.11	0.14	-0.17	0.39	0.44
Left temporal cortex															
T_0	-0.11	0.06	-0.23	0.01	0.08	-0.06	0.06	-0.18	0.06	0.36	-0.05	0.05	-0.15	0.05	0.31
T_1	0.01	0.07	-0.13	0.15	0.87	0.01	0.07	-0.13	0.15	0.83	-0.002	0.05	-0.10	0.10	0.96
T_2	-0.17	0.06	-0.29	-0.05	0.01*	-0.02	0.07	-0.16	0.12	0.79	-0.15	0.05	-0.25	-0.05	0.01*
T_3	0.10	0.06	-0.02	0.22	0.10	0.06	0.06	-0.06	0.18	0.31	0.03	0.05	-0.07	0.13	0.45
Right parietal cortex															
T_0	-0.01	0.07	-0.15	0.13	0.93	-0.09	0.06	-0.21	0.03	0.12	-0.01	0.04	-0.09	0.07	0.83
T_1	0.03	0.06	-0.09	0.15	0.68	-0.03	0.06	-0.15	0.09	0.66	0.05	0.05	-0.05	0.15	0.26
T_2	0.02	0.06	-0.10	0.14	0.78	-0.10	0.06	-0.22	0.02	0.12	0.11	0.05	0.01	0.21	0.02*
T_3	0.09	0.06	-0.03	0.21	0.13	0.07	0.06	-0.05	0.19	0.24	0.02	0.05	-0.08	0.12	0.68

Table 5: Multiple comparison for each treatment group within each time point. (* < 0.05; **<0.01; *<0.001).**

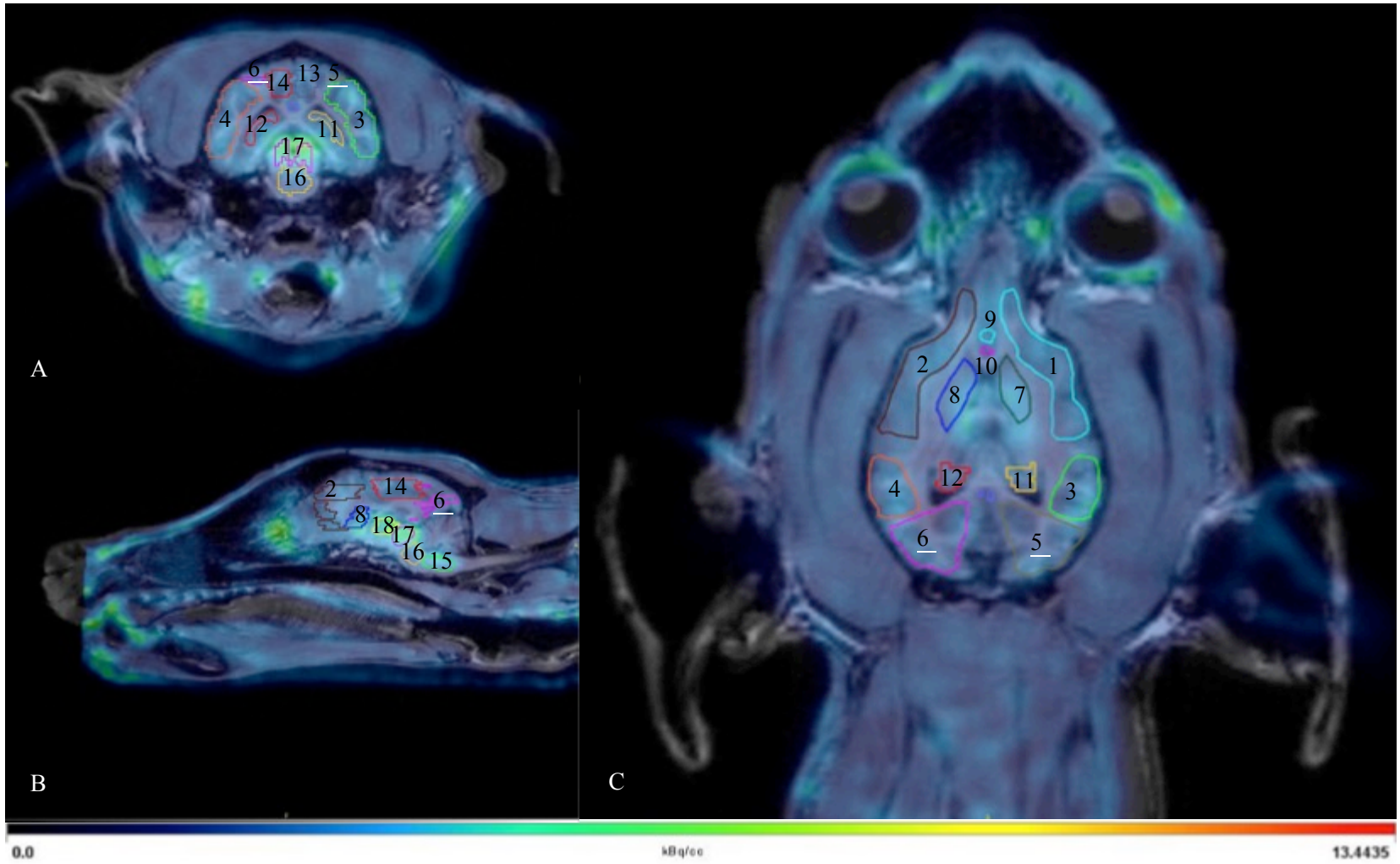

Fig. 1: Transversal (A), sagittal (B) and dorsal (C) fusion image ([^{11}C]DASB PET scan and MRI). 1: right frontal cortex, 2: left frontal cortex, 3: right temporal cortex, 4: left temporal cortex, 5: right occipital cortex, 6: left occipital cortex, 7: right caudate nucleus, 8: left caudate nucleus, 9: presubgenual anterior cingulate cortex, 10: subgenual anterior cortex, 11: right hippocampus, 12: left hippocampus; 13: right parietal cortex, 14: left parietal cortex, 15: medulla oblongata, 16: pons, 17: midbrain, 18: left thalamus.

Chapter 7: The amount of HF-rTMS sessions determines an increase or decrease in central monoamines

The amount of HF-rTMS sessions determines an increase or decrease in central homovanillic acid and 3,4-dihydroxyphenylacetic acid

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Abstract:

Repetitive transcranial magnetic stimulation (rTMS) is thought to exert its anti –depressant action through the serotonergic and dopaminergic system. Nonetheless, only the immediate effects on these systems have been thoroughly studied. Therefore, this study aimed to evaluate the effects - immediate and long-lasting - of an acute and chronic accelerated high frequency repetitive rTMS (aHF-rTMS) on dopamine, serotonin and their metabolites in the cerebrospinal fluid (CSF) and serum of healthy beagle dogs.

It was hypothesized that aHF-rTMS, over the left frontal cortex, modulates the concentrations of dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the CSF and serum. In addition, it was expected to detect differences in induced changes between an acute (1-day session) and chronic (4 days session) aHF-rTMS treatment

22 dogs were randomly divided into 3 unequal groups: 5 active sessions (1-day session, n=10), 20 active sessions (4 days sessions, n=8), 20 sham sessions (4 days sessions, n=4). Each dog received an anatomical MRI accompanied by neuronavigation, followed by an aHF-rTMS treatment over the left frontal cortex. At baseline, 24 hours, 1 month and 3 months after the last stimulation session serum and CSF were collected. Concentrations of DA, HVA, DOPAC, 5-HT and 5-HIAA were measured. Significance was set at 0.05.

This study shows that an active accelerated HF-rTMS protocol (aHF-rTMS) over the left frontal cortex can induce either an increase or decrease in HVA and DOPC in the CSF of dogs. An acute aHF-rTMS protocol provokes an immediate increase, which lasts up to 3 months, whereas chronic treatment provokes a delayed decrease in HVA. Between both active treatments a statistical difference was found in HVA in the CSF. No changes were noticeable in the serum.

The results imply that a single and four-day active HF-rTMS over the left frontal cortex provoke contrasting effects on the dopaminergic system. Nonetheless, both active protocols could still achieve clinical improvement by activating different neurobiological pathways that influence the dopaminergic system.*:*

Introduction

Propagation of the action potential between two neurons is performed by electrochemical transaction between the presynaptic axon terminal and the postsynaptic dendrites of the cell body. When the action potential reaches the axon terminal, a release of a neurotransmitter into the synaptic cleft is provoked. Thereupon, the neurotransmitters bind onto their specific receptor of the postsynaptic neuron and thereby alter the membrane potential and metabolism of the postsynaptic neuron.

Neurotransmitters can be divided into four groups: amino acids, acetylcholine, neuropeptides and monoamines. The group of monoamines holds two catecholamines (dopamine (DA) and noradrenaline (NAD)) and an indolamine (5-hydroxytriptamine (5-HT) or serotonin). The catecholamines are formed from the amino acid tyrosine, which undergoes hydroxylation and decarboxylation to become DA [1]. After synthetisation, DA is transported into vesicles. Upon neuronal excitation, the vesicles are emptied into the synaptic cleft and DA binds to its pre or postsynaptic receptor. The dopaminergic transmission is terminated by a re-uptake or degradation of DA [2-4]. After re-uptake into the presynaptic neuron, mediated by the dopamine transporter (DAT), DA is stored in vesicles or, when accumulated freely in the cytosol, broken down by monoamine oxidase (MAO). The extracellular DA is also taken up by the glial cells surrounding the synaptic cleft, where it is degraded by MAO and catechol-O-methyl-transferase (COMT) [4]. The 2 main breakdown products of DA are 3,4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) [5-7]. Serotonin on the other hand, is synthesized from the essential amino acid L-tryptophan. Tryptophan is hydroxylated and decarboxylated to form sequentially 5-hydroxytryptophan (5-HTP) and serotonin (5-HT). As DA, 5-HT is stored in vesicles prior to neuronal activation. After its release, the serotonergic neuronal transmission is ended by removal of 5-HT from the synaptic cleft, mediated by the serotonin transporter (SERT). Serotonin that is not stored in vesicles is degraded by MAO and a dehydrogenase to form 5-hydroxy-3-indolacetic acid (5-HIAA) [8, 9]. The breakdown products of both DA (HVA and DOPAC) and 5-HT (5-HIAA) are diffused from the neuronal or glial cells into the extracellular space towards the cerebrospinal fluid [10].

The concentration of the metabolites of DA and 5-HT resembles the turnover rate of the parent molecules. In addition, DOPAC, HVA and 5-HIAA have been put forward as biomarker for diseases in the central nervous system [11]. Although inconsistent results, a recent meta-analysis found a significant decrease in CSF HVA in depressed patients compared to healthy controls. Thus HVA in the CSF could be a valid biomarker for depression [12]. Indeed, the most well-known ethological hypothesis for depression psychopathology is related to deficiencies of the monoaminergic system [12, 13]. As such, most psychopharmaceutical treatment modalities (eg. SSRI's) for depression are based on normalizing monoaminergic neurotransmission.

