Inaugural-Dissertation zur Erlangung der Doktorwürde der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Dog Electroencephalogram for Early Safety Seizure Liability Assessments and Investigation of Species-Specific Sensitivity for Neurological Symptoms

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München, 2018

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Angefertigt bei AbbVie Deutschland GmbH & Co. KG,
67061 Ludwigshafen
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Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D
Berichterstatter:	Univ.-Prof. Dr. Heidrun Potschka
Korreferenten:	Univ.-Prof Dr. Bernhard Aigner Prof. Dr. Andrea Fischer

Tag der Promotion: 10. Februar 2018

Meinen Großeltern Lisi und Dieter, meinen Eltern Kirsten und Josef und meiner Schwester Marie.

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I. List of abbreviations

°C	Degree Celsius
µ	Micro
3R	Replace, Reduce, Refine
5HT	5-Hydroxytryptamine (Serotonine)
a/d	analogue-digital
AAALAC	Association for assessment and accreditation of laboratory animal care
AD	Alzheimer's disease
ADMA	Asymmetric dimethylarginine
ADME	Absorption, distribution, metabolism, excretion
ADR	Adverse drug reaction
AE	Adverse Event
AED	Anti-epilepsy drug
Arg	Arginine
AUC	Area under the curve
aVF	Augmented voltage foot (Goldberger ECG deviation)
aVL	Augmented voltage left (Goldberger ECG deviation)
aVR	Augmented voltage right (Goldberger ECG deviation)
AZ	"Aktenzeichen"/ file reference number
BCS	Body conditioning score
BL	Baseline
bpm	Beats per minute
BSP	Burst suppression pattern
C	Central (electrode position)
C	Carbon atom
ca.	*circa*
cc	cubic centimeter
C_{free}	Free concentration in plasma
CLC	Communication link controller
C_{max}	Maximum plasma concentration
CNS	Central nervous system
CRI	Constant rate infusion
CRO	Contract research organization
CSF	Cerebrospinal fluid
CT	Computer tomography
D	Dopamine
D. rerio	*Danio rerio,* Zebrafish
DMA	Dimethylarginine
DMPK	Drug metabolism and pharmacokinetics
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleinic acid
DSI™	Data Science International

List of abbreviations

DSS	Data and Statistical Science
e.g.	for example
ECG	Electrocardiogram
ECoG	Electrocorticogram
EDTA	Ethylene diamine tetraacetic acid
EEG	Electroencephalogram
EMA	European Medicines Agency
EMG	Electromyogram
EOG	Electrooculogram
EPSP	Excitatory post-synaptic potential
et al.	*et alii*
etc.	*et cetera*
EU	European Union
F	Frontal (electrode position)
FDA	Food and Drug Administration
FEAB	Fentanyl-etomidate-anesthetized beagle
FELASA	Federation for Laboratory Animal Science Associations
FFT	Fast Fourier transformation
FHD	First human dose
FIH	First in human
FOB	Functional observation battery
Fp	Fronto-polar (EEG electrode position)
Fu (p)	Fraction unbound (in plasma)
GABA	Gamma-aminobutyric acid
GHB	Gamma-hadroxybutyrate
GLP	Good laboratory practice
GV-Solas	GV = Gesellschaft für Versuchstierkunde Solas = Society for laboratory animal sciences
HPMC	Hydroxypropylmethylcellulose
hr	hour
HR	Heart rate
Hz	Hertz
i.v.	intravenous
ICH	International Committee on Harmonization of technical requirements for registration of pharmaceuticals for human use
IE	Idiopathic epilepsy
IED	Interictal epileptic discharge
iEEG	intracranial Electroencephalography
ILAE	International League Aganist the Epilepsies
Inc.	Incorporation
ISPS	Inhibitory post synaptic potential
JET	Jacketed external telemetry
kg	Kilogram
LA	left arm (Einthoven ECG deviation)
LF	Left foot (Einthoven ECG deviation)

List of abbreviations

LFP	Local field potential
LOAEL	Lowest observed adverse effect level
LOEL	Lowest observed effect level
MEA	Micro electrode array
MES	Maximal electroshock test
MEST	Maximal electroshock threshold test
Met	Methionine
mg	Milligram
min	minute
miRNA	Micro ribonucleinic acid
ml	Milliliter
MRI	Magnetic resonance imaging
MTD	Maximum tolerated dose
n	Nano
NaCl	Natrium (engl. sodium)-chloride, saline
NCE	New chemical entity
NOAEL	No observed adverse effect level
NPS	Neuropsychiatric symptoms
NSAID	Non-steroidal anti-inflammatory drug
O	Occipital (electrode position)
OECD	Organization for economic cooperation and development
P	Parietal (electrode position)
p.o.	*per os*
PC	Phosphatidylcholine
PD	Pharmacodynamics
PI	Principal Investigator
PK	Pharmacokinetic
PoE	Power over Ethernet
PPB	Plasma protein binding
PTZ	Pentylenetetrazole
RA	Right arm (Einthoven ECG deviation)
RF	Radio-frequency
RR	Respiratory rate
s.c.	subcutaneous
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SE	Status epilepticus
SM	Sphingomyelin
SNE	Subdermal needle electrode
SOP	Standard operation procedure
SUD	Substance use disorders
SWD	Spike-wave discharge
SWS	Seizure warning system
T	Temporal (EEG electrode position)
$t_{1/2}$	Half-life

List of abbreviations

TI	Therapeutic index
TierSchG	“Tierschutzgesetz” (German law on animal welfare)
TierSchHuV	“Tierschutzhundeverordnung” (German law on dog welfare)
TierSchVersV	„Tierschutzversuchsverordnung“ (German law on animal experiments)
TK	Toxicokinetic
T_{max}	Time of maximal plasma concentration
TRH	Thyreoid releasing hormone
TRX	DIS's digital transcievers
TSH	Thyroid stimulating hormone
Tyr	Tyrosine
USA	United States of America
V	Volts
V.	*Vena*
z	zero
α	Alpha-Frequency
β	Beta-Frequency
γ	Gamma-Frequency
δ	Delta-Frequency
θ	Theta-Frequency
ς	Sigma-Frequency

II. Introduction

Neurological liabilities are a major concern during drug development and seizures especially are considered a severe adverse effect (Zaccara *et al.*, 1989; Easter *et al.*, 2007; Easter *et al.*, 2009; Fonck *et al.*, 2015). Not only can they progress into convulsions but repeated seizures can cause lasting damage to the brain (Walker & Kovac, 2015). Drugs of different pharmaceutical classes and therapeutic indications can have the potential to induce seizures, but the highest risk are compounds from central-nervous-system (CNS) indications (Easter *et al.*, 2009; Authier *et al.*, 2016).

Preclinical detection of seizure risk is important to guarantee the safety of participants in clinical trials and ultimately human and animal patients. In preclinical drug development, seizure liability is often a chance finding in toxicological studies at high doses (Easter *et al.*, 2009; Backes, 2016). In clinical studies symptoms of non-convulsive seizures such as dizziness, hallucinations, blurred vision and mood changes have been reported. Detecting such symptoms in animals and relating them to seizures is challenging (Metea *et al.*, 2015). Clinical observations of animals are only conducted at pre-defined time points and therefore frank convulsions may also be missed (Metea *et al.*, 2015; Backes, 2016). In addition, there are often no clear clinical forewarning signs preceding convulsions (Elander, 2013).

Dedicated studies to explore CNS effects of drug candidates fall within the field of safety pharmacology and different models for assessment of seizure liability exist. As it is the only method to reliably capture non-convulsive seizures, the current gold-standard for seizure liability assessments is video – electroencephalography (EEG) (Easter *et al.*, 2009; Authier *et al.*, 2014b). In human medicine, the EEG is a valuable tool for diagnosis of neurologic or psychiatric disease. In veterinary medicine, EEG is more challenging as strongly developed masticatory muscles cause a higher degree of artefacts as compared to human EEG recordings. Moreover, animals do not always tolerate the procedure, and diagnostic activation techniques are not easily adapted (Brauer *et al.*, 2011).

In regulatory safety studies, the dog is the standard non-rodent species and the goal of this thesis was to evaluate the use of dog EEG for assessments of neurological symptoms, especially seizure liability, in drug development. The design of such studies should enable a maximized detection of neurological symptoms and their correlation to drug plasma concentrations and biomarkers, while minimizing animal numbers and distress.

Paroxysmal EEG activity prior to clinical convulsions has been reported during a period that could be sufficient for prophylactic anticonvulsive treatment (Dürmüller *et al.*, 2007). This could provide a basis for refinement of seizure liability testing in mammals. In addition, clinical symptoms occurring at the same time as abnormal, drug induced, EEG patterns are also of interest to identify reliable premonitory signs of convulsions.

Six adult beagle dogs, three from each gender, were implanted with telemetric EEG transmitters. Three of them already had cerebrospinal fluid (CSF) ports, and feasibility to combine the two implant types was explored. To this aim, a set of reference compounds was tested first. When compatibility of implants was confirmed, three in-house model-compounds were selected. It is a general belief that the dog is more sensitive for neurological symptoms than other species. Still, it has successfully been used in EEG seizure studies (Dürmüller *et al.*, 2007; van der Linde *et al.*, 2011b; Authier *et al.*, 2014b; Authier *et al.*, 2015; Authier *et al.*, 2016). From the selected previous drug candidates, data was already available from rodent and non-rodent safety studies, so that further evaluation of species-specific sensitivity for neurological symptoms was possible.

Also, a shortened experimental time could potentially reduce the distress of animals used for research. Use of the intravenous route of administration could be a way to achieve this. Reducing experimental time would also enable continuous clinical observations thereby increasing symptom detection rate and allowing immediate veterinary treatments in case of severe symptoms. In addition, sample collection during presence of symptoms could lead to an improved correlation of drug plasma concentration to clinical effects, thereby enabling a better calculation of safety margins. Dose levels for toxicology studies could be adapted accordingly and occurrence of severe neurological symptoms in a larger subset of animals could be prevented. In the development of compounds that have a high risk to induce such symptoms exploratory pilot studies as investigated here could help to refine regulatory toxicological studies.

III. Literature

1 Seizure and Convulsion Liability Assessments in Drug Development

A thorough evaluation of the adverse effect profile of novel drugs is a major pillar of drug development. The aim of safety assessment studies is risk identification in relation to exposure. This allows calculation of safety margins that ensure patient safety. If a sufficient safety window (therapeutic index, TI) cannot be established, further development is stopped. Early detection of adverse effects is important to guide project decisions for selection of the best development candidate (Easter *et al.*, 2009; Butler *et al.*, 2017). Seizure liability is a dangerous adverse CNS effect, but Easter *et al.* (2009) observed that it is often only investigated after convulsions have been observed in toxicology studies. Later discontinuation of a project implies that more animals have been used without creating a benefit for patients. Attrition is especially high in certain indications and one of them is neuroscience (Mead *et al.*, 2016). Many companies have stopped investing in this field, reducing the chance to close therapeutic gaps in diseases like Alzheimer's diseases (AD) or schizophrenia (Pangalos *et al.*, 2007). The complexity of the nervous system, with many pathways not being completely understood, is one reason for this (Scott *et al.*, 2013). Both, drugs that target the CNS and drugs that do not, have been related to seizure induction (Zaccara *et al.*, 1989; Easter *et al.*, 2007; Easter *et al.*, 2009; Fonck *et al.*, 2015). Easter *et al.* (2009) retrospectively evaluated investigative drugs from AstraZeneca (1999-2008) with preclinical findings of seizure liability and found that 50% of them were not from CNS indications. As with other adverse effects, seizure liability needs to be evaluated in relation to exposure (FDA, 2001) and therapeutic range (Löscher, 2009; Backes, 2016) in order to determine the appropriate safety margin. Convulsions are a severe finding and regulators require a high safety margin of additional 10x from the no observed effect level (NOAEL) for animals (Elander, 2013). Determination of exposure levels at the time point of seizures or convulsions therefore is beneficial for calculation of safety margins.

1.1 Definitions: Seizures and Convulsions

In literature, the terms seizure and convulsion are often used interchangeably (Elander, 2013). According to Fisher *et al.* (2005), "an epileptic seizure is a transient occurrence of signs and/ or symptoms due to abnormal excessive or synchronous neuronal activity in the brain." (Fisher *et al.*, 2005) and a convulsion can potentially be a clinical symptom of this (Easter *et al.*, 2007; Easter *et al.*, 2009). A convulsion is defined as an episode of abnormal and pronounced muscle contractions (Blume *et al.*, 2001; Fisher *et al.*, 2005; Easter *et al.*, 2009). Its quality can be tonic, clonic or tonic-clonic (Blume *et al.*, 2001; Fisher *et al.*, 2005; Fonck *et al.*, 2015). "Tonic" describes an increase in muscle tone for a few seconds to minutes (Blume *et al.*, 2001). Repetitive prolonged myoclonic muscle contractions are referred to as clonic convulsion or rhythmic myoclonus (Blume *et al.*, 2001). According to

Löscher (2009), convulsions are an unspecific symptom of many developmental drugs. The relation between seizures and convulsions is expressed in the, according to Metea *et al.* (2015), frequently used formulation: “not all seizures result in behavioral convulsions and not all apparent convulsions are related to seizures” (Metea *et al.*, 2015).

Historically, seizures were first defined in the context of human epilepsy in 1964 by the “International League Against the Epilepsies”(ILAE) (Berendt *et al.*, 2015). They first introduced a unified classification scheme for seizures in the variants of human epilepsy in 1969 (Gastaut, 1969). This classification scheme differentiates main categories of seizures according to their mode of onset (partial, generalized, unclassified) and classifies each one into different subgroups. It has then been subject to multiple revisions, e.g. (Merlis, 1970; Angeles, 1981; Dreifuss, 1989; Fisher *et al.*, 2005; Engel, 2006; Berg *et al.*, 2010).

In veterinary medicine, the “international veterinary epilepsy task force” adapts the human terminology to animal epilepsy to provide a common language for veterinary neurologists and create consensus between animal and human epilepsy understandings (Berendt *et al.*, 2015). Their first proposal has been published in 2015 (Berendt *et al.*, 2015). In veterinary medicine, Fischer *et al*. (2013) considered the clinical value of a classification scheme for seizures as limited, due to multiple causes: first, diagnosis often is based on descriptions or video recordings provided by the patient owners only, instead of on EEG recordings (Fischer *et al.*, 2013). Treatment options in animal epilepsy are limited, so the scheme is not needed to guide selection of anti-seizure-drugs (Fischer *et al.*, 2013). Lastly, there are differences in progression and severity of epilepsy between dog breeds and some epilepsy syndromes are unique to certain breeds (Fischer *et al.*, 2013).

Walker & Kovac (2015) pointed out that the ILAE definition of a seizure is not comprehensive, neither to describe epileptic seizures in humans nor when applied to pharmacological research. According to them, it is important to consider that not all seizure activity results in signs or symptoms visible for an external observer and that preclinical assessment of seizure liability is done in different *in vitro* assays, e.g. hippocampal slices, and in animal models (Walker & Kovac, 2015). Therefore, their proposal for the definition of a seizure was: “A seizure is the escalating synchronization of neurons originating from the CNS [thereby including its occurrence in cell cultures or other in vitro assays]. *In vivo*, a seizure may elicit specific symptoms like, for example convulsions, depending on the affected brain region or species” (Walker & Kovac, 2015).

1.2 Causes of Seizures: Symptomatic and Reactive Seizures

The mechanisms that cause the occurrence of a seizure or a convulsion cannot always be identified (Delanty *et al.*, 1998; Elander, 2013). Synchronous firing of neurons is generated if the balance between inhibitory and excitatory neurotransmission is disrupted (Delanty *et al.*, 1998; Fonck *et al.*, 2015). This can happen as a consequence of structural abnormalities of the CNS, metabolic disturbances or drug actions (Delanty *et al.*, 1998). Epilepsy is defined by

the occurrence of at least two spontaneous seizures (Fischer *et al.*, 2013). In the diagnosis of epilepsy, primary (or idiopathic), secondary (or symptomatic) and reactive seizures are differentiated. Idiopathic seizures are the "true epileptic" form, resulting from a functional impairment of the brain, whereas "symptomatic" applies if other diseases are causative (Podell *et al.*, 1995; Fischer *et al.*, 2013). In cases in which the origin of the disorder cannot be identified, "epilepsy with unknown cause" is diagnosed, (Fischer *et al.*, 2013). "Reactive" seizures are induced in a normal brain by external causes, mainly toxins or metabolic alterations (Podell *et al.*, 1995; Fischer *et al.*, 2013). Drug-induced seizures thereby fall in this latter category. According to Podell *et al*. (1995), recurrence of reactive seizures does not justify the diagnosis of epilepsy, as they are reactions of a normal brain without structural or functional abnormalities.

1.3 Symptoms of Seizures

Symptoms of a seizure can be variable depending on extent and localization within the CNS (Easter *et al.*, 2009; Elander, 2013; Fischer *et al.*, 2013). As described in human medicine, signs of non-convulsive seizure, both generalized or focal, range from mood changes or impaired vision (Easter *et al.*, 2009), over headache to hallucinations. In non-convulsive status epilepticus, alterations in mental state of different degrees are an always present symptom (Husain *et al.*, 2003). These alterations "can range from mild confusion to profound impairment of consciousness" (Husain *et al.*, 2003). In epileptic patients it has been shown that electrophysiological seizure activity can be present hours in advance of a visible manifestation (Litt *et al.*, 2001).

In animals, signs of non-convulsive seizures induce unspecific or subtle behavioral changes that are difficult to detect and even more challenging to interpret (Baird *et al.*, 2015; Metea *et al.*, 2015). Alterations in the level of consciousness in animals can be expressed in reduced or abnormal activity, like tail-chasing or aggressiveness (Dodman *et al.*, 1996). Myoclonic twitches or muscle rigidity can also be signs of seizures, both drug induced (Metea *et al.*, 2015) and epileptic (Fischer *et al.*, 2013). Also, autonomic signs like increased salivation, vocalization and ocular abnormalities can be symptoms of seizures (Fischer *et al.*, 2013).

In drug development, such signs might not be recognized as ongoing seizure activity by the study personnel (Easter *et al.*, 2009; Metea *et al.*, 2015) and it is challenging to judge them as potential premonitory signs for convulsions (Elander, 2013; Metea *et al.*, 2015). Also, due to their short duration or occurrence at times when the animals are not observed (e.g. at night), seizures and seizure-related convulsions can be missed (Metea *et al.*, 2015; Backes, 2016).

The ultimate detection of a seizure is with the EEG (Privitera *et al.*, 1994; Podell *et al.*, 1995; Elander, 2013; Fonck *et al.*, 2015). EEG signs that precede seizures, and thereby indicate a lowered seizure threshold, are a general increase in synchrony, isolated or repetitive sharp waves, isolated spikes or isolated spike-waves (Metea *et al.*, 2015; Authier *et al.*, 2017).

Seizures in the EEG consist of mainly spike-wave-complexes as spike trains. “The ictal nature of observed EEG abnormalities” can also be indicated by “other EEG forms such as paroxysmal spikes, fast ripples, or postictal depression” (Fonck *et al.*, 2015). In cases of convulsive seizures, it can be determined via EEG whether the convulsion was centrally or peripherally mediated (Elander, 2013).

1.4 Preclinical Seizure Liability Assessments: Methods in Drug Development

1.4.1 In silico Methods

Computational prediction of a drug’s pharmacological profile, especially its capability to penetrate the blood-brain-barrier, can help to calculate the risk of a new chemical entity (NCE) (Easter *et al.*, 2009; Butler *et al.*, 2017). Computers can match a compound’s structure to pathways known to be related to seizure generation. Easter *et al.* have identified 53 targets with potential seizure risk and based on this, Zhang et al. (2011) developed a support vector machine method that detects seizure liability with an accuracy of 86.9%[1]. The foundation of computational tools is a thorough review of the literature. In general, literature research is one of the first steps in target safety assessments (Butler *et al.*, 2017). Limitation of computational approaches, as concluded by Easter *et al.* (2009), is the lack of ultimate understanding of seizure generation.

1.4.2 In vitro Methods

In vitro profiling can reveal a candidate drug’s affinity for a receptor or interaction with other CNS pathways known to be involved in seizure generation (Easter *et al.*, 2009; Elander, 2013). Affinity studies detect binding potential, whereas functional *in vitro* assays bring further information on the consequent effects, like interaction with neurotransmitters or action as either agonist or antagonist (Easter *et al.*, 2009; Elander, 2013). According to Scott *et al*. (2013), “Patch clamp electrophysiology is the gold standard method of detecting compounds that perturb electrical activity”; usually one ion channel is investigated at a time so they are less suited as general screening tools.

Culturing neuronal cells, mixed with glia cells, on microelectrode arrays (MEA) is possible with cells of different origin to test influences on different pathways in the neuronal network (Scott et al., 2013). *In vitro* methods can be conducted as early screening methods at low costs, have a high throughput and usually low compound needs (Easter et al., 2009; Markgraf et al., 2014). However, according to Scott *et al.* (2013), investigation of CNS effects is “challenging to study *in vitro* due to the large number of cell types that exist in the brain and the complex anatomical and functional networks that underlie human behaviors” (Scott et al., 2013). In addition, *in vitro* methods do not allow determination of systemic influences on seizure generation (Cole et al., 2002).

[1] Currently, the link to this tool is corrupt and the author was contacted to investigate whether this is due to technical or scientific reasons

1.4.3 Ex vivo Methods

For assessment of seizure liability *ex vivo*, brain slice preparations, most commonly the rat hippocampal brain slice assay, have been developed (Easter *et al.*, 2009; Elander, 2013; Markgraf *et al.*, 2014). Predictability for the performance of 16 reference compounds was 89% in the rat hippocampal slice assay (Easter *et al.*, 2009). Easter *et al*. (2009) note that the use of slices originating from other brain areas and multi-electrode arrays could increase assay performance. Fonck *et al*. (2015) add, that "the most appropriate preparation is likely to be dependent on the exact mechanism of drug induced seizure" (Fonck *et al.*, 2015).

1.4.4 In vivo Zebrafish Locomotor Assay

Early *in vivo* screening of drug candidates can be done in the larval zebrafish (*Danio rerio*) locomotor assay (Winter *et al.*, 2008; Easter *et al.*, 2009; Koseki *et al.*, 2014; Cassar *et al.*, 2017). Larval zebrafish are exposed to the test compound souled in fish water and their swimming behavior is tracked (Winter *et al.*, 2008; Easter *et al.*, 2009). Within this assay, reference compounds have a good predictivity in different laboratories: Winter *et al.* (2008) report 72% predictivity and Cassar *et al*. (2017) achieved 70% sensitivity, 100% specificity, 100% positive predictive value and 57% negative predictive value (Cassar *et al.*, 2017). With drug candidates with known preclinical seizure liability, assay performance was poor: Despite exposure levels comparable to reference compounds, 2 out of 9 candidates were false negative (22% sensitivity, 100% specificity, 100% positive predictive value and 46% negative predictive value) (Cassar *et al.*, 2017).

1.4.5 In vivo Rodent and Non-Rodent Methods

Convulsive drug action can possibly be detected in rodent behavioral observation assays. For improved detection or further characterization, specialized tests have been developed. Most of them are based on induction of convulsions in different animal models and derive from efficacy testing of anti-epileptic drugs (Easter *et al.*, 2009). In general, the candidate drug is administered in connection with a convulsive stimulus and the latency to convulsions or the dose necessary to induce them is compared to controls. To induce convulsions, either chemicals (e.g. pentylenetetrazole, PTZ) or electricity can be applied. Repeated electrical stimulation is also used to permanently lower the seizure threshold (kindling effect) to increase the animals' sensitivity for proconvulsive agents (Potschka *et al.*, 2000). Alternatives to kindled animals are genetically seizure-prone animals (Löscher, 2009). EEG recordings from these animals can be included to further increase the sensitivity of this approach (Elander, 2013).

The gold standard for seizure detection is the video-EEG (Easter *et al.*, 2009). It allows seizure classification and detection of absence or non-convulsive seizures (Russo *et al.*, 2011; Hamdam *et al.*, 2013) as well as it can preclude seizure activity in cases of unclear symptoms (Easter *et al.*, 2009). In addition, the EEG can show CNS effects at low doses and improve

calculation of safety margins (Elander, 2013). EEG seizure liability studies have been successfully performed in rodents (Easter *et al.*, 2009; Markgraf *et al.*, 2014; Authier *et al.*, 2016), as well as in non-rodents (Braitman & Sparenborg, 1989; Dürmüller *et al.*, 2007; Authier *et al.*, 2009; van der Linde *et al.*, 2011a; van der Linde *et al.*, 2011b; Authier *et al.*, 2016). Hamdam *et al*. (2013) describe video-EEG an "emerging technique", as "simultaneous assessments of new compounds on behavior (via video), seizure liability and disruption of sleep patterns (via EEG)" has been facilitated (Hamdam *et al.* (2013), referring to Authier *et al.* (2009)). A safety pharmacology animal model that uses the EEG to investigate CNS symptoms is the fentanyl-etomidate-anesthetized beagle (FEAB) (Van Deuren *et al.*, 2009; van der Linde *et al.*, 2011a; van der Linde *et al.*, 2011b). According to the authors, its advantages are the possibility to simultaneously evaluate central-nervous, cardiovascular and respiratory parameters (van der Linde *et al.*, 2011b). Disadvantages of anesthetized animal models are potential pharmacodynamic and pharmacokinetic interactions between the anesthetic and the test compound. Also, anesthetics themselves influence CNS activity and pro- or anticonvulsive effects have been described (Reddy *et al.*, 1993; Baraka & Aouad, 1997).

1.5 Preclinical Seizure Liability Assessments: Current Practice in the Pharmaceutical Industry

It has been pointed out that an early detection of seizure risk is advantageous to enable chemical modification of the molecule and guide the project strategy on an early stage (Pugsley *et al.*, 2008; Easter *et al.*, 2009; Hamdam *et al.*, 2013). Early-stage screening methods (computational, *in vitro*, *ex vivo,* rodent behavioral observations) do not guarantee detection of seizure liability and the first adverse findings are convulsions in advanced-state toxicology studies (Easter *et al.*, 2009). At this stage of the development process, many resources and animals already have been used and the finding of a convulsion can complicate and potentially stop further development of a drug candidate (Easter *et al.*, 2009; Elander, 2013; Fonck *et al.*, 2015). In addition, the requirements by the ICH S7A are not specific, so no standardized approach for seizure liability testing is established (Porsolt *et al.*, 2002; Easter *et al.*, 2009; Markgraf *et al.*, 2014; Fonck *et al.*, 2015; Metea *et al.*, 2015). Currently, there is no single method that guarantees adequate assessment of seizure liability or proconvulsive action, so usually a combination of methods is used (Easter *et al.*, 2009; Löscher, 2009; Elander, 2013). Experience from previous interactions with regulatory authorities can guide the strategy to advance the program (Authier *et al.*, 2013).

Elander (2013) assembled parameters that need to be considered after the occurrence of convulsions. Before choosing follow-up tests, a detailed description is needed referring to duration, quality (tonic, clonic, tonic-clonic), loss of consciousness and recovery (Elander, 2013). Concomitant stress factors that might lower the seizure threshold like noise, handling or altered clinical chemistry parameters should be considered as well (Elander, 2013). Also, impurities of the test compound have to be excluded (Elander, 2013). At necropsy,

additional histopathological methods, like special stains, can be used to identify astrocyte and microglial changes or neurodegeneration that have been linked to convulsions (Elander, 2013). A dedicated seizure liability study should allow "determination of drug plasma levels at the time of symptoms and confirm the NOAEL, identify premonitory signs that can be translated to clinical trials, confirm that the seizure is self-limiting and can be treated with standard anticonvulsants" (Authier *et al.*, 2013; Authier *et al.*, 2014b). Also, the therapeutic indication and the targeted patient population are factors that need to be considered for a thorough risk assessment (Fonck *et al.*, 2015).

1.5.1 Species Selection for in vivo Seizures Liability Studies

Rodents are the routine species used as long as no pharmacological indication necessitates the use of a non-rodent species (Authier *et al.*, 2009; Authier *et al.*, 2014b; Authier *et al.*, 2016). There are ethical concerns about the use of animals in drug development and especially the use of non-rodents is often questioned in the scientific community and the general public (Spielmann & Gerbracht, 2001; Bailey *et al.*, 2013). So far use of rodents and non-rodents remains a regulatory requirement (FDA, 2005) and several authors showed better translatability of results from non-rodent than from rodent testing (Broadhead *et al.*, 2000; Olson *et al.*, 2000; Spielmann & Gerbracht, 2001).

In regular safety testing, the dog is often referred to as the standard non-rodent species (Broadhead *et al.*, 2000; Hasiwa *et al.*, 2011; Bailey *et al.*, 2013) and next to the rat, it is the most commonly used species in toxicology (Easter *et al.*, 2009). One advantage of the dog as a preclinical species is that it has long been a companion animal to humans. Due to this close relation, dogs show symptoms more reliably than animals with a lesser degree of domestication and study personnel is accustomed to interpreting dog behavior (Backes, 2016). Appropriate housing conditions can be offered to dogs in a research facility, and handling is in general well tolerated. Providing good conditions for research animals is demanded for ethical and legal reasons as well as it is the base for good results (Clark & Pomeroy, 2010). An extensive literature background exists on dogs (Clark & Pomeroy, 2010) and authorities worldwide accept data generated in this species. Physiological parameters compare well to humans, and therefore the dog has been used as a model for many human diseases, including genetic diseases (Reid *et al.*, 1996; Clark & Pomeroy, 2010).

Dogs are generally believed to be overly sensitive to convulsions (Redman & Weir, 1969; Edmonds *et al.*, 1979; Easter *et al.*, 2009; Hasiwa *et al.*, 2011) and that they are therefore less suited as a relevant model to assess human seizure liability (Authier *et al.*, 2014b). On the other hand, authorities require testing drug effects in the most sensitive species (FDA, 2005) and therefore, according to Authier *et al*. (2013), the use of the dog can be specifically requested. Easter *at al.* (2009) hypothesized that the increased susceptibility of the dog for seizures is due to its genetic predisposition to epilepsy. This disease is the most common neurologic disorder in dogs (Volk, 2015) and currently it is estimated that 0.5-1% of the population is affected (Fischer *et al.*, 2013). The incidence of idiopathic epilepsy is higher in

certain breeds (Fischer *et al.*, 2013; Hülsmeyer *et al.*, 2015) and the beagle dog, that is most often used in pharmaceutical research, is amongst them (Bielfelt *et al.*, 1971; Fischer *et al.*, 2013). Canine epilepsy is very comparable to the human disease: the incidence is similar, many drugs are effective in both species and EEG recordings from human and dog epileptics show the same characteristics (Berendt *et al.*, 1999; Potschka *et al.*, 2013). As a result, the epileptic dog has also been discussed as a natural model of the human disease (Potschka *et al.*, 2013). Limitations of canine epilepsy as a natural disease model are the lack of routine EEG investigations in the characterization of dog epilepsy (Brauer *et al.*, 2011) and the very limited documentation of temporal lobe epilepsy in the veterinarian literature. Also, species differences in pharmacokinetics, e.g. the shorter elimination time in dogs compared to humans (Potschka *et al.*, 2009), are drawbacks of the canine model in the testing of novel anti-epileptic drugs (Potschka *et al.*, 2013).

Considering species similarities of dogs and humans with regards to epileptic seizures and the concomitant experience with seizures in dogs (detection, literature references, and treatment options), the dog can be considered as a model for seizures liability studies in drug development. Also, Olson *et al*. demonstrated that results from dog studies predict human toxicities to a higher degree (63%) compared to rodents (43%) (Olson *et al.*, 2000). One disadvantage of the use of rodents compared to dogs is the smaller size that does not account for detection of some signs related to non-convulsive seizure liability like nystagmus. Retrospective analysis of internal data could not confirm a higher convulsion susceptibility of the dog compared to the NHP (Backes, 2016) and this mitigates concerns about false positive results.

The background incidence of spontaneous seizures should be considered in any dedicated seizure liability study (Easter *et al.*, 2009; Elander, 2013) as spontaneous convulsions have not only been observed in beagles (Edmonds *et al.*, 1979) but also in rats (Nunn & Macpherson, 1995) and for some strains of NHPs natural occurrence of epilepsy with photosensitivity is also known (Meldrum *et al.*, 1975; Szabó *et al.*, 2005).

1.6 Clinical Seizure Liability

In a clinical setting, it is very rare that the occurrence of a seizure in a patient can be related to a single drug (Murphy & Delanty, 2000). There are many factors that can promote seizure risk. Only to mention a few, there are interactions with other drugs or alcohol, a special predisposition due to the illness itself, inherited neurological disorders or other comorbidities (Delanty *et al.*, 1998; Murphy & Delanty, 2000; Löscher, 2009; Fonck *et al.*, 2015). The risk for drug-induced seizures is higher with epilepsy patients (Fonck *et al.*, 2015) and in other conditions with a reduced seizures threshold (Dickey & Morrow, 1990), e.g. with an impaired blood-brain barrier (Fonck *et al.*, 2015). The probability that a new-onset generalized seizure in humans is drug – induced was estimated to be 6.1% (Pesola & Avasarala, 2002).