On the other hand, repetitive transcranial magnetic stimulation (rTMS), a non-pharmaceutical, non-invasive antidepressant treatment, applied to the dorsolateral prefrontal cortex is an FDA approved, non-invasive technique for drug-resistant major depression. Even though its exact anti-depressant action mechanism remains largely unclear, there is evidence that its therapeutic effect could be mediated by an enhancement of monoaminergic neurotransmission [14-16]. Functional imaging in healthy and depressed humans showed a transient increase of striatal dopamine after a single rTMS session over the left frontal cortex [17, 18]. Even more, single rTMS stimulation over the motor cortex can evoke central and peripheral release of dopamine [19, 20]. Further, low frequency rTMS over the motor cortex induces a decrease in HVA in the CSF of people with Parkinson's disease [21]. The release of striatal dopamine after a single rTMS treatment was confirmed in anesthetized macaque monkeys by functional brain imaging [22]. In the rodent rTMS model, micro dialysis revealed a dopamine increase in the dorsal hippocampus, the shell of the nucleus accumbens and the dorsal striatum after acute rTMS [14-16, 23-25]. Even more, acute rTMS provokes a significant increase of the DA turnover rate in the frontal cortex and decrease in the striatum and the hippocampus [16]. Besides DA, acute rTMS increase 5-HT and 5-HIAA in the rat's hippocampus, its catabolic turnover rate was not affected [16]. Furthermore, acute rTMS over the left frontal cortex induced changes in the tryptophan/5-HT metabolism in the limbic system of healthy people [26]. On the other hand, Kanno et al. (2003) [27] found in rats that acute rTMS inhibited an induced release of 5-HT. Nonetheless, there is also evidence that acute rTMS might have no significant effect on the 5-HT level in rats [14]. Sibon et al (2006) [26] found, in comparison to stimulation of the left occipital cortex, that the tryptophan/5-HT metabolism was significantly

decreased left parahippocampal gyrus and the right insula whereas an increase was found in the right cingulate gyrus and cuneus. These findings indicate that an acute rTMS treatment can modulate the dopaminergic and serotonergic system, which could be noticeable in the brain and reflected periphery [20, 28]. Nonetheless, only the immediate effects of an acute rTMS treatment were evaluated and, to our knowledge, no studies were performed that combined central and peripheral assessment of the monoaminergic system neither in human studies nor in animal models. It has to be kept in mind that most of the rTMS studies on the monoaminergic system were performed in rodents and that extrapolating this data to humans remain difficult [29]. Recently, dogs have been subjected to rTMS using human frameless neuronavigation system and a human figure-of-eight coil [30-32]. These studies evaluated immediate and long-term effect on an accelerated HF-rTMS protocol over the left frontal cortex in dogs. Due to the possibility using human appliances in a canine TMS model, the extrapolation of results to humans would improve. It is therefore that this study aimed to investigate the effect of a single and 4-day accelerated high frequency TMS (aHF-rTMS) protocol on DA, 5-HT and their metabolites (DOPAC, HVA, 5-HIAA) in the CSF and serum. It was hypothesized that in the CSF changes would occur in the monoaminergic metabolism. More specifically that an increase would be found in DA and 5-HT or their metabolites in the CSF. Secondly, we hypothesized that the changes in the central monoaminergic metabolism would be reflected in the serum. Finally, differences between both active treatment groups were expected.

Materials and methods

Ethics

The Ghent University Ethical Committee approved this study (approval number EC 2015/140; date of approval).

Animals

Twelve healthy Beagle dogs (5 females neutered, 1 female intact, 5 males neutered, 1 male intact) were used in this study. For practical reasons, 10 of these 12 dogs were randomly selected for reuse. Only after a three-month washout period (equal to 6 months after the last stimulation session)

and a return to baseline, the dogs were reused and considered as a new test subject. Hence, 22 dogs (12 used and 10 reused) dogs entered the study. All dogs were owned by the department of veterinary medical imaging and small animal orthopaedics and the department small animals of the faculty of veterinary medicine. The dogs were permanently housed in groups of 8 on an internal surface of 15 m2, with permanent access to an outside area of 15 m2. The floor coverings in the inner part consisted of wood shavings. Frequently, toys such as Kongs were given to the animals and they were twice a day released onto an enclosed play area. In addition, students of the faculty of veterinary medicine regularly walked the dogs.

Neuronavigation

The study aimed to apply an aHF-rTMS treatment over the left frontal cortex. Therefore, a frameless neuronavigation system was used to provide the external localisation of the left frontal cortex of each dog. First, a tomographical dataset (3T MRI) was acquired; thereafter neuronavigation was performed as described by Dockx et al. (2017) [30].

The stimulation protocol

The 22 dogs were randomly divided into 3 unequal groups. The first group consisted of 10 dogs (5 neutered males and females). The second group held 8 dogs (3 neutered females, 1 intact female, 3 neutered males, 1 chemically neutered male). The last group consisted of 4 animals (2 neutered males, 1 chemically neutered male, 1 neutered female). Several months prior to the stimulation, positive reinforcement was used to accustom all dogs to the researcher, the experimental room, the placing of the coil and the sound of the coil.

All stimulations were applied under general anaesthesia. Premedication consisted of butorphanol IV (0.2 mg/kg; Dolorex1; Intervet Belgium NV). After onset of sedation, anaesthesia was induced intravenously by administering midazolam (0.2 mg/kg; Dormicum; Roche Nederland B.V.) immediately followed by propofol (Propovet Multidose, Abbott Laboratories, Berkshire, UK, 1±2 mg/kg given to effect). General anaesthesia was maintained with isoflurane (Isoflo, Abbott Laboratories, Berkshire, UK) in oxygen using a rebreathing system. Immediately following the induction of anaesthesia, the motor threshold of the left motor cortex was determined. A motor

threshold (MT) of 100% was defined as the machine output (Magstim Company Limited, Wales, UK) that could provoke 5 out of 10 visible muscle contractions in the right upper front limb.

Group 1 received 5 daily sessions, whereas group 2 and 3 received 20, active or sham respectively, sessions (5 daily sessions during consecutive 4 days). Each session contained 40 trains of 1.9 seconds each. The trains were separated by a 12 second intertrain interval (in total 1560 pulses were given per session). The time interval between sessions was 10 to 15 minutes. This protocol (20Hz, 110% MT) was an exact copy of an accelerated HF-rTMS (aHF-rTMS) treatment protocol performed in MDD patients at our medical university hospital [33].

The anesthetic depth was clinically monitored (ventral position of the eye and absence of the eyelid-reflex). When deemed necessary, the isoflurane dose was adjusted to maintain the same depth.

CSF and serum sampling

At baseline, 24 hours, 1 month and 3 months post treatment, a CSF and serum tap were performed. The CSF tap was acquired at the cisterna magna using a 19 G needle after the dogs were positioned in right lateral recumbence. While under general anaesthesia and right lateral recumbence, a 21G needle was used to draw blood from the vena jugularis externa. An antioxidative mixture containing 0.1M perchloric acid (Merck, Darmstadt, Germany), 0.05% Na2EDTA (Sigma Aldrich, Saint Louis, USA) and 0.05% sodium metabisulfite (Merck, Darmstadt, Germany) was made. 900 µl and 25 µl of this mixture was added to 100 µl serum and 100 µl CSF respectively. The diluted samples were immediately frozen (-80 °Celsius) until further analysis. Prior to the analysis, the samples were thawed and centrifuged at 15000 rpm for 15 minutes. The supernatant was transferred and diluted 1/2 (CSF) and 1/5 (serum) with 0.5 M acetic acid (Fisher scientific, Bishop meadow road, UK).

Monoamine analysis

Total dopamine (DA), 3,4-dihydroxyphenylacetic (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were measured in CSF and serum based on previously reported methods [34, 35].In summary, the samples were injected automatically on a reversed phase liquid chromatography system (autosampler ASI-100 and HPLC pump P680 A HPG/2, Dionex, Amsterdam, The Netherlands) with

electrochemical detection (potential= + 700mV) (Amperometric Detector LC-4C, BAS, Indiana, USA). The separation was achieved using a narrowbore C18 column (Alltech®, AlltimaTM, 5 µm, 150 x 2.1mm, Grace, Deerfield, IL, USA). The mobile phase buffer contained 0.1 M sodium acetate (Carl Roth GmbH + Co, Karlsruhe, Germany), 20 mM citric acid (Sigma Aldrich, Saint Louis, USA), 1 mM sodium octane sulfonic acid (Carl Roth GmbH + Co, Karlsruhe, Germany), 1 mM dibutylamine (Sigma Aldrich, Saint Louis, USA) and 0.1 mM Na2EDTA adjusted to pH 3.7 (mobile phase composition: 97 buffer / 3 methanol (v/v)). The sample concentration was expressed as ng monoamine /100 µl.

Statistical analysis

Rstudio 1.1.456 (R: A Language and Environment for Statistical Computing; R Core Team; R Foundation for Statistical Computing, Vienna, Austria, 2016, https://www.R-project.org/) with packages MASS (version 7.3-50), doBy (version 4.6-2), sommer (version 3.0), stats (version 3.4.2) and emmeans (version 1.3.0) were used to compute all analyses.

Two datasets were created containing either the results in the CSF or serum. Onto each data set a linear mixed model was fitted. Serum and CSF concentrations of DA, HVA, DOPAC, 5-HT and 5-HIAA were set as response variables. Treatment group and time points were set as fixed-effect factors. The presence of a time point by treatment interaction was also considered in the model. In addition, time and animal were set as random factors. A random intercept was included into the model. The type I error was set at 0.05. Normality of the error terms, linearity of the regression function and homoscedasticity of the error terms were checked using diagnostic plots and statistical tests (Bartlett test of homogeneity of variances and the Shapiro-Wilk normality test).

A linear mixed model with heterogeneous unstructured variance was fitted. The model was written as $E(Yt|T_1, T_2) = \beta_0 + \beta_1 t_1 + \beta_2 t_2 + \beta_3 t_3 + \beta_4 T_1 + \beta_4 T_2 + \beta_6 t_1 T_1 + \beta_7 t_2 T_1 + \beta_8 t_3 T_1 + \beta_9 t_1 T_2 + \beta_{10} t_2 T_2 + \beta_{11} t_3 T_2$ with Yt as response variable. The serum and CSF concentrations were set as response value whereas time and treatment (both categorical) were set as predictor value. The factor time (continuous) and animal (categorical) were set as random factors. The predictor time (t) denotes the different timepoint with t_1 the first of three (k-1 = 4-1 = 3) dummies (= 1 if time point = “24 hours

post" or 0 otherwise), t_2 the second dummy (= 1 if time point = "1 month post" or 0 otherwise), t_3 (= 1 if time point = "3 months post" or 0 otherwise. The treatment predictor (T) indicated the different treatment modalities with T_1 the first of two (k-1 = 3-1 = 2) dummies (= 1 if treatment = "20 sessions active" or 0 otherwise) and T_2 (= 1 if treatment = "5 sessions active" or 0 otherwise). The reference level (for each region) was set as the concentration at baseline in the control group (intercept). Post hoc, linear contrasts were set up within each response variable for which the previous model indicated a significant time by treatment interaction.

Results

In the CSF, the concentrations of DA and 5-HT remained below the detection limit. Therefore, no statistical analysis was performed on the DA and 5-HT concentrations in the CSF. For the response variables HVA and DOPAC the linear mixed model found a significant time by treatment interaction (Table 1). No significant time by treatment interaction was found for 5-HIAA. The linear contrasts revealed for HVA an immediate increase within the 5-session group that lasted significant to 3 months after the stimulation. In addition, the immediate increase and increase at 3 months were also noticeable for DOPAC (Table 2). In contrast, within the 20 active sessions group, a decrease was seen after 1 month for HVA. This decrease persisted until 3 months after the last stimulation session was given (Table 2). No changes, except for a marginally significant decrease at 1 month, in DOPAC were found for the 20 active sessions group. HVA and DOPAC concentrations in the CSF remained constant within the 20 sessions sham group. Between both active treatments, baseline differences were noticeable for both HVA and DOPAC, although the baselines of the active treatments did not differ significantly from the baseline of the sham treatment. Three months after the active treatments were given, a significant difference in the CSF of HVA was present. At that time point a difference of 12.37ng/100 µl CSF (95% CI [1.71; 23.04]; *P*-value = 0.02) was found between bot active treatments.

In the serum, the concentration of DA remained below the detection limit. No time by treatment interactions were noticeable for the response variables HVA, DOPAC, 5-HT and 5-HIAA.

Discussion

The results of this study show that active aHF-rTMS over the left frontal cortex can induce either an increase or decrease in HVA and DOPAC in the CSF of dogs. An acute aHF-rTMS protocol (single day) induces an immediate increase, which lasts up to 3 months. In contrast, chronic aHF-rTMS treatment protocol (4 consecutive days) induces a delayed decrease in HVA. Between both active treatments a statistical difference was found in HVA in the CSF. No changes were noticeable in the serum.

The results indicate that the metabolism of this DA was altered by an active aHF-rTMS treatment. This modification of the metabolism of DA could have been achieved by: influencing its synthesis in the presynaptic neuron, storage in the synaptic vesicles, release in the synaptic cleft, binding and recognition by the target receptor, re-uptake and metabolic inactivation (thus resulting in a prolonged and/or higher concentration of DA in the synaptic cleft). Evidence of an alteration by rTMS on the dopamine transporter was provided by Ikeda et al. (2005, [36]). They found that a single HF-rTMS treatment decreased the expression of the DAT whereas chronic HF-rTMS augmented its expression. This augmentation remained present 10 days after the last stimulation session was given [36] and thus implies an increase in DA metabolism due to higher intracellular DA availability for MAO enzyme. Recently, Peng and his team [37] found in rats that a chronic treatment with TMS (5/10 Hz and 0.84/1.26 Tesla, during 7 days over the vertex) induces a reduction of the MAO-A enzyme in the prefrontal cortex. Since chronic HF-rTMS induces a prolonged increased expression of DAT, the recycling of DA might get the overhand of its metabolism by MAO due to the inhibition of MAO-A by the chronic HF-rTMS and therefore explain the decreased HVA and DOPAC found in this study. It has to be kept in mind that the role of COMT in the catabolism of DA plays only a secondary role under normal conditions due to its extra neuronal localisation (astrocytes, capillary walls, and postsynaptic dendritic spines) [4, 38]. The COMT mediated catabolic pathway can become of more relevance in conditions with altered DAT expression. Consequentially, a decrease in DAT expression, induced by an acute HF-rTMS protocol, could lead to more diffusion of DA from the synaptic cleft

into the extra synaptic compartment to be metabolized [39]. Therefore, causing an increase in HVA and DOPAC.

rTMS is, as other antidepressant treatments, able to enhance the biosynthesis of cholecystokinin (CCK) [40]. Muller et al. (2000) [40] found that the expression of CCK was augmented 20 hours after 11 weeks of TMS. During those 11 weeks, 8250 pulses (20 Hz, 130% resting motor threshold) were in total applied over the left frontal cortex in rats. In comparison, during our study 7800 pulses were given during the single day aHF-TMS treatment (20Hz, 110% resting motor threshold). Both studies were able to induce effects on either the expression of CCK or dopamine metabolism. Since CCK has the ability, by binding to its receptor, to directly affect the release of DA, it is plausible that the effect provoked by the active single day treatment was mediated by an increased CCK synthesis [41]. An induced release or inhibition of DA by CCK depends on the brain region and the present CCK receptor. Under certain circumstance, one pathway might dominate thereby provoking an increased or decreased release of DA [42]. This could account for the contrasting effects of the aHF-rTMS protocols on HVA and DOPAC. It must be kept in mind that CCK is also co-localized with gamma-aminobutyric acid (GABA). This inhibitory neurotransmitter is able to downregulate the release of dopamine [43], which could account for the fact that our 4-day active TMS treatment decrease the HVA concentration in the CSF, thus, provoking a deactivation of the dopaminergic neurotransmission. This assumption is strengthened by the fact that glutamate is decreased after a single day treatment whereas a 5-day treatment increases glutamine (precursor glutamate) levels [44, 45]. Glutamate is besides an activating neurotransmitter, also the precursor of GABA. Besides, an augmentation of GABA in the medial prefrontal cortex of depressed patients was accompanied by clinical improvement after chronic rTMS treatment (25 sessions, 5 weeks, 80-120% resting motor threshold) [46].

Finally, changes in central HVA and DOPAC are, based on the results of Hausmann et al (2002) [47] and Kuroda et al (2010) [48], less likely caused by an increase or decrease in the synthesis of DA.

Peripheral DA en HVA have been validated as biomarker for the central DA metabolism [28, 49, 50]. However, our study was not able to find any change in DA or its metabolites in the serum similar to

the findings in the CSF. This could be explained by the fact that peripheral DA does not solely originate from the brain. Peripheral DA mainly originates from the adrenal medulla and neuroendocrine cells [49, 51]. Therefore, physiological changes in the peripheral metabolism of DA could mask induced changes by the central DA metabolism. It is only when peripheral inputs of DA and its metabolites into the blood are controlled that peripheral and central measurements correlate [28, 50, 52-54]. This is the first limitation of our study. Besides fasting, no remedial measures, such as administering debrisoquin or sampling from the internal jugular vein, were applied. Another limitation of our study is that the aHF-rTMS protocol was only applied on a small sample size of 12 dogs, which were reused in different groups. Nonetheless, significant alterations on the DA metabolism were noticed only within both active treatment groups. Table 3 indicates that no differences in baseline measurements were noticed between groups, thereby indicating that the reuse of the dogs during the experiment had minimal risks of carry-over effects. During the sham treatment, an active coil was placed tilted, 90 degrees, over the left frontal cortex. An active coil placed in this manner can provoke minor voltages in the underlying cortical tissue [55]. Despite this fact, no changes in DA metabolism were observed within the sham treatment group. Another limitation is that all interventions were performed under general anaesthesia whereas sedation provokes larger changes in rCBF after an aHF-rTMS protocol [32]. Therefore, the results obtained in this study should be re-evaluated under different anaesthetic protocols and during full consciousness, albeit this last option is difficult to achieve. DA and 5-HT remained below the detection limit. This could have been because DA and 5-HT are, after release, re-used or broken down, therefore resulting in very low concentrations in the CSF. A final limitation is the use of reversed phase HPLC to determine the concentrations in the CSF and serum. A combination of HPLC with mass spectrometry would have been the method of choice due to an increase in selectivity [56]. In addition, the possibility rises that low CSF concentrations of DA and 5-HT can be measured using this technique. Despite these limitations, our study was able to induce changes in the dopaminergic neurotransmission with active aHF-rTMS protocols.

Conclusion

Depending on the number of applied active sessions, aHF-rTMS either increases or decreases the central DA catabolism. Thereby two of our initial hypotheses were confirmed. However, the precise pathway through which rTMS exerts its actions on the dopaminergic system remains unclear as well as the link with therapeutic efficiency. Due to a vast number of peripheral monoamines and their metabolites, the induced central changes were not detectable in the serum. Further research is therefore needed to explore the exact pathway of the rTMS effect on the dopaminergic system and the use of peripheral monoamines as biomarker for rTMS treatment.

Acknowledgements

This research was funded by Ghent university special research fund (BOF 01J05715)x. We thank the division of veterinary nuclear medicine (department of medical imaging and small animal orthopaedics, university of Ghent) to provide access to their infrastructure. We would like to thank Mrs. Ria Berckmans (Centre for Neurosciences, Vrije Universiteit Brussel) for her excellent technical assistance during the analysis of the serum and CSF samples.

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	HVA	DOPAC
Intercept	<0.0001	0.000
Time	0.05	0.06
Treatment	0.18	0.03*
Time:Treatment	0.003**	0.04*

Table 1: Output main effect and interaction test for the predictors time and treatment group. ****P*-value <0.05; *****P*-value <0.01

		20 sessions sham					5 sessions active					20 sessions active				
		Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
HVA																
	T_1 -T_0	5.66	7.20	-8.70	20.03	0.43	9.98	4.24	1.52	18.44	0.02*	-7.14	4.88	-16.87	2.60	0.48
	T_2 -T_0	-3.04	5.61	-14.24	8.15	0.59	8.51	4.06	0.41	16.60	0.04*	-12.17	4.72	-21.60	-2.75	0.01*
	T_3 -T_0	-2.71	6.84	-16.36	10.95	0.69	19.39	4.75	9.91	28.87	0.0001**	-11.03	5.19	-21.39	-0.67	0.04*
	T_2-T1	-8.70	7.43	-23.53	6.12	0.25	-2.82	8.42	-19.62	13.99	0.74	-5.03	5.28	-15.57	5.50	0.34
	T_3 -T_1	-8.37	7.48	-23.29	6.56	0.27	9.41	4.47	0.48	18.34	0.04*	-3.89	5.10	-14.07	6.28	0.45
	T_3 -T_2	0.34	3.93	-7.51	8.18	0.93	10.89	2.79	5.31	16.46	0.00**	1.14	2.96	-4.77	7.05	0.70
DOPAC																
	T_1 -T_0	-0.18	0.22	-0.62	0.27	0.43	0.22	0.12	-0.03	0.46	0.09	-0.07	0.14	-0.34	0.20	0.61
	T_2 -T_0	-0.20	0.16	-0.51	0.12	0.22	-0.19	0.15	-0.49	0.12	0.22	0.09	0.12	-0.15	0.33	0.44
	T_3 -T_0	-0.18	0.17	-0.52	0.17	0.31	0.44	0.11	0.21	0.66	0.0004**	0.16	0.11	-0.05	0.37	0.14
	T_2-T1	-0.02	0.17	-0.36	0.32	0.91	-0.01	0.19	-0.39	0.37	0.96	0.16	0.13	-0.09	0.41	0.20
	T_3 -T_1	0.00	0.25	-0.49	0.49	0.99	0.22	0.15	-0.08	0.53	0.15	0.23	0.14	-0.05	0.51	0.11
	T_3 -T_2	0.02	0.23	-0.44	0.49	0.92	0.34	0.15	0.03	0.65	0.03*	0.06	0.15	-0.24	0.37	0.67