Evaluation of results acquired in the course of clinical trials can be of special value to estimate seizure liability of released drugs. In contrast to case reports, they are assessed in controlled conditions (Rosenstein, 1993). If a seizure occurs in the course of a clinical study as an adverse event (AE), which is seldom the case, its relation to the study drug is examined thoroughly by the principal investigator (PI) and by physicians from both, pharmaceutical company and FDA (Food and Drug Administration) (Alper *et al.*, 2007). The overall safety of first-in human doses seems to be guaranteed, as shown in a retrospective analysis by Bayer (Wensing *et al.*, 2010). This report covers the years 2000-2005 but reflects, as to the authors, experience from a longer period: the most frequent symptom in phase I studies was headache and none of the adverse effects required hospitalization or led to lasting impairments (Wensing *et al.*, 2010). Still, the recent example of BIA-10247 in which 5 healthy volunteers suffered from irreparable brain damage, in one case leading to death (Kaur *et al.*, 2016) implicates "that drug development will always have risks" and that "It is not possible to identify all 'high-risk' compounds that need elevated risk assessment" (Eddleston *et al.*, 2016).

The definite safety profile of a drug can only be defined after its release, when it has been used by a larger number of patients, in a more of more diverse population and potentially in off-label or multiple-drug use conditions (Fung *et al.*, 2001; McNaughton *et al.*, 2014). Reports about seizures occurring post – marketing can be found with drugs from all kinds of pharmaceutical classes and fields of indication (Dickey & Morrow, 1990). Fonck *et al.* (2015) observed that seizures in relation with use of a marketed compound generally occur at (brain) exposure levels exceeding the recommended limits. The highest risk is reported for drugs which directly access the CNS, e.g. antidepressants or neuroleptics (Dickey & Morrow, 1990; Easter *et al.*, 2009; Kumlien & Lundberg, 2010). Among neuroactive compounds, antidepressants seem to imply a specifically high risk, being the leading cause of seizures reported to poison control centers in 1988-1989 (29%, (Olson *et al.*, 1993)) and in 2003 (49% (Thundiyil *et al.*, 2011)). 31% of drugs withdrawn for safety reasons from the market between 1960 and 1999 were from CNS indications (Fung *et al.*, 2001). The safety issues that were causative of this were not seizures but mainly hepatic and hematologic liabilities, followed by cardiovascular, dermatologic and carcinogenic; often, multiple toxicities were the cause (Fung *et al.*, 2001). Neurologic reasons contributed to only 4.1% of withdrawals, followed by psychiatric and abuse (Fung *et al.*, 2001).

2 The Electroencephalogram

An electroencephalogram is a recording of the spontaneous electric activity of the brain (Kirschstein, 2008) measured by electrodes placed above different brain regions. Cortical pyramidal neurons are the main generators of the EEG (Teplan, 2002; Kirschstein, 2008). Glial cells also contribute to the EEG as they have close electrical coupling between each other, thereby amplifying the neuronal signal (Holliday & Williams, 1999). To be exact, the term EEG only refers to recordings derived from scalp electrodes, whereas an electrocorticogram (ECoG) is measured directly from the cortical surface and an electrogram is a recording from depth electrodes (Teplan, 2002). In literature however, the term EEG is frequently used for any recording of electric brain activity, regardless of the recording technique.

The electric activity is generated at synapses, when the action of different neurotransmitters induces either an inhibitory (IPSP) or excitatory (EPSP) potential at the postsynaptic neuron through changes in the local distribution of ions (Holliday & Williams, 1999; Kirschstein, 2008). Action potentials in the axons do not significantly influence the EEG due to their multi-directional course relative to the surface that results in a net potential equaling zero (Holliday & Williams, 1999).

Cortical neurons function as a dipole, so depending on the site of the electric excitation, mainly its distance to the recording electrode, the signal measured on the skull can either be positive or negative (Kirschstein, 2008). An ESPS in deep sites will induce positivity below a surface electrode whereas an EPSP just below that electrode will cause negativity (Holliday & Williams, 1999). A single post-synaptic potential is too small to be recorded but pyramidal cells have a multitude of synapses that fire with some synchrony, sum up and facilitate recording with an EEG electrode (Holliday & Williams, 1999).

The anatomic organization of the brain in gyri and sulci has the effect that the electric currents that run horizontally below the electrode are not completely captured (Kirschstein, 2008). The recorded signal is the sum of all inhibitory and excitatory postsynaptic potentials, predominantly from cells on the top of one gyrus (Kirschstein, 2008). Thereby, the signals acquired via EEG are field potentials that have no definite direction like muscle potentials, that pass through a fiber in a direction relative to an electrode (Holliday & Williams, 1999).

2.1 The Electroencephalogram in Veterinary Medicine

2.1.1 *Historical Overview*

The British physiologist R. Caton was the first person to measure electric brain activity from animals in 1875 (Caton, 1875; Brass, 1959; Teplan, 2002). Brass (1959) reviewed the consequent research activities using animals that where then initiated to expand the knowledge about the electric currents of the brain (Brass, 1959). According to him, the first term for this novel technique, “electrocerebrogram”, was introduced in a review on electric

brain activity by Práwdicz-Neminski in 1925 (Brass, 1959). He was also the first to comprehensively describe spontaneous electric activity of the dog's brain; for this, dogs were immobilized with curare and their EEG was measured invasively through holes in the skull (Brass, 1959). In 1924, Hans Berger was first to record the human scalp EEG (Teplan, 2002). For linguistic reasons (not to mix Greek and Latin), he introduced the term "electroencephalogram" in 1929 (Berger, 1929; Haas, 2003). Berger described the distinct EEG waveforms and their changes in different vigilance and disease states (Brass, 1959).

Animal EEG was mostly used for experimental, not diagnostic, purposes (Brass, 1959). Fields of interest were experimentally induced epilepsy or investigation of drug substance influences on the EEG, mainly of anesthetics (Brass, 1959). The first introduction of the EEG as a diagnostic technique in clinical veterinary medicine was by Brass (Brass, 1959; Schütt-Mast & Stephan, 1996; Holliday & Williams, 1999). He established EEG reference values by collecting data from healthy dogs and comparing them to recordings from patient dogs, using five electrodes. He concluded that the main application of EEG in veterinary medicine is for localization of focal pathologies as hematomas, neoplasms or inflammatory processes and their differentiation to epileptic seizures to guide treatment decisions or surgery planning (Brass, 1959).

One year after Brass' first description of the use of the EEG in the veterinary clinic, McGrath published a textbook with guidelines for neurologic examination of dogs that that included a description of the EEG as diagnostic tool in veterinary medicine as well (McGrath, 1960). In 1962, Croft described electroencephalography as a "young and growing science" and already noted the lack of standardization of recording and interpretation techniques in veterinary medicine (Croft, 1962). Croft contributed to the knowledge of pathological EEG patterns like the flat EEG in encephalitis (Croft, 1970a) or spike-wave patterns in epilepsy (Croft, 1962), changes after head trauma (Croft, 1970b), in cerebrovascular diseases (Croft, 1971), and space-occupying lesions (Croft, 1972). Like Brass, Croft and other clinicians at that time preferred recording EEGs from un-anesthetized animals with different degrees of mechanical restraint (Brass, 1959; Croft, 1962; Redding, 1964).

While Brass and Croft used needle electrodes (Brass, 1959; Croft, 1962), Redding proposed the use of alligator clamps in veterinary medicine and described new recording coordinates (Redding, 1964). Advantage of alligator clamps over needle electrodes is that clipping of the hair, to which some owners might oppose, is not necessary for proper fixation (Redding, 1964). He used local anesthetics to anesthetize the facial muscles to both, prevent artefacts and block pain induced by the clamps (Redding, 1964). Redding also published the "Atlas of Electroencephalography in the Dog and Cat" in 1984 (Redding & Knecht, 1984). 15 years later, Holliday published a comprehensive review on clinical EEG focusing on the dog (Holliday & Williams, 1999). Recently, Hasegawa reviewed methods in veterinary neurology and proposed a standard montage and sedation protocol in veterinary medicine (Hasegawa, 2016).

The first EEGs were obtained using paper-bound polygraphs (Holliday & Williams, 1999). The introduction of computers for recording and storing EEG data brought eminent progress regarding interpretation (e.g. by allowing digital change of montages), computational analysis, data storing and data sharing among physicians (Bergamasco *et al.*, 1999), (Kellinghaus, 2013). Computerization of the bioelectrical signal from the brain enables to analyze data in different ways: in the time, the frequency or in the space domain (Bergamasco *et al.*, 1999).

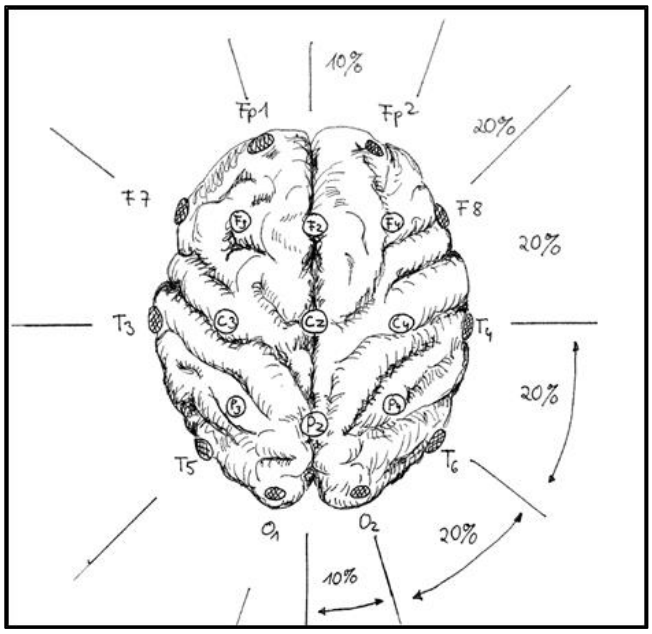
An early report on quantitative electroencephalography (qEEG) in veterinary medicine has been published by Klemm (Klemm, 1969), and since then this technique has been used by several other veterinarian clinicians and researchers, e.g. (Greene *et al.*, 2002; Bergamasco *et al.*, 2003; Jeserevics *et al.*, 2007). This technique enables characterization of background activity beyond visual interpretation. It can be used for example to obtain objective criteria for the central action of anesthetics (Greene *et al.*, 2002). Guidelines for quantitative EEG analysis in dogs have been proposed by Bergamasco *et al.* in 2003 (Bergamasco *et al.*, 2003). However, the clinical use of quantitative EEG parameters is confounded by a lack of reference values and standardized protocols (Jeserevics *et al.*, 2007). Quantitative EEG data have been described for some breeds like Labrador retrievers (Moore *et al.*, 1991) or beagle dogs (Jones & Greufe, 1994). Another variable is anesthesia which has either been avoided (Jones & Greufe, 1994) or induced by different drugs like isoflurane (Moore *et al.*, 1991), medetomidine (Itamoto *et al.*, 2001), (Jeserevics *et al.*, 2007) or propofol (Bergamasco *et al.*, 2003). Anesthetics themselves can have proconvulsive effects (Reddy *et al.*, 1993; Baraka & Aouad, 1997), thereby influencing the EEG and especially qEEG parameters.

2.1.2 *Status Quo and Comparison to Human Medicine*

In 1968, Klemm reviewed methods so far described for animal EEG recording in veterinary medicine and tried to standardize them (Klemm, 1968; Steiss, 1988). He recommended a first-line montage and compared different electrode types and restraint methods (Klemm, 1968). In human medicine, the EEG recording technique had already been harmonized by the introduction of the „ten-twenty-system“(10-20) system in 1958 (Jasper, 1958; Klem *et al.*, 1999; Milnik, 2009).

Electrode Number and Positions

In human EEG recordings, electrode placement considers anatomical structures of the skull that can be reliably identified in different individuals (nasion, inion, preauricular points) and relative to individual skull size (Jasper, 1958; Klem *et al.*, 1999; Milnik, 2009) (*compare* **Figure 1** *and* **Figure 2**). Electrode names refer to the brain area they monitor: frontopolar (Fp), frontal (F), central (C), parietal (P), occipital (O) and temporal (T). Assignment to the right or left hemisphere is done by adding even (right hemisphere) or odd (left hemisphere) numbers. The second letter “z” indicates electrodes along the midline (“zero”) (Milnik, 2009).



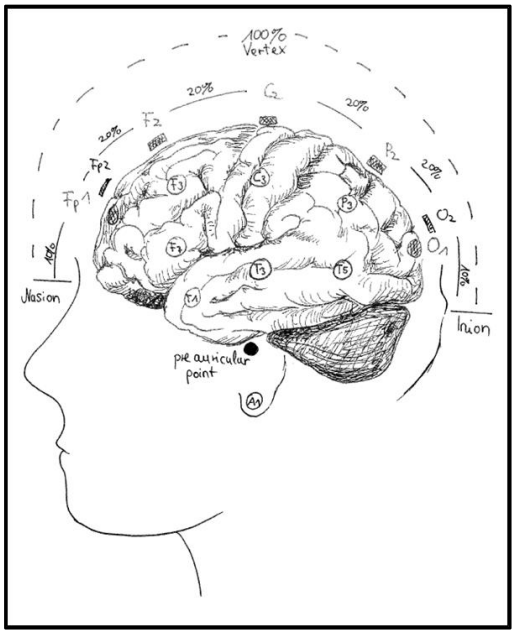


Figure 1 and **Figure 2**: Topographical illustration of electrode positions according to the 10-20 system Even numbers indicate electrodes on the right side, odd numbers stand for electrodes on the left side. All electrodes are placed within 10% or 20% of the total (100%) distance between anatomical structures or electrodes,the midline e.g. connects inion and nasion: nasion + 10% = Fpz; +20% =Fz: +20%=Cz; +20%= Pz, +20%= Oz, +10% = Inion *Drawings adapted from Milnik (Milnik, 2009) and Jasper (Jasper, 1958), (Klem et al., 1999).*

Coordinates for electrode placement in veterinary medicine are adapted from the 10-20 system (Pellegrino & Sica, 2004; Bunford *et al.*, 2017)**.** Steiss found that in 1988, the most common maontage was according to or adapted from the one described in the atlas of electroencephalography by Redding and Knecht (see (Redding & Knecht, 1984), Steiss, 1988). Examples of alternative placements in dogs that have been described are shown in **Table 1**. One reason for this variability is the anatomy of different species and different breeds, especially in case of the dog (Pellegrino & Sica, 2004; Bunford *et al.*, 2017). In humans, at least 16 electrodes are commonly placed (Bunford *et al.*, 2017). In dogs, usually a lesser number is used (compare **Table 1**) which is due to the smaller size of the head and stronger developed muscle layer covering the skull (Bunford *et al.*, 2017).

Reference	Indication for EEG	Electrode type, number	Fixation type	Dogs
(Brass, 1959)	ME	SNE (steel), n=5	training, dark quiet room; n.s.	Healthy dogs; patients, n = n.s.
(Redding, 1964)	ME	Alligator clamps	Mechanical restraint	normal dogs
(Herin *et al.*, 1968)		SNE, n=8	Gallamine triethiodide	n.s., n=40
(Moore *et al.*, 1991)	MD qEEG	SNE, n=21	Isoflurane	Healthy Labrador Retrievers, n=8
(Kersten, 1993)	MD	SNE, n=8	n.s.	Healthy and patient dogs, n = n.s.
(Morita *et al.*, 2002)	Epilepsy	SNE, n=12	s.c. lidocaine; i.m. Xylazine	Epileptic Shetland Sheep dogs, n=11
(Bergamasco *et al.*, 2003)	MD qEEG	SNE, n=16	Propofol	Healthy beagle dogs, n=10
(Pellegrino & Sica, 2004)	Epilepsy	SNE, n=8	Xylazine	Normal dogs n=50, epileptic dogs, n=9
(Jeserevics *et al.*, 2007)	Epilepsy	SNE, n=16	Medetomidine (dark, quiet room)	Healthy (n=16) and epileptic (n=15) Finnish Spitz dogs
(Raith *et al.*, 2010)	Status epilepticus	SNE, n=5	Propofol or pentobarbital infusion	Patient dogs (n=7) and cats (n=3)
(James *et al.*, 2011)	ME for long term recording	SNE, SWE, GCE	none	Epilepsy patients
(Davis *et al.*, 2011)	ME iEEG	Intracranial, n=16	none	Epilepsy patients
(Cauduro *et al.*, 2017)	Seizures	SNE, n=12	Dexmedetomidine, propofol	Patient dogs (n=200) n.s.

Table 1*:* Examples of indications, electrodes, restraint methods and subjects from the veterinary literature. SNE = Subcutaneous needle electrode; SWE = Subdermal wire electrode; GCE = Gold-cup electrode; ME = Method establishment; MD = Method description; n.s. not specified; iEEG = intracranial Electroencephalogram; n = number; SE = Status epilepticus.

Electrodes

Different electrode types are used in human medicine, e.g. needle electrodes, disposable gel electrodes or reusable disk electrodes (Teplan, 2002). For veterinary medicine, Steiss (1988) found that in 1988, the most commonly used electrode was the subdermal needle electrode (SNE). One alternative, as mentioned above, is the alligator clamp, introduced by Redding (Redding, 1964). Most new publications from the field of veterinary EEG describe the use of needle electrodes, too (compare **Table 1**). James *et al*. compared applicability of subdermal needle electrodes, subdermal wire electrodes and gold cup electrodes for long-term recordings in dogs and concluded, that the differences found “may not be clinically relevant” (James *et al.*, 2011).

Artefacts

Two categories of artefacts can be differentiated which are physiological artefacts and technical artefacts. Physiological artefacts originate from the recorded subject and technical artefacts are caused by the equipment or external sources (Milnik, 2011).

Amongst physiological artefacts, muscle artefacts are a frequent finding in dog EEGs. As opposed to humans, dogs have a prominent muscle layer between electrodes and skull which causes a comparably higher level of artefacts (Brass, 1959; Bunford *et al.*, 2017). Another cause of artefacts in dog EEGs are ear movements (Brass, 1959). Even in anesthetized dogs, activity from temporal muscles transiently occurs (Klemm, 1968) and therefore the proposal made by Bunford *et al*. (2017) to record EEGs from sleeping dogs only would not completely solve that issue. Another approach to reduce artefacts (and simultaneously block pain from electrode application) is injection (Redding, 1964), (Itamoto *et al.*, 2001; Morita *et al.*, 2002) or topic application (Ward *et al.*, 2016) of local anesthetics. Not only facial muscles are sources of artefacts, but "the EEG is extremely sensitive to any other source of electrical activity, including muscle, cardiovascular and ocular activity of the patient" (Wrzosek, 2016). Ocular and cardiovascular activity can be monitored with the help of additional electrodes, which is commonly done in human EEG recordings and has also been adapted by veterinarians in the literature, e.g. (Holliday & Williams, 1999).

In human medicine, movement, muscle and ocular artefacts can be reduced by instructing the patient accordingly. In veterinary medicine, this is not possible, and some animals need to be sedated or anesthetized to tolerate the procedure. Whereas the earliest reports on EEG in veterinary medicine describe the use of trained animals (Brass, 1959), mechanical restraint (Redding, 1964) or muscle relaxants (Herin *et al.*, 1968), the majority of veterinary neurologists seem to routinely use sedation or anesthesia when recording an EEG. The use of different protocols contributes to the low level of standardization of electroencephalographic proceedings in veterinary medicine (compare **Table 1**). Holliday *et al.* (1999) recommend sedation rather than anesthesia, as the confounding effect on EEG is smaller and different vigilance states can still be captured.

The high level of artefacts makes interpretation of animal EEGs challenging, especially as there are no established quantitative procedures for automated artefact rejection in dog studies as for human EEGs (Bunford *et al.*, 2017). In human EEG recordings, the use of a large number of electrodes allows changing the montage in various ways. Thereby, the "logical filed distribution" of EEG signs can be controlled to distinguish between artefact or real symptom (Milnik, 2011). Using a sufficient number of recording electrodes, despite of the different anatomy of the dog compared to the human, therefore also helps artefact identification in veterinary medicine.

Activation techniques

Standard activation techniques are well established in human medicine like the "Berger reaction", photic stimulation, sleep deprivation, anti-epileptic drug (AED) withdrawal or hyperventilation. They are used to provoke occurrence of epilepsy-typical potentials within interictal recordings and thereby increase the diagnostic value of the EEG. Their adaption to veterinary medicine is challenging and has rarely been investigated (Brauer *et al.*, 2011; Brauer *et al.*, 2012; Wrzosek *et al.*, 2017). Neither did passive hyperventilation during general anesthesia (Brauer *et al.*, 2012) nor did photic stimulation in medetomidin-sedated dogs increase the frequency of epileptic discharges (Wrzosek *et al.*, 2017). One exception is the naturally activating nature of sleep, as this state has been found to increase the incidence of epileptic discharges (Holliday & Williams, 1999; Wielaender *et al.*, 2017). A pharmacological activation technique to increase the probability of recording epileptic EEG is the intravenous administration of chlorpromazine, eventually combined with photic stimulation (Holliday *et al.*, 1970; Holliday & Williams, 1999).

Indications

Like in human medicine, seizures of unknown origin are the most common reason to write an EEG from animals (Holliday & Williams, 1999). It is also used for monitoring and treatment control of status epilepticus in dogs and cats (Raith *et al.*, 2010). Quantitative analysis of EEGs of epileptic dogs before and after phenobarbital treatment showed comparable changes in relative power of delta, alpha, theta and beta activity in two different studies (Jeserevics *et al.*, 2007; Bocheńska *et al.*, 2014). This could be used in monitoring the effects of antiepileptic treatment apart from clinical symptoms (Bocheńska *et al.*, 2014).

Long-term EEG monitoring of the epileptic brain in human medicine is done for different reasons: in the preparation of epilepsy surgery, for treatment monitoring or automated seizure forewarning. This is done with the help of portable EEG devices which can either be non-invasive or involve chronic implantation of electrodes. A non-invasive EEG device for ambulant monitoring of epileptic dogs has only recently been described in the veterinary literature which enables capturing rare seizures for accurate diagnosis and enables diagnosis of myoclonic seizures and absence seizures in veterinary clinics (James *et al.*, 2011; James *et al.*, 2017; Wielaender *et al.*, 2017; Wielaender *et al.*, 2018). So far, recordings from epileptic patient dogs were mostly acquired in the interictal state, with few exceptions (Raith *et al.*, 2010; Davis *et al.*, 2011; Coles *et al.*, 2013; James *et al.*, 2017; Wielaender *et al.*, 2017; Wielaender *et al.*, 2018). For human patients with refractory epilepsy surgical resection of the epileptic focus can be the only remaining treatment option (Davis *et al.*, 2016; Hasegawa, 2016) and this has also been considered for dogs with drug-resistant epilepsy (Hasegawa, 2016). Implantation of intracerebral electrodes for exact determination of the epileptic focus in the pre-surgical workup is then commonly done in humans (Hasegawa, 2016). In animals, cortical or depth electrodes have mostly been used experimentally (Hasegawa, 2016), e.g. for investigation of a stereotactic system for implantation of

stimulating and recording depth electrodes in dogs (Long *et al.*, 2014). One exception were studies by Davis *et al*. (2011) and Coles *et al*. (2013): They tested an algorithm-based device that predicts seizure occurrence in dogs with naturally occurring epilepsy (Davis *et al.*, 2011) and then confirmed feasibility in pet dogs (Coles *et al.*, 2013).

Applications of EEG beyond assessment of seizures is monitoring of anesthetic depth which is frequently done in humans (Teplan, 2002; Bruhn *et al.*, 2006). Commercially available devices for humans have been tested in animals (March & Muir Iii, 2003; March & Muir, 2005; Bollen & Saxtorph, 2006; Ribeiro *et al.*, 2008) but the authors concluded that further validation would be needed for clinical use.

Commonness

According to James *et al.*, "most veterinary neurologists" use the EEG (James *et al.*, 2011). She refers, however, to a survey dating back to 1988 that indicated that 17 out of the 19 participants used the EEG (Steiss, 1988). Other authors claim that the EEG has not the status of a routinely used technique in veterinary medicine (Brauer, 2010; De Risio *et al.*, 2015; Wrzosek, 2016). No newer surveys on the frequency at which EEG recordings are made in veterinary medicine today were found but Brauer (2010) states that there is a demand for the technique, especially in the diagnosis of epilepsy (Brauer, 2010). Currently, availability of EEG equipment is limited to specialized institutes which leads to an on average restricted knowledge of this method among veterinarians (Jeserevics *et al.*, 2007).

It has been assumed that use of the EEG has decreased in veterinary medicine since imaging methods have become widely available (Brauer, 2010). In human medicine, innovations in the field of imaging techniques have not markedly reduced the amount of electroencephalographic examinations and an EEG remains a standard tool in the diagnosis of seizure patients (Milnik, 2010). Compared to other methods, it has advantages in humans: the EEG can be easily obtained in a non-invasive fashion without any risk for the patient, repeated recordings can be made, e.g. for treatment monitoring, and the costs are comparably low (Milnik, 2010).

In human medicine, the EEG has been combined with other methods to increase the diagnostic value of both methods. One example is EEG-triggered fMRI that relates cerebral oxygen consumption to epileptic EEG activity (Hasegawa, 2016). As this can be performed in anesthetized animals, this method could be used in future in the diagnostic workup of epileptic animal patients (Hasegawa, 2016). Other EEG-derived disciplines in human medicine are the study of evoked potentials, brain computer interfaces and EEG biofeedback (see (Teplan, 2002)).

It can be concluded that Klemm's attempt to standardize EEG recording techniques in veterinary medicine (Klemm, 1968) was not successful, which is also mentioned by several authors (Pellegrino & Sica, 2004; Jeserevics *et al.*, 2007; Brauer, 2010; Hasegawa, 2016; Bunford *et al.*, 2017). This lack of standardization, the introduction of other diagnostic

methods in neurology and limited availability of recording equipment are reasons why it is seldom used. However, the EEG is a recognized technique also in veterinary medicine. Main interest in this method in the veterinary clinic is for characterization and treatment of epileptic disorders (Hasegawa, 2016).

2.2 EEG in Drug Development

In drug development, EEG studies are not required from regulators, but they can be included as safety or efficacy biomarker. In safety studies, main applications of the EEG are, according to Authier *et al.* (2016), sleep and seizure studies. For both endpoints, it is considered the current gold standard (Authier *et al.*, 2014a; Authier *et al.*, 2014b). EEG symptoms are generally considered to have a high degree of translatability between species, including humans (Fonck *et al.*, 2015). Concerning efficacy studies, EEG has been proposed as a translational biomarker in various disorders like schizophrenia, Alzheimer's disease, depression or attention-deficit disorders (for a review, see Leiser *et al*. (2011)) or as a PD biomarker (Wilson *et al.*, 2014). Quantitative EEG is used to confirm a drugs central bioavailability and determine duration of central drug action (Irwin, 1982; Porsolt *et al.*, 2002).

Especially in the development of antiepileptic drugs, the EEG has also played an important role: first in the characterization of animal models of epilepsy (e.g. (Grossman, 1963; Girgis, 1978; Opdam *et al.*, 2002; Szabó *et al.*, 2005)) and second in the testing of antiepileptic treatments in these models (e.g. (Lockard *et al.*, 1975; Turski *et al.*, 1984; Akman *et al.*, 2011)). Anxiolytic drugs have also been characterized in the EEG (Krijzer & Van der Molen, 1987).

Another application of the EEG has been the testing of anti-pain medications. In minimally anesthetized animals, noxious stimuli are reflected in the EEG and thereby efficacy of drugs can be evaluated (LeBlanc *et al.*, 2016). This model has also been used in veterinary medicine to evaluate pain during surgical procedures (Haga *et al.*, 2001; Kaka *et al.*, 2015; Kaka *et al.*, 2016).

In pharmaceutical research, electrodes are mostly implanted into the skull (Authier *et al.*, 2016). Mounted EEG caps with the data storage medium directly connected to the electrodes, tethered EEG or telemetry can be used. Telemetry with internalized implants facilitates long term recordings in freely moving animals, which has several advantages. It allows that animals can be grouped housed and exhibit their normal behaviors, increases the chance of detecting rare events with unpredictable onset and the quality is higher than with EEGs from restraint animals (Morton *et al.*, 2003; Authier *et al.*, 2014b). However, forms of restraint, e.g. the use of a sling, have still been used in order to minimize artefacts or to allow continuous infusion (Dürmüller *et al.*, 2007). Only few authors describe the use of non-invasive EEG measurements in drug safety testing (Authier *et al.*, 2015).

2.3 Technical Definitions

The difference in the electric activity that is recorded beneath two electrodes is termed a "derivation" and is displayed in one "channel" of the recording (Holliday & Williams, 1999). In a "reference montage", one electrode is placed over electrically active brain tissue ("active electrode" or "exploring electrode") and compared to that of an "indifferent" – or "reference" – electrode placed over electrically inactive sites (Holliday & Williams, 1999). A reference electrode over active brain tissue can also be chosen and then, the montage is called "bipolar" (Holliday & Williams, 1999). A third ground electrode "is needed for getting differential voltage by subtracting the same voltages showing at active and reference points" (Teplan, 2002). In order to record the activity from different brain areas, multiple electrodes are compared amongst each other. Therefore, they are arranged in transversal or longitudinal chains over the skull (Holliday & Williams, 1999). In a bipolar montage, each electrode is used twice: in two adjacent channels, it is used once as reference electrode and once as active electrode (Holliday & Williams, 1999). In clinical neurophysiology, differential amplifiers that potentiate the difference in voltage between two connected electrodes are used (Holliday & Williams, 1999; Kellinghaus, 2013). By international definition, electrodes should be connected with the amplifier in a way, that a negative signal from the electrode that is named first in the derivation is displayed upwards (Holliday & Williams, 1999). The amplifier transforms the "microvolt signals into the range where it can be digitized accurately" (Teplan, 2002) and therefore, sensitivity of the amplifier should be highest in the range of the electrical brain activity (0.5Hz to 70Hz) (Holliday & Williams, 1999). After signal amplification, an analogue-digital converter (A/D) enables storing the EEG on a computer (Teplan, 2002). According to the Nyquist theorem, half the sampling rate is the highest reliably presentable frequency in the EEG (Kellinghaus, 2013).

2.4 Visual EEG Analysis

Traditionally, the first step of EEG interpretation is visual analysis (Holliday & Williams, 1999). A trained and experienced interpreter is required for this in contrast to automatic analysis that uses definite computer settings (Holliday & Williams, 1999).

The graphic display of the EEG is a change of voltage with time (Klemm, 1969). The term "spontaneous EEG" is used when there is no intentional external stimulation of the activity of the brain (Klemm, 1969). Different waveforms, each defined by a certain frequency (unit: Hertz (Hz)) and amplitude (unit microvolts (µV)), can be visually identified (alpha, beta, delta, theta and gamma). They form the appearance of the ongoing background activity, which first indicates different stages of vigilance. The background activity varies between the different regions of the brain (Roesche, 2012) and changes with age (Pampiglione, 1963; Holliday & Williams, 1999; Milnik, 2010; Roesche, 2012).

Upon this background activity, different transient patterns can be superimposed (Holliday & Williams, 1999). They can be characteristic for different stages of vigilance or neurologic

diseases. Examples of transient EEG patterns are sleep spindles and K-complexes that are present in drowsiness or sleep, or spike-wave complexes characteristic for epileptic disorders. Artefacts can be mistaken for symptomatic EEG changes, as their nature is transient as well (Holliday & Williams, 1999). The duration of transient events is usually less than a few seconds (Holliday & Williams, 1999). Automated analysis of paroxysmal EEG patterns is possible with software solutions, e.g. detection of spike wave patterns or spike trains.

2.5 Quantitative EEG Analysis

Quantitative analysis of the EEG (qEEG) brings information on the amount of the individual spectral components that form the EEG signal (Bergamasco *et al.*, 1999; Holliday & Williams, 1999). This can be used to investigate changes in background activity that would not be evident upon visual analysis alone (Bergamasco *et al.*, 2003) but does not provide information on specific graphoelements as paroxysmal activity or artefacts (Bergamasco *et al.*, 1999; Holliday & Williams, 1999).

Quantitative analysis is achieved by applying the Fast Fourier Transformation (FFT) to selected episodes (Bergamasco *et al.*, 1999). These episodes need to be cleaned from artefacts as these highly impact the result of frequency analysis (Bergamasco *et al.*, 1999). Also, vigilance state needs to be controlled for correct application of qEEG (Porsolt *et al.*, 2002; Wilson *et al.*, 2014). Parameters that need to be defined are the lower and upper limits of the frequency bands and usually, the common EEG rhythms are selected (delta, theta, sigma, alpha, beta and gamma) but the exact bandwidths are differently defined in literature. Results of quantitative analysis can be displayed in absolute (μV^2) or relative (%) values. The absolute power is calculated from the square of the amplitudes of each frequency band (Moore *et al.*, 1991). Relative power is defined as the absolute power of one band, divided by the power of the entire spectrum and then multiplied with 100 to express the result as percentage (Jeserevics *et al.*, 2007).