Table 2: Multiple comparison for each time point within each treatment group. (* < 0.05; **<0.01; *<0.001).**

		5 sessions active - 20 sessions sham					20 sessions active - 20 sessions sham					5 sessions active - 20 sessions active				
		Estimate	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*	*Estimate*	*SE*	*LCL*	*UCL*	*p-value*
HVA																
	T_0	-5.66	6.00	-17.63	6.31	0.35	12.39	6.29	-0.16	24.94	0.05	-18.05	5.10	-28.22	-7.87	0.001***
	T_1	-1.34	9.25	-19.79	17.11	0.89	-0.41	9.68	-19.74	18.92	0.97	-0.93	7.16	-15.22	13.35	0.90
	T_2	5.89	4.63	-3.35	15.12	0.21	3.26	4.91	-6.55	13.07	0.51	2.63	4.06	-5.47	10.73	0.52
	T_3	16.44	6.59	3.28	29.60	0.02*	4.06	6.69	-9.29	17.41	0.55	12.37	5.34	1.71	23.04	0.02*
DOPAC																
	T_0	-0.28	0.14	-0.56	-0.01	0.04*	0.02	0.14	-0.25	0.30	0.86	-0.31	0.10	-0.51	-0.10	0.004**
	T_1	0.11	0.19	-0.26	0.48	0.56	0.13	0.19	-0.25	0.52	0.49	-0.02	0.14	-0.29	0.25	0.859
	T_2	0.01	0.17	-0.34	0.35	0.96	0.31	0.18	-0.05	0.68	0.09	-0.31	0.15	-0.61	0.00	0.05
	T_3	0.33	0.24	-0.15	0.81	0.17	0.36	0.23	-0.11	0.83	0.13	-0.03	0.18	-0.40	0.34	0.88

Table 3: Multiple comparison for each treatment group within each time point. (* < 0.05; **<0.01; *<0.001).**

Chapter 8: General discussion

Summary of the findings

Changes in canine cerebral perfusion after accelerated HF-rTMS: a proof of concept study

This proof-of-concept study was the first study that applied an accelerated HF-rTMS protocol in healthy dogs. Five sessions of the protocol were applied over the left frontal cortex for 1 day. After aHF-rTMS rCBF was increased in the left frontal cortex (stimulation site) and in the subcortical regions. No behavioural changes or side effects were noticed.

Accurate external localization of the left frontal cortex in dogs by using pointer based frameless neuronavigation

Due to skull size differences, optimalization of TMS target localisations in dogs is mandatory. Therefore, similar to the human TMS applications, a feasibility and accuracy frameless stereotactic neuronavigation study was set up. The findings indicated a median measurement error of 0.26 cm between the internal and external localisation of the left frontal cortex. In detail, an average lateral deviation and rostro-caudal was found of respectively 0.17cm (sd = 0.14cm) and 0.33cm (sd = 0.19 cm).

Cortical motor threshold determination in dogs

Besides the need of a good accuracy in identifying the target region when applying a TMS protocol, the application with the appropriate stimulation intensity setting is also of high importance. Since the stimulation intensity is based on the cortical motor excitability, an accurate and reliable determination of the CMT is of essence. It was found that the daily determination of the CMT remained constant over the time period of the study and was not influenced by the active or sham accelerated HF-rTMS protocol. A higher CMT was found when the CMT assessment was performed using EMG or under anaesthesia compared to the visual method or under sedation. Male dogs tended to have a higher CMT and coil-cortex distance than females. Finally, a positive associaton was found between the CMT and the coil-cortex distance.

Anaesthesia, not number of sessions, influences the magnitude and duration of an aHF-rTMS in dogs

TMS is conducted in awake humans. When applying rTMS in animals, anaesthesia or sedation may be needed. This placebo controlled study conducted in healthy dogs induced changes in the regional cerbral blood flow under anaesthesia and as well as under sedation. The magnitude of this increase was higher but shorter lived under sedation when compared to anaesthesia. No effect of aHF-rTMS amount of sessions (one day versus 4 day stimulation) was found on the rCBF after applying both aHF-rTMS protocols.

Acute accelerated high frequency rTMS causes an immediate local and remote increase in the serotonin transporter binding index, measured with [^{11}C]DASB

This sham controlled aHF-rTMS study showed immediate and long-term changes on the SERT binding index in healthy dogs. More specifically, changes were found in the left frontal cortex, the left hippocampus, pons, left thalamus, presubgenual cortex, subgenual cortex, the left temporal and right parietal cortices. Differences in SERT BI between two active protocols, differing in amount of sessions, were found in the left frontal cortex, the pons, the left hippocampus, $_{pre}$sgACC, the sgACC, the left temporal cortex and the right parietal cortex.

The amount of HF-rTMS sessions determines an increase or decrease in central homovanillic acid and 3,4-dihydroxyphenylacetic acid

In humans and rodent studies, rTMS can induce a release of central dopamine and serotonin. In order to find a peripheral biomarker for rTMS in dogs, this sham-controlled study aimed to find changes in the central and peripheral dopaminergic and serotonergic metabolism. Only changes in the central dopaminergic metabolism were observed. An increase in dopamine metabolites was found for the single day aHF-rTMS protocol whereas a decrease was noticed for the 4-day aHF-rTMS protocol.

Discussion

aHF-rTMS over the left frontal cortex induces local and remote changes in the cerebral perfusion in dogs measured with [^{99m}Tc]HMPAO SPECT. These findings are identical to what was found in human cerebral perfusion studies [1-4]. In contrast, HF-rTMS in rats showed a generalized hypoperfusion [5]. The authors state that the inability to provoke a focal effect rather than a generalized hypoperfusion was due to the large size of coil, even though the authors used the smallest commercially available rodent coil. The use of the dog as a TMS animal model has one less hurdle to overcome in comparison to rodents since a human figure-of-eight coil can induce focal and remote changes in the canine rCBF. Induced changes in the cerebral perfusion are some of the mechanisms behind the beneficial action of TMS in depression [1, 6, 7]. Only in chapter 2 (proof of concept study) remote changes in the rCBF were found in combination with an elevated rCBF in the left frontal cortex. In chapter 5, only the rCBF of the left frontal cortex was elevated. When comparing the results of chapters 2 and 5, it must be kept in mind that the experiments were performed under sedation in chapter 2 whereas under general anaesthesia in chapter 5. This could explain the absence of remote perfusion effects in chapter 5. It is know both in humans as in rodents that the TMS effect and its propagation depend upon the neuronal activity [8-11]. A loss of consciousness induced during midazolam (used in chapter 5) could explain why the initial response at stimulation site was not propagated to remote regions [8, 9]. Aside from midazolam, all volatile anaesthetics can affect neuroplasticity, reduce excitatory and augment inhibitory neuronal transmission. Finally, the [^{99m}Tc]HMPAO SPECT studies did not show any (linear) dose-response relationship. This implies that, in dogs, an increase in applied aHF-rTMS sessions over the left frontal cortex does not influence the magnitude of the rCBF.

Accurate localization of the left frontal cortex in dogs was achieved using a human frameless based stereotactic neuronavigation system. Thereby implying that a human frameless neuronavigation system can be used in dogs. A median measurement error of less 0.3 cm was achieved. A measurement error of 0.3 cm was set as a limit when using neuronavigation systems to obtain intracranial biopsies in humans[12-14]. In rodents, accuracy is increased by using fixed stereotactic

frames, which is accompanied by pain and distress [11]. The dog as TMS animal model enables the use of high accuracy frameless stereotaxy and decreases animal suffering. An accurate localisation of the left frontal cortex is of great essence since the canine frontal lobe is considerable smaller in volume when compared to its human counterpart [15]. The focality of the applied aHF-rTMS protocols on the left frontal cortex was confirmed by the [^{99m}Tc]HMPAO SPECT studies. These studies showed an immediate increase in rCBF at the stimulation site. No adjacent cerebral regions to the left frontal cortex were immediately influenced by the created electromagnetic field of the coil.

The applied intensity of the electromagnetic fields plays, besides its accuracy, a crucial role. The CMT assessment in dogs was based on the visual contraction of the upper limb whereas in rodents this is done, in the majority of publications, based on the visual contraction of both hind limbs [16, 17]. Once again this implies that the canine TMS model resembles more the TMS setting used in human research and clinical settings. More, the CMT remains constant in healthy dogs over a period of 1 year. Thereby confirming current human CMT research [18-20]. In contrast to human studies, the visually measured CMT held a lower machine output than when measured with EMG [21-23]. This discrepancy could be explained by the fact that in comparison a larger coil was used, thereby activating a larger part of the motor cortex and subsequently activating muscles with a lower threshold, thus causing a lower visual CMT. Factors influencing the CMT were the state of consciousness, the gender, age and the coil-cortex distance. As confirmed by chapter 5, general anaesthesia decreased the cortical excitability. The results of anaesthesia on the CMT were parallel to human and animal studies [24-26]. The electromagnetic field produced by a TMS coil declines exponentially with the distance to the coil [27]. This implies that any factor that increases the coil cortex distance could potentially affect the delivered intensity of the magnetic stimulation [28]. Since male dogs have a larger coil-cortex distance, due to sexual dimorphism, they also have a higher CMT. Older individuals portray atrophy of the grey matter, accompanied by a decreased neuronal activity, and a reduction in regional brain volume. Therefore, older dogs show a higher CMT than younger dogs. Both gender and age influence the coil CMT by modifying the coil-cortex distance. Therefore, it is logical that in dogs the coil-cortex distance was positively associated with the CMT.

An aHF-rTMS protocol alters the monoaminergic neutransmission in healthy dogs. Evidence was found that the density of the SERT was altered. Depending on the brain region, either an increase or decrease in SERT BI was achieved. A decrease in SERT BI, as present in pons and (pre)subgenual cortex after stimulation, could have originated from a down regulation of the SERT mRNA [29]. Other possibilities for a drop in SERT BI are either an internalization of the SERT protein or an elevated 5-HT in the synaptic cleft. However, all three explanations for the decrease in SERT BI should theoretically lead to alterations in the central 5-HT, or its metabolites. Despite, no changes in the central or peripheral 5-HT concentrations were noticed as in rodent TMS studies [30]. This absence could be explained by a too small effect size. Because alterations on the SERT were noticed in both directions (increase and decrease in different brain regions) there was no or a very small net effect on the serotonin metabolism. In contrast, alterations were found in the central dopaminergic metabolism. More specifically, an increase was induced by the single day protocol whereas a decrease was seen after the 4-day protocol. These alterations in DA metabolites could be caused by influencing the synthesis rate of DA, its storage into vesicles, its release in the synaptic cleft, its binding to its receptor, its re-uptake and its metabolic deactivation.

Within the PET and monoamine studies (chapters 6 and 7) significant differences between both active aHF-rTMS protocols were found. This contrasted with what was found during the perfusion (SPECT) studies. Therefore, implying when adding more sessions to an aHF-rTMS treatment, other cerebral responses would be achieved. Although hypothetical, it is possible that both active protocols yield similar clinical results by activating different pathways in the brain. No differences in response rates could be noticed since only healthy individuals were used during these studies.

The results concerning SPECT and monoamines in healthy dogs were confirmed in two anxious aggressive dogs (own unpublished data) subjected to the one-day protocol. Normalisation of their regional cerebral perfusion and an elevation in HVA and DOPAC in the serum was noticeable after a single day aHF-rTMS protocol over the left frontal cortex. In analogue with chapter 2, remote changes in the rCBF were observed. The rCBF changes were accompanied by an improvement of their anxious aggressive behaviour. Compared to chapter 5, the anaesthetic protocol, used in both anxious

dogs, was identical. However, no remote changes were found in chapter 5. Here, the studies were performed on healthy dogs with no abnormal baseline cerebral perfusion in contrast to the regional alterations found in the anxious aggressive dogs, eg hyporperfusion of the left frontal cortex. Hypoperfusion of the left frontal cortex is associated with a better clinical response [3]. Therefore, it is plausible that within an abnormal canine brain the initial response at the stimulation could be propagated to remote regions even under general anaesthesia.

Limitations

Small sample sizes, the uses of a human figure-of-eight coil, sedation or anaesthesia are the major limitations when interpreting the results presented in this doctoral thesis. Despite the small sample sizes, within each study a statistical significance was achieved. A Beagle's head is considerably smaller than a human head with a frontal lobe that occupies approximately half the volume. However, the size and the shape of the skull do not influence the distribution of the magnetic field, created by the coil [31]. The smaller size of the frontal cortex did not hamper the precision of stimulation, as no neighbouring cortical areas were stimulated. Sedation and anaesthesia can also influence the outcome an rTMS paradigm as they depress the neuronal activity. Since they are inevitable for rTMS applications in dogs, the influence of different forms of sedation and anaesthetics on rTMS should be researched.

In the placebo-controlled studies, it must be kept in mind that an active coil, tilted 90 degrees was used as a sham condition. An active coil held this way, can provoke minor voltages in the underlying cortical tissue [32]. However, no changes in the rCBF, SERT BI and monoamines were noticed in the control group. In these studies, animals were re-used but no differences in baseline measurements were found. Indicating that 6 months after stimulation the risk of carry over effects was severely reduced, thereby justifying the re-use of the dogs.

Finally, besides fasting the animals, were imposed to control the peripheral concentration of DA and its metabolites. This could account for the lack of changes in the serum.

Future directives

Future research concerning TMS in dogs should be focused on three objectives. First, gain better insights into the working mechanism of TMS in healthy dogs and dogs with a behavioural disorders. Second, a refinement of the used treatments in order to reduce the total number of applied anaesthesia's and the working costs. Finally, large-scale placebo-controlled studies should be conducted in dogs, e.g. dogs with pathological anxiety.

A better insight into the working mechanism of TMS can already be achieved by reprocessing the data obtained by the SPECT, PET and monoamine studies. The SPECT images were processed using only 12 ROI's whereas onto the PET images 22 ROI's were delineated. If the same number of ROI's should be drawn onto the SPECT images, a possible association could be found between the perfusion and SERT BI of certain ROI'S. Thereby linking a region's cerebral perfusion with its SERT density. If the data obtained from the monoamine study would be added, a possible association could be found between the cerebral perfusion, SERT BI and the metabolism of DA. Even better, the data set could be expanded by functional imaging (PET or SPECT) of the dopamine transporter (DAT) after an aHF-rTMS protocol. Therefore, more information about the altered dopaminergic transmission and the associated brain regions following an aHF-rTMS would be available. Furthermore, significant changes were found in the SERT BI but not in the central 5-HT metabolism. Therefore, the question remains which mechanism could be responsible for the absence of changes in the central 5-HT metabolism. Functional brain imaging of the 5-HT_{1A} and 5-HT_{2A} after an aHF-rTMS protocol would provide information concerning the serotonergic autoregulation and signal transduction. The central and peripheral monoaminergic dataset should be expanded with more cases and if possible re-analysed. The effect of the aHF-rTMS protocols on the central and peripheral monoamines and their metabolites could have been too small to be detected whereby no association was found between the central and peripheral monoamines. It should also be opted to use the reversed phase HPLC in combination with mass spectrometry instead of electrochemical detection [33].

There is a high need to refine the TMS protocol in dogs. First, each step in the process of applying a TMS treatment to a dog requires general anaesthesia. Even more if TMS would be applied

clinically in dogs, each TMS treatment would be accompanied by a general anaesthesia. General anaesthesia decreases or completely abolishes the excitation level of neurons and therefore plausibly diminishes the clinical TMS effect. A replacement could be found for the neuronavigation. Nonetheless, identifying the TMS target with surface distance measurements would reduce the number of needed general anaesthesia's. Sedation or full consciousness should be considered instead of general anaesthesia. Of course, both alternatives hold risks for the patient and researcher applying the TMS protocol, especially when dogs with behavioural problems are included. Therefore, instead of abolishing general anaesthesia, remedial measures could be introduced in order to reduce the number or duration of the needed general anaesthesia's. The effect of intermittent TBS should be compared to that of the used aHF-rTMS protocol. To reduce the duration of a stimulation session, and thus general anaesthesia. intermittent theta burst stimulation (iTBS) could be considered. Although in human clinical research both protocols may have similar clinical outcomes, the effects of intermittent TBS should be compared to that of the used aHF-rTMS protocol in dogs as well. It has been found that tDCS can precondition the motor cortex for a subsequent rTMS protocol [34-36]. These studies indicated that a subtreshold 5-HF-TMS protocol can provoke lasting changes in the corticospinal excitability when it was preceded by a conditioning session of tDCS. Even more simultaneous application of tDCS and rTMS can prolong the rTMS induced neuroplastic changes [36]. Therefore, simultaneous application of rTMS and tDCS over the left frontal cortex may facilitate and prolong the functional and clinical changes in dogs. Subsequently the treatment frequency in dogs and thereby the amount of needed general anaesthesia's could be reduced.

Finally, a large-scale sham-controlled study in dogs with pathological anxiety should be performed in order to validate the dog as natural animal model for TMS.

Conclusions

This dissertation found that an aHF-rTMS protocol in, sedated or anesthetized, dogs can be accurately and safely applied over the left frontal cortex with a human figure-of-eight coil. In addition, functional brain imaging modalities have identified that the used aHF-rTMS protocols provoke similar changes in cerebral perfusion and the SERT BI in healthy dogs as in humans. The action of aHF-rTMS

on the dopaminergic system reflects the findings in human studies. When applying aHF-rTMS protocols that differ in the amount of sessions, different results on the cerebral perfusion, SERT BI and monoaminergic metabolism can be expected in healthy dogs. Besides these changes, a single day aHF-rTMS protocol was able to induce long-term amelioration of anxious aggressive behaviour. HVA and DOPAC presented themselves as possible biomarker for the therapeutic effect of TMS. These findings combined with the similarities in neurophysiology and neuropsychiatric diseases between humans and dogs imply that the dog could be used as a natural animal for TMS research. Even more, dogs with behavioural deficits might benefit from a TMS treatment.

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Summary

Transcranial magnetic stimulation (TMS) is a non-invasive brain modulation technique that is used for the treatment of medication resistant depression. Although its exact neurobiological mechanism is unclear, strong evidence exists that its antidepressant action is mediated by the serotonin transporter and subsequently by the normalisation of the monoaminergic system. The majority of studies investigating the neurobiological mechanism of TMS are performed in rodents. When extrapolating results obtained through rodent TMS studies, some hurdles such as handling, focality and coil size must be overcome. Even more, current rodent animal models for depression have to be artificially induced, thus imposing ethical considerations. In contrast to the induced rodent animal models, the dog has been proven to be a valid natural occurring animal model for several diseases including neuropathological afflictions such as epilepsy, obsessive-compulsive disorder and anxiety. Even more, dogs present themselves for longitudinal functional imaging modalities such as PET and SPECT since they are less susceptible to the long-term effects of radiation. This dissertation therefore aimed to investigate the feasibility of a canine animal model for an acute and chronic aHF-rTMS paradigm over left DLPFC, using human TMS equipment, to provide new insights into its neurobiological mechanism. Besides, it was hypothesized that dogs suffering from anxiety could be benefitted by the paradigm. Accelerated HF-rTMS in dogs can be applied using similar targeting and intensity setting strategies as in humans. It has to be kept in mind that the needed required anaesthesia or sedation influences the neurobiological effect of TMS. Nonetheless, as in humans aHF-rTMS in dogs alters the regional cerebral perfusion at the stimulation target (left DLPFC) and remote (sub)cortical regions. Even more, depending on the number of applied sessions, acute or chronic, aHF-rTMS raises or decreases the availability of the SERT and the central monoamines. To conclude, aHF-rTMS can be applied safely over the left frontal cortex in healthy dogs by using a human frameless stereotaxy system and CMT assessment strategies. In addition, the canine brain shows similar neurophysiological changes (perfusion and SERT availability) as a response to aHF-rTMS. Consequentially, aHF-rTMS over the left frontal cortex in dogs could be used as a natural animal model to assess the antidepressant effect of TMS.

Samenvatting

Transcraniële magnetische stimulatie (TMS) is een niet-invasieve neuro-modulatie techniek die wordt ingezet bij de behandeling van therapieresistente depressie. Het exacte neurobiologische werking van rTMS is nog niet volledig gekend. Toch zijn er sterke aanwijzingen dat het antidepressieve effect gemedieerd zou worden door de serotonine transporter en een normalisatie van het monoaminerge systeem. De meeste studies omtrent de neurobiologisch werking van TMS werden uitgevoerd op knaagdieren., Het extrapoleren van deze resultaten naar de mens wordt bemoeilijkt door de grootte van de TMS coil, de focaliteit van het elektromagnetisch veld, de nood aan anesthesie of sedatie, enz.. Bovendien berusten de huidige knaagdier modellen op het kunstmatig induceren van depressie en angst bij deze dieren, wat ernstige ethische vragen oproept. .Hier tegenover staat de hond, die reeds gevalideerd is als een natuurlijk dier model voor verscheidene humane neuropathologische aandoeningen zoals epilepsie, obsessief-compulsief gedrag en angst. Deze doctoraatsthesis beoogt dan ook om de hond te onderzoeken als mogelijks natuurlijk dier model voor TMS. Dit door middel van een acuut en chronisch aHF-rTMS protocol toegediend over de linker frontale cortex, met TMS toestellen voor humaan gebruik. Met behulp van functionele beeldvorming werd de neurobiologische werking van TMS onderzocht bij gezonde honden.. TMS geeft in honden gelijkaardige neurofysiologische reacties (perfusie en SERT beschikbaarheid) als bij de mens. Net zoals bij de mens veroorzaakt TMS een verandering van de cerebrale perfusie van het stimulatie gebied en geconnecteerde (sub)corticale regio's. De verkregen neurofysiologische effecten worden beïnvloed door verschillende factoren zoals sedatie, narcose en het aantal toegediende TMS sessies. Sedatie en narcose beïnvloeden in verschillende mate het effect van het TMS protocol. Het aantal toegediende TMS sessies, acuut of chronisch, beïnvloedt de beschikbaarheid van de serotonine transporter en de centrale monoaminen. Finaal kan besloten worden dat TMS bij honden gebruikt kan worden als een natuurlijk diermodel voor de studie van de antidepressieve werking na TMS.