For dogs, Kis *et al*. (2014; 2017) report that absolute power is variable between individuals as a consequence of varying skull size and skull thickness in different breeds (Kis *et al.*, 2014; Bunford *et al.*, 2017; Kis *et al.*, 2017). Relative ratios between the power bands are less variable and have therefore often been preferred for comparison (Bergamasco *et al.*, 1999), (Kis *et al.*, 2014; Kis *et al.*, 2017). Bunford *et al*. (2017) also point out that calculation of relative power is “common practice in human EEG studies because absolute EEG power is less psychometrically sound than relative EEG power” (Bunford *et al.*, 2017).

3 Reference Compounds for Validation of a Telemetry EEG System

For establishment of a seizure liability assay, selection of a set of positive and negative reference compounds from literature is a possible approach and has been chosen for validation of mouse EEG (Easter *et al.*, 2007), hippocampal brain slices (Easter *et al.*, 2007), the zebrafish locomotor assay (Winter *et al.*, 2008) and a computational support vector machine (Zhang *et al.*, 2011). For the establishment of dog EEG for seizure liability assessments, the use of PTZ has been described by e.g. Dürmüller *et al*. (2007) and Baird *et al*. (2015). In rodent models (e.g.(Irwin, 1968)) for evaluation of CNS effects of drug candidates however, other reference compounds are commonly used. For identification of sedative effects, diazepam, haloperidol and morphine are often chosen (Roux *et al.*, 2004). For hyperexcitability, the use of D-amphetamine is commonly described (Roux *et al.*, 2004). Other reference compounds exist, and the testing protocols vary between laboratories (Authier *et al.*, 2016).

3.1 Midazolam

Midazolam is a benzodiazepine and belongs to the same chemical class as diazepam. In rodent CNS tests, diazepam is more commonly used (Roux *et al.*, 2004). However, midazolam has been reported to be superior in the treatment of convulsions via other routes than the intravenous one (McMullan *et al.*, 2010; Schwartz *et al.*, 2013) especially as intramuscular diazepam administration is not feasible (Schwartz *et al.*, 2013). Midazolam also is the standard anticonvulsant for the treatment of drug-induced convulsions in toxicological studies. Therefore, this drug was also selected for treatment of convulsions in the following experiments with in-house compounds. In the veterinary clinic, it is also used as anticonvulsant, as well as a sedative or as premedication for induction of general anesthesia (Reves *et al.*, 1985). Concerning EEG effects, benzodiazepines have been well characterized: According to Wilson *et al*. (2014), who reviewed applications of pharmaco-EEG in animals and men, benzodiazepine-induced EEG changes are best described in literature. These changes include mainly an increase in beta and a reduction in alpha power (Porsolt *et al.*, 2002; Wilson *et al.*, 2014). In dog EEG, midazolam was described in combination with medetomidine (Itamoto *et al.*, 2002), flumazenil (Artru, 1989) and lidocaine-induced seizures (Horikawa *et al.*, 1990).

3.2 Propofol

Propofol is not commonly used in rodent CNS tests, but its effects on dog EEG have been well described in the literature (Artru *et al.*, 1992; Kusters *et al.*, 1998; Bergamasco *et al.*, 1999; Bergamasco *et al.*, 2003; Lopes *et al.*, 2008; Ribeiro *et al.*, 2008; Akos *et al.*, 2012). Also, results of quantitative EEG analysis of beagle dogs have been published (Bergamasco *et al.*, 2003). This enabled comparison of results with literature data. Propofol is well tolerated in healthy dogs (Bergamasco *et al.*, 2003) and therefore, it is also widely used in veterinary

medicine for induction and maintenance of general anesthesia. (Hall & Chambers, 1987; Watkins *et al.*, 1987; Morgan & Legge, 1989). It is administered intravenously, and then has a fast onset of action. Through rapid hepatic metabolism, it does not accumulate and narcotic depth can therefore be easily adjusted. It has also been described that spikes in the EEG could appear in propofol anesthesia (Reddy *et al.*, 1993; Baraka & Aouad, 1997). This would allow EEG pattern recognition without causing severe distress to the research dogs.

3.3 Apomorphine

In the veterinary clinic, apomorphine is commonly used to induce emesis when poisoning is suspected. Apomorphine is an unselective D1 and D2 receptor agonist. In drug development, apomorphine is known as a stimulant reference compound that induces stereotyped behavior in different species, namely lip-smacking and chewing movements in NHP (Meldrum *et al.*, 1975; Korsgaard *et al.*, 1985) and rodents (Kropf *et al.*, 1989). The "apomorphine-induced climbing and stereotypies test" in rodents is used in the testing of antipsychotic drugs (Bardin *et al.*, 2006). There is a dose-dependency to this effect: lower doses (0.05 - 0.1 mg/kg) of apomorphine were reported to block non drug-induced stereotypies whereas higher doses (0.25 - 1.0 mg/kg) induce them (Korsgaard *et al.*, 1985). An increase in motor activity was also frequently described after apomorphine injection with a concomitant increase in EEG artefacts from muscle activity (Meldrum *et al.*, 1975).

Apomorphine effects on the EEG were investigated in epileptic baboons (Meldrum *et al.*, 1975), rats (Kropf *et al.*, 1989; Sebban *et al.*, 1999) rabbits (Brücke *et al.*, 1957) and healthy volunteers (Luthringer *et al.*, 1999). No reports on its effects on dog EEG were found. Wilson *et al*. (2014) compared results of a study that explored effects of apomorphine on the rat EEG (Wilson *et al.*, 2014) to a study of a low dose (0.75 mg total) in healthy human volunteers (Luthringer *et al.*, 1999; Wilson *et al.*, 2014). They stressed the importance to control the vigilance state to exclude an influence of natural changes in the level of wakefulness on EEG results (Wilson *et al.*, 2014). Visually assessable changes in the EEG after apomorphine administration had the characteristics of an "arousal reaction", namely a decrease in amplitude and rise in frequency (Brücke *et al.*, 1957; Meldrum *et al.*, 1975).

3.4 Quinpirole

Quinpirole is, as D-amphetamine, known to have an EEG stimulating effect in rodents (Arnt *et al.*, 1987). Clinical signs in rodents are "dose dependent hyperactivity (locomotion, sniffing, head movements and rearing), whereas licking/ biting was only seen occasionally." (Arnt *et al.*, 1987). Also, it is a selective D-2 agonist, and testing quinpirole enabled comparison of selective agonists on different dopamine receptors (D2 and D3). Pharmacologic properties of quinpirole in dogs were investigated by Whitaker & Lindstrom with a 0.2 mg/kg dose: They found that excretion was mostly via urine (94% of the dose), and only a minor part was detected in the feces (3%) within 72 hours (Whitaker & Lindstrom,

1987). They chose the intravenous route as the substance class is known to induce emesis in dogs (Whitaker & Lindstrom, 1987). EEG effects of quinpirole were investigated in rats by Sebban *et al*. (1999). They discovered that the effects of D2 agonists on EEG power are dose-dependent, with an increase of power at low (0.01 mg/kg) and a decrease at higher (0.5 mg/kg) doses (Sebban *et al.*, 1999).

4 In-House Compounds

Substances available for the EEG studies were developed for the treatment of neuropsychiatric diseases, being schizophrenia (compound 1 and 2) and neuropsychiatric disorders in AD and related dementias (compound 3). Modes of action varied between compounds, so the use of the EEG under different conditions could be evaluated. Molecular targets were the dopamine-receptor 3 (D3), glycine-transporter 1 (GlyT1) and the serotonin (5HT) receptor, subtype 2c (5HT2c).

It was known from previous studies that these compounds induce different neurological symptoms in preclinical species at high doses. Pre-existing rodent and non-rodent data were available and for two of them (compound 1 and compound 3) data from NHP infusion studies existed. Route of administration was the same as intended for the EEG studies and therefore, comparison of exposure related to neurological symptoms across species was enabled. Neurological symptoms, mode of action and indication are summarized in **Table 2**.

Compound Code	Mode of Action	Indication	Symptoms in non-rodent preclinical studies	Symptoms in rodent preclinical studies
1	D3 receptor agonist	schizophrenia, substance abuse	Dogs: ataxia, jerks, emesis, convulsions	Sedation, decreased activity
2	Glycine transporter 1 agonist	schizophrenia	Dogs: Head tremor NHPs: stereotypies, aggressiveness, ataxia, tremor, twitches	decreased activity, palpebral closure, walking on toes ataxia, sedation, convulsions
3	5HT2c receptor agonist	neuropsychiatric disorders in AD and related dementias	NHP: Decreased activity, emesis, salivation, tremors, nystagmus, convulsions	decreased activity, tremors, convulsions, ataxia

Table 2: Overview on in-house compounds. Mode of action, indication and neurological symptoms in preclinical species are shown.

4.1 Compound 1

Convulsions were a symptom seen in dogs in the previous toxicology studies with compound 1 and they occurred at a maximal plasma concentration (C_{max}) of 7875 ng/mL. In rodent CNS safety assessments, a dose dependent decrease in spontaneous locomotor activity was seen at doses higher than 300 mg/kg corresponding to a mean total C_{max} of 24660 ng/mL (plasma protein binding rat: 87.1%). In the PTZ test for evaluation of pro- or anticonvulsant activity statistically significant increases in latency to tonic convulsions were observed in rats receiving oral doses between 30 (corresponding to a mean total C_{max} of 5740 ng/mL) and 300 mg/kg. At 10 mg/kg, no anticonvulsive effects were observed. Compound 1 had already been used as a model compound to explore species-specific sensitivity to convulsions in a NHP infusion study (Backes, 2016) and to establish a zebrafish locomotor assay (Cassar *et al.*, 2017).

4.2 Compound 2

With regards to compound 2, this drug candidate induced a distinct symptom in the dog, which was a tremor predominantly affecting the head. As the cause of this observation was unclear, one goal of the thesis was to explore whether the tremor was an expression of seizure activity by use of the EEG. From clinical observation, it was suspected that underlying seizures were not the cause, as the level of consciousness of dogs was not or only mildly affected and as the head tremor was unresponsive to midazolam treatment. The NOAEL for dogs was set at a dose of 3 mg/kg, which corresponded to a C_{max} of 294 ng/mL and an $AUC_{0\text{-}24\ hr}$ of 3872 ng*hr/mL. In NHPs no head tremor or convulsions were observed and the NOAEL was higher. Neurological symptoms induced by compound 2 in NHPs were stereotypic (gnawing, circling, grooming, and forepaw padding) and aggressive behavior, occasional ataxia, tremor and twitching. The NOAEL for NHPs in a 2 week study was 100 mg/kg/day for males and females corresponding with an $AUC_{0\text{-}24}$ of 34200ng*hr/mL and a C_{max} of 6620 ng/mL. In rats, decreased activity, palpebral closure, walking on toes and ataxia were main clinical signs. At doses equal to or greater than 300 mg/kg severe sedation, hunched posture and clonic convulsions (at 600 mg/kg) were observed. The NOAEL in rats was defined for male and female rats differently: 665 ng/mL was set for male rats, whereas 1290 ng/mL was determined for female rats.

4.3 Compound 3

In safety pharmacology studies in rodents, compound 3 produced a range of CNS/ neurobehavioral effects: decreased spontaneous locomotor activity, reduced grooming behavior and convulsions (oral dose 300 mg/kg, total plasma exposure 2000 ng/mL). In the PTZ seizure assay in rats, proconvulsant activity was observed at 10 mg/kg (660 ng/mL). Toxicology studies with compound 3 were conducted in rats and NHP (cynomolgus (*macaca fascicularis*)). Symptoms observed in rats were ataxia (≥20 mg/kg/day), tremors (≥60

mg/kg/day), reduced food consumption and mortality at 120 mg/kg/day. In NHP toxicology studies, convulsions were seen and the lowest exposure related to this finding was 1030 ng/mL. Other symptoms in NHPs were tremors, emesis and increased salivation. A neurological symptom of special interest in this species was nystagmus. To explore if it was a potential premonitory sign for convulsions, a dedicated infusion study was conducted. In this study, convulsions were observed at plasma drug levels between 1500 and 2000 ng/mL and nystagmus could be observed in all animals prior to convulsions. A dedicated rat EEG study was also designed to explore seizure liability and it was found that spike-wave discharges were present in the EEG in the absence of clinical symptoms preceding clinical convulsions.

IV. Materials and Methods

1 Leading Questions

1. *Can electroencephalographic premonitory signs of convulsions be identified that facilitate prophylactic anticonvulsive treatment and avoid the occurrence of convulsions in toxicology studies, thereby reducing the burden of animals?*
2. *Is combination of two procedures possible to enable simultaneous EEG recording and CSF sampling for analysis of potential biomarkers of seizure liability?*
3. *Can intravenous administration of escalating doses reduce inter-animal variability so that use of fewer animals is justified?*
4. *Is the dog a relevant animal model for seizure liability assessments and how does the dog's sensitivity for neurological symptoms compare to other preclinical species?*
5. *Can quantitative and automated analysis of dog EEG support investigation of CNS effects of development drugs?*

2 Literature Research

Literature from Public Resources

The literature research was conducted using the following search engines: PubMed®, go3R®, Google Scholar® Key words were "EEG dog", "Seizure dog", "EEG preclinical safety", "EEG safety pharmacology", "EEG sleep", "EEG veterinary medicine", "epilepsy dog", "drug induced seizures", "drug induced convulsions", "seizure classification", "EEG changes drugs", "seizure prediction", "seizure liability methods", "zebrafish seizure", "guidelines safety testing", "species selection", "drug induced neurological symptoms", "EEG apomorphine", "EEG propofol", "EEG quinpirole", "EEG midazolam", "dog apomorphine", "dog quinpirole" and variations of them. The database Pharmapendium® was used to search for reports on preclinical seizure liability of various reference compounds in the context of establishment of a zebrafish locomotor assay and knowledge from this research was also used for the selection of reference compounds.

The reference lists of relevant publications were additionally searched to find further references. The first review of the literature was done in June 2015. Ongoing literature work was conducted throughout working on this thesis.

Literature from Internal Resources

Internal databases that were used with the same key words as listed above were Pro Quest Dialogue® and ClinicalKey®. Data from toxicological studies with in-house compounds were originally collected in the Prestima Production tool (different versions; current version 6.4).

After termination of a study, all collected data were uploaded and stored in the eDocs system. This database was accessed using the current version (eDocs 4). Also, the project-specific folders stored on AbbVie servers were searched.

3 Animal Welfare

3.1 Animal Research Allowance

An animal research allowance was approved by the responsible authority (Landesuntersuchungsamt Rheinland-Pfalz, Referat 23, Mainzer Straße 112, 56068 Koblenz). Title of this permit translates as “Investigating non-rodent species-selection in neuroscience: species-specific sensitivity of the dog for neurological symptoms” (“Untersuchungen zur Speziesauswahl beim Nichtnager im Bereich Neurowissenschaften: Speziesspezifische Sensitivität des Hundes für neurologische Symptome”. It was approved in 2014 under the reference number AZ: 23177-07/G14-9-081 (G2/14). EEG recording was requested as an extension to the original permit and approval was granted in September 2015.

Study design and conduct were in accordance with the EU directive 2010/63/EU and the following acts from the legislation of the Federal Republic of Germany:

- Ordinance on the protection of animals used for experimental or scientific purposes published on 1st August 2013 (German implementation of EU directive 2010/63; BGBl. I S. 3125, 3126) [TierSchVersV]
- Commission Recommendation 2007/526/EC on guidelines for the accommodation and care of animals used for experimental and other scientific purposes published on 18 June 2007
- The German Animal Welfare Act as published on 18 May 2006, amended according to Art. 20 G from 9 December 2010; amended by Art. 1G from 4 July 2013; amended by Art. 141 from 29. March 2017. [Deutsches Tierschutzgesetz, TierSchG]
- Act on the Welfare of Dogs published on 2nd May 2001; amended by Art 3 on 12. Dec. 2013 [Deutsche Tierschutz-Hunde-Verordnung, TierSchuHuV]

Also, AbbVie is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and participates in a rehoming-program.

3.2 Study Plans

To align study procedures internally, two study plans were written according to internal standards. Study plans define the housing conditions according to species, responsibilities (e.g. study director, deputy study director, lead technician, veterinarians etc.) and timelines. Also, details on the test item (formulation, storage conditions, and dose) are assembled. All data that need to be collected during the study (body weights, food consumption, plasma

samples for PK, etc.) are defined, together with the corresponding methods (e.g. preparation and storage of samples) and timelines. The first plan defined proceedings of the surgical implantation of EEG transmitters; the second study plan determined the procedure for the testing of reference and in-house compounds. If changes were made to the original study plan, these were announced in “Amendments” and shared latest on the day of study conduct. Additional “Study Communications” containing updates on the current status of an experiment, were sent via eMail. All information related to an ongoing study is stored internally on a shared drive and in print in a raw data folder.

3.3 Veterinary Care

On days without experiments, laboratory animals were supervised by animal care technicians with at least one attending veterinarian on site on week days. During the weekends, one veterinarian was on-call.

During experiments, at least two veterinarians were present. The surgical implantation of EEG transmitters was done by a trained veterinary surgeon, invited solely for this purpose.

General veterinarian examinations were done during animal selection, prior to and after surgery and prior to and after all EEG experiments. A neurological examination was done at animal selection, after surgery and after experiments with in-house compounds. Clinical pathology and hematology was controlled prior to surgery, after surgery, prior to the testing of in-house compounds and after the testing of compound 2. Clotting parameters were controlled prior to surgery.

In addition, a regular health check with control of clinical chemistry and hematology parameters is done for all dogs kept at the research facility every six months. Once a year, an examination according to FELASA standards is conducted. Dogs are vaccinated according to current recommendations by the “German Committee of Clinical Veterinarians” [free translation of “Bundesverband praktizierender Tierärzte”].

Veterinary treatments during the experiments to treat neurological or other clinical symptoms were allowed by the study plan. They consisted of intravenous midazolam (Midazolam-ratiopham®, 15mg/3mL), subcutaneous metoclopramide (Emeprid® Injection, Cewa) or maropitant (Cerenia maropitant, 10mg/mL Zoetis Belgium SA) and subcutaneous metamizole (Metamizol Natrium, 500mg/ml, WDT). Details are described in the section of the respective experiment.

4 Animal Selection, Housing and Identification

4.1 Animal Selection

Adult intact Beagle dogs (4 males, 3 females; Marshall Bio Resources, North Rose, USA.) were selected from the in-house colony. Inclusion criteria of dogs were pre-implanted CSF ports, mild temperament, a normal general and neurologic veterinary examination and normal clinical chemistry and hematology parameters. Age of selected dogs varied between 30 and 48 months at the start of the experiment. Only male dogs with pre-implanted CSF ports were available. The method of CSF port implantation was described by M. Wilsson-Rahmberg *et al*. (1998). Ports are especially designed screws with a central opening. For CSF collection, a cannula with a stylet is inserted first through the covering skin and then through the port. Each dog requires cannulas with specific lengths. The dogs selected for this study had their ports implanted in 2013 for pharmacokinetic (PK) studies. They were not naïve but had been used in PK studies with very low compound doses and a minimal distress level. Wash-out time after PK studies is at least one month in general, and has been longer for selected dogs. For the pilot study, in which compatibility of CSF ports and EEG electrodes was explored, one dog with CSF ports that had lost patency was selected.

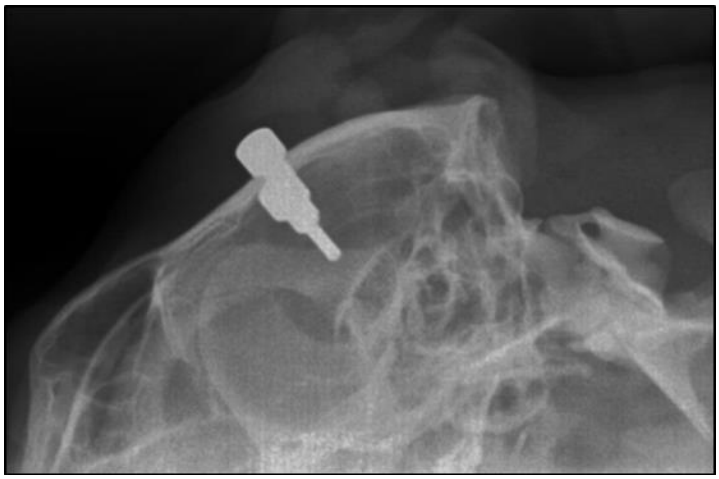


Figure 3: X-ray of a dog with CSF port. Two implants pointing in the direction of the lateral ventricles are fixed in the skull.

Mean age of selected dogs was 3.25 years which is according to Fischer *et al*. (2013) within the range of onset of idiopathic epilepsy. Changes in the thyroid hormone levels have been reported to indicate idiopathic epilepsy (von Klopmann *et al.*, 2006) and therefore, they were evaluated. Genetic predisposition to epilepsy was of interest as well, so the breeder of the research dogs was contacted for information on the incidence of epilepsy in their colony. The Marshall beagle® is a purpose-bred dog breed and its genetic background should be well known to guide selection of parent animals. Unfortunately, no answer to this request was received. However, epilepsy was not observed in any of the dogs during the study and in the whole colony for the past years.

4.2 Animal Housing and Enrichment

Dogs were group housed with males and females being kept separately in kennels with outdoor runs. Single housing of dogs was done during feeding or whenever warranted by veterinary advice or study design needs. Dogs were kept on a standard diet (Ssniff Hd Ereich, Extrudat: Ssniff Spezialdiäten GmbH, Ferdinand-Gabriel-Weg 16, 59494 Soest, Germany) which was offered once daily. Changes were made in the feeding regime only for conduction of experiments and are described later. Unfiltered tab water was available *ad libitum* at all times.

Lighting in the animal rooms consisted of daylight with additional artificial light provided in a 12 hours light cycle from 6 a.m. to 6 p.m.. Inside temperature averaged 22 +/- 4°C and was controlled together with air exchange rate and air humidity. All indoor and outdoor kennels were cleaned daily. As enrichment, dog toys, claws and chewing bones were offered.

4.3 Animal Identification and Group Assignment

All dogs had a microchip implanted and an individual number tattooed in the right ear. Individual cage cards that were kept in the animal rooms provided additional information about e.g. breeder, date of birth, responsible personnel, study number, allowance reference number etc. Male dogs were identified by odd study numbers (1001, 2001, 3001), females received even study numbers (1002, 2002, 3002). Animal assignment to the different experiments was randomly and based on animal weight, substance availability, and wash-out times and is shown in **Table 3**. In between experiments the washout-time was at least two weeks and minimum 5 half-lives of the respective test compounds.

Animal number	Sex	Compound
1001	Male	Midazolam, Apomorphine, Compound 2
1002	Female	Midazolam, Apomorphine, Compound 2
2001	Male	Propofol, Quinpirole, Compound 1
2002	Female	Propofol, Quinpirole, Compound 3
3001	Male	Compound 3
3002	Female	Compound 1

Table 3: Dog assignment to EEG experiments. As the same person was responsible for study planning, study conduct and EEG interpretation, blinding was not performed in these pilot studies.

5 Implantation of EEG Transmitters

A complete list of the material and medication used is provided in **Appendix 1 and 2**. Prior to surgery, the functioning of the each transmitter was controlled and a protocol was created in the recording program to facilitate intra-surgical recording of EEG to control compatibility of CSF and EEG implants, correct electrode placement and reconfirm implant functioning. A trained veterinary surgeon (invited for this purpose), one sterile and one non-sterile assistant performed the surgery, while one or two additional veterinarians and a veterinary

nurse prepared the next dog, given multiple surgeries on one day, and supervised recovery from anesthesia.

5.1 Preparation of Dogs for Implantation

General health of dogs was assessed by evaluation of hematology and clinical chemistry parameters and a general veterinary examination.

Antibiotic treatment (Baytril® Flavour, Bayer Vital GmbH, 5 mg/kg p.o.) was started on the day prior to surgery and continued for a total of seven days. Together with the antibiotics, dogs received oral doses of caprofen (Rimadyl®, Zoetis Deutschland GmbH, 4 mg/kg p.o.) once daily. On the day of surgery, buprenorphine (Buprenovet®, Bayer Vital GmbH, 0.02 mg/kg, i.m.) was administered and subsequent doses were given every six hours for two to three days following surgery, depending on the individual dog's clinical appearance. Additional premedication was midazolam (Midazolam-ratiopharm® 15 mg/3ml, 0.1 mg/kg, i.v.).

Dogs were fasted overnight prior to surgery. General anesthesia was induced with intravenous propofol (Propovet™ Multidose, Abbott Laboratories Ltd, 4 mg/kg for induction of anesthesia; subsequent doses of approximately 1 mg/kg with regards to clinical effect, i.v.) administered via catheter placed in the right *vena cephalica*. Prior to moving the dog from the preparation to the surgery room, it was intubated and its head and neck where shaven. Eye lubricant (Bephanthen® Augen – und Nasensalbe) was administered to both eyes. Inside of the surgery room, the dog was placed on a heating pad with the extremities supported by towels. A local anesthetic (lidocaine; Xylocaine® Pumpspray, AstraZeneca GmbH, one pump spray) was administered into the ear canals prior to insertion of the stereotaxic ear bars. Then the dog's head was placed in a stereotaxic frame (**Figure 4**). Surgical fields were prepared with Braunoderm® (B. Braun Melsungen AG), Braunol® (B.Braun Melsungen AG) and Kodan® (Schülke & Mayr GmbH). Dogs were covered with sterile foil. Anesthesia was maintained with inhalant isofluran (1.5-2.3% Isofloran CP, 1mL/mL, CP Pharma GmbH). During surgery, intravenous fluids were administered (Ringer-Lactat nach Hartmann B. Braun; B. Braun Melsungen AG, 10mL/kg/h i.v.). Vital parameters (ECG, heart rate, respiratory rate, SpO_2, CO_2, temperature) were monitored (LifeVet®, Eickemeyer®), observed continuously and recorded in a protocol by the non-sterile assistant.

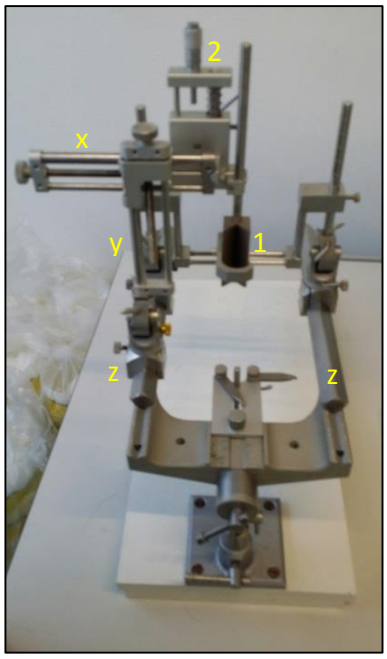


Figure 4: Stereotaxic frame used for implantation of EEG transmitters in dogs. Scaling on the three axes (x, y, z) enables the surgeon to localize exact positions for implantation. Stereotaxic coordinates for implantation were chosen by the surgeon (after external consultation). Ear bars (not shown here) are used to stabilize the animals head for an exact localization of the stereotaxic coordinates. The hand piece of the drill is attached to the stereotaxic as well (1). The drill enters the bone only in a pre-defined depth ("brake" on the stereotaxic (2)).

5.2 Pilot Study to Explore Compatibility of EEG and CSF Implants

After placing the dog for the pilot study in the stereotaxic frame, the surgical field on the head was aseptically prepared, as described above. A second field over the hip was also prepared, as it was not known whether a bone graft would be required after CSF port removal.

An incision was performed over the midline and muscle tissue was bluntly dissected from the skull. Positions for screw placement were determined using the stereotaxic frame and marked with a sterile skin marker (Mediware Skin Marker, servoprax GmbH). For this dog (and the following dogs with CSF ports), the screws that served as EEG electrodes (0-80X1-8, Plastics one) were placed 17-20 mm rostral from the inter-aural line and 10 mm on either side from the midline in the frontal bone. Prior to drilling, intravenous dexamethasone (1 mg/kg Dexamethasone Injektionslösung ad us. vet. 2mg/mL; i.v.) was administered. Then, the hand piece of the drill (Foredom®, H.30) was attached to the stereotaxic and the holes were carefully drilled. The drill bit was rinsed with sterile saline (NaCl 0.9% B. Braun Melsungen AG) by an assistant to prevent overt heating of tissue. The stereotaxic had an automated stop to prevent the drill from entering the cranial cave. One implant (DSI™ PhysioTel® Digital implants M-01) was removed from its sterile package and rinsed in sterile saline. The first centimeter of isolation was removed from both biopotential cables, bent and inserted into the holes in the skull. The screws then were manually drilled in. Thereby, the tips of the cables enrolled themselves around the screws.

The implant was turned on and identified by the software to record approximately five minutes of intra-surgical EEG. The EEG was evaluated by two persons. After the recording

was complete, EEG electrodes and CSF implants were removed in this dog. No bone graft or other sealing methods were deemed necessary. The wound was closed via subcutaneous absorbable stitches and skin staples.

5.3 EEG-Transmitter Implantation

The procedure for permanent implantation was the same in most aspects with the following exceptions: An additional field on the left side of the neck was shaven and aseptically prepared. The coordinates on the head were determined as described above and the holes were drilled. Coordinates for dogs with CSF ports were the same as used in the pilot experiment. For dogs without CSF ports, screws were inserted 5 mm rostral from inter-aural line and 10 mm on either side from the median line. Then, an incision was made in the neck and the biopotential leads were tunneled subcutaneously by using a trocar to the incision side on the head. The lengths of the leads were determined according to the individual dog's size. After the recording of control EEG, screws were drilled in and a pouch was created between the neck muscular layers to hold the telemetry transmitter. Topic bupivacaine (bupivacaine 0.25% Jenapharm® Mibe GmbH Arzneimittel) was used as an additional analgesic on muscle layers. Cables were then fixed on the skull using surgical glue (Surgibond tissue adhesive, SMI AG). Screws were covered using bone cement (Smartset GHV, DePuy CMW, Johnson+Johnson). Then, both wounds were closed with subcutaneous stitches and skin staples.

5.4 Recovery

After wound closure, isoflurane was discontinued. Spontaneous respiration was controlled in the wake up period. As the first swallowing was observed, the intratracheal tubus was removed and the dog was transferred to a heated box. Fluids were discontinued and the dog was continuously observed until it was able to maintain an upright position. Oxygen was provided via mask and in some dogs an additional heat source (3M™, Bair Hugger™) was used. When dogs were alert and able to walk, they were transferred to their home kennels. Nutri-Cal® (Albrecht GmbH) paste and canned food (Hill's™, a/d™ Restorative Care) were then offered. Clamps were removed 14 days after surgery. Hematology and clinical chemistry parameters were controlled for each dog three days after surgery.

5.5 Maintenance of Asepsis during Multiple-Day Surgeries

Establishment of aseptic conditions was challenging, as not all parts of the material could be autoclaved. This included parts of the stereotaxic frame and the drill. The hand piece of the drill was sent to an external company for ethylendioxide-sterilization, kept in an autoclaved bag from which it was only removed by the surgeon during surgery. The power cable was covered with sterile plastic tubes (Medical Technologies GmbH; camera cover folded with paper insertic aid, 13x242cm, sterile). Drilling was controlled with a foot piece, so there was

no need to touch the motor of the drill. An additional sterile hand piece was kept separate in case of potential disruption of sterile conditions that would necessitate exchanging the device. Parts of the stereotaxic frame that could not be autoclaved were bathed in a strong disinfectant (Cidex® OPA; Advanced Sterilization Products®, Johnson + Johnson) prior to surgery and then rinsed with AMPUWA. Potency of the disinfectant solution was controlled with Cidex®OPA test stripes and exchanged accordingly.

6 Setup of the Video-EEG recording Unit

For recording telemetric EEG with synchronized video, two normal dog home-kennels were equipped with custom-made attachments to hold the necessary hardware (described below). Special attention was paid to ensure that all components were out of the animals' reach and did not hinder daily cleaning procedures. Also, to avoid interferences with the signal, the metal bars of the dogs' kennels were partially replaced with a polycarbonate window.

6.1 DSI™ PhysioTel Digital Implants, M series, type M-01

The DSI™ PhysioTel Digital Implants are able to record one biopotential (one channel bipolar EEG recording), body temperature and activity. Probes for temperature and activity recording are internalized in the device. Each implant has a unique identification number to allow assignment of signals to the respective animal. Lashes around the transmitter allow its fixation during surgery. Its small size with a volume of 11 cc and a weight of 13.7 g implies minimal impairment of the animals. Usability of the implants is limited to their battery life that allows 40 days of constant measuring. Implants can be switched on by swiping a magnet over them; turning off is possible either by another magnet swipe or via software.

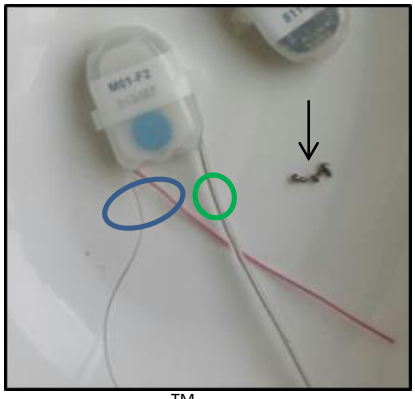


Figure 5: DSI™ PhysioTel Digital Implants M-01. blue circle: biopotential leads; green circle: antenna. Arrow: screw electrodes.

6.2 DSI™ PhysioTel Digital Transcievers (TRX-1)

Inside of the kennel, the TRX is needed for the reception of the radio – frequency (RF) signals from the implants and for their transmission to the "Communication Link Controller" (CLC). To enable acquisition, different premises have to be met: the minimum distance between the animal and the transceiver cannot be less than 40 centimeters, maximal distance is 3-5 meters. In order to obtain best quality signals, the recommended 90° angle between two transceivers was realized (see **Figure 6 and Figure 7**).

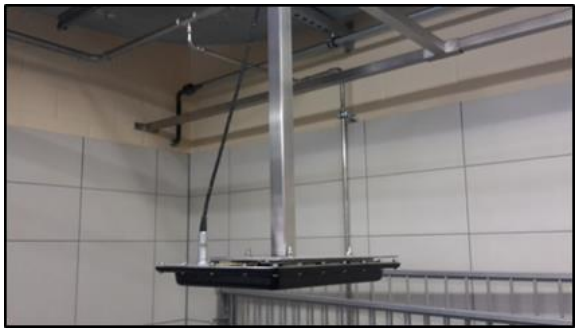


Figure 6 and **Figure 7:** Two digital transcievers; a 90° angle was chosen to realize optimal signal transmission. One is hung from the ceiling the other is at one side of the recording kennel. Metal bars have been replaced with a polycarbonate plate to minimize interferences.

6.3 The Communication Link Controller (CLC)

One CLC is able to receive signals from up to four implants via the TRX-1. It transfers them to the acquisition computer but also works bidirectional and thereby allows implant management (e.g. determination of recording properties, switching implants off).

6.4 Additional Network Components

A common router (CISCO®) was used to create the network in which the different components of the video telemetry recording unit communicate. To enable adding the multiple components to the network while minimizing use of cables, a switch, which provides power over Ethernet (PoE), was used.

The video recording unit consisted of color cameras (Axis®, P1365). They were connected to the network via switch to allow synchronization of video and EEG. Two cameras were placed in each kennel to allow 360° view of the animal.

To verify that the magnetic swipe has turned the implants on, a PhysioTel™ "Digital Power-On Detector" was used. Upon implant activation, this device gives a light and sound signal.

6.5 Setup of an EEG Protocol

EEG and video data were collected on a mobile workstation (Dell Inc. Precision M4800) with the DSI™ software Ponemah (Version 5.20) and the Noldus Media Recorder (Version 2).

The hardware needs to be assembled in a certain order: First, the router has to be powered up. Then it can be connected to a non-PoE on the switch. Next, the computer is started and connected to a second non-PoE port on the switch. The transceivers are connected to the CLC before the CLC is linked with the router, using a PoE port. The cameras are individually connected to the switch. Then, the Ponemah software (with the license dongle inserted) is started and identifies the different components. With the help of the software, a scan detects the individual implants to enable assigning them to a CLC. Then, a protocol needs to be created that defines the correct assembly of the hardware components as well as technical parameters for recording and details about the individual study. Settings that need to be adjusted prior to EEG acquisition are listed in **Table 4**.

The option to synchronize two videos with one EEG signal does not exist in Ponemah. A work-around was developed: an additional not implanted transmitter was used to enable recording from the second angle. In the protocol, a second group was created and assigned to this camera.

Parameters in the Ponemah study protocol	
Definition of information submitted per implant	Each implant can record one bio-potential, activity and temperature. In addition, information on battery life (recording days left, remaining battery capacity) can be submitted to the computer. To increase battery life, channels that are not of interest for the current study can be switched off. In the experiments described here, biopotential, temperature and activity were always sampled.
Sampling Rate	Sampling rate is chosen for biopotential, temperature and activity separately. 500 Hz were chosen for the EEG, making it priority. Secondary sampling rate for temperature and activity was 50 Hz.
Group assignment	Each implant is assigned to one group automatically. One group consists of the three channels (biopotential, temperature and activity) submitted by one implant. The video camera corresponding to one animal needs to be assigned to one group for synchronization.
Experimental protocol	Optional: Information on the individual study can be entered like: date, test substance, dosage, animal number, animal sex, responsible scientist etc. ***Note:*** Care must be taken that group name and animal identifier are the same. Otherwise, the video will not be saved for offline-analysis in DSI™ Neuroscore.
Definition of events	Events can be defined and added during the acquisition. Examples for events are: “Jumping”, “Emesis”, “Dosing”, “Animal is drinking” etc. This tool was not used in the experiments described here. Instead, all events were noted in a separately written protocol.

Table 4: Setup of a protocol in DSI™ Ponemah.

7 EEG Experiments

7.1 Baseline and Sleep Recording

The functioning of the system was controlled two weeks after surgery. Baseline recordings of 20 minutes were obtained from all dogs. The EEG from only one animal at a time was recorded in the first control EEGs. To further evaluate if the setup was suitable to visualize alterations in the EEG signal, normal sleep was recorded. Two of the three grouped housed male dogs were recorded simultaneously, starting at 6 p.m. in the evening. The recording was continued until 7 a.m. on the next day.

7.2 EEG Recording with Subcutaneous Needle Electrodes

A telemetry transmitter was connected to stainless-steel needles to test whether this would enable to record minimal-invasive EEGs with the same equipment as used with the implanted telemetry. One female dog with implanted EEG transmitters was chosen for this experiment and the signals sampled by the implanted and the non-implanted transmitter were recorded simultaneously.

The dog was fastened overnight and electrodes were placed under propofol (Propovet™ Multidose, Abbot Laboratories) anesthesia. 4 mg/kg were initially administered, with subsequent doses of about 1 mg/kg after effect to keep the dog anesthetized for a period of 20 minutes. Eye lubricant was administered on both eyes (Bepanthen® Augen- und Nasensalbe, Bayer) and the dog was placed on a mattress in lateral recumbence. A local anesthetic (Lidocainhydrochloride 2%, 2 x 1 mL s.c.) was injected subcutaneously. The two needle electrodes were then inserted subcutaneously above the implanted screw electrodes (5 mm rostral from inter-aural line and 10 mm on either side from the median line). Needle electrodes were fixed with tape (Hansaplast® Fixierpflaster Classic). A net bandage with holes cut for the ears was used to further stabilize the electrodes and hold the transmitter body at the side of the neck. The one-channel EEG was recorded for a period of 20 minutes under propofol anesthesia from implanted and not-implanted electrodes. Propofol administration was then discontinued and the dog was allowed to recover from anesthesia. Recording was continued for 15 minutes before the needle electrodes were removed.

7.3 Reference Compounds

A set of reference compounds was selected from literature for different purposes: the first aim was to explore whether drug effects can be differentiated from normal baseline activity with bilaterally implanted electrodes in dogs with and without CSF ports. Then, the technical readiness of the telemetry equipment could be evaluated under real study conditions. Lastly, the testing of relatively harmless reference compounds provided an option for training the processes of an EEG study and EEG interpretation. This included the induction of symptoms

often seen in toxicology studies, like emesis, and their differentiation from neurological symptoms.

To avoid testing a substance like PTZ that would induce severe symptoms and limit the reuse of dogs, compounds and doses were selected that were considered to be well tolerated. Two CNS stimulants, apomorphine and quinpirole, and two CNS depressants, midazolam and propofol, were chosen after reviewing the literature. Selection was limited to some degree, as the use of e.g. morphine or d-amphetamine is legally restricted.

Doses for reference compounds were based on the manufactures prescription (midazolam, propofol, apomorphin) or on literature reports (quinpirole, (Whitaker & Lindstrom, 1987)).

One male and one female dog were chosen for each experiment with reference compounds. A general veterinary examination was conducted prior to all experiments. Prior to dosing, 20 minutes of baseline EEGs were recorded. All experiments were performed in the morning, with compound administration between 8.00 and 12.00. Catheters for intravenous administration were inserted into the right *V. cephalica* in the respective experiments (midazolam, propofol, quinpirole). The investigator was not blinded to treatment or dose.

7.3.1 Midazolam

Midazolam was administered intravenously as ready-to use formulation (Midazolam-ratiopharm® 15mg/3ml) at a dose of 0.2 mg/kg. The EEG was then recorded for 20 minutes. Clinical symptoms were observed by two observers and recorded via video. Due to wrong protocol setup, the video was not saved and was not available for offline analysis. The second dog was dosed after the recording of the first one was finished on the same day.

7.3.2 Propofol

The dogs selected for this experiment were fasted overnight. Propofol (Propovet™ Multidose, Abbot Laboratories) was administered via catheter, placed in the right *V. cephalica*. After intravenous injection of a starting dose (4 mg/kg), subsequent doses (approx. 1 mg/kg) were administered by the attending veterinarian based on clinical signs to maintain anesthesia for 20 minutes. During this time, the dog was placed in lateral recumbence and covered with a towel. Eye lubricant was administered on both eyes (Bepanthen®, Augen- und Nasensalbe, Bayer). After propofol was discontinued, the EEG was recorded until the dog was awake and walking. The dogs were recorded in the morning on two different days. Clinical signs were controlled by two veterinarians and recorded with video.

7.3.3 Apomorphine

Emesis is a common finding in dog toxicology studies; therefore the potential EEG artefacts related to this symptom were of interest. As one of the available in-house compounds was a

D3 agonist, comparison of EEG effects of unselective and selective agonists was of interest as well. The two dogs were given a third of their usual amount of food half an hour prior to the start of the experiment. This was done as the effects of emesis on the EEG should be evaluated by injecting apomorphine and vomiting on an empty stomach was considered more stressful. Apomorphine was administered subcutaneously (0.8 mg/kg; Emedog, 1 mg/mL, Laboratoire TVM, s.c.). After 30 minutes, dogs were treated with metoclopramide (1 mg/kg, Emeprid® cewa, s.c.). EEG recording was then discontinued. Dogs were under veterinary observation until emesis stopped. They were fed normally after the experiment. EEGs of both dogs were recorded on the same day, one after the other.

7.3.4 Quinpirole

Quinpirole (Quinpirole-hydrochloride, Sigma-Aldrich) for intravenous administration was prepared freshly at the day of each experiment. It was initially dissolved in sterile saline (NaCl 0.9%, B. Braun) at a concentration of 6.6 mg/mL and sterile filtered through a 0.22 µm Sterivex®-GP, Millipore polyethersulfone (Merck Millipore) under a laminar flow hood.

After intravenous administration of quinpirole, clinical observations and EEG recordings were continued for the duration of symptoms. Both dogs were recorded on separate days. The male received a dose of 0.2 mg/kg which was lowered for the female dog to 0.1 mg/kg. Dogs received subcutaneous metoclopramide (1 mg/kg; Emeprid®, cewa) 30 minutes after Quinpirole administration. Total recording time was seven hours for the male and four hours for the female dog.

7.4 In-House Compounds

The use of drug candidates from the field of neuroscience enabled evaluation of the EEG for assessment of seizure liability under real-life conditions. The selected compounds had different modes of action (***see Literature, chapter 4***). The investigator was not blinded to treatment in the experiments and was also responsible for offline EEG analysis.

7.4.1 *Compound 1*

Experiments with compound 1 were conducted on two separate days, each starting in the morning at 9 a.m.. On each day, one male and one female dog received escalating intravenous doses of compound 1.

Dose Selection

Doses were selected based on pre-existing data from toxicology studies in dogs and NHPs. Previous studies in dogs used the oral route of administration, whereas NHPs had received escalating intravenous infusions. Neurological symptoms were seen in both species. Dose selection was based on the following goal: the first dose targeted plasma concentrations not

correlated with neurological symptoms. The two subsequent escalating doses targeted plasma concentrations associated with the occurrence of neurological symptoms. To determine which dosages would be needed to reach these levels, pharmacokinetic modeling experts were consulted. They established a computational two-compartment model using Phoenix software (Phoenix 64; Build 6.4.0.768), based on data from a toxicology dog study with oral administration and from a DMPK study with intravenous and oral dosing. The calculated doses were the following: 12 mg/kg (from 0-10 minutes) to target an initial mean C_{max} of 2.5 µg/mL; 27 mg/kg (from 30-40 minutes) to target a mean C_{max} of 7.5 µg/mL; 29 mg/kg (from 60-70 minutes) to target a mean C_{max} of 11.5 ug/mL and 30 mg/kg (from 90-100 minutes) for target mean C_{max} of 15 µg/mL (**Figure 8**).

Based on experiences from the first experiment with the male dog, the protocol was adapted for the female dog to reduce the overall duration and the burden of the procedure for the animal: The number of dosing steps was reduced to doses of 30 mg/kg for a target exposure of 7.5 µg/mL and 40 mg/µL for 11.5 µg/mL.

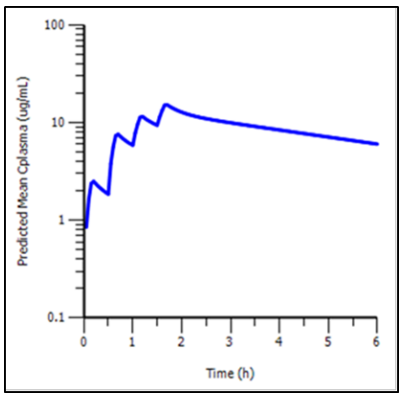


Figure 8: Dosing scheme Compound 1: Doses were modeled to achieve increasing target exposures.

Formulation

Compound 1 (succinate) was initially dissolved in DMSO (5 DMSO : 95 saline: NaCl 0.9% B. Braun) at a concentration of 20 mg/mL. The solution was then stirred in an ultrasound bath at 37°C until visually clear. Next, sterile saline was added to achieve the desired concentration of 15 mg/mL. This was done on the day before the study. Overnight, the solution was kept in a glass bottle with screw cap at 4°C. Aseptic filtering was done freshly on the next day by the pharmaceutical lab. The solution was sterile filtered through a 0.22 µm Minisart High-Flow syringe Filter (PES Sartorius 16532) under a laminar flow hood using aseptic techniques. Final concentration was determined and found to be 16.9 mg/ mL in the first and 15.9 mg/mL in the second experiment. Infusion rates were adapted accordingly (**Table 5**). Until administration, the solution was kept at room temperature.

Dog	Dose (mg/kg)	Conc. (mg/mL)	Dosing Scheme	Sample Time (min)	Target Plasma Concentration (µg/mL)	Volume (mL)	Infusion Rate (mL/h)
M	12	16.9	10 min infusion + 20 min observation	12	2.5	7.1	42.4
	27	16.9	10 min infusion + 20 min observation	42	7.5	15.9	95.4
	29	16.9	10 min infusion + 20 min observation	72	11.5	17.15	102.9
	30	16.9	10 min infusion + 20 min observation	102	15	n/a	n/a
F	30*	15.9	10 min infusion + 20 min observation	12	7.5	16.98	102
	40*	15.9	10 min infusion + 20 min observation	42	11.5	22.64	135

Table 5: Experimental design compound 1 M = male, f = female; * = adjusted dosages, based on experience from experiment 1.

Study Design

After 20 minutes of baseline recording, administration of the test compound was started using an infusion pump (B.Braun Perfusor FM, B. Braun Melsungen AG). During the time of infusion, one person was in the dog kennel and described all clinical symptoms. They were recorded by a second person who also observed the EEG trace. The handwritten records were transferred to Excel (Microsoft Office 2010) for later evaluation after the experiment. Heart rate and other physiological parameters were assessed at irregular intervals by a veterinarian during infusions and in the observation time in between. After administration of the calculated dose was finished, the infusion line was removed. Two minutes later, the dog was taken out of the kennel for collection of blood samples. For the remaining 18 minutes of this 20 minutes observation time, the dog was left in the recording kennel and supervision was continued from the outside. After these 20 minutes, the second and third doses were administered following the same procedure. A CSF sample was collected from the male dog through the implanted CSF port 100 minutes after the start of the experiment. Recording was continued for both dogs as long as clinical symptoms were present for five more hours after administration of the last dose. A control-EEG with duration of 30 minutes was recorded from the male dog to confirm recovery two days after the experiment.

7.4.2 *Compound 2*

Dose Selection

Doses were derived from previous oral toxicology studies (**Table 6**). There, doses >10 mg/kg induced the head tremor and resulted in a C_{max} of 1208 ng/mL and 1086 ng/mL for males and females respectively. Doses up to 30 mg/kg were well tolerated. Mean oral bioavailability of compound 2 in dogs was 78.4% and $t_{1/2}$ (after an oral dose of 1 mg/kg) was 5.4 hours. Modeling of doses for intravenous administration was done, but only the free

base form of the compound was available. Due to poor solubility, this form was not suitable for intravenous administration and therefore, the oral route was chosen. The calculated intravenous dose necessary to reach a target plasma concentration of 1000 ng/mL would have been 2 mg/kg. The following dose of 3 mg/kg would have reached the next target concentration of 2000 ng/mL. Each dose would have been administered over a period of 10 minutes with 20 minutes of observation time in between. An oral dose of 14 mg/kg was selected based on earlier studies. Pharmacokinetic parameters are shown in **Table 6** and **Figure 10.**

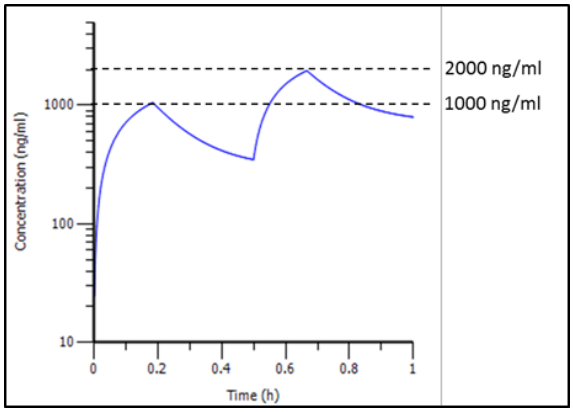


Figure 9: Modeling of intravenous doses of compound 2. Due to poor solubility, the oral route was chosen.

Sex	Dose Capsule (mg/ kg/day)	C_{max} (ng/mL)	AUC (ng x hr/mL)	$T_{½}$ (hrs)
Male	10	1208.0	9335.4	5.5
Female	10	1086.0	12689.2	6.0

Table 6*:* Plasma exposure in earlier toxicology studies with compound 2. Male and female dogs received oral doses of 3, 6, 10 and 30 mg/kg. Head tremor was observed with oral doses >6 mg/kg.

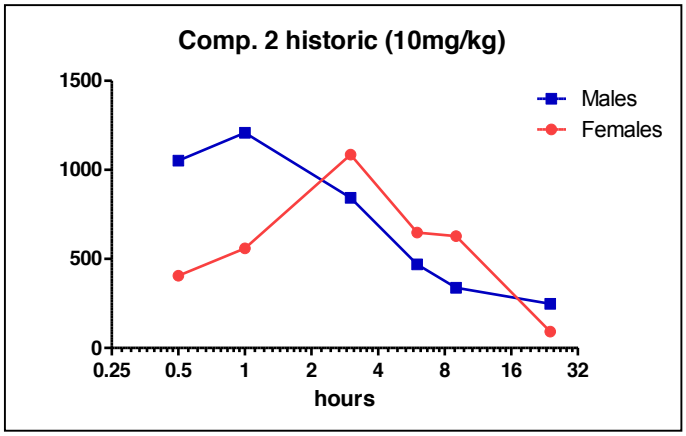


Figure 10*:* Plasma concentration of compound 2 after oral administration (10 mg/kg) to male and female dogs.

Formulation

Compound 2 was prepared freshly on the day of the experiment. The substance was dissolved in 0.2% HPMC (10 mg/mL) and then stirred continuously until administration. For administration, the appropriate volume based on actual body weight (10 mL for the female, 15.4 mL for the male) was filled in gelatin capsules to achieve the selected dose of 14 mg/kg.

Study design

After 30 minutes of baseline recording, compound 2 was administered *p.o.* via capsule (14 mg/kg). The EEG equipment was installed outside of the animal room and observations were primarily via video. The animal room was entered for collection of blood (30, 50, 120 minutes and at 6 and 24 hours after dosing) and veterinary examinations. One CSF sample was collected from the male dog 50 minutes after dosing. All study events like the time of dosing or the onset of symptoms were documented. As the male dog had not resumed normal behavior in the evening, the recording was interrupted for 10 minutes for data saving before it was restarted and continued overnight until 6 a.m. on the following day.

7.4.3 *Compound 3*

Dose Selection

Dosages of compound 3 were calculated by AbbVie pharmacokinetic modeling experts based on data from two individual DMPK studies with intravenous dosing in dogs. The modeling software was Phoenix 64 (Build 6.4.0.768) and a fitted PK two compartment model with population parameters V, V2, Cl, Cl2 was applied. Selection of target exposures was based on target exposures previously chosen for the NHP infusion study (500 ng/mL, 1000 ng/mL, 1500 ng/mL and 2000 ng/mL) under consideration of species specific protein binding. No previous experience existed with high doses of this compound in dogs. In oral NHP toxicology studies, individual animals had convulsions after single doses at lower plasma levels of 1030 ng/mL and 1390 ng/mL. **Figure 11** shows the exposure simulation that determined intravenous doses of 8 mg/kg, 6 mg/kg and 9.5 mg/kg to result in target exposures of 750 ng/mL, 1000 ng/mL and 1600 ng/mL.

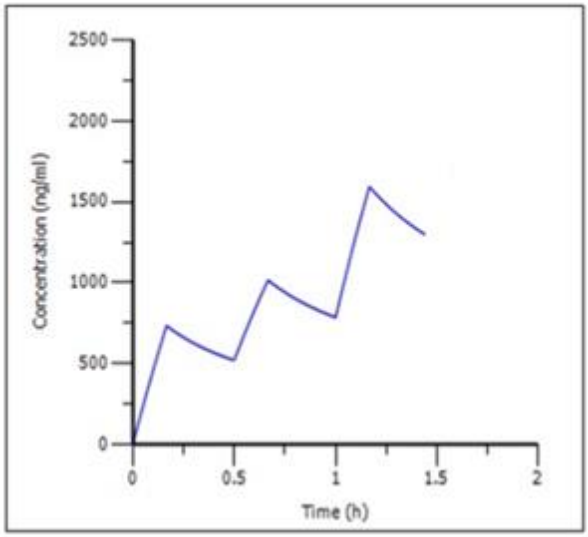


Figure 11*:* PK modeling of the dosing scheme for compound 3 Intravenous doses: 8.0, 6.0 and 9.5; target exposures: 750, 1000, 1600 ng/mL.

For the second experiment with compound 3, the model was adapted by considering the plasma concentrations measured in the first experiment. Also, higher total plasma concentrations were targeted. The first dose of 16.5 mg/kg was calculated to result in a total plasma concentration of 1500 ng/mL and the second dose of 11.0 mg/kg should reach an exposure of 2000 ng/mL (**Figure 12**). The male dog from the first experiment was selected based on higher sensitivity compared to the female due to the CSF ports.

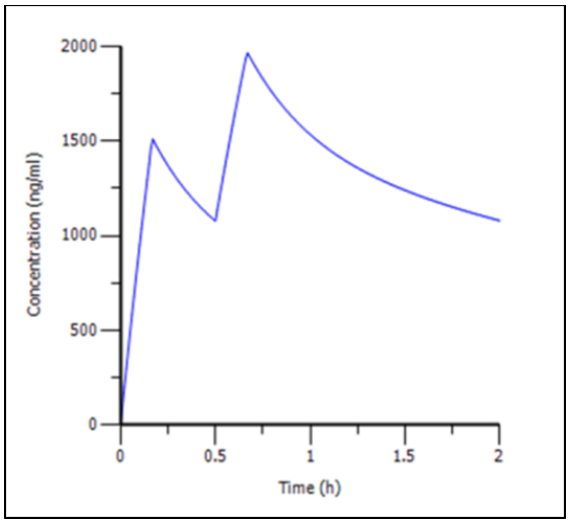


Figure 12: Second simulation for compound 3. First dose: 16.5 mg/kg (target 1500 ng/mL); second dose: 11.0 mg/kg (target 2000 ng/mL).

Dog	Dosage	Target Exposure (µg/mL)	Volume (mL)	Infusion Rate (mL/h)
3001	8.0	750	14.54	87.27
3001	6.0	1000	10.9	65.45
2002	8.0	750	9.69	56.0
2002	6.0	1000	7.27	43.63

Table 7*:* Infusion rate calculation for the first experiment with compound 3.

Dog	Dosage	Target Exposure (µg/mL)	Volume (mL)	Infusion Rate (mL/h)
3001	16.5	1500	32.0	196.2
3001	11.0	2000	22.0	132.0

Table 8*:* Infusion rate calculation for the second experiment with compound 3.

Formulation

Compound 3 was initially dissolved in D5W (5% Glucose, Deltaselect®) at a concentration of 6 mg/mL. The pH was measured with a pH meter (inoLab, pH720, WTW) and adjusted to 7.4 using 0.1 mM NaOH or HCl. Then, osmolality was controlled with an osmometer (OM 815, Typ M 10/25 µl, Löser) to ensure that the compound was suitable for intravenous administration. Osmolality was adapted to physiological conditions (290 +/- 10 mosmol) using AMPUWA® (Fresenius Kabi Deutschland GmbH). On the experimental day, the solution was sterile filtered using a 0.22 µm Sterivex® filter.

Study Design

After 20 minutes of baseline recording, the male dog received the two consecutive intravenous doses of 8 mg/kg and 6 mg/kg over 10 minutes, with a 20 minutes observation time in between. Blood samples were taken at 12 and 32 minutes, two minutes after termination of each infusion step respectively. A CSF sample was collected from the male dog 50 minutes after dosing. The EEG of the female dog was also recorded during the experiment with the male dog, so the baseline was extended by 60 additional minutes. After termination of the experiment with the first dog, dosing scheme was repeated with the female dog. Clinical observations of the dogs were recorded during the time of the experiment and transferred to an Excel sheet (Microsoft® Excel 2010). Additional blood samples were collected 24 hours after the start of dosing for both dogs individually.

ECG Recording and Analysis

EEG recording and infusion scheme were the same for the second experiment with compound 3. In addition ECG recording was performed to monitor effects of compound 3 on the cardiovascular system that had been observed in the first study. For this measurement, a jacketed external telemetry system was used (DSI™ JET – jacketed external telemetry, Prior to initiation of this experiment, the dog was habituated to wearing the jacket (Lomir Biomedical Inc.). The setup consisted of the recording laptop computer (Dell Inc., Latitude

E66440) the ECG transmitter (Model JET-3ETA-BP), a Bluetooth receiver (Bluegiga Technologies Inc., WRAP Access Server™) and commercially available adhesive electrodes (ConMed® Corporation, Softrace® Medium repositionable ECG electrodes). The recording software was, as for the EEG, Ponemah (DSI™). As described for the EEG, a protocol had to be configured prior to recording. For parallel EEG and ECG recordings, two computers were necessary, so ECG time synchronization was not automatic via software. As the individual computer times differ from each other, exact starting and stopping times of the recording were documented to enable relating both signals with each other. The dog's hair was clipped in places where the electrodes were applied. Then, the skin was cleaned with alcohol to enable secure hold of the electrodes. Electrodes were placed at the right (RA = right arm electrode, red) and left (LA = left arm electrode, yellow) forelimb and at the left hind limb (LF = left foot electrode, green). The ground electrode was positioned at the right hind limb. Electrodes were then connected with the transmitter which is stored in a pocket on the outer layer of the jacket. The inner layer consists of softer fabric and protects the electrodes. Ponemah (DSI™) records the ECG according to Einthoven (I., II, and III deviation) and Goldberger (aVR (augmented voltage right), aVL (augmented voltage left) and aVF (augmented voltage foot). Sampling rate was 500 Hz and epoch time was five seconds. First, ECG recordings were visually evaluated. The recording software Ponemah (DSI™) extracts HR and QTcv automatically and the derived data can be copied to Excel for further analysis. Prolongation of the QT interval is used as an indicator for pro-arrhythmic drug effects (El Amrani *et al.*, 2016). QTcv means that the QT interval is mathematically corrected by heart rate (Van de Water *et al.*, 1989; Prior *et al.*, 2009; El Amrani *et al.*, 2016). Graph Pad Prism (Version 5) was used to calculate median HR and QTcv.

7.5 Visual Video-EEG Analysis

Visual analysis of EEG was done during the experiment in Ponemah and then retrospectively using DSI™ Neuroscore (Version 3.2). In this program, a 1 Hz high-pass filter and a 50 Hz power-line filter were used on all EEG recordings.

All recordings were split into a baseline period and a period covering the time after substance administration. If multiple doses were administered, further divisions were made.

Recordings were visually examined in 10 second windows. Artefacts and episodes with signal drop-outs were identified visually and marked. Background activity was evaluated by considering wave morphologies, frequency and amplitude. Episodes at which the animal was asleep were marked accordingly as well as transient abnormal EEG patterns. Video was displayed simultaneously with the EEG trace during both, online- and offline viewing.

Due to the limited animal numbers, no statistical evaluation of EEG data was done. The interpreter was not blinded to animal or treatment.

7.6 Automatic Seizure Detection

DSI™'s Neuroscore software (Version 3.2) was extended with a module for automatic seizure detection. For internal validation of this seizure detection module, EEG data from mice that had received intraperitoneal PTZ was used. There are two possibilities by which the program identifies seizures and both were explored. The first option is to set an absolute threshold. In order to do this, the mean amplitude of the background activity has to be determined. The program then identifies all discharges with a higher voltage. Additional parameters can be defined to refine the automated detection, e.g. maximum voltage, which eliminates the detection of high-amplitude artefacts or signal drop-outs. The identifiers of spike-trains can also be defined manually through minimal spike-train duration and minimal/ maximal distance between spikes. The second option for automatic seizure detection is to define a dynamic threshold, which is useful in recordings with an unstable baseline.

7.7 Quantitative EEG Analysis

After artefacts and episodes with altered vigilance state had been visually identified and marked, a quantitative analysis of EEG was done in Neuroscore. Epoch time was set to two seconds for all recordings and relative EEG power was calculated for the frequency ranges defined in **Table 9.** Calculation was done by using Fast-Fourier Transformation (FFT), which is a build-in feature of the Neuroscore software.

Frequency band	Spectrum (Hz)	Color code	
Delta	0.5-4	Light blue	
Theta	4-8	Dark Blue	
Alpha	8-12	Green	
Sigma	12-16	Violet	
Beta	16-24	Orange	
Gamma	24-50	Yellow	

Table 9*:* Power bandwidths selected for quantitative EEG analysis via FFT.

Relative power [%] of EEG frequencies was then displayed in a signal grid in Neuroscore. Artefact coverage [%] was calculated for each 2 seconds epoch. Also, episodes with reduced vigilance were marked in the recording and coverage of sleep markers was calculated and added to the signal grid as well. This table was then exported to Excel (Microsoft Office 2010) where lines with an artefact or sleep marker coverage >0% were deleted.

50 to 100 artefact-free two-second-epochs were selected in Excel (Excel 2010, Microsoft®) from the baseline period and around TK sampling time points. Further data processing was done using Graph Pad Prism (version 5). For each experiment, scatter plots that show changes in the single power bands from baseline to post treatment (see supplementary data) were created. Within the scatter plots, means +/- standard deviation (SD) are shown. To investigate changes in relative power bands in the baseline period and after treatment between dogs, the median of each power band was calculated. One plot was created for

each experiment respectively showing the median power changes in each frequency band of two dogs from baseline to post treatment. Due to the small animal numbers, results are presented in a descriptive way.

7.8 Blood Sampling for Drug Level and Biomarker Analysis

Blood samples were collected two minutes after termination of each infusion step for compounds 1 and 3 (at 12 and 32 minutes; and for dog the male dog in addition at 62 minutes in the experiment with compound 1). As compound 2 was administered orally, samples were collected according to clinical symptoms 30, 50, 90, 480 minutes after compound administration. In all experiments, one last sample was drawn 24 hours after dosing. Samples for determination of drug levels were collected in a K3 EDTA S-Monovette® (Sarstedt AG & Co) and centrifuged to get plasma. Plasma samples were then stored -20°C until test item concentrations were analyzed. Serum samples were collected in a Z-Gel Monovette® (Sarstedt AG & Co). After allowing 30 minutes of clotting time at room temperature, samples were centrifuged (10 minuntes, 3000 U/min). Serum samples were then stored at -80°C prior to further use for biomarker research.