Dankwoord

In 2015 kreeg ik de kans om gedurende 4 jaar te werken omtrent het ontwikkelen van een translationeel hersenstimulatie model. Het succesvol beëindigen van dit werk zou niet mogelijk geweest zijn zonder de hulp van mijn collega's, vrienden en familie.

Als eerste zou ik mijn promotor Dr. Kathelijne Peremans en Prof. Dr. Chris Baeken willen bedanken om mij 4 jaar geleden blindelings het nodige vertrouwen te geven. Kathelijne, je hebt mij gedurende mijn gehele doctoraat niet alleen gestuurd, onderwezen en bijgestaan door dik en dun. We hebben ook samen moeilijke en blije periodes gekend. We hebben samen personen moeten afgeven maar er zijn ook personen bijgekomen. Hierdoor ben je voor mij meer dan een promotor geworden. Om het met de woorden van Ian te zeggen, je bent als het ware moeder de gans die niet alleen mij maar al haar doctoraatsstudenten liefdevol onder haar vleugels neemt. Chris, bedankt om er altijd en overal ter wereld klaar te staan voor mij. Als ik het echt niet meer zag zitten kon ik steeds bij jou terecht. Ik mocht jou steeds alles vragen, geen enkele vraag was te dom of taboe. Jouw deur stond steeds open. Je bleef dan altijd nuchter en kalm en gaf mij advies op een manier die jij alleen meester bent. Hierdoor creëerde je bij mij niet enkel nieuwe motivatie maar ook veel bewondering voor jou als persoon. Onze band is alleen maar sterker geworden nadat we elk vader geworden zijn.

Prof. Dr. Ingeborgh Polis en Prof. Dr. Filip De Vos, dank u om beide in mijn begeleidingscommissie te zetelen. Jullie advies en commentaren hebben mij vanuit een aan ander perspectief doen kijken naar mijn verkregen resultaten. Ingeborgh, ik zou jou willen bedanken om mij weg wijs te maken in de anesthesie van de kleine huisdieren. Indien ik vragen had of iemand nodig had om mij the helpen bij mijn anesthesieen, op de scinti of het UZ, stond je steeds paraat. Filip, door jou voelde ik mij steeds gedreven om kwaliteitsvolle papers af te werken zodanig dat ik ze niet zou hoeven publiceren in de Woef.

Naast mijn promotoren zou ik graag de leden van de examencommissie bedanken voor het nalezen en kritisch in vraag stellen van mijn proefschrift: Prof. Dr. Ernst Rietzschel, Prof. Dr. Marie-Anne

Vanderhasselt, Prof. Dr. Dierckx, Prof. Dr. Rik Achten, Prof. Dr. Robrecht Raedt en Dr. Tim Waelbers.

Geen onderzoek is mogelijk zonder funding dus zou ik graag het bijzonder onderzoeksfonds willen bedanken voor het financieren van mijn project.

Lise Vlerick, bestie, we hebben samen niet hard maar heel hard gewerkt. Desondanks, voelde dit niet zo aan omdat we ons werk combineerde met het nodige amusement. We stonden steeds klaar voor elkaar en vulden elkaar perfect aan. Op het einde van deze vier jaar kennen we elkaar door en door en kunnen we elkaar perfect inschatten.

Dr. Andre Dobbeleir, ik weet niet goed hoe ik jou moet bedanken. Ik kan de avonden dat we samen op het UZ gescand hebben niet meer tellen. Je hebt me niet alleen bijgestaan door de scanner te bedienen en scans te verwerken, ook hielp je mij om de honden in slaap te doen en CSF te nemen wanneer er niemand anders aanwezig was. Ook tijdens moeilijke periodes bleef je komen om mijn scans te laten doorgaan. Zonder jou was het tijdig en succesvol beëindigen van mijn doctoraat zeker en vast niet gelukt. Indien jij er echt niet kon bij zijn, kon ik rekenen op Dr. Yves D'Asseler, waarvoor dank. Dr. Romain Duprat, without your help I would not have know how to apply TMS in dogs. You were there with me when we started to stimulate the first dogs. I hope I can pass this knowledge through to Ian in the same manner and level of expertise as you did.

Prof. Dr. Jimmy Saunders, dank u voor uw steun en vertrouwen de voorbije 4 jaar en mij de kans te geven te mijn carrière te laten starten aan uw vakgroep.

Nick en Glenn, het was ontzettend aangenaam samen te werken met jullie. Ik heb enorm veel van jullie geleerd en genoten van onze scans op het UZ. Bieke, ik apprecieer het enorm wat je allemaal voor mij gedaan hebt. De dienst anesthesie wil ik bedanken om mij steeds telefonisch of fysiek te helpen met de anesthesie. Jullie konden mij steeds geruststellen wanneer ik belde omdat ik rare dingen zag gebeuren tijdens de anesthesie. Tim, Tom en Anna I would like to thank you for fitting my scans in the UZ in your agenda's even if it meant scanning during the evening, my sincere apologies to your

partners. Eva, Emmelie, Josefien, jullie waren de eerste collega's waarmee ik samenwerkte op beide vakgroepen. Jullie hebben mij direct een warm welkom gegeven en stonden steeds klaar om mij te helpen. Sara, jouw kennis omtrent psychologie en liefde voor honden vormde de basis voor een mooie samenwerking.

Prof. Dr. Ilse Smolders, Prof. Dr. Ann Van Eeckhaut, Prof. Dr. Dimitri De Bundel en Ria, de samenwerking met jullie heeft reeds enkele mooie resultaten opgeleverd. Dank jullie voor jullie inzet, tijd, adviezen en geduld, dit voornamelijk wanneer ik onverwacht binnen viel met enkele stalen ter analyse. Prof. Dr. Ingeborg Goethals en Prof. Dr. Sofie Bhatti, Dank u voor de toffe samenwerking. Pieter, met jou was het steeds gedreven maar ontspannen werken met hier en daar een cynische noot.

Een dikke merci voor mevrouw Ann Crombé. Ann, dank u voor al mijn papierwerk in orde te brengen en alles te regelen. Wetende dat jij het ging regelen stelde mij ten zeerste gerust. Ook Sandra, Claudine, Marleen, Franky, Liz, dank u voor jullie logistieke steun. Chiara, Chris en Jan jullie staan mee aan de basis van TMS als behandeling voor honden met angstproblemen. Dank jullie voor jullie inzet en vertrouwen gedurende het verloop van de studie. Annemie met jou heb ik steeds fijne ontspannende babbels gehad evenals de nodige emotionele steun en het nodige hart onder de riem.

Ian, brother, you are the newest asset at our research team. You will continue my work. I wish you the very best during your PhD.

Zonder de steun van familie en vrienden was dit doctoraat er niet geweest. Ma, Lobke, Jan, bedankt voor alle steun en leuke herinneringen de voorbije jaren. Jullie kregen het hard te verduren tijdens mijn opleiding tot dierenarts, vooral wanneer ik het niet meer zag zitten. Helaas hebben we onverwachts afscheid moeten nemen van ons oma. Ze zou nochtans heel fier geweest zijn op hare poulin en ik op haar. Oma, jij bent de fundering waarop niet alleen mijn persoonlijkheid maar ook opleiding is gebaseerd. Ik zie ons nog steeds zitten in de Kliniekstraat en de Pussemier, ik die mijn biologie aan u probeer uit te leggen of jij die mij al mijn Latijnse, Franse en Engelse vocabulaire aan het opvragen bent. Jo, Sofie, Frederik en Guillaume, dank u voor de nodige ontspannende spelletjesavonden. Ik zeg het vaak, we doen te weinig. Jo, merci om er als beste vriend te zijn en al

mijn gezaag en geklaag te aanhoren. Frederik, bedankt voor de vele avonden lessen in statistische verwerking van mijn data.

Finaal zou ik mijn vriendin willen bedanken. Vaak stond ik naar het oneindige te kijken en mij groen te voelen. Dan zeg je dat je mij begrijpt en doordat ik zo jong ben niet weet wat ik moet. Ik zou mij geen beter leven kunnen inbeelden zonder jou in ons gezinnetje.

Curriculum vitae

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ADDITIONAL INFORMATION

Publications

Dockx, Robrecht, Kathelijne Peremans, Lise Vlerick, Nick Van Laeken, Jimmy Saunders, Ingeborgh Polis, Filip De Vos, and Chris Baeken. 2017. "Anaesthesia, Not Number of Sessions, Influences the Magnitude and Duration of an aHF-rTMS in Dogs." Plos One 12 (9). Impact factor: 2.766, category: MULTIDISCIPLINARY SCIENCES, rank: 15/64.

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repetitive transcranial magnetic stimulation positively influences the behavior, monoaminergic system and cerebral perfusion in anxious aggressive dogs: a case study". Journal of Veterinary Behavior Clinical Applications and Research (under review)

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Pauwelyn, Glenn, Nick Van Laeken, Jeroen Verhoeven, Filip De Vos, Kathelijne Peremans, **Robrecht Dockx**, Chris Baeken, Benedicte Descamps, Christian Vanhove, Ken Kersemans, and Ingeborg Goethals. 2018. “The Signature of the Serotonin System in the Chronic Corticosterone Depression Model : a Study with [18F]MPPF, [18F]altanserin and [11C]DASB.” Neuropsychiatry 8 (4): 1377–1390.

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Vlerick, Lise, Kathelijne Peremans, **Robrecht Dockx**, Kurt Audenaert, Chris Baeken, Bart De Spiegeleer, Jimmy Saunders, and Ingeborgh Polis. 2018. “The Influence of Subanaesthetic Ketamine on Regional Cerebral Blood Flow in Healthy Dogs Measured with 99mTc-HMPAO SPECT.” Plos One 13 (12).

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Glenn Pauwelyn; Lise Vlerick; **Robrecht Dockx**; Jeroen Verhoeven; Andre Dobbeleir; Ingeborgh Goethals; Tim Bosmans; Kathelijne Peremans; Christian Vanhove; Filip De Vos; Ingeborgh Polis. “ PET quantification of [18F]MPPF in the canine brain using blood input and reference tissue modeling” Plos One (under review)

Volckaert, Veerle, Eva Vandermeulen, Emmelie Stock, Luc Duchateau, Sylvie Daminet, Myriam Hesta, Jimmy Saunders, **Robrecht Dockx**, and Kathelijne Peremans. 2017. “Week-to-week Variation of Scintigraphic (semi-)quantitative Thyroid Variables in 14 Healthy Experimental Cats.” Research in Veterinary Science 115: 382–386. Impact factor: 1.616, category:

VETERINARY SCIENCES, rank: 31/140.