7.9 CSF Sampling for Biomarker Analysis

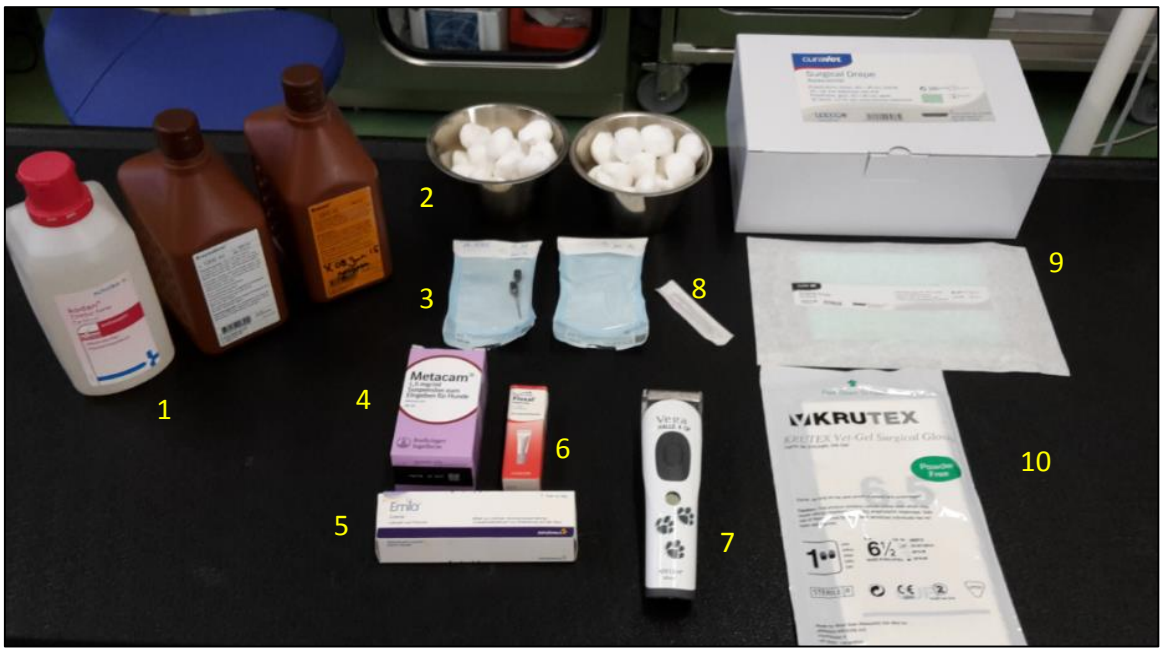


Figure 13: Materials used for collection of CSF via implanted port (1) different disinfectants (Kodan®, Braunol®, Braunoderm®); (2) sterilized gauze (3) autoclaved collection needles; (4) pain medication (Metacam® 1.5 mg/mL ad us. Vet, Boehringer Ingelheim GmbH), only in case of multiple sampling sessions per day; (5) local anesthetic (Emla®, AstraZeneca GmbH), (6) local antibiotic ointment; (7) electric razor (e.g. Aesculap® Vega/ Isis B. Braun Melsungen AG); (8) sterile cannula 20G; (9) sterile covers (e.g. CuraVet); (10) sterile gloves (e.g. KruTex Vet-Gel surgical gloves).

Male dogs used in this study had previously been implanted with CSF ports. Prior to punctuating a CSF port, a field on the animal's head was clipped. 30 minutes prior to sample collection, a liniment containing a local anesthetic was applied (Emla®, AstraZeneca GmbH). Directly prior to sampling, the field on the head was disinfected wearing sterile gloves. Three different disinfectants were used and each of them was applied three times (Braunol®, B. Braun, Braunoderm®, B. Braun, Kodan®, Schülke). After these preparations, a sterile 30G cannula was used to puncture the skin over the port. For sample collection, sterile custom-made stainless steel cannulas were used. Their lengths were previously determined according to the anatomical position of the lateral ventricles of each dog. Collection of CSF was done by inserting the cannula through the port with the dog's head in an upright position. Next, the stylet was removed and the person holding the dog bended its neck so it looked in a downward direction. CSF then was collected in autoclaved Eppendorf® tubes (1.5 mL, Eppendorf AG). The first drop was discarded as blood contamination from superficial vessels can occur. Prior to removing the cannula, the dog's head was brought to an upright position and the stylet was inserted. After instrument removal, an antibiotic ointment is administered. CSF samples were then stored at -80°C until biomarker analysis.

8 Biomarker Analysis

Aim of the biomarker analysis was identification of changes in the metabolomic profile as early indicators of neurotoxicity. Serum samples were collected two minutes after completion of each infusion step from each dog in the experiments with intravenous administration. In the experiments with compound 2, time points were according to clinical signs. Baseline serum samples from each dog had been collected at an earlier time point, simultaneous with samples for the health check prior to each experiment. For dogs treated with compound 1, baseline samples were not analyzed to enable inclusion of additional quality controls. As the number of CSF samples was limited, statistical analysis of serum sample measurements was prioritized The analysis of serum samples for biomarkers was done in cooperation with the department of DMPK, AbbVie, GmbH & Co.KG, Ludwigshafen. The statistical analysis of the high-dimensional metabolomics data was done by the data and statistical science (DSS) department, AbbVie GmbH & Co.KG, Ludwigshafen.

8.1 Technical Methods

Serum and CSF samples were frozen at -80°C. They were thawed once for aliquot preparation. 15 µL of serum were distributed to protein-low bind Eppendorf® tubes (1.5 mL, Eppendorf AG). After finalization of the last EEG experiment, they were submitted to the DMPK department for metabolomics phenotyping with the Absolute IDQ® p180 Kit (Biocrates Life Sciences, Insbruck, Austria). This kit analyzes up to 188 endogenous metabolites of different classes (acylcarnithines, aminoacids and biogenic amines,

monosaccharides, sphingolipids and glycerophospholipids) (see: List of Metabolites: http://www.biocrates.com/images/ListofMetabolites_p180.pdf; (BIOCRATES, 2017)).

8.2 Statistical Methods

Software for statistical analysis was R version 3.3.2 run in RStudio version 1.0.143. The package "limma" was used to stabilize the variance across metabolites and reduce the influence of outliers. Time points between animals were not exactly identical, so the time points with the most similar exposure levels were chosen. With compound 2 and compound 3, pre- and post-treatment levels of all metabolites were compared using a paired t-test for each post-treatment time point. For compound 1, no baseline metabolite levels were available from the same animals, so baseline data from other dogs were used and a 2-sample unpaired t-test was performed for each time point. A Benjamani-Hochberg procedure was used to control the false-discovery rate (FDR). Results are presented for significant differences at an FDR of $q < 0.05$ and, in some cases, for $p < 0.05$. Significantly changed metabolites were compared amongst the three treatments for potential overlaps by creating a Venn diagram. The relation of identified metabolites for a specific pathway was then investigated. Results are presented with fold changes, which are the ratios of the metabolite values from treated samples to untreated samples.

V. Results

1 Animal Selection: Assessment of General and Neurological Health

All dogs were of good general health and had no neurologic abnormalities before surgical EEG implantation and after recovery. Body temperature before surgery ranged between 37.8°C and 38.7°C. Capillary refill time was below two seconds in all dogs. Heart rate was variable, ranging from 87 to 112 bpm. Hematological and clinical chemistry parameters before and after surgery were within normal limits. The thyroid function of all dogs was normal.

2 Surgical Implantation of EEG Transmitters

2.1 Pilot Study: Intra-surgical EEG Evaluation and Removal of CSF Implants

Implantation of EEG electrodes in addition to existing CSF ports was possible. The coordinates were adapted and the EEG recorded during the pilot study was evaluated by two researchers. Good EEG signal quality was observed (**Figure 14**).

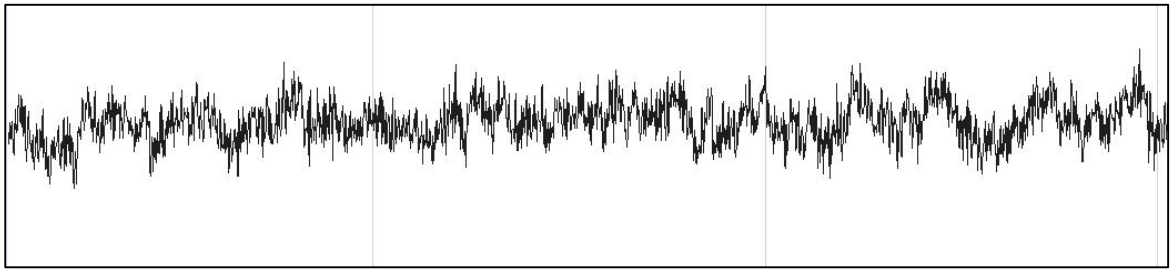


Figure 14: EEG recorded during the pilot study. Signal quality was evaluated by two different researchers.

Removal of EEG and CSF implants in this dog was possible. Duration of the procedure was three hours from induction of anesthesia till extubation. Neurological examination of the dog was normal after the procedure. The dog was kept for an additional 6 weeks for recovery, then it was submitted successfully to an adoption program. Control X-rays were made four weeks after surgical implant removal (**Figure 15** and **Figure 16**). In the ventro-dorsal recording (**Figure 15***, to the left*), a round brightening on the right side could indicate the former position of one of the CSF ports, but is too large compared to the actual screw size. Also, no corresponding spot is visible on the other side. Therefore, good healing of the implantation sites was diagnosed. Former implantation sides of the EEG electrodes are not visible in the x-ray.

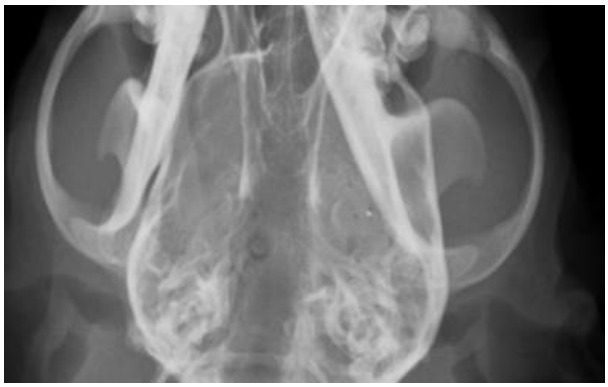



Figure 15 (left) and **Figure 16** (right): Ventro-dorsal and latero-lateral control X-rays 4 weeks after surgical removal of CSF ports and EEG electrodes of the dog used in the pilot study. X-rays indicate a good healing process.

2.2 Implantation of EEG Transmitters in Dogs with and without CSF Ports

DSI™ does not recommend electro cautery during implantation of telemetry devices and will not account for proper device functioning if this is disregarded. We used electro cautery prior to transmitter implantation to stop bleeding of superficial vessels and no negative effects on device functioning were observed. EEG implants are well tolerated and have kept patency for 18 months so far.

Duration of implantation was 1.8 hours (mean, range 1.30 hours – 2.03 hours). All dogs resumed sternal recumbence within 20 – 40 minutes after extubation. Female dogs recovered better from anesthesia and were transferred to their home cages faster (mean: 4.6 hours; range 3.5-6hours). One male dog had self-resolving vertical nystagmus for a period of five hours following surgery and was transferred to his home cage after six hours. Surgery of male dog 2001 was in the afternoon, and it was decided to keep him in the waking box overnight. Male dog 3001 was transferred to his home kennel after 10 hours.

As recovery of all dogs was good, buprenorphine (Buprenovet® Multidose 0.3 mg/mL,Bayer Vital GmbH) was discontinued after four administrations (three on the day following surgery, one on the second day following surgery). Caprofen and enrofloxacin were continued for seven days following surgery. Clamps were removed after 14 days. The post-surgical clinical chemistry and hematology parameters were within normal limits and indicated good recovery. Control-EEGs after removal of clamps were normal.

3 EEG Experiments

3.1 Baseline Recordings

3.1.1 Visual Analysis

During the second week after surgery, function of the EEG transmitters was controlled and 15 - 20 minutes of EEG were recorded from each dog. By visual analysis, no abnormal transient EEG patterns could be identified. Recording quality varied, depending on the grade of activity of the dog which corresponded to an increase in artefact incidence. Best recordings were obtained when the dog was left alone and sat or lied down. The predominant pattern in wake dogs was beta activity, with average amplitude of 30 µV and a frequency of 20 Hz (**Figure 17**). Alpha activity indicated a reduction in the vigilance level (**Figure 18**).

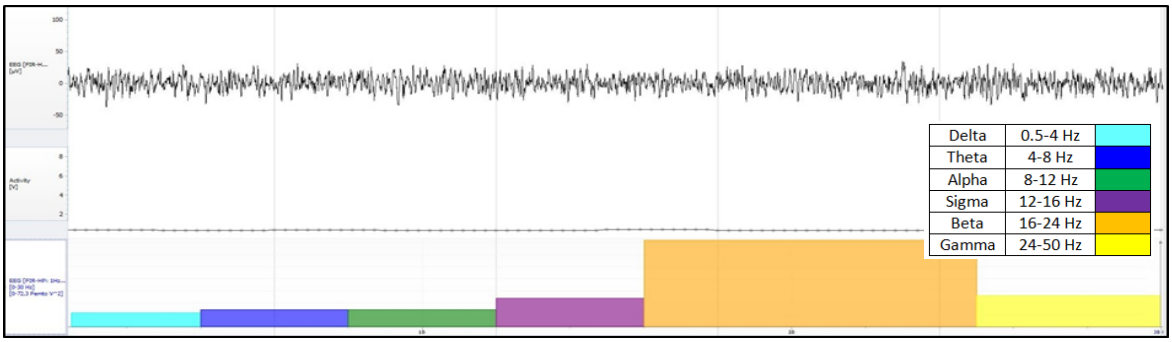


Figure 17: 10 seconds baseline EEG of an awake dog: no artefacts, beta activity is confirmed by FFT analysis (beta = orange).

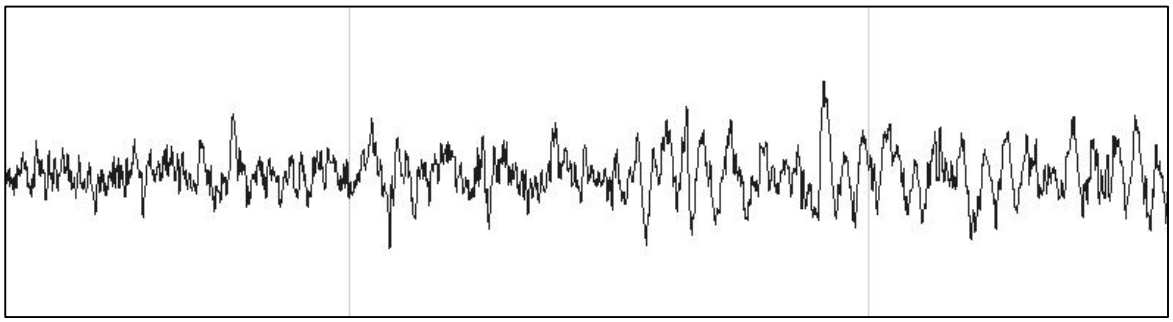


Figure 18: 10 seconds of baseline EEG: no artefacts, beta and alpha activity, indicating a reduced vigilance level.

Artefacts were present in all EEG recordings. Movement artefacts were the most common ones and often exclusion of up to several minutes of EEG data was necessary. Movement artefacts are not only a result of muscle activity, but turning of the dog's head causes the cables underneath the skin to bend and thereby the electric input is altered. This could be observed with normal dog movements or when the dog was handled. Artefacts were distinguished from normal EEG activity by relating the animals' activity recorded by the transmitter and the video to abnormal EEG patterns. Paroxysmal EEG activity simultaneous

to increased animal movements or certain behaviors was considered as artefact. In **Figure 19,** the correlation between increased movements and occurrence of EEG artefacts is illustrated.

The implanted accelerometer detects movements of the whole animal along the three axes of the room. Movements of single body parts are not reflected on the activity trace but can induce changes in the EEG, e.g. when the whole body moves along with tail movements and causes bending of cables. On the contrary, animal movement does not always induce overt EEG artefacts. This can be seen in **Figure 19** as well.

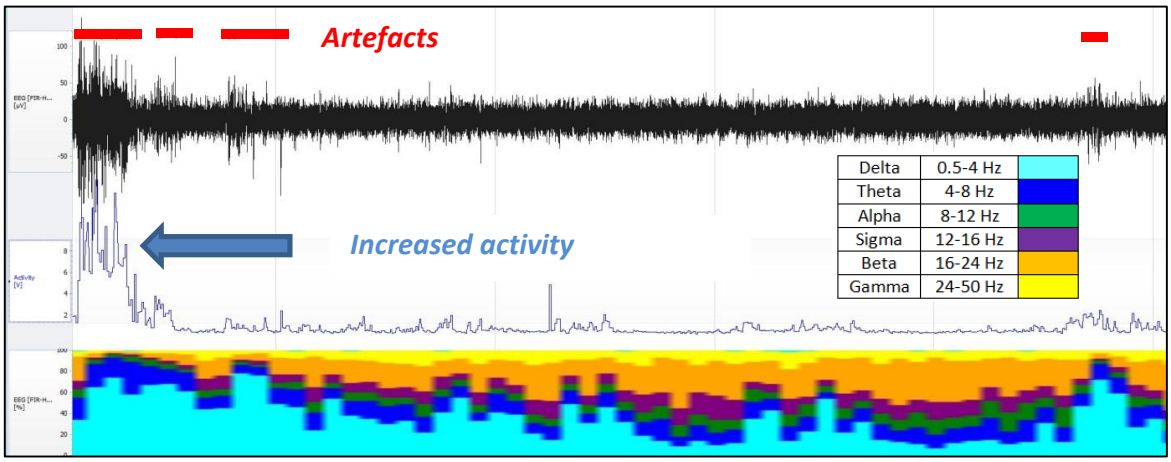


Figure 19: 10 minutes of baseline recording Movement artefacts marked red. The arrow points at the activity trace; a good correlation of increased movements to EEG artefacts is illustrated. In addition, the influence of artefacts on spectral analysis is demonstrated: in unaffected regions, beta activity dominates whereas there is an increase in delta activity during animal movements.

Artefacts from muscle activity were also amongst the most common ones. Muscle potentials have a high voltage up to millivolts. This distinguishes them from brain activity which is in the microvolt range. Muscle activity can completely hide brain potentials. High-voltage activity in the EEG with no behavioral correlation was estimated to result from activity of the *m. masseter* or tongue movements. Occurrence of such artefacts could be provoked when the dogs were fed (**Figure 20**) or drinking.

In some cases, the video does not show a clear origin of the artefact. This is the case with eye movements that cause slow waves in the EEG (**Figure 21**). The eye ball functions as a dipole and eye movements therefore are reflected in the EEG. In human EEG recordings, an EOG is therefore often recorded along with the EEG. This was not done in the studies for this thesis and it was found that video is not sufficient to monitor eye movements.

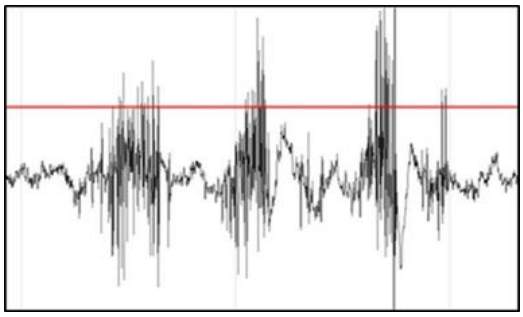


Figure 20*:* Muscle artefacts (4 seconds EEG, male dog). Dog was fed so artifacts are from jaw muscles and the tongue.

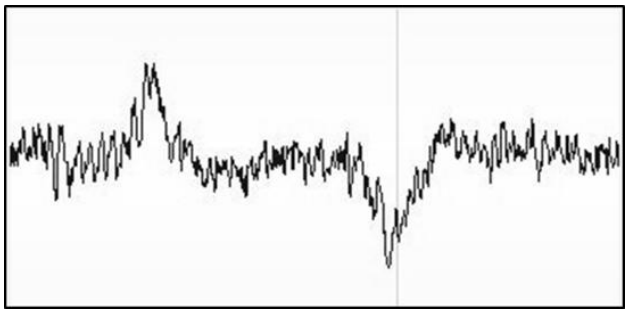


Figure 21:Eye movements (2.5 seconds EEG, male dog).

EEG artefacts can also be caused by the electric activity of the heart muscle and are a common finding in clinical EEG recordings. Therefore an ECG is often written simultaneously with the EEG to monitor appearance of artefacts simultaneous with QRS complexes. No abnormal EEG patterns that could be due to ECG artefacts were identified in all of the recordings. In one study, an ECG was written simultaneous to the EEG but was not timely synchronized and could therefore not be used to identify artefacts.

Isolated signal drop outs occurred in most recordings. Their duration varied between several seconds to some minutes at maximum. Signal drop-outs occurred predominantly at the beginning of the recording period and their cause could not be determined. They were excluded for qEEG analysis.

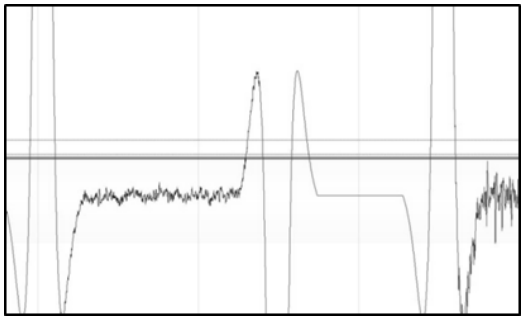


Figure 22: Signal drop out (6 seconds EEG, female dog). Some seconds of normal EEG activity can be distinguished.

3.1.2 Quantitative Analysis of Baseline Recordings

To control whether quantitative EEG analysis was possible, two baseline recordings were selected from each dog to check for inter-animal variability. After removing artefacts, a Kolmogorov–Smirnov test was run on each individual baseline to check for normal distribution (95%) and the result was that neither relative nor absolute power were normally distributed. Then, medians of the individual power bands from the two separate baseline recordings were calculated for each dog. Results are shown in **Figure 23**. The medians for each relative power band are similarly distributed in two individual baseline recordings for all the dogs. No further statistical tests were run, due to the small animal numbers.

However, with these results it was assumed that baseline qEEG parameters were stable enough to enable detection of possible treatment effects.

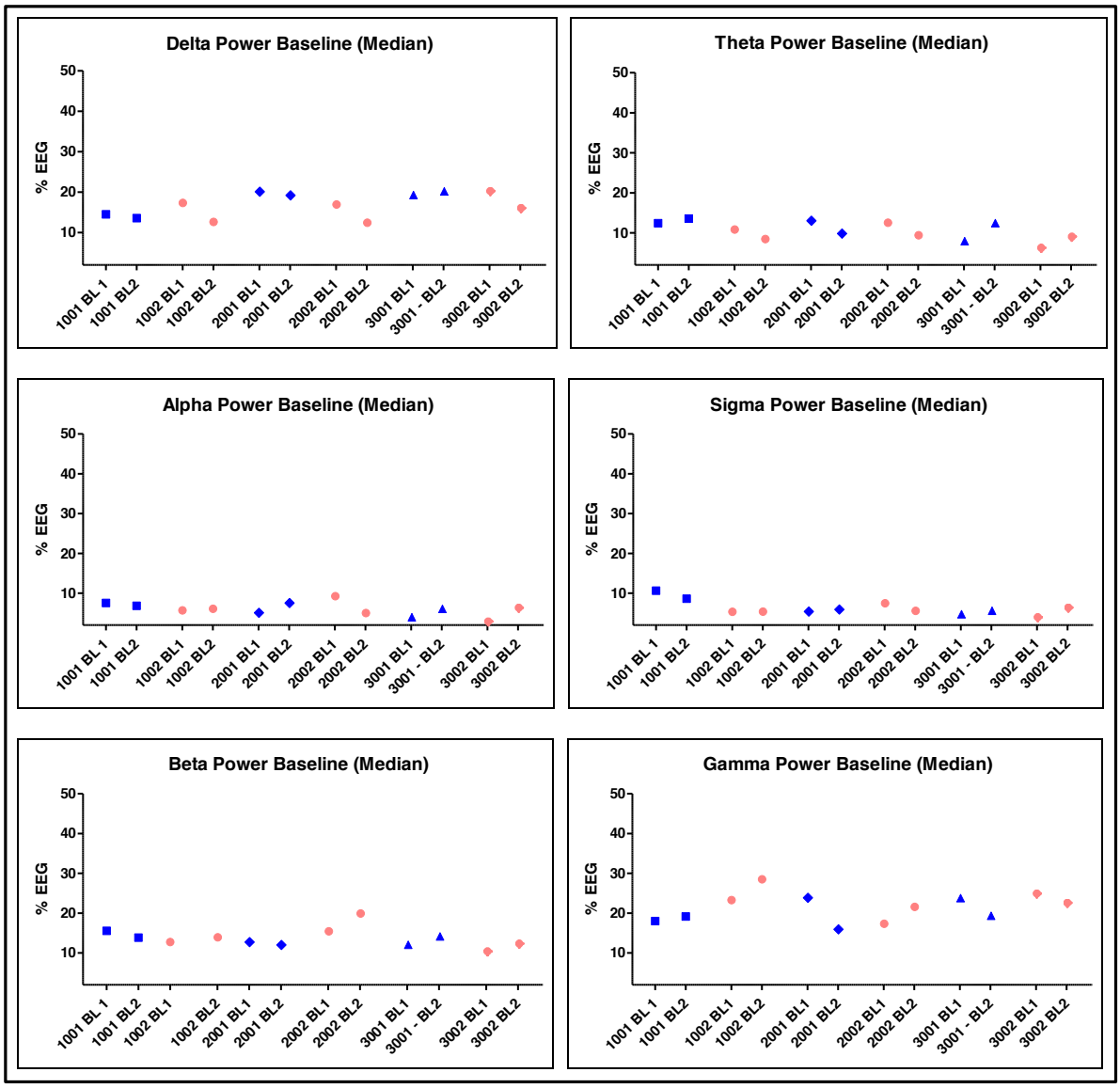


Figure 23*:* Median relative power of 2 individual baseline recordings from each dog

3.1.3 Sleep Recordings

In overnight recording sessions, different sleep stages were recorded. A hypnogram, as in **Figure 24**, shows the transition between the different sleep stages over time. Sleep stages can be defined differently, depending on species or focus of a study. Neuroscore (DSI^{TM}) allows differentiation of six different stages in large animals, that are displayed on the y-axis of the hypnogram (top-down: active wake = A, wake = W, REM = R, 1, 2, 3). In preclinical research, the differentiation between two deep sleep stages (2 and 3) is often not done, e.g. if only changes in REM duration in treated animals relative to control animals is investigated.

For creation of the hypnogram (**Figure 24**), the sleep stages 1 (light slow-wave sleep), 2 (deep slow wave sleep) and REM (rapid eye movement sleep) were differentiated from "wake". A decrease in vigilance is paired with an increase in the slower frequency components of the EEG (theta, delta, alpha > sigma, beta, gamma). In light (**Figure 26**) and deep (**Figure 27**) sleep, spindles could be identified as well as K-complexes. Spindles had an average amplitude of 100 µV. REM sleep (**Figure 28**) visually resembles the wake state as the background is dominated by faster activity. However, theta activity is present at the same time and can be used to identify this sleep stage.

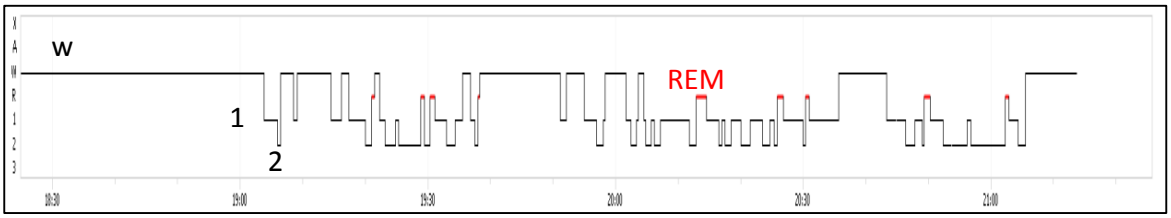


Figure 24*:* 4 hour hypnogram w=wake, 1=light sleep, 2=deep sleep, REM=rapid eye movement sleep.

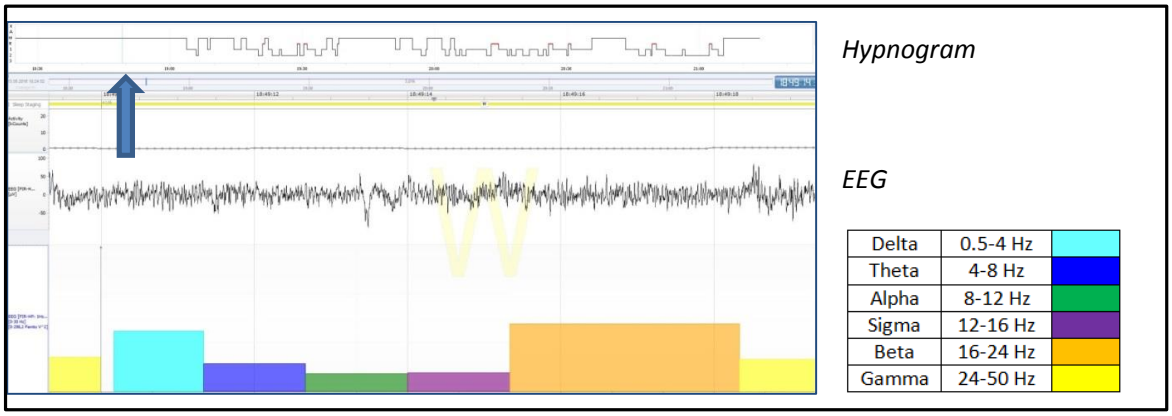


Figure 25: 10 seconds of EEG of a wake dog. The hypnogram (4 hours) is shown above. ↑ indicates the position of the EEG in the hypnogram. FFT analysis shows a predominance of beta activity.

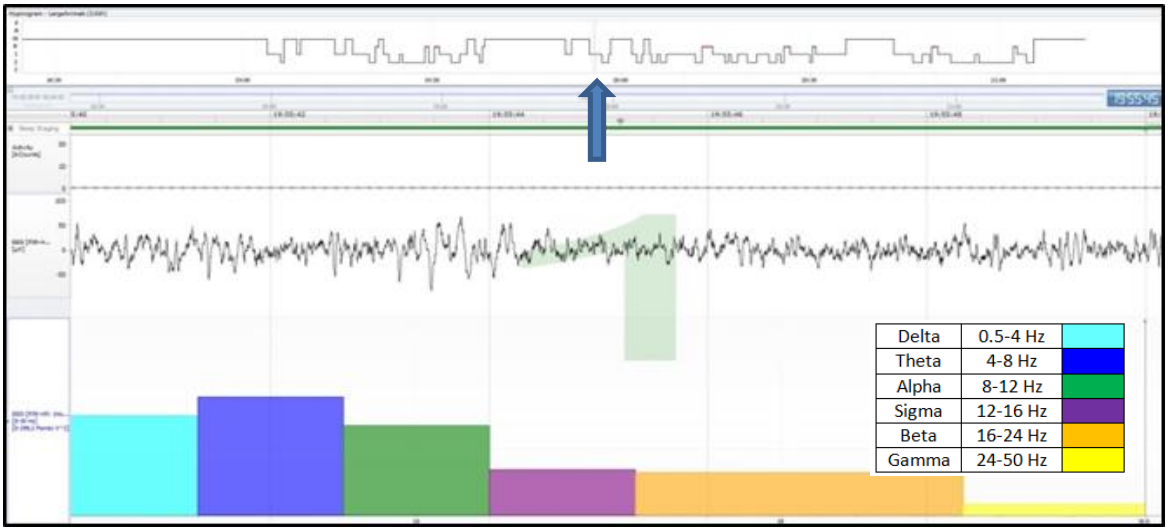


Figure 26*:* 10 seconds EEG during light sleep: ↑indicates where the excerpt is in the hypnogram.

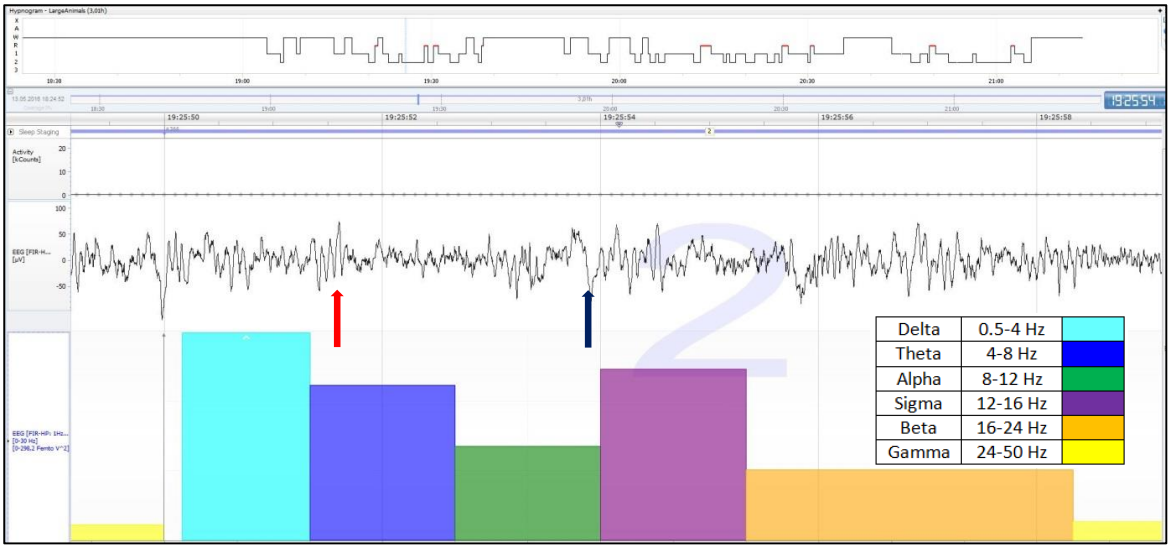


Figure 27: 10 seconds of EEG from a dog in deep sleep. Sleep spindles (red ↑), K-complexes (blue ↑) The light blue ↑indicates where in the hypnogram the 10 seconds of EEG are located.

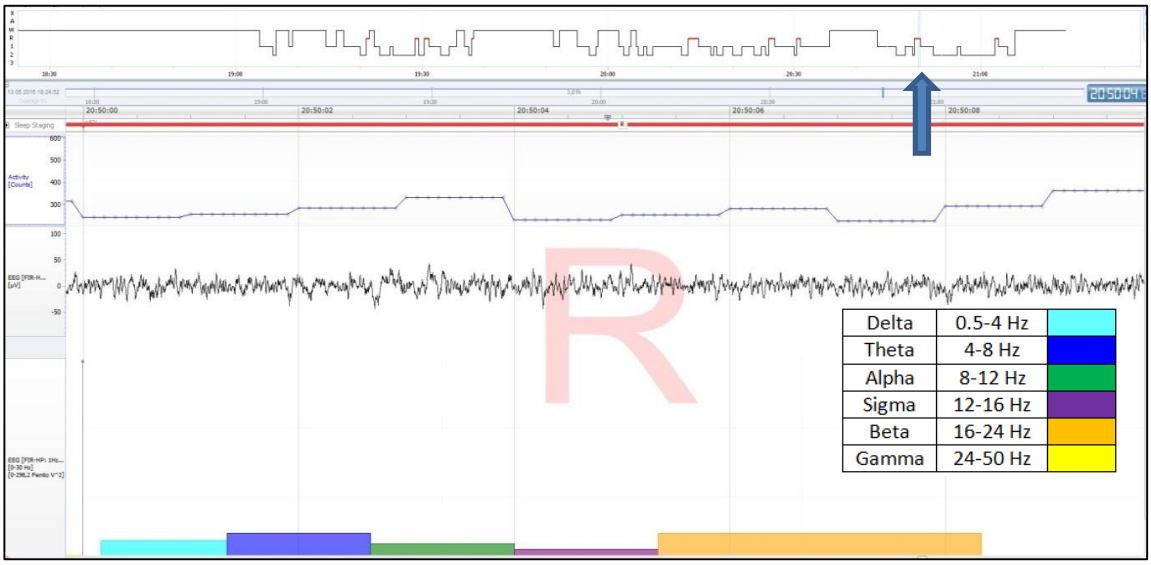


Figure 28: 10 seconds of EEG from a dog in REM sleep. ↑indicates where in the hypnogram the 10 seconds of EEG shown are located.

3.1.4 Comparison of EEGs Recorded with Subcutaneous and Implanted Electrodes

Simultaneous recording of the EEG from implanted and subcutaneous needle electrodes was possible. Amplitudes of the subcutaneously recorded signal differed by a factor 3-5 to the signal recorded with implanted electrodes. Peak voltages are 50 µV at implanted electrodes compared to 10 µV at subcutaneous electrodes. Number of artefacts is higher in the recording from the subcutaneous needle electrodes than in the recording from the implanted electrodes. Some artefacts were present in both EEG traces and appeared simultaneously (**Figure 29**). In the subcutaneously recorded EEG, artefacts caused the baseline to oscillate with very high amplitudes. In cases in which artefacts were limited to

the EEG from subcutaneous needle electrodes, they were probably caused by movement of the needles or the transmitter.

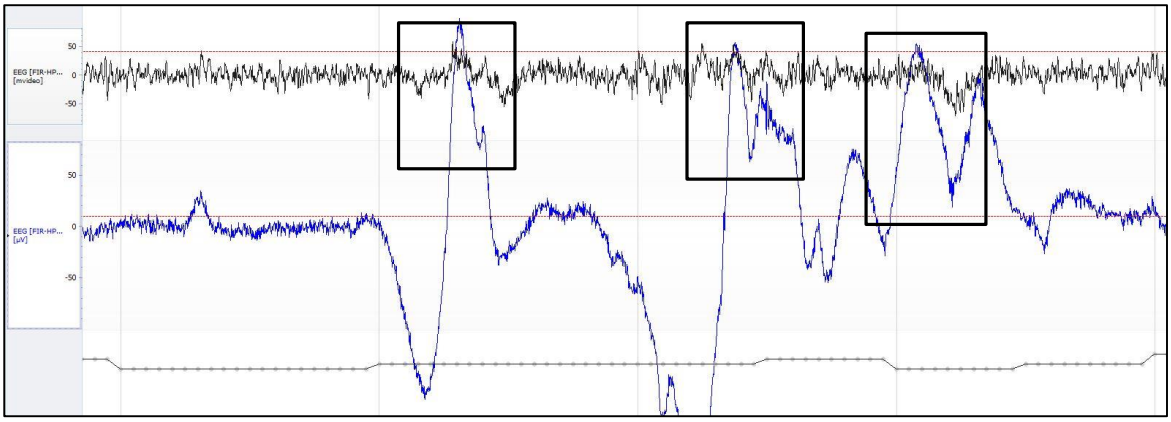


Figure 29: Simultaneous recording of EEG from implanted and s.c. needle electrodes. The upper trace is from implanted electrodes, below is the recording from s.c needle electrodes. Artefacts are present in both with s.c. > implanted, but sometims seem to be “mirrored” (rectangles).

Propofol anesthesia resulted in a decreased frequency and higher amplitude in the EEG trace recorded with the implanted electrodes. Also, typical transients like spindles could be identified. In the recording from the subcutaneous needle electrodes, an increase in amplitude to 20 µV was induced by the anesthetic. The increase inamplitude and the elimination of artefacts lead to similar graphical appearance of both traces. Frequency seemed to be unaffected (**Figure 30**).

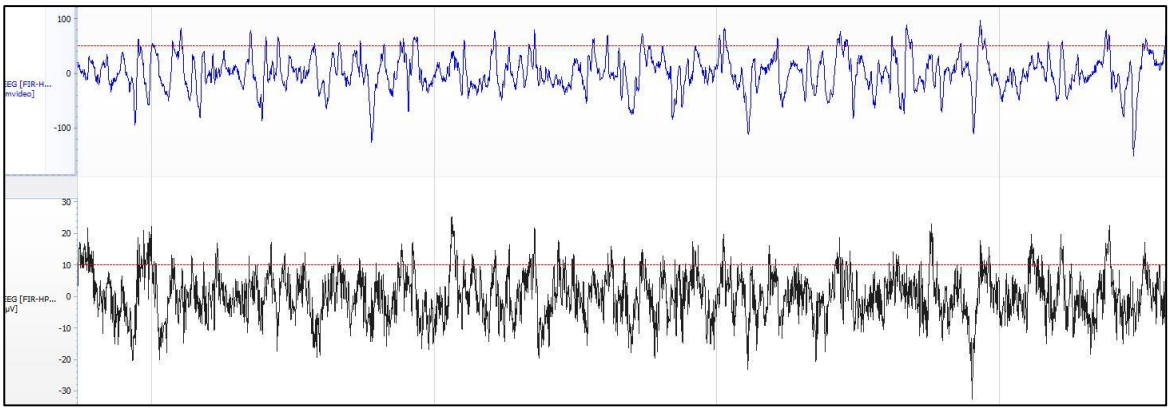


Figure 30: EEG from implanted and subcutaneous electrodes under propofol anesthesia. A slowing of background activity is evident in the upper trace (implanted electrode). With the subcutaneous needle electrode (lower trace), only an increase in amplitude can be observed compared to baseline recordings.

3.2 Reference Compounds

3.2.1 Midazolam

Clinical Observations

After intravenous midazolam, the male and the female dog showed an initial mild sedation lasting less than one minute which was followed by a paradox reaction involving increased sniffing and excitation. This effect was more prominent in the female dog than in the male and lasted for approximately 10 minutes. There were no other clinical observations.

EEG Results: Visual Analysis

No abnormal EEG activity was noted during baseline recordings (**Figure 31**).

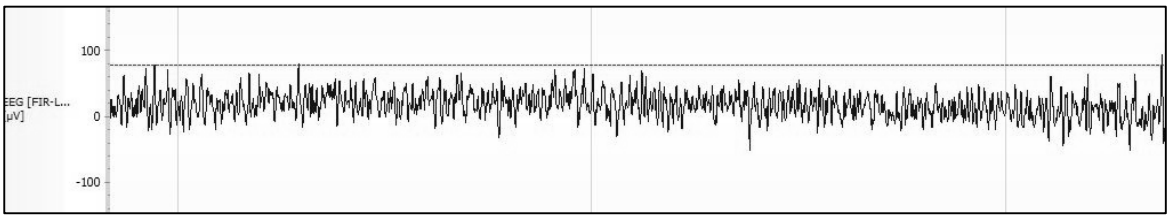


Figure 31*:* Baseline prior to midazolam adminstration (10 seconds, male dog).

A mild slowing of the EEG occurred temporarily with peak amplitudes of 50 µV. Spindle-like patterns could be distinguished within the first 5 minutes after midazolam administration (**Figure 32**). They had a low incidence (3-4 per dog after midazolam administration) and their amplitude was around 80 µV. A transient slowing of background activity appeared within one minute after intravenous administration of midazolam that lasted 20 seconds (**Figure 33**). Similar episodes were identified at irregular intervals during the next ten minutes.

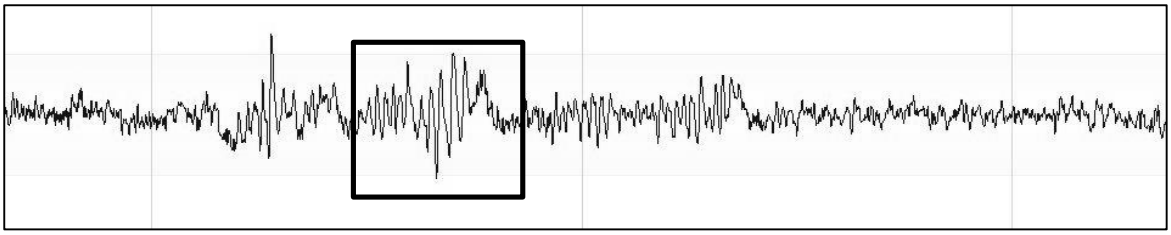


Figure 32*:* 10 seconds of EEG recording (female dog) with spindle-activity after midazolam administration. Spindles (marked) were present within the first five minutes after midazolam administration.

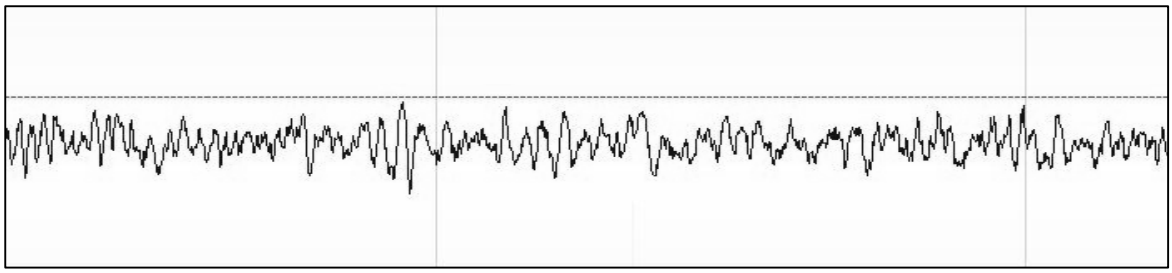


Figure 33*:* 10 seconds of EEG recording (female dog) with sinusoidal, slow waves: This pattern appeared within the first minute after intravenous midazolam administration and lasted 20 seconds. Similar episodes were observed for the next ten minutes following midazolam administration.

EEG Results: Quantitative Analysis

In the male dog, an increase in relative delta power was observed after administration of midazolam. In the female dog, the slower frequencies showed a slight decrease while faster frequencies increased. Results for both dogs are shown in **Figure 34**. No homogenous changes were seen. Raw data plots are provided in the supplementary data (**Appendix 4**).

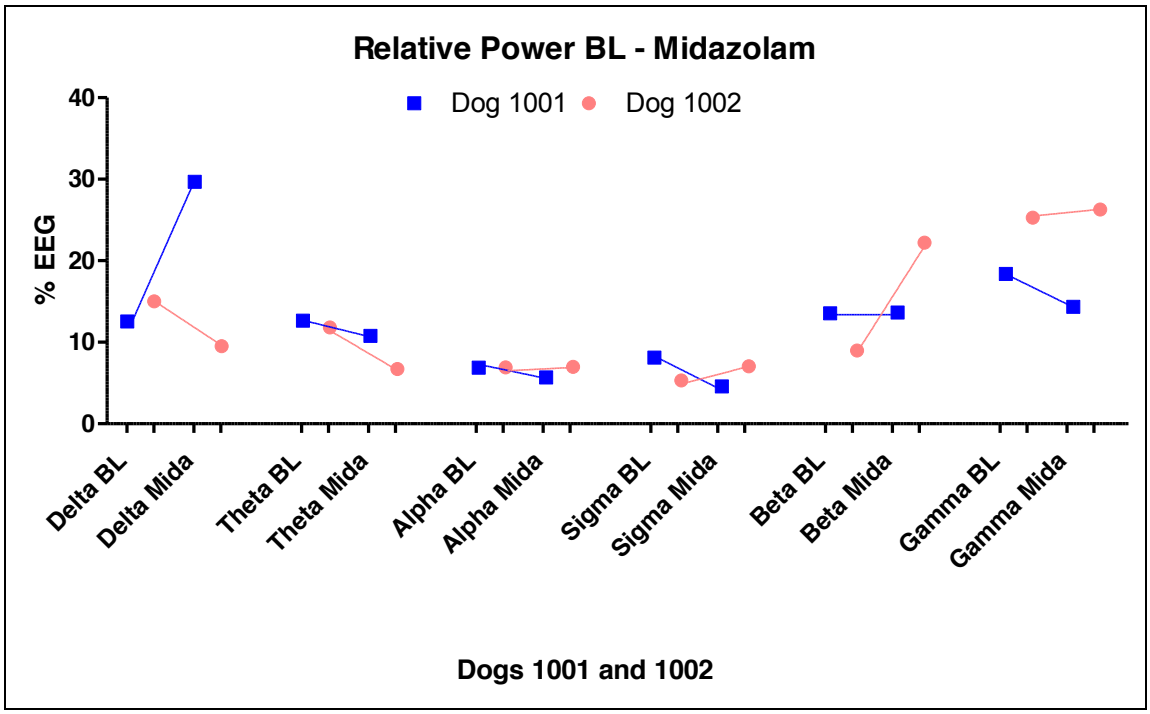


Figure 34: Median relative power changes in male and female dog after administration of midazolam.

3.2.2 Propofol

Clinical Observations

Intravenous injection of propofol resulted in rapid loss of consciousness. Breathing rate and responses to external stimuli, e.g. lid reflexes, were regularly controlled by a veterinarian. When the lid reflex could be induced, a subsequent dose of propofol was administered and thereby anesthesia was maintained for 20 minutes. After the last dose was administered,

both dogs regained consciousness rapidly within five minutes. They were returned to their home kennels after approximately another 30 minutes.

EEG Results: Visual Analysis

EEG during baseline recording was normal (**Figure 35**). Changes after propofol administration were as expected from literature reports (Bergamasco *et al.*, 2003). The low amplitude (around 50 µV) and high frequency that are characteristics of beta activity changed and delta activity became predominant. This consisted of low frequencies coupled with high amplitudes around 100 µV (**Figure 36** and **Figure 37**). Sleep spindles (**Figure 37**), K-complexes and vertex low waves could be identified. A hypnogram was created (**Figure 38**) to reflect the varying levels of anesthesia, namely the progression to lighter plane before administration of a subsequent dose.

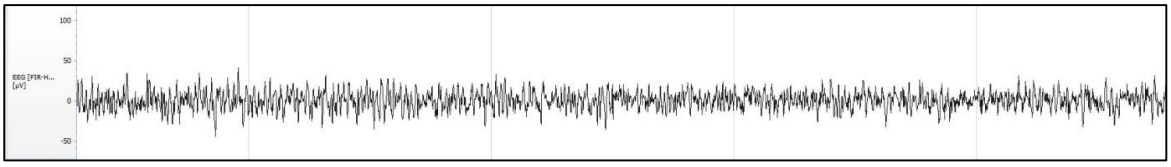


Figure 35*:* Normal baseline EEG (10 seconds, male dog).

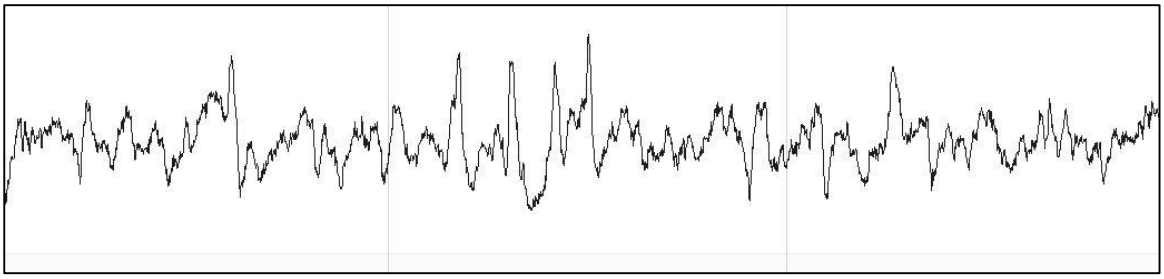


Figure 36*:* Delta waves present after administration of propofol (6 seconds, male dog).

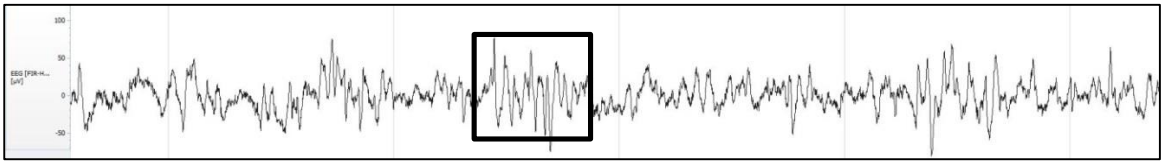


Figure 37*:* Delta waves and spindle activity (marked) after administration of propofol, (10 seconds, male dog).

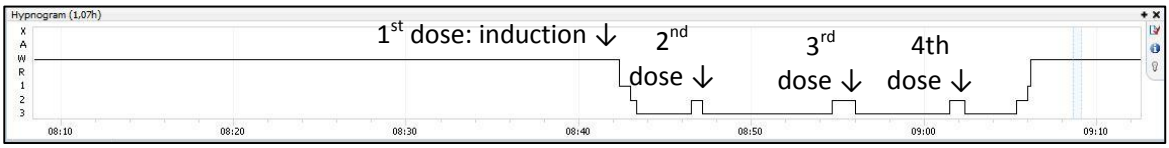


Figure 38*:* Hypnogram during propofol anesthesia. A lighter anesthetic plane can be detected with the EEG and the EEG depressant inflence of the subsequent dose of propofol can be illustrated.

EEG Results: Quantitative Analysis

Median relative power was calculated for the baseline and during propofol anesthesia. Frequency changes of both dogs show the same trend for all power bands. After propofol, an increase in the lower frequencies (delta, theta, alpha) is evident for both dogs. Relative beta and gamma power were reduced during propofol anesthesia (**Figure 40**). Raw data plots are provided in the supplementary data (**Appendix 4**).

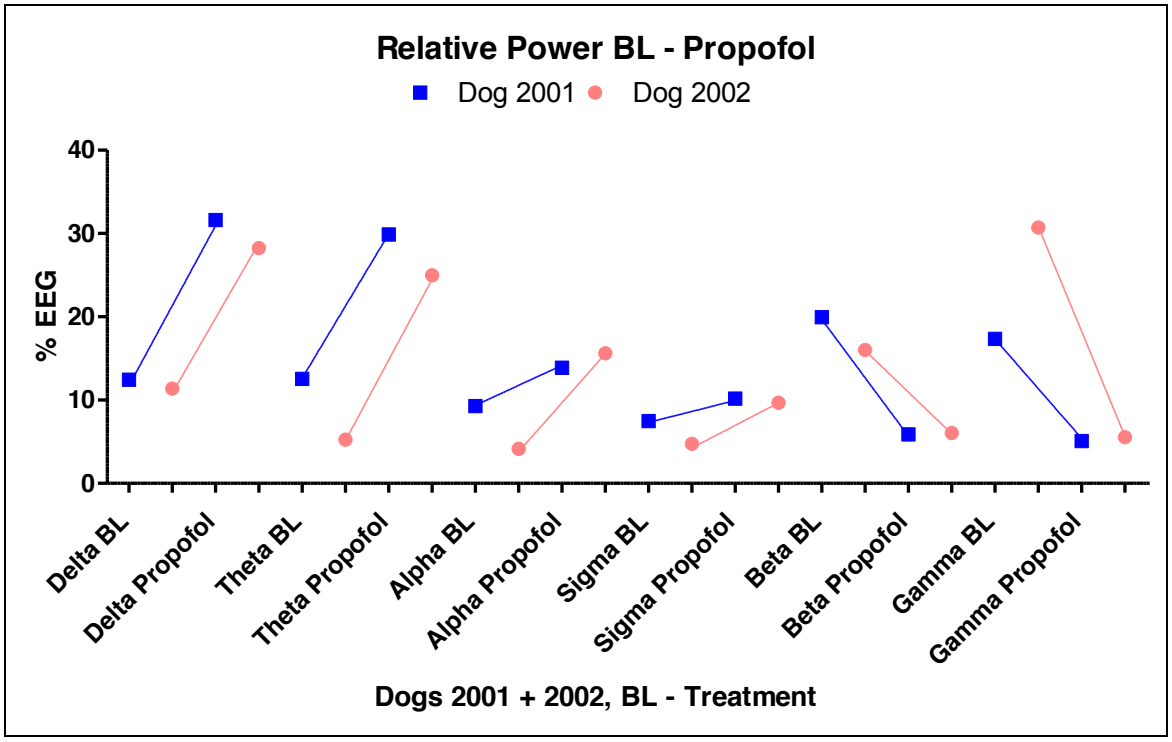


Figure 39: Median relative power changes in male and female dog after administration of propofol.

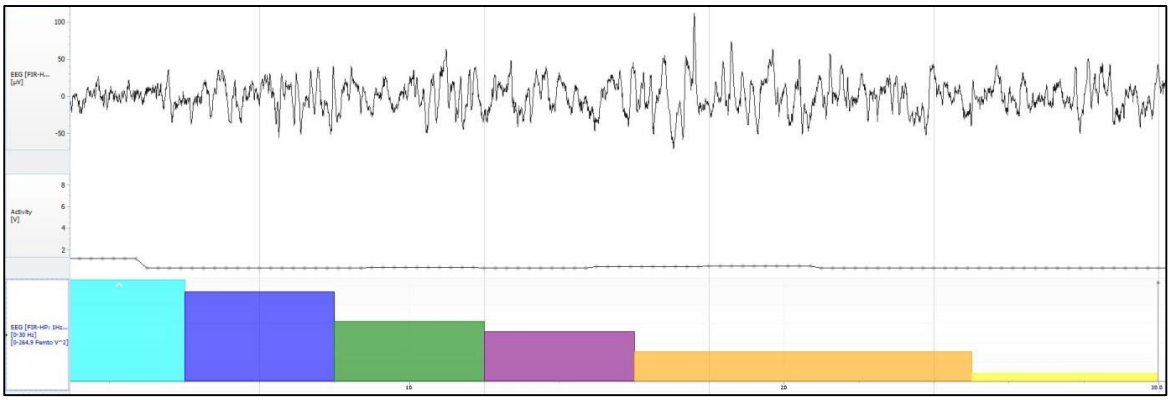


Figure 40*:* qEEG two minutes after propofol administration: dominant slow (delta, theta) frequencies.

3.2.3 Apomorphine

Clinical Observations

Subcutaneous apomorphine administration caused repeated emesis approximately 15 minutes after administration in both dogs, lasting approximately 30 minutes. Increased licking and swallowing were observed prior to vomiting. After 30 minutes, antiemetic medication (Cerenia maropitant, 10 mg/mL Zoetis Belgium SA) was administered and no further emesis was observed. Dogs were returned to their home kennels after a total recording time (BL + after apomorphine treatment) of one hour.

EEG Results: Visual Analysis

Visual EEG changes after apomorphine administration were not pronounced. An increase in amplitude to 150 µV with increased synchrony could be observed. These episodes lasted between 2 and 20 seconds and did not remarkably differ from baseline recordings.

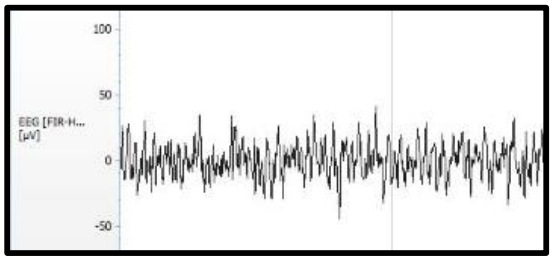


Figure 41: Normal Baseline EEG (2.5 seconds, male dog).

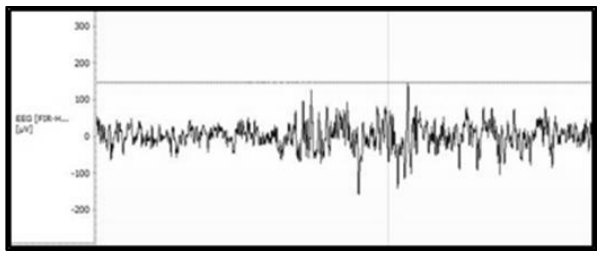


Figure 42: Transient increase in amplitude after apomorphine administration (2.5 seconds, male dog).

No EEG changes preceded emesis. After emesis, EEG muscle artefacts (amplitude >150 µV), most probably from swallowing and tongue movements (licking), appeared in the EEG.

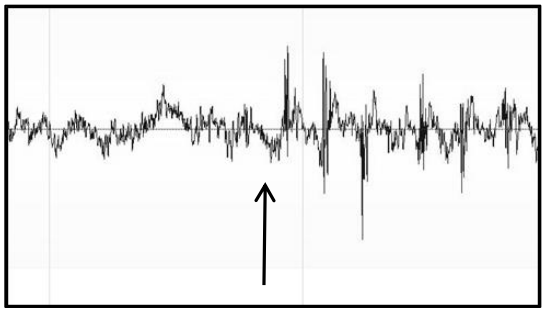


Figure 43: EEG after emesis (↑).

As tongue movements or activity of the jaw muscles are known to induce artefacts in the EEG, an increase in the artefact coverage was one of the expected outcomes in this experiment. In contrast, neither emesis itself nor the prodromal signs resulted in a total increase in the artefact rate compared to baseline. Even an opposite trend could be

observed, as the movement activity of the dogs, especially running and jumping, was markedly reduced which led to an increase in EEG quality.

EEG Results: Quantitative Analysis

The median of the relative power bands of two dogs was calculated for each spectral band. Then, baseline relative power was plotted against relative power post treatment. An increase in delta power was seen in the male, but not in the female. Also a decrease in gamma power could be observed in the female, whereas in the male no change from baseline was induced by apomorphine injection. Results of qEEG differ between dogs in this case, which does not correlate to clinical symptoms. The raw data plots of the two dogs are provided in the supplementary data (**Appendix 4**).

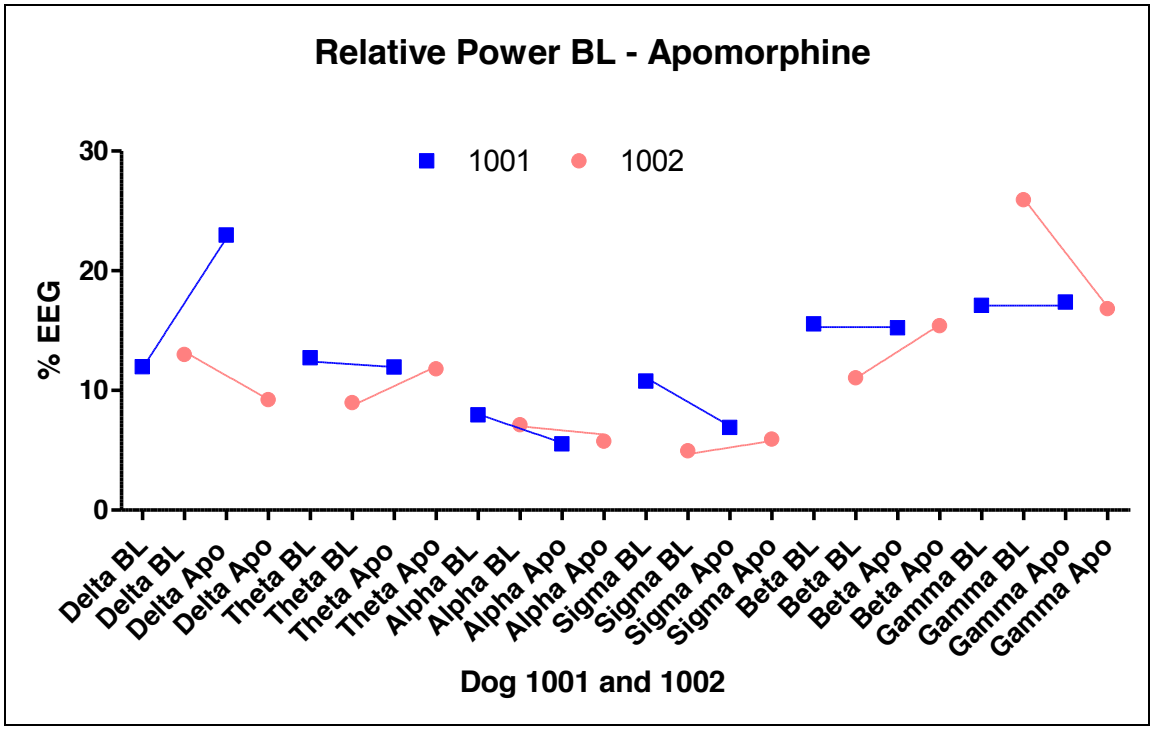


Figure 44*:* Median relative power changes in male and female after administration of propofol.

3.2.4 Quinpirole

Clinical Observations

Intravenous administration of quinpirole caused decreased activity and emesis in both dogs, starting 10 minutes after administration. The male was resting for five hours after compound administration. Due to this effect, the dose was reduced to 0.1 mg/kg for the female. Poor coordination and ataxia were observed in the female after administration of quinpirole. Body temperature of both dogs dropped below physiological values (37.5-39.0°C).

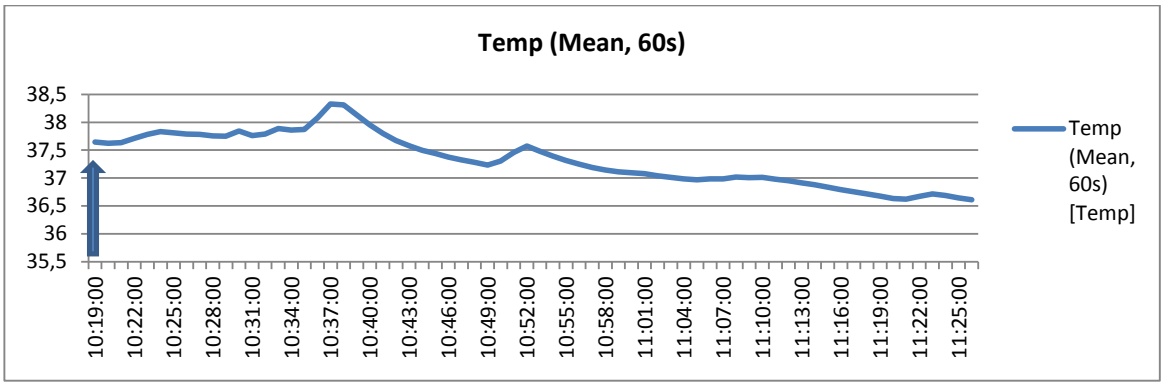


Figure 45: Changes in body temperature after quinpirole administration (↑), (female dog).