Doctoral schools

1.1 Vereist voor het behalen van het getuigschrift en/of facultair verplicht

Basisstatistiek voor onderzoekers: Basiscursus statistiek
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Basisstatistiek voor onderzoekers: Proefopzet

1.2 Optioneel (niet vereist voor het getuigschrift noch facultair verplicht)

Advanced Clinical Training in Small Animal Neurology and Neurosurgery
Translational Biomedical In Vivo Imaging

.3 Communicatievaardigheden
Public speaking for researchers
.4 Onderzoek en valorisatie

Basisstatistiek voor onderzoekers: Basiscursus R

Conferences

2015

Research in psychiatry day (Leuven, Belgium) - Poster presentation - The dog as natural animal model for high-frequency transcranial magnetic stimulation of the left frontal cortex.

Non invasive brain stimulation techniques (NIBS) for psychiatric disorders: first euoprean meeting (Gent, Belgium)

European Association of Nuclear Medicine conference 2015 (Hamburg, Germany) - Poster presentation - Dockx, Robrecht, Chris Baeken, Romain Duprat, Filip De Vos, Bart De Spiegeleer, André Dobbeleir, Jimmy Saunders, Ingeborgh Polis, Kurt Audenaert, and Kathelijne Peremans. 2015. “Regional Cerebral Blood Flow Changes After Accelerated Repetitive Transcranial Magnetic Stimulation of the Canine Frontal Cortex.” In European Journal of Nuclear Medicine and Molecular Imaging, 42:S290–S290.

2016

International Conference on Mental Health (Brussel, Belgium) - Oral presentation - The dog as natural animal model for HF-rTMS

European Association of Nuclear Medicine conference 2016 (Barcelona, Spain) - Poster presentation -Dockx, Robrecht, Chris Baeken, Lise Vlerick, Romain Duprat, Filip De Vos, Bart De Spiegeleer, André Dobbeleir, Jimmy Saunders, Ingeborgh Polis, Eric Achten, Kurt Audenaert, and Kathelijne Peremans. 2016. “Using Tc-99m-HMPAO SPECT as Tool to Compare Two Transcranial Magnetic Stimulation Protocols and Their Long-term Effects in Dogs.” In European Journal of Nuclear Medicine and Molecular Imaging, 43:S614–S614. Impact factor: 7.277, category: RADIOLOGY, NUCLEAR MEDICINE & MEDICAL IMAGING, rank: 3/126.

2018

3rd European Conference on Brain Stimulation in Psychiatry (Lyon, France) - Poster presentation - Dockx, Robrecht, Kathelijne Peremans, Lise Vlerick, Jimmy Saunders, Ingeborgh Polis, Chris Baeken, Ilse Smolders, and Ann Van Eeckhaut. 2018. “TMS Improves Anxious Aggressive Behaviour in Dogs: a Case Study.”

3rd European Conference on Brain Stimulation in Psychiatry (Lyon, France) - symposium - Brain stimulation for psychiatric disorders: insight from animal models

Devriendt, Nausikaa, Matan Or, Kathelijne Peremans, Lise Vlerick, Robrecht Dockx, and Hilde De Rooster. 2018. “Changes in Regional Cerebral Blood Flow in Dogs with Different Grades of Hepatic Encephalopathy Berofe and After Successful Closure of Extrahepatic Portosystemic Shunts.” In ECVS Congress. Athen, Greece.

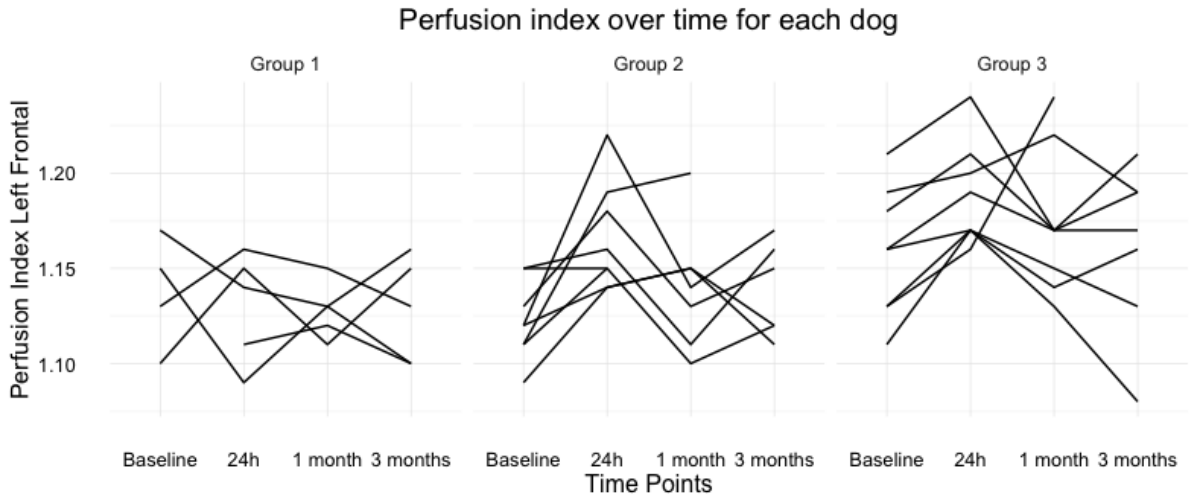
Pauwelyn, Glenn, Nick Van Laeken, Robrecht Dockx, Jeroen Verhoeven, Benedicte Descamps, Ken Kersemans, Kathelijne Peremans, Chris Baeken, Ingeborg Goethals, Christiane Vanhove, and Filip De Vos. 2018. “The Signature of the Serotonin System in the Chronic Corticosterone Depression Mode: a Study with (18F)MPPF, (18F)altaserin and (11C)DASB.” In San Sebastian,Spain.

Vlerick, Lise, Kathelijne Peremans, Robrecht Dockx, Kurt Audenaert, Chris Baeken, Bart De Spiegeleer, Jimmy Saunders, Tim Bosmans, and Ingeborgh Polis. 2018. “99mTc-HMPAO SPECT After Single and Repeated Subanaesthetic Ketamine in Healthy Dogs. .” In Venetie.

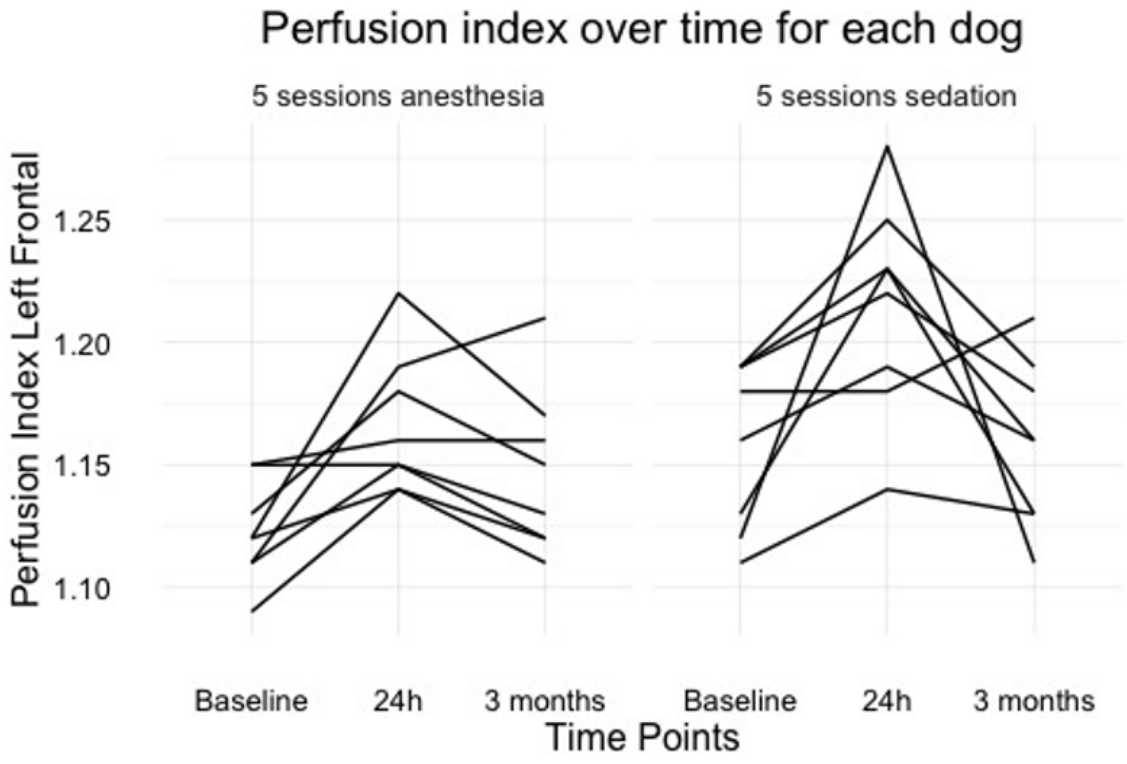
Awards

Poster Price - 3rd European Conference on Brain Stimulation in Psychiatry (Lyon, France) - Poster presentation - Dockx, Robrecht, Kathelijne Peremans, Lise Vlerick, Jimmy Saunders, Ingeborgh Polis, Chris Baeken, Ilse Smolders, and Ann Van Eeckhaut. 2018. “TMS Improves Anxious Aggressive Behaviour in Dogs: a Case Study.”