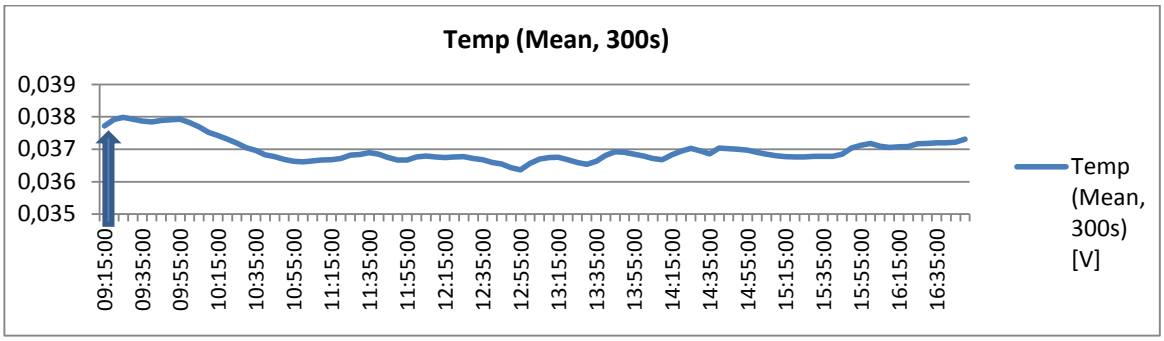


Figure 46*:* changes in body temperature after quinpirole administration (↑), (male dog).

EEG Results: Visual Analysis

No marked EEG changes could be identified after quinpirole administration. Artefacts after emesis could be identified that had the same appearance as shown after apomorphine administration. Within 10 minutes of recording, EEG voltage increased and frequency decreased, indicating a decrease in vigilance state (**Figure 48**).

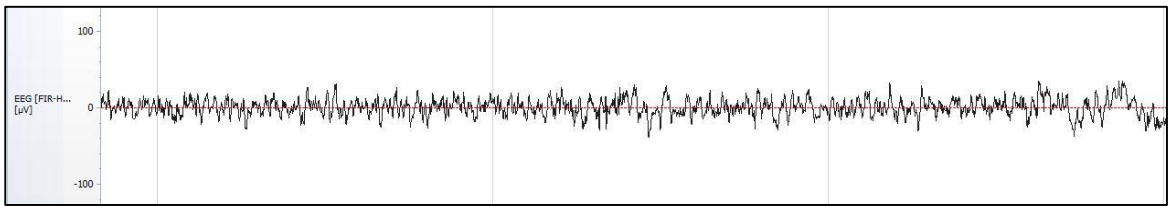


Figure 47: Baseline EEG (6 seconds, male dog).

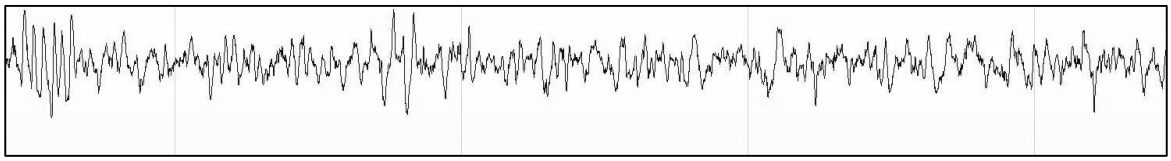


Figure 48: Slowing of background activity after administration of quinpirole (10 seconds, female dog).

EEG Results: Quantitative Analysis

Median relative frequencies were calculated for both dogs for the baseline and the post-dosing period. Delta power of both dogs decreased after compound administration, whereas gamma power increased. The other medians are changed in the same direction in both dogs, but these changes are more subtle. The raw data plots, showing the medians +/-SD for each dog and each frequency band individually, are provided in the supplementary data (**Appendix 4**).

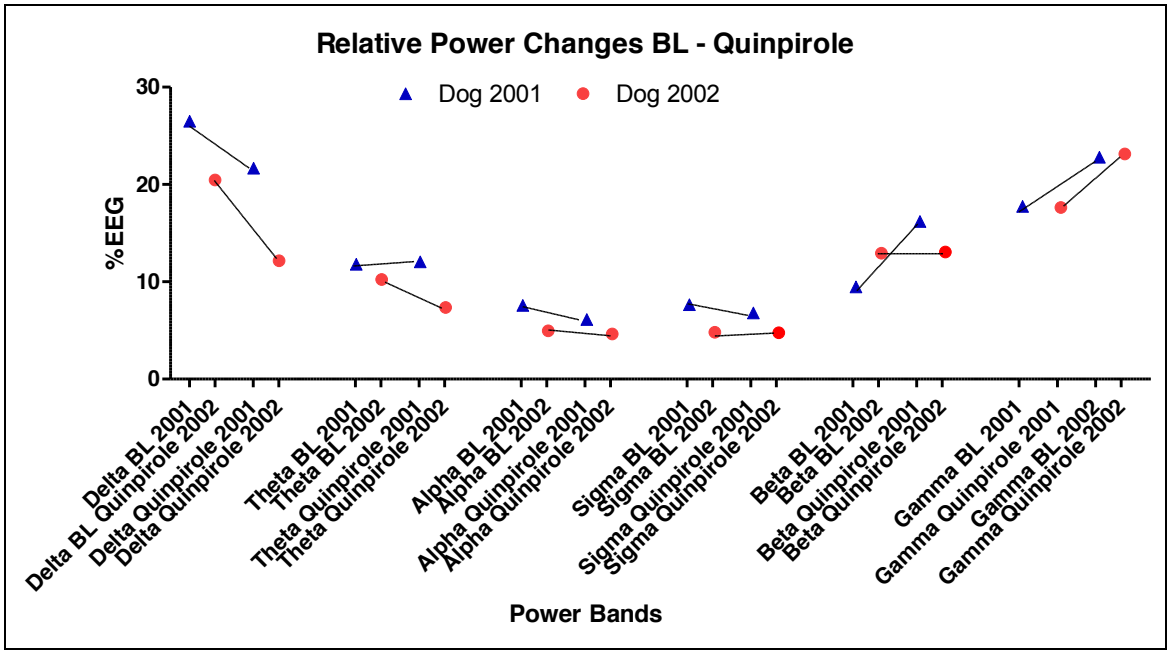


Figure 49*:* Quantitative changes after quinpirole administration in dogs (median of BL – post treatment for each individual frequency band).

3.3 In-House Compounds

3.3.1 Compound 1

Clinical Observations and Exposure in the Male Dog

Intravenous administration of the first dose (12 mg/mL over 10minutes) of compound 1 was well tolerated and resulted in plasma levels of 2362.3 ng/mL which was close to the targeted concentration of 2500 ng/mL. During the following 20 minutes of observation time, the only finding was transient lip licking. The second dose (27 mg/kg, administered over 10min) achieved a plasma concentration of 5894.4 ng/mL which was lower than the targeted 7500 ng/mL. Symptoms observed during infusion and during the following 20 minutes observation time were salivation, emesis, unstable gait and repeated urination. The third infusion of compound 1 (29 mg/kg over a 10 minutes) resulted in a total plasma exposure of 7667.1 ng/mL which was lower than the target concentration of 11500 ng/mL. Clinical symptoms during infusion were facial twitches, a mild tremor of the hind limbs and progressive ataxia.

Also, the dog appeared agitated. During sample collection for PK analysis, a tonic convulsion occurred. The tonic phase with signs of lost consciousness lasted approximately 15 seconds. Then, tonic-clonic limb movements started during which autonomic signs were present (salivation, urination, and defecation). Anticonvulsive treatment (midazolam, 0.2 mg/kg i.v.) was administered immediately after start of the tonic phase. Treatment was efficacious within seconds and total duration of the convulsion was below one minute. Symptoms after the convulsion were increased respiration rate, constricted pupils and emesis. Subcutaneous metoclopramide (1 mg/kg; Emeprid®, cewa) was administered and observation and EEG recording were continued for another seven hours during which no further clinical signs were noted. Clinical symptoms and correlating exposures are summarized in **Table 10**.

Dose (mg/kg)	Volume (mL)	Infusion Rate (mL/h)	Sample Time (min)	Target Exposure (ng/mL)	Plasma Concentration Male (ng/mL)	Neurological (and autonomic) Symptoms
12	7.1	42.4	12	2500	2362.3	None
27	15.9	95.4	42	7500	5894.4	Urination, salivation, (emesis)
29	17.15	102.9	62	11500	7667.1	Tremor, facial twitches, limb twitches, tonic, then tonic- clonic convulsion
n/a	n/a	n/a	1440	n/a	3009.3	None

Table 10: Comparison between target exposure, measured exposure, and symptoms after compound 1, male dog . Actual exposure was lower than calculated values. n/a= not applicable.

Clinical Observations and Exposure in the Female Dog

The dosing scheme for the female dog was adapted in order to reduce the incidence of emesis and the experimental time, thereby lowering the burden for the animal. A higher initial dose was chosen (30 mg/kg over 10 minutes) which resulted in a plasma concentration of 6966.21 ng/mL. This was lower than the target of 7500 ng/mL. Clinical symptoms observed during dose administration and observation time were salivation and emesis. The second dose (40 mg/kg over 10 minutes) induced neurological symptoms which were ataxia, a fine tremor of the head, facial jerks and hypersensitivity to touch. In addition, the female seemed to be agitated. A tonic, then tonic-clonic convulsion occurred eight minutes after start of the second infusion while the dog was still connected to the infusion line. Anticonvulsive treatment (midazolam, 0.2 mg/kg i.v.) was administered immediately, so total duration of the convulsion was less than two minutes. A subsequent dose of midazolam (0.1 mg/kg i.v.) was administered 90 seconds after the first dose, as it was unclear if the convulsion might reoccur. Blood for compound level determination was sampled within two minutes after the convulsion and analysis showed that the exposure at that time was 13292.6 ng/mL. This was higher than the targeted 11500 ng/mL. The dog had tachycardia (160 bpm) and tachypnoe (40/min) directly after the convulsion, lasting for 10 minutes.

Other symptoms were poor coordination, constricted pupils and a fine head tremor. 1.5 hours after the convulsion, no clinical symptoms were observed and the dog appeared normal. Observation was continued for the next five hours and no further treatment was necessary.

Dose (mg/kg)	Volume (mL)	Infusion Rate (mL/h)	Sample Time (min)	Target Exposure (ng/mL)	Plasma Concentration Female (ng/mL)	Neurological (and autonomic) symptoms
30	16.98	102	12	7500	6966.21	Salivation, emesis
40	22.64	135	42	11500	13292.6	Ataxia, tremor (head), facial jerks, increased sensitivity to touch, tonic, then tonic-clonic convulsion
24h	n/a	n/a	1440	n/a	4511.26	none

Table 11*:* Plasma exposure of compound 1 and clinical symptoms, female dog) n/a= not applicable.

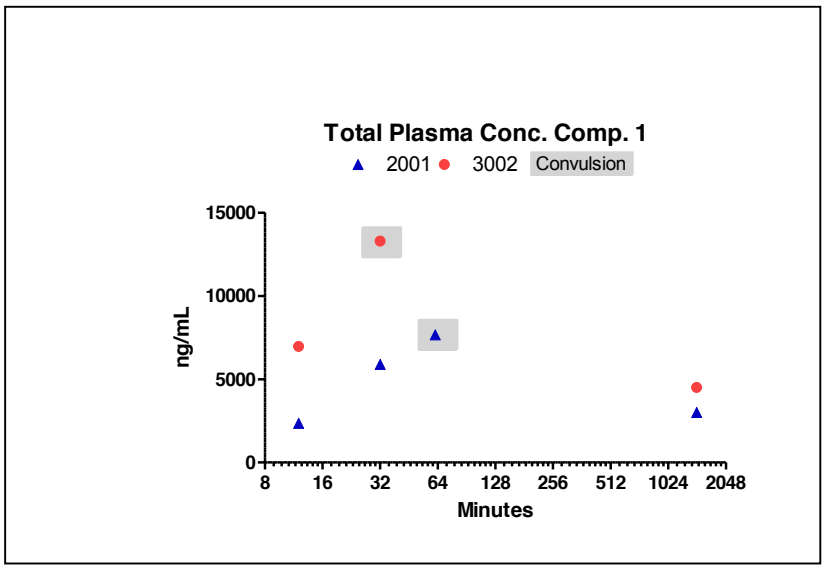


Figure 50*:* Total plasma exposure of compound 1. Doses were not the same for the two dogs (male: 12 mg/kg, 27 mg/kg and 29 mg/kg; female: 30 mg/kg and 40 mg/kg). The sample time point closest to convulsion is highlighted.

Symptoms observed in the EEG study were compared with symptoms recorded in earlier toxicology studies in dogs. Studies with multiple dosing (5 days, 2 weeks, 13 weeks) were chosen. **Table 12** lists the symptoms and indicates, whether they were also observed with intravenous dosing of compound 1 in the dog EEG study.

Symptoms in dog toxicology studies	Symptoms in EEG studies?	Comment
Activity Decreased	Not clearly	
Activity Increased	Yes	Excitation/ agitation in both dogs in the EEG study
Ataxia	Yes	Grade in toxicology studies: severe Grade in EEG study: mild - moderate
Aggressive Behavior	(Yes)	Male dog in EEG study: growling after 3rd dose
Convulsions	Yes	Convulsion type in toxicology study: tonic; in EEG study: tonic, evolving to tonic-clonic
Dissociative behavior	Yes	EEG study: sniffing, trying to hide
Emesis	Yes	/
Head Movements	Yes	Female dog
Jerks	Yes	/
Salivation	Yes	/
Sedation	(Yes)	After convulsion and midazolam treatment, dogs were calm for the rest of the afternoon, possible influence of midazolam
Sensitivity to noise increased	Yes	Evident when dogs outside were barking; in addition: sensitivity to touch increased in the female dog
Tremor (mild)	Yes	/

Table 12: Symptoms with compound 1 in dog toxicology studies and EEG studies.

Compound 1 has previously been tested in a NHP infusion study. Samples in this study were also collected after clinical convulsion. **Figure 54** shows total plasma concentration of compound 1 in the NHP infusion study (145000 ng/mL, 135000 ng/mL and 146000 ng/mL) and in the dog EEG study (7667.1 ng/mL and 13292.6 ng/mL). In **Figure 55**, free plasma levels are shown. Due to the large difference in plasma protein binding (dog 0.3, NHP 0.012), free concentrations are similar in both species (NHPs 1740 ng/mL, 1620 ng/mL, 1752 ng/mL and dogs 2300.13 ng/mL, 3987.78 ng/mL).

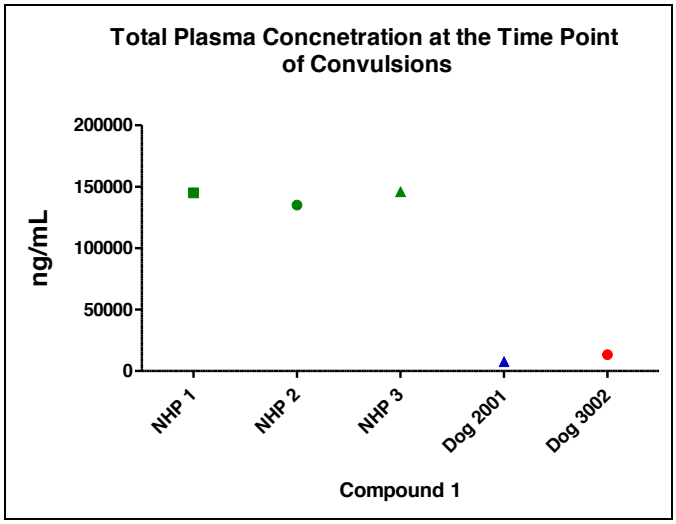


Figure 51: Total plasma concentration of compound 1 at which convulsions occurred in dogs and NHPs.

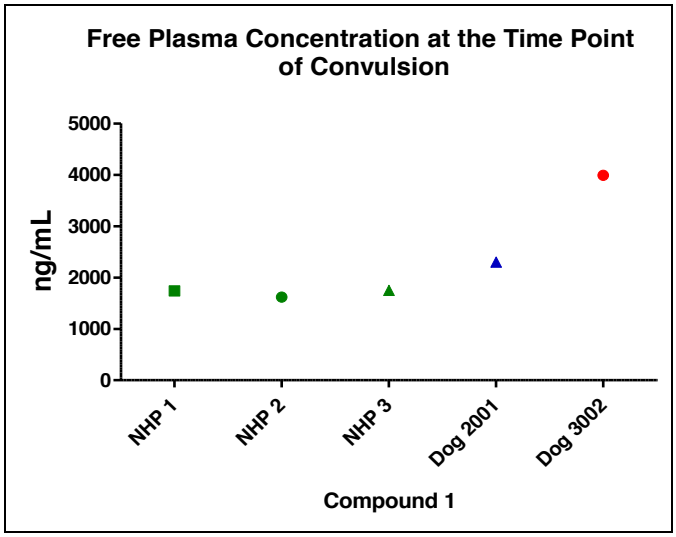


Figure 52*:* Free plasma concentration of compound 1 at which convulsions occurred in dogs and NHPs. When total exposure is considered, the dog appears to be more sensitive. This is reverted when free compound levels are evaluated.

EEG Results: Visual Analysis

The EEG from the baseline period was normal in both dogs (example from the male dog, **Figure 53**). Incidence of artefacts was high during baseline. After start of the first dose, emesis was the first observed symptom and each event was accompanied by EEG artefacts as observed with apomorphine or quinpirole. After administration of the first intravenous dose, an increase in synchrony was observed in the EEG (**Figure 54**). This effect was even more expressed and accompanied by voltages of up to 200 µV during administration of the second dose (**Figure 55**).

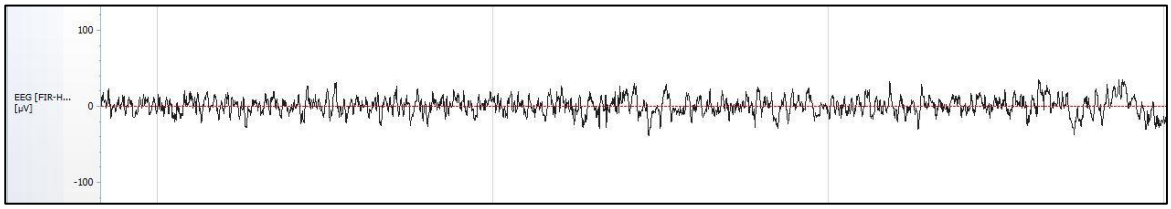


Figure 53*:* Baseline recording (10 seconds, male dog).

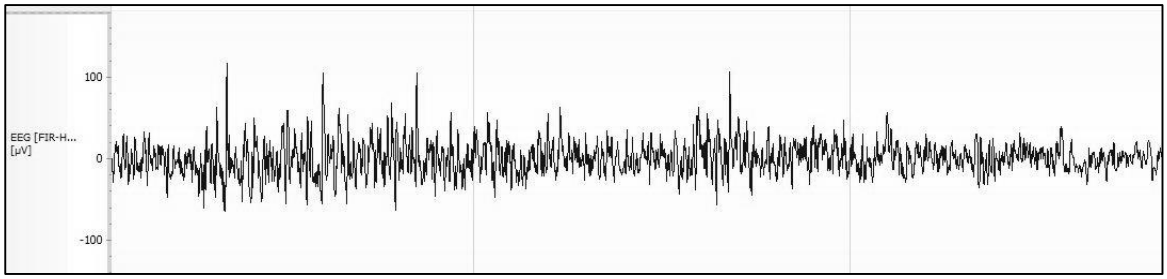


Figure 54*:* EEG recorded at the end of the first infusion step of comp. 1 (8 seconds, female dog). Synchronized background activity with peak amplitudes of 100 µV.

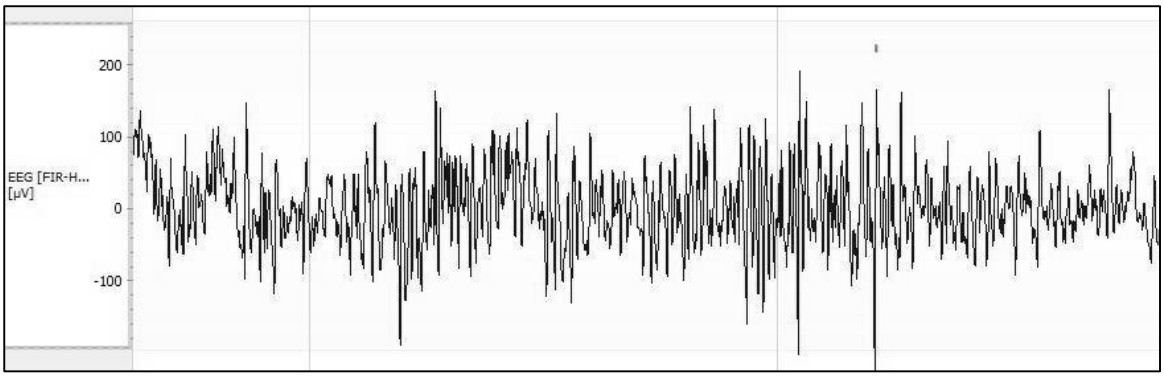


Figure 55: EEG recorded during the second infusion of compound 1 (8 seconds, female dog). Synchronized background activity with peak amplitudes of 200 µV can be seen.

In the male dog, transient paroxysmal activity started during administration of the third dose approximately two minutes before the onset of clinical convulsion. In the female, two minutes of paroxysmal activity preceded the convulsion, too. Due to signal loss and artefacts, it was not possible to identify this during live-view of the EEG but only retrospectively during offline-analysis.

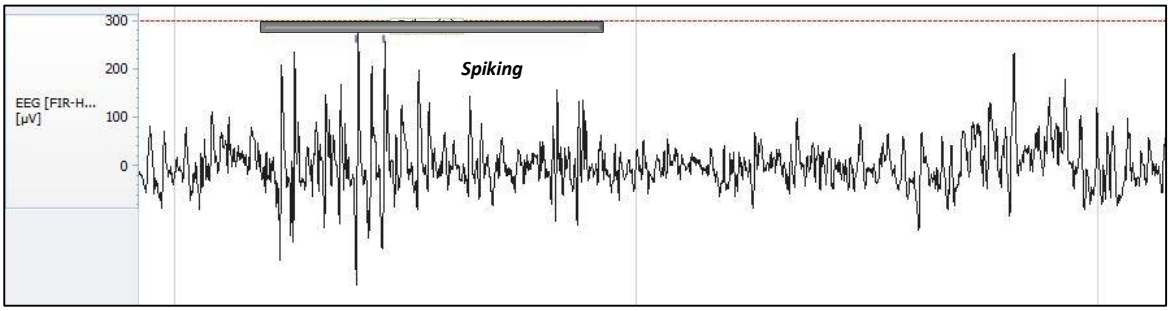


Figure 56: EEG, two minutes prior to the onset of convulsion (10 seconds, female dog). Spikes can be identified, peak amplitude is 300 µV.

The paroxysmal activity in both dogs was characterized by an increase in synchronization and mean amplitude of around 150 µV. Single spikes as well as spike trains with peak amplitudes of 300 µV could be identified. The first spikes were not correlated with muscle contractions; however, prior to the onset of convulsions, spikes were overlaid by high-frequency muscle potentials that corresponded to visible jerks in the video. During generalized convulsion, peak amplitudes in the EEG were 1.3 mV which also indicates that most probably muscle activity overlies brain potentials.

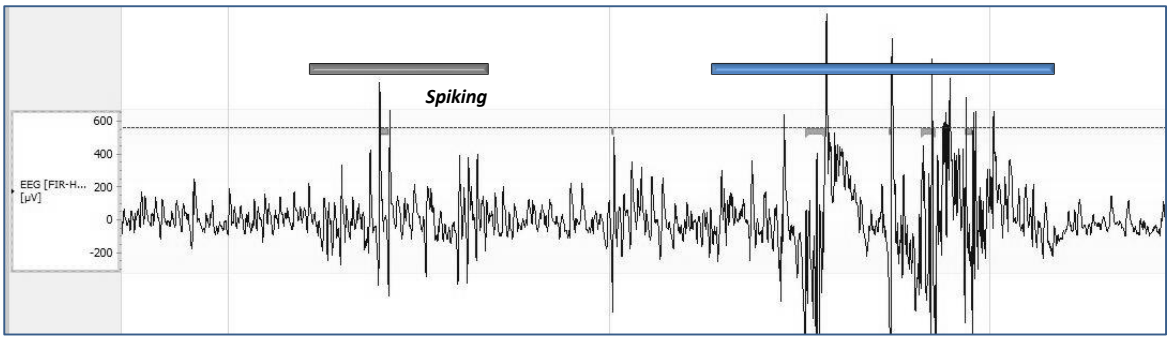


Figure 57: EEG with muscle activity (10 seconds, female dog) Amplitudes above 1 mV indicate that muscle activity overlies the EEG (marked blue).

In the male, the first paroxysmal spikes appeared about one minute prior to the onset of convulsion. Duration of the clinic convulsion was 30 seconds. Midazolam administration was effective to stop the convulsions (**Figure 58**).

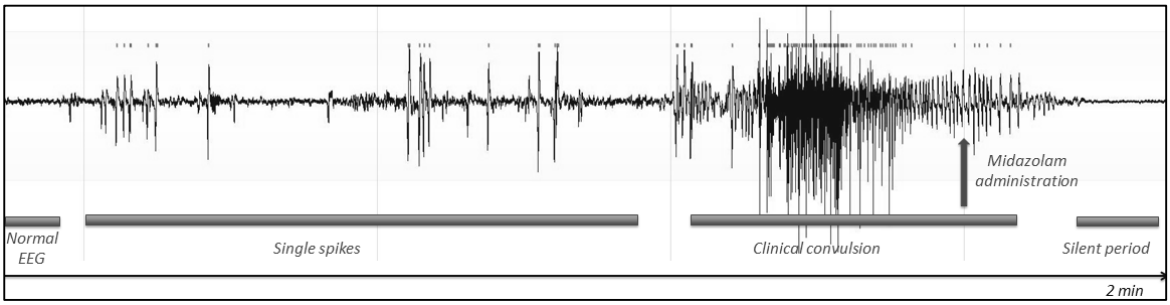


Figure 58: Convulsion timeline (2 minutes, male dog). Starting one minute prior to clinic convulsion, spikes can be identified in the EEG. The high amplitude, corresponding to peak amplitudes during convulsions indicates that muscular twitches overly EEG activity. Midazolam administration stops the convulsion. Duration of convulsion according to EEG is 15-30 seconds.

In the female, the signal was lost for a period of 3.8 seconds directly prior to the onset of convulsion for unclear reasons (**Figure 59**). The time of the seizure was approximately one minute but was difficult to determine, as artefacts, originating from handling of the dog, overly its end (**Figure 60**) and hinder EEG analysis for a time of 30 seconds. After an artefact-free signal was restored, amplitude still averaged 70 µV, with peaks of 100 µV for another 20 seconds. Then, very low amplitudes appeared, with mean amplitude of 20 µV.

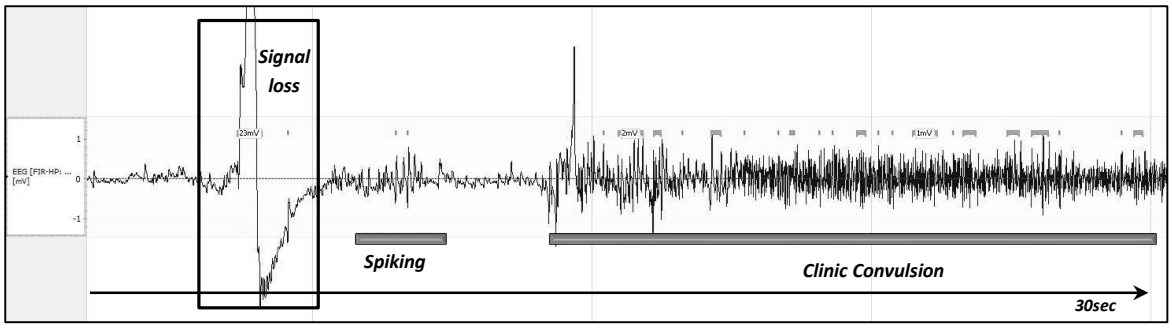


Figure 59*:* EEG at onset of convulsion (30 seconds, female dog). EEG signal was lost for approx. 3.8 seconds (marked). Single spikes can be identified prior to convulsion. Peak amplitude during convulsion was 2 mV, indicating muscle activity contributes to the signal.

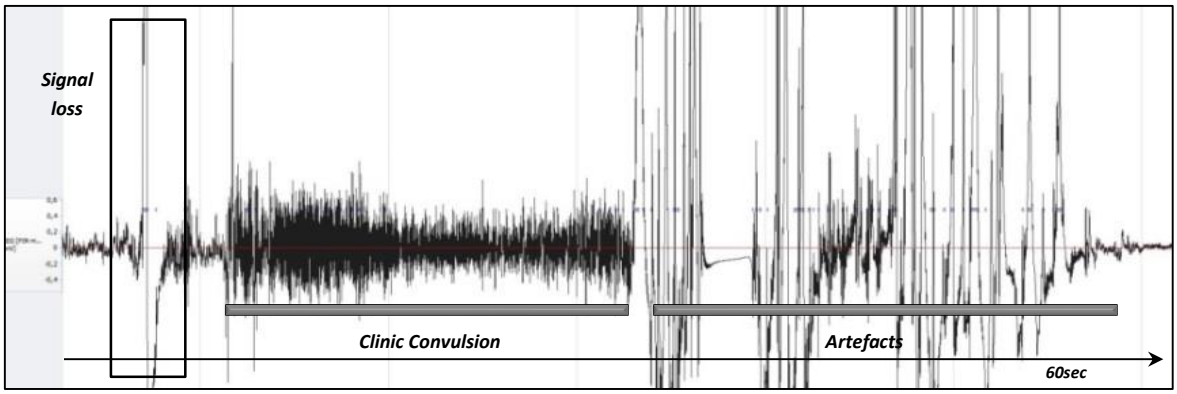


Figure 60: Artefacts in EEG at the end of convulsion. High amplitude activity masks EEG; cause could be handling of the dog (60 seconds of EEG, female dog).

After the generalized convulsion, amplitudes fell below baseline values in both dogs (**Figure 61**)**.** Amplitudes were below 20 µV, respectively for both dogs.

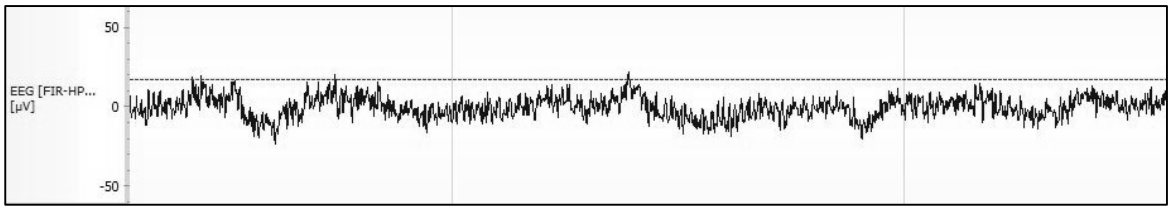


Figure 61: EEG after tonic-clonic convulsion and midazolam treatment (10 seconds, male dog). The axis line is set to 20 µV. This is below baseline values that usually range between 30 µV and 70 µV.

EEG results: Quantitative Analysis

Median relative power was calculated from baseline and post-dosing relative frequencies. Plasma levels of both dogs were compared and time points at which exposure was comparable between both dogs were selected for FFT analysis. E1 for the male is 5894.4 ng/mL and for the female it is 6966.21 ng/mL. E2 qEEG data were derived from the time when the male dog had a drug plasma exposure of 7667.1 ng/mL and the female had

13292.6 ng/mL. In both dogs, an increase in the beta and theta frequency band was observed, along with a decrease in gamma. Raw data plots are provided in **Appendix 4**.

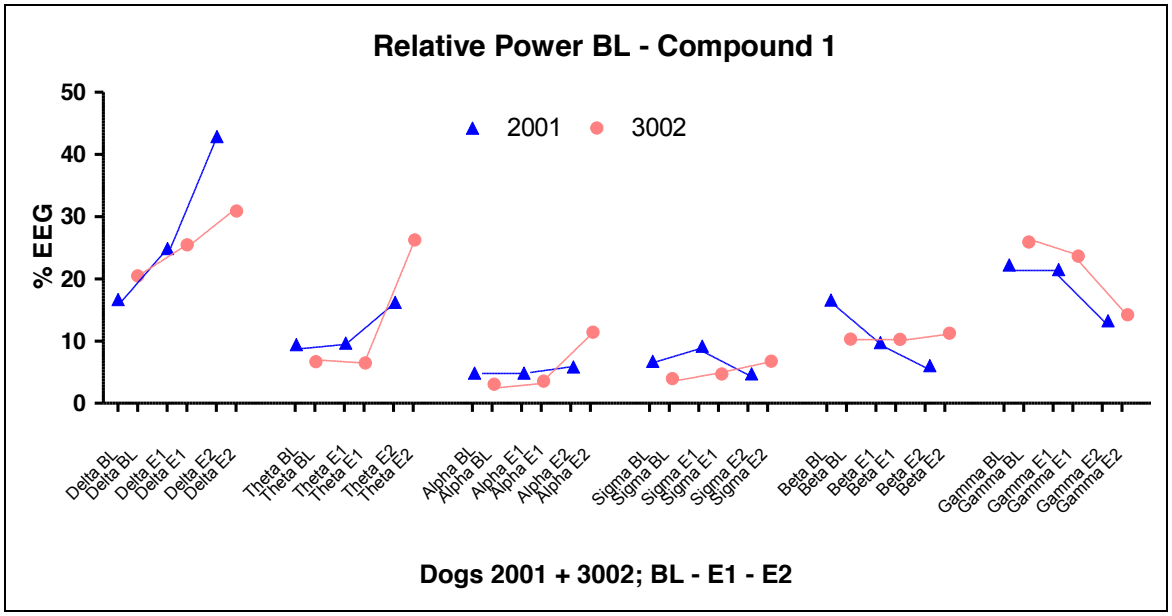


Figure 62*:* Median power of the male and female dog at baseline, at the time of E1 (5894.4 ng/mL and 6966.21 ng/mL) and E2 (7667.1 ng/mL and 13292.6ng/mL).