Addendum



S1 Fig.: Line plot for each individual dog in each treatment group



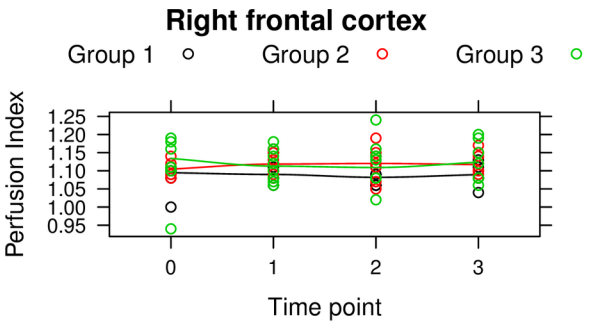
S2 Fig.: Line plot for each individual dog in each treatment group

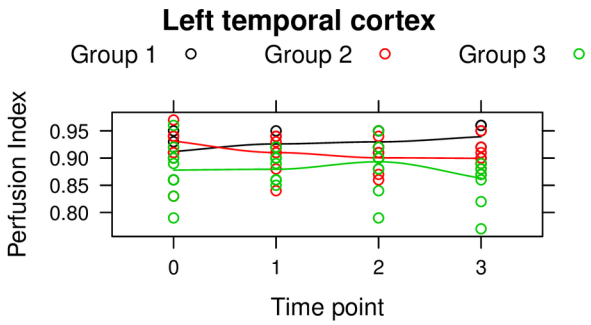
	Baseline				24 hours post				1 month post				3 months post			
	Mean	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*
Group 1[*] (n=4)	1.14	0.03	1.10	1.17	1.13	0.03	1.09	1.16	1.13	0.01	1.11	1.15	1.13	0.03	1.10	1.16
]2y-4y]	1.17	NA	NA	NA	1.14	NA	NA	NA	1.13	NA	NA	NA	1.16	NA	NA	NA
]4y-6y]	1.13	NA	NA	NA	1.16	NA	NA	NA	1.15	NA	NA	NA	1.13	NA	NA	NA
]6y-8y]	1.13	0.03	1.10	1.15	1.12	0.03	1.09	1.15	1.12	0.01	1.11	1.13	1.12	0.03	1.10	1.15
Group 2 (n=8)	1.12	0.02	1.09	1.15	1.17	0.03	1.14	1.22	1.14	0.03	1.10	1.20	1.14	0.02	1.11	1.17
Males	1.12	0.02	1.09	1.15	1.17	0.03	1.14	1.22	1.15	0.03	1.11	1.20	1.14	0.02	1.11	1.17
Females	1.14	0.02	1.10	1.15	1.15	0.01	1.14	1.15	1.12	0.04	1.10	1.15	1.12	0.00	1.12	1.12
]2y-4y]	1.14	0.01	1.12	1.15	1.18	0.04	1.15	1.22	1.12	0.02	1.10	1.14	1.15	0.03	1.12	1.17
]4y-6y]	1.11	0.02	1.09	1.12	1.14	0.00	1.14	1.14	1.15	0.00	1.15	1.15	1.12	0.01	1.11	1.12
]6y-8y]	1.14	0.04	1.11	1.20	1.17	0.02	1.15	1.19	1.16	0.06	1.11	1.20	1.15	0.02	1.13	1.16
Group 3 (n=8)	1.16	0.03	1.11	1.21	1.19	0.03	1.16	1.24	1.16	0.04	1.13	1.24	1.16	0.04	1.08	1.21
Males	1.16	0.04	1.13	1.21	1.19	0.04	1.16	1.24	1.17	0.05	1.14	1.24	1.16	0.03	1.13	1.19
Females	1.18	0.03	1.11	1.21	1.19	0.02	1.17	1.21	1.17	0.04	1.13	1.22	1.16	0.06	1.08	1.21
]2y-4y]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
]4y-6y]	1.20	0.03	1.17	1.22	1.21	0.03	1.19	1.24	1.19	0.03	1.17	1.22	1.18	0.01	1.17	1.19
]6y-8y]	1.19	0.07	1.14	1.24	1.18	0.02	1.16	1.21	1.17	0.04	1.13	1.24	1.15	0.05	1.08	1.21

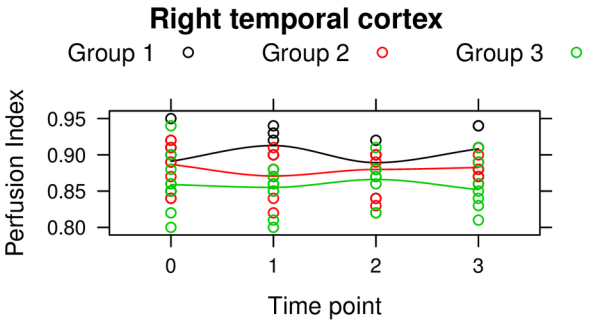
S1 Table: Descriptive statistics for the left frontal cortex perfusion index for each treatment group under anaesthesia (* only male beagle dogs were included assigned to this group).

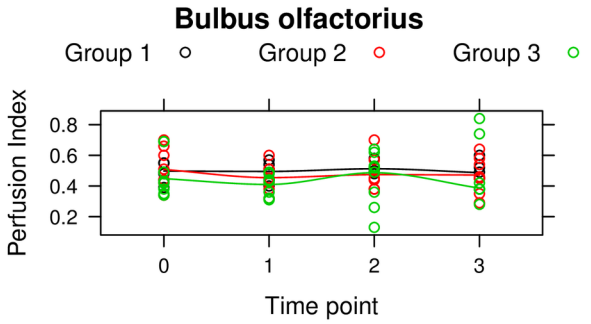
	Baseline				24 hours post				3 months post			
	Mean	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*
Group 2 (n=8)	1.12	0.02	1.09	1.15	1.17	0.03	1.14	1.22	1.15	0.03	1.11	1.21
Males	1.12	0.02	1.09	1.15	1.17	0.03	1.14	1.22	1.16	0.03	1.11	1.21
Females	1.14	0.01	1.12	1.15	1.15	0.01	1.14	1.15	1.12	0.00	1.12	1.12
]2y-4y]	1.15	0.03	1.13	1.18	1.18	*NA*	1.18	1.18	1.15	*NA*	1.15	1.15
]4y-6y]	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
]6y-8y]	1.14	0.03	1.11	1.19	1.17	0.02	1.15	1.19	1.17	0.04	1.13	1.21
Group 4 (n=8)	1.16	0.03	1.11	1.19	1.21	0.04	1.14	1.28	1.16	0.03	1.11	1.21
Males	1.15	0.03	1.11	1.18	1.19	0.04	1.14	1.23	1.16	0.04	1.13	1.21
Females	1.20	0.05	1.12	1.28	1.25	0.03	1.22	1.28	1.16	0.04	1.11	1.19
]2y-4y]	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
]4y-6y]	1.2	0.03	1.16	1.25	1.19	0.03	1.16	1.21	1.19	0.03	1.16	1.21
]6y-8y]	1.14	0.03	1.11	1.19	1.21	0.05	1.14	1.28	1.14	0.03	1.11	1.18

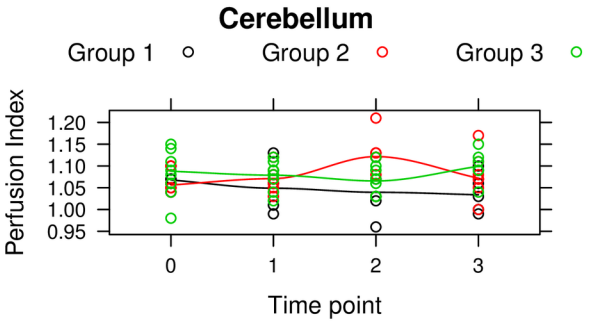
S2 Table.: Descriptive statistics for the left frontal cortex perfusion index for each treatment group that underwent 5 aHF-rTMS sessions.

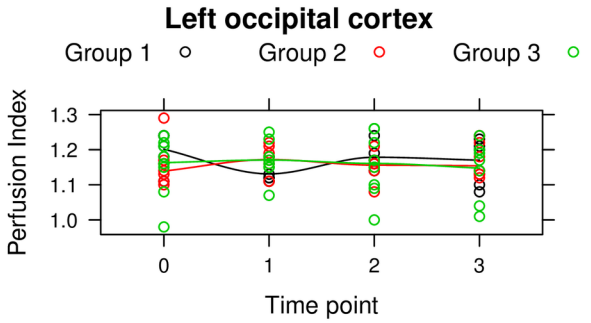

Right frontal cortex
Group 1
Group 2
Group 3
Perfusion Index
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0
1
2
3
Time point

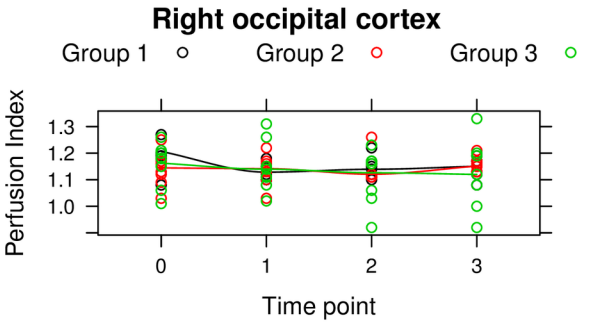

Left temporal cortex
Group 1
Group 2
Group 3
Perfusion Index
0.95
0.90
0.85
0.80
0
1
2
3
Time point

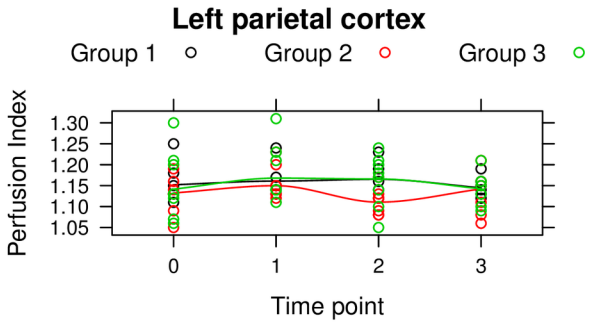

Right temporal cortex
Group 1
Group 2
Group 3
Perfusion Index
0.95
0.90
0.85
0.80
0
1
2
3
Time point

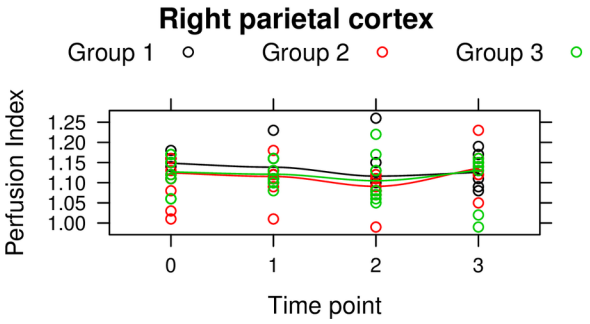

Bulbus olfactorius
Group 1
Group 2
Group 3
Perfusion Index
0.8
0.6
0.4
0.2
0
1
2
3
Time point

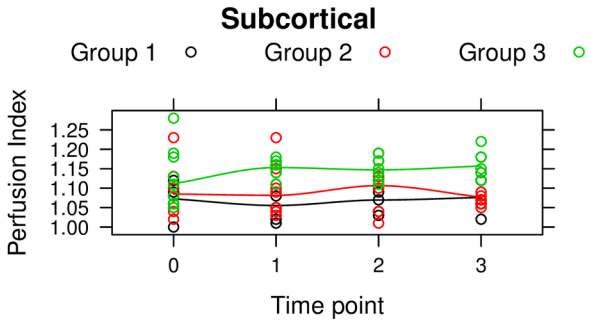

Cerebellum
Group 1
Group 2
Group 3
Perfusion Index
1.20
1.15
1.10
1.05
1.00
0.95
0
1
2
3
Time point


Left occipital cortex
Group 1
Group 2
Group 3
Perfusion Index
1.3
1.2
1.1
1.0
0
1
2
3
Time point


Right occipital cortex
Group 1
Group 2
Group 3
Perfusion Index
1.3
1.2
1.1
1.0
0
1
2
3
Time point


Left parietal cortex
Group 1
Group 2
Group 3
Perfusion Index
1.30
1.25
1.20
1.15
1.10
1.05
0
1
2
3
Time point


Right parietal cortex
Group 1
Group 2
Group 3
Perfusion Index
1.25
1.20
1.15
1.10
1.05
1.00
0
1
2
3
Time point


Subcortical
Group 1
Group 2
Group 3
Perfusion Index
1.25
1.20
1.15
1.10
1.05
1.00
0
1
2
3
Time point