3.3.2 Compound 2

Clinical Observations and Exposure

Oral administration of compound 2 at a dose of 14 mg/kg via gelatin capsule induced tremor of the head. In the male dog this symptom was observed starting 30 minutes after administration, in the female it was first noted after 50 minutes. The neck muscle contractions appeared to be transmitted to the rest of the body, as seen by swinging movements. The tremor was more pronounced and longer lasting in the male than in the female. This relates to plasma exposure, which reached higher levels in the male than in the female with a measured peak plasma concentration of 1357.85 ng/mL 50 minutes after compound administration. In the female, peak concentration was 950.71 ng/mL in the 50 minutes sample. The plasma concentrations were therefore in the range and above the target plasma concentration of 1000 ng/mL. An additional plasma sample was collected from the female at 1.5 hours as the clinical expression of the symptom was higher at that time point than at the 50 minutes sample. In the male, the intensity had remained the same at that time point. The female was also able to control the head movements, for example when study personnel were present in the animal room. Duration of the tremor in the female dog was four hours. Additional symptoms in the male were salivation and stereotyped behavior such as biting the bars of his kennel. Both dogs appeared conscious during the whole observation time as they responded to external stimuli and were able to eat and drink. As the symptoms were still present in the male dog in the evening, EEG and video recording were continued during the night. Analysis of the video showed that the dog remained lying

and got up in the morning. Then, the head tremor had disappeared and the dog moved normally. Blood samples for evaluation of clinical pathology and hematology parameters were collected approximately 22 hours after dosing and were within normal limits for both dogs.

In previous toxicology studies, the symptoms observed in dogs were similar to those detected in the EEG study. The sex difference had already been observed with oral dosing. In the NHP studies, head tremor was not observed (**Table 14**).

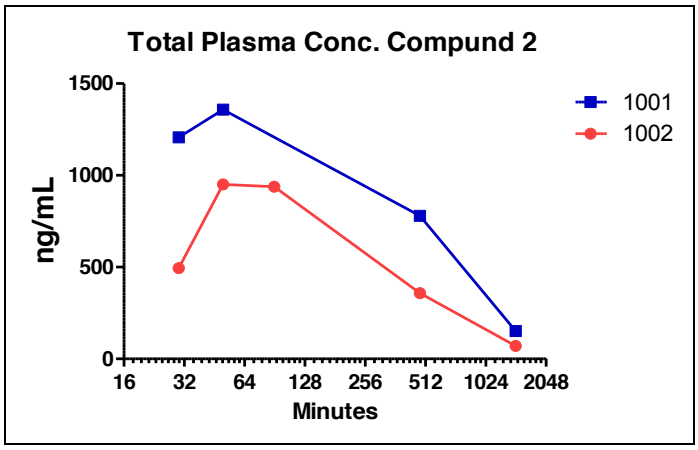


Figure 63*:* Total plasma concentration of compound 2 in the male (blue) and female (red) dog.

Dose Capsule (mg/ kg/day)	Sample Time Point (hrs after dosing)	Plasma Concentration (ng/mL) Male	Plasma Concentration (ng/mL) Female
14	0.5	1206.91	493.90
	1.0	1357.85	950.71
	1.5	Not sampled	937.55
	7.5	779.37	357.,75
	24	151.60	70.62

Table 13*:* Total plasma concentrations of compound 2 The target exposure of 1000 ng/mL was reached in the male but not in the female dog.

Dose (mg/kg)	Route	Clinical signs NHP	C_{max} (day 1) (ng/mL)	Clinical signs Dog	C_{max} (day 1) (ng/mL)
10	p.o.	stereotypical behavior (gnawing, circling), pupils dilated, eyelids partially/completely closed	M: 643 F: 653	Head tremor, imbalance, salivation (female) pupils dilated	M:1208 F:1086
30	p.o.	All signs observed at 10 mg/kg, in addition: excessive grooming, increased activity/hyper-reactivity, hunched posture, aggressive behavior	M: 1420 F: 1510	Head tremor, Ataxia (female), Salivation, pupils dilated	M: 3758.2 F:3103.2
100	p.o.	Signs observed at lower doses, in addition: decreased activity, ataxia, tremors, stereotypy (forepaw padding), inappetence	M: 4260 F: 4810	Not tested	Not tested

Table 14*:* Clinical symptoms and pharmacokinetics observed after 10 mg/kg, 30 mg/kg and 100 mg/kg oral doses of compound 2 in NHPs, compared to the dog.

Body temperature of the male increased to over 39.5°C in the late afternoon (6 hours after dosing) and is presented in (**Figure 64**). Maximal temperature was 39.7°C. A consequent fall to physiological values happened during the night. The time frame from 17.30-18.15 was not measured, as recording was interrupted then to secure the data collected during the day.

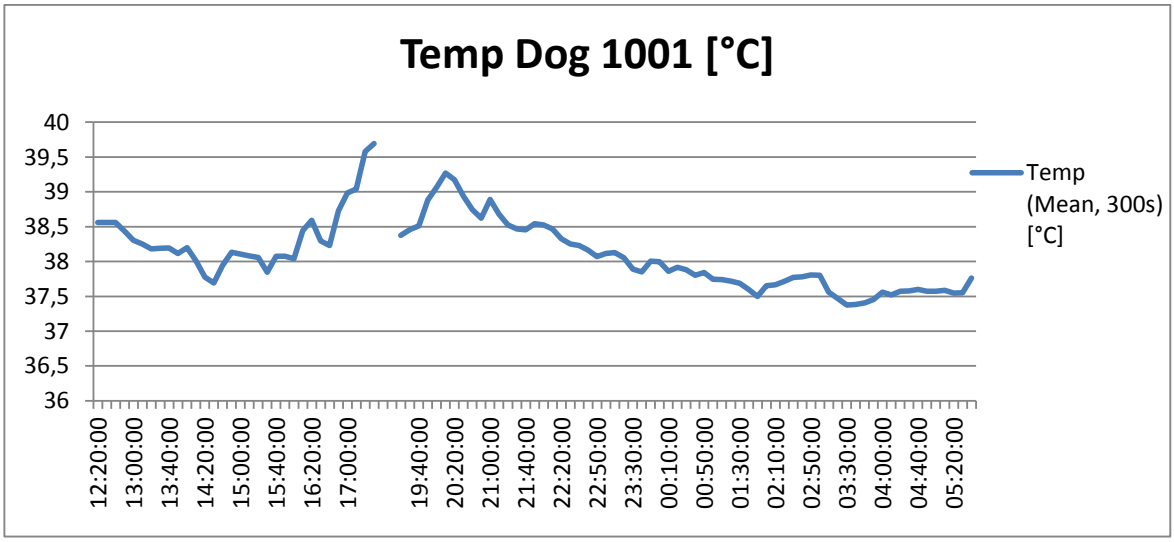


Figure 64*:* Body temperature changes with compound 2 (measured by implant, male dog). Shown is the time from the start of the experiment till the next morning (17.30-18.15 not measured: interruption of recording for data saving).

The female did not react with changes in body temperature as shown in **Figure 65.** Values were within physiological limits during the experiment and video showed that behavior of this dog was normal during the night.

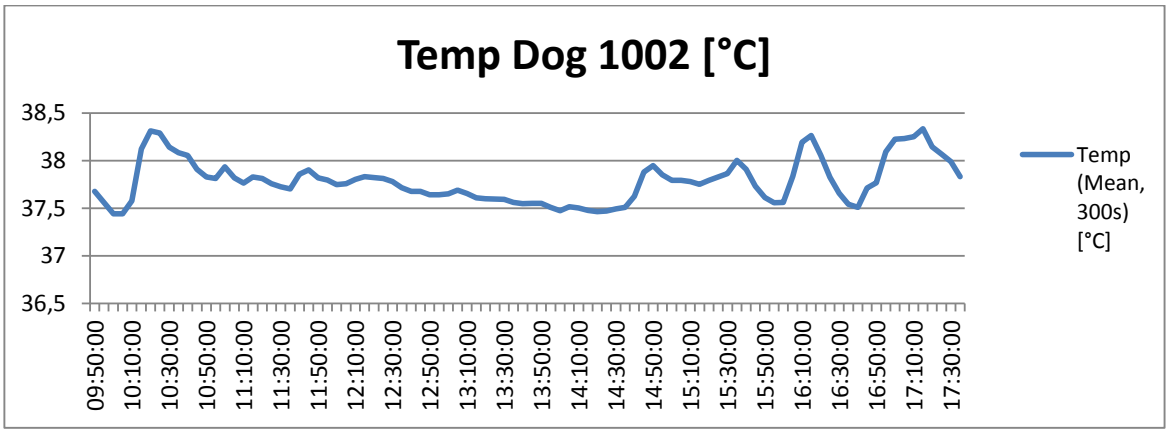


Figure 65: Body temperature changes with compound 2 (measured by implant, female dog). Shown is the time from the start of the experiment till the evening.

EEG Results: Visual Analysis

Normal EEG activity was observed in both dogs during the baseline period (example from the female dog, **Figure 66**). Abnormal EEG activity was present in both dogs at the onset of clinical symptoms. For the male, this was about 30 minutes after oral compound administration. The female had a later onset of paroxysmal EEG activity, around 50 minutes after dosing. In both dogs, the first change in EEG was an increase in amplitude to peak values of 170 µV, coupled with a high degree of synchronization (**Figure 67**).

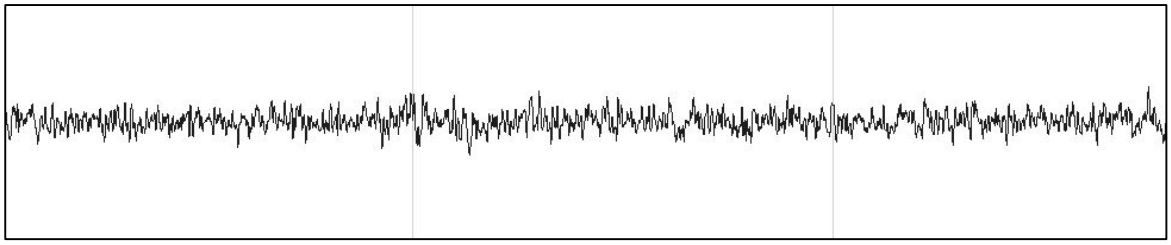


Figure 66: Baseline recording of the female dog (6 seconds).

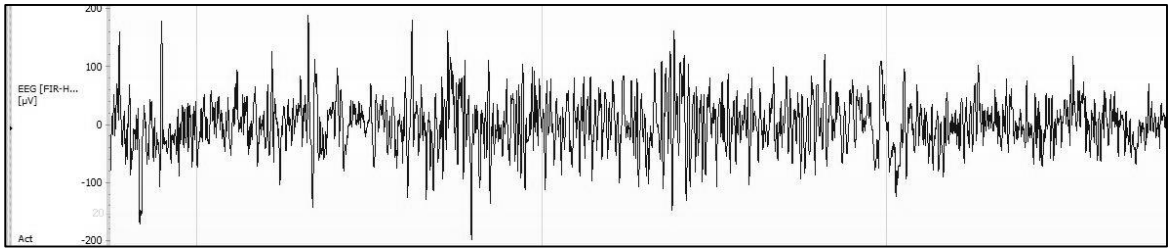


Figure 67: EEG after compound 2 administration (10 seconds, female dog). Peak amplitudes are higher than the average in baseline recordings, with peaks of 170 µV.

EEG changes in the male consisted of the appearance of rhythmic, synchronized sharp transients resembling bilateral triphasic waves, one hour after compound administration. A low amplitude wave builds up into a high-amplitude upward spike that is then followed by a symmetrical downward deflection (**Figure 68**). Peak amplitude of these waves was 130 µV. Duration of these episodes was up to two minutes and they were interrupted by baseline-like EEG rhythms. In the female dog, fast activity with peak amplitudes over 200 µV was present 1.5 hours after compound administration (**Figure 69**). These intervals lasted up to two minutes each and were also interrupted by activity resembling baseline activity.

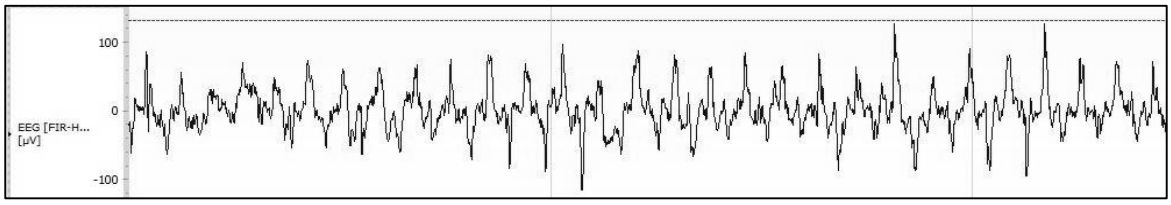


Figure 68: Paroxysmal activity, appearing in intervals of up to two minutes starting one hour after administration of compound 2 (10 seconds, male dog); Peak amplitude 130 µV.

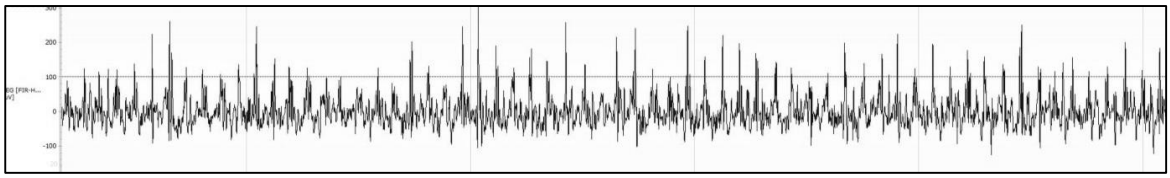


Figure 69: EEG activity 1.5 hours after compound 2 (10 seconds, female dog); amplitude >200 µV.

Seven hours after compound administration, an oscillating, symmetrical pattern was observed in the male dog. Peak amplitudes were above 200 µV: periodiogram and FFT analysis showed that these slow oscillations were predominantly in the range of 4-8 Hz which is in the theta band (**Figure 70**).

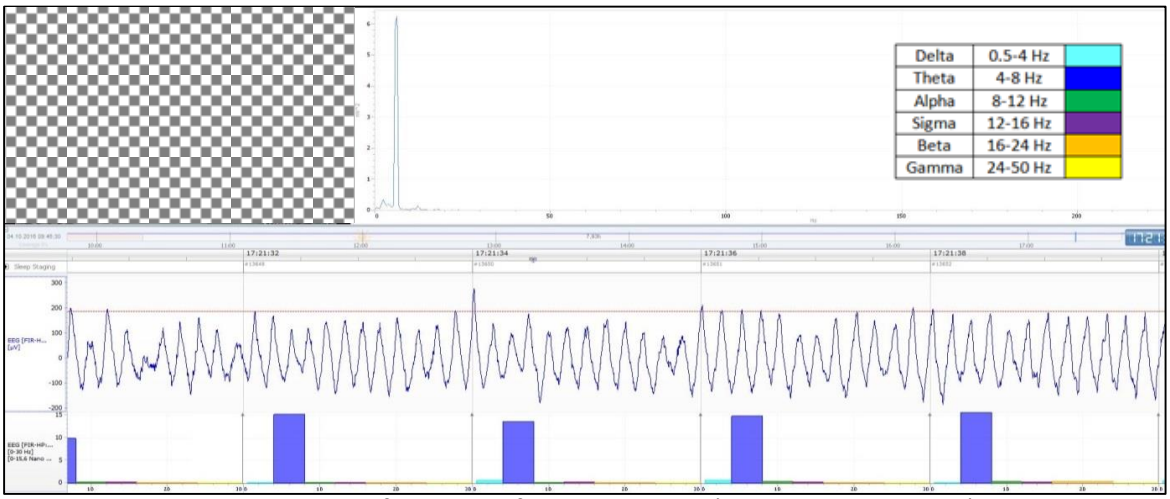


Figure 70: Sinusoidal oscillations five hours after compound 2 (10 seconds, male dog) Amplitudes are above 200 µV and frequency is 10 Hz. The FFT power bands confirm that the EEG has a dominant frequency in the theta band.

Another, faster variant of symmetrical EEG paroxysms after administration of compound 2 in the male dog were high amplitude, fast, oscillating deflections. They also appeared in intervals eight hours after compound administration. Peak amplitude was 200 µV. This was observed at several intervals during the first eight hours of the night-time recording.

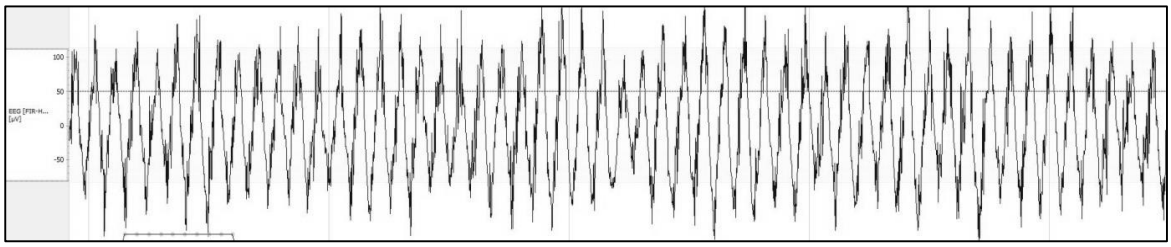


Figure 71*:* Paroxysmal activity after compound 2 (10 seconds, male dog); peak amplitude 200 µV.

In the female dog, the EEG had a normal appearance five hours after compound 2 had been administered. Latency to sleep for dog 1002 was five hours, but sleep was frequently interrupted during the first half of the night.

In the male, EEG symptoms lasted almost the whole night. Appearance of slow background activity superimposed with spindles around 14 hours after compound administration indicated progression to sleep (**Figure 72**). The first episode of sleep lasted less than two minutes, but sleep reoccurred at shorter intervals and in increasing lengths during the night-time. They were interrupted by episodes of normal waking background activity and intervals with a markedly increased amplitude and high synchrony (**Figure 74**).

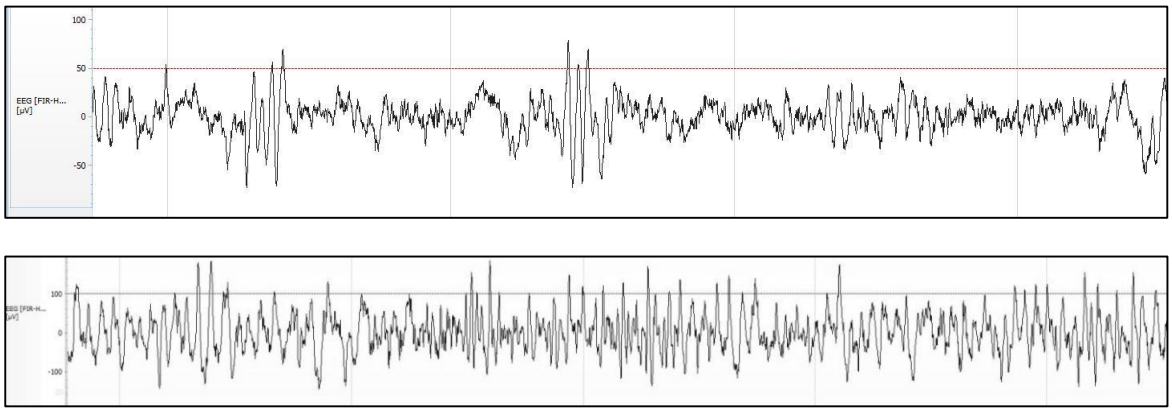


Figure 72 (above) and **Figure 73***:* Examples of EEG activity during the night-time recording after compound 2 (10 seconds, male dog). These episodes first appeared at 2 a.m. and the first ones lasted around two minutes. Consequent similar episodes were recorded with rising lengths and increased frequency during the rest of the night.

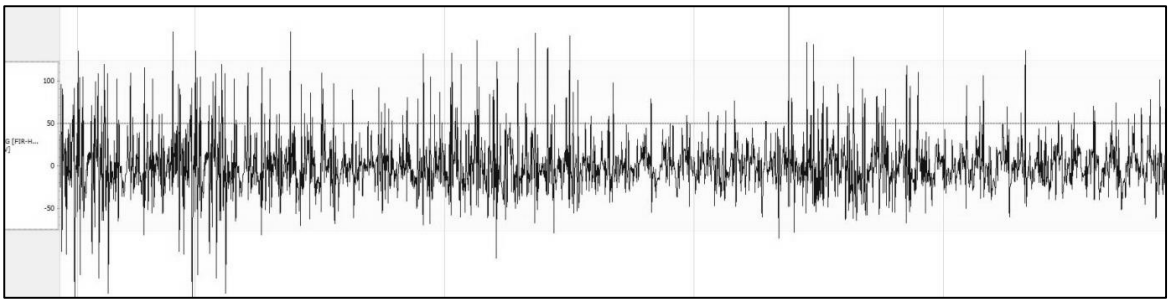


Figure 74: Highly synchronized EEG after compound 2 (10 seconds, male dog). This EEG was recorded during the night. Peak amplitudes are above 100 µV.

EEG results: Quantitative Analysis

Time points for calculation of FFT relative power were chosen based on exposure: E1 is the peak exposure of both dogs respectively (male: 1357.85 ng/mL and female: 950.71 ng/mL). E2 is the evening time point (7.5 hours after compound administration: male 779.37 ng/mL; female 357.75 ng/mL). Quantitative analysis of EEG showed no remarkable changes for the female when baseline values were compared to post treatment values. In the male dog, a marked increase in relative theta power was observed during the duration of the recording. The difference between the dogs also correlates to clinical symptoms.

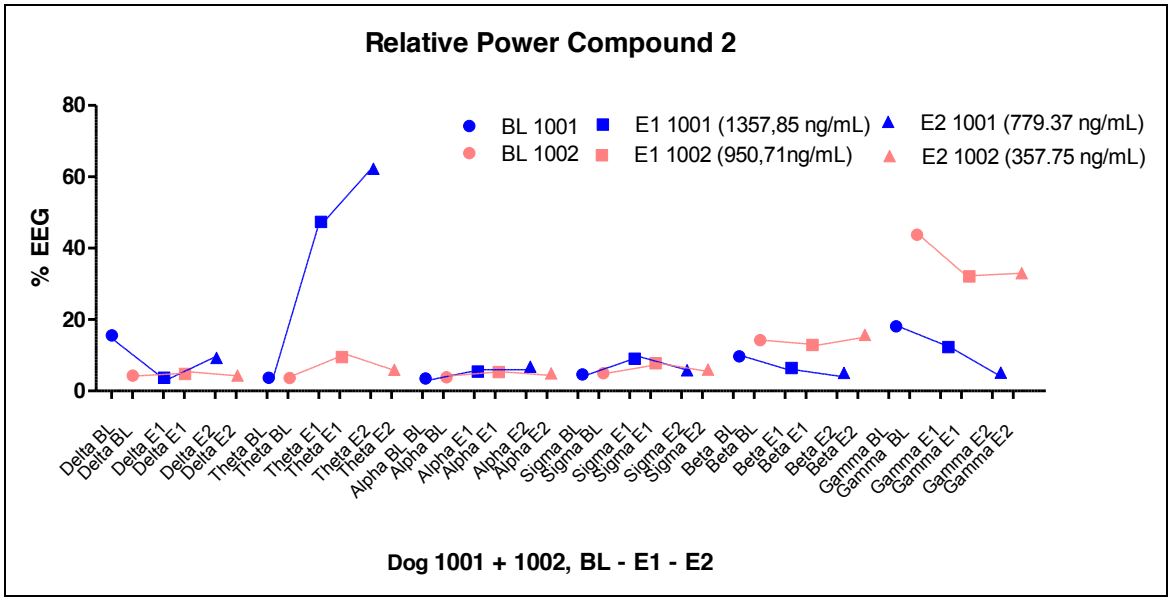


Figure 75: Median power changes with compound 2. An increase in theta power is the most prominent finding in the male dog (blue). In the female dog (red), no obvious changes were induced in the quantitative EEG power by compound 2.

3.3.3 Compound 3

Clinical Observations and Exposure in the First Experiment

Compound 3 was administered by continuous intravenous infusion to one male and one female dog on the same day. First, the experiment with the male dog was conducted. The first symptoms, appearing three minutes after the start of the infusion (8.0 mg/kg over 10 minutes), were licking, smacking and a careful gait. Then, reddening of the oral mucosal membranes and a rise in respiration and heart rate (160 bpm) were noted. At the end of the first infusion, the male was resting in a lying position. Plasma level of compound 3 after termination of the first infusion was 1116.54 ng/mL in this dog which was above the targeted exposure of 750 ng/mL.

The female dog kept standing during the first infusion (8.0 mg/kg over 10 minutes), but her gait seemed unstable. The increase in heart rate was minor compared to the male dog (120 bpm). The plasma exposure of compound 3 in the female measured 12 minutes after the start of the infusion was 710.84 ng/mL and was below the targeted 750 mg/mL.

In both dogs, eye squinting was observed, while all ocular reflexes remained normal. Red oral mucosal membranes and an increase in the pulsation intensity of the femoral vessels were observed in both dogs during the first infusion. During the observation time between infusions, reduced activity was noticed as well as tense facial muscles and erected ears. Observation time was increased from 20 to 30 minutes for the male dog to ensure recovery from cardiovascular symptoms. Prior to the start of the second infusion (6.0 mg/kg over 10 minutes), the heart rate had normalized and the dog had resumed a standing position.

Symptoms in both dogs during the second infusion were lip licking, fine tremors of the hind legs, tachypnoe and tachycardia. During infusion, the male dog rested in sternal recumbence with hunched posture and retracted tail while the female remained in a standing position.

After the stop of the infusion, the male dog was lying and appeared to be sedated. The nictitating membrane was prolapsed and the pupils reacted slightly delayed. Cardiovascular parameters normalized after 15 minutes. The dog was located on a mattress and appeared to be sedated for a period of three more hours. In the female dog, sedation was also present for two more hours but there were no ocular findings. The second infusion resulted in plasma concentrations of 1527.87 ng/mL in the male and 1295.48 ng/mL in the female dog respectively. Both values were above the targeted 1000 ng/mL. The third dose was not administered due to the unexpected cardiovascular symptoms.

Dog	Dose (mg/kg)	Volume (mL)	Infusion Rate (mL/h)	Sample Time (min)	Target Exposure (ng/mL)	Measured Exposure	Symptoms
M	8	14.54	87.27	12	750	1116.54	licking, smacking, careful gait, red oral mucosal membranes, tachypnoe, tachycardia, eye squinting, tense facial muscles, erected ears
	6	10.9	65.45	52	1000	1527.87	lip licking, fine tremors hind legs, tachypnoe tachycardia, resting, hunched posture
	n/a	n/a	n/a	60	n/a	806.87	sedation
	n/a	n/a	n/a	1440	n/a	18.19	none
F	8.0	9.69	56.0	12	750	710.84	unstable gait, eye squinting, tachypnoe tachycardia, tense facial muscles, erected ears
	6.0	7.27	43.63	42	1000	1295.48	lip licking, fine tremors, tachypnoe tachycardia
	n/a	n/a	n/a	60	n/a	576.53	sedation
	n/a	n/a	n/a	1440	n/a	5.76	none

Table 15*:* Total plasma concentration of compound 3 and clinical symptoms of the male (M) and female (F) dog in the first experiment with compound 3.

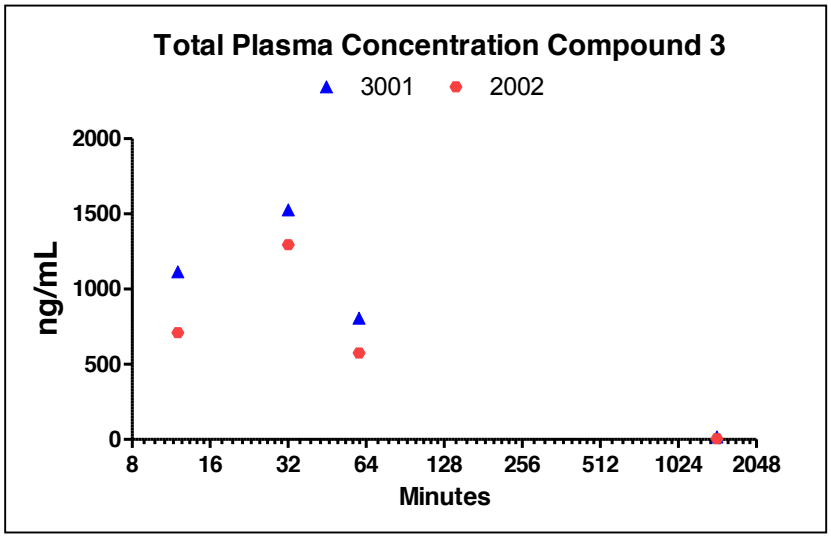


Figure 76: Total Plasma Exposure of compound 3 (male: blue, female: red) after the first experiment.

Administration of compound 3 resulted in a drop in body temperature in both dogs. Minimum temperature of the male was 35.87°C six hours after the first infusion; for the female dog minimum was 36.42°C approximately three hours after start of the first infusion. EEG and temperature recording were not continued overnight, but the temperature for both dogs was within physiological limits on the next day.

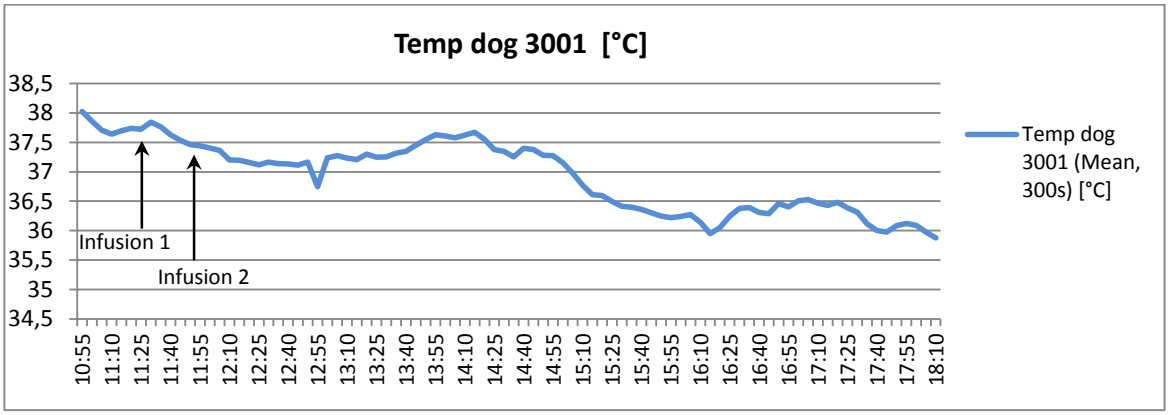


Figure 77: Body temperature after compound 3 (male dog, measured by implant). The left arrow marks the approximate start of the 1st infusion; the right arrow marks the start of the 2nd infusion.

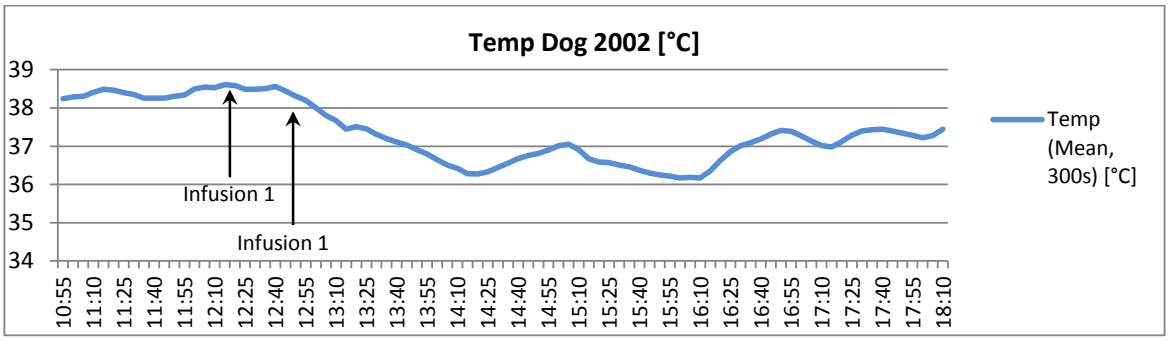


Figure 78: Body temperature after compound 3 (female dog, measured by implant). The left arrow marks the approximate start of the 1st infusion; the right arrow marks the start of the 2nd infusion.

Clinical Observations and Exposure in the Second Experiment

Due to unexpected cardiovascular symptoms, it was decided to repeat the experiment with compound 3 with one dog and include ECG recording. As the male dog appeared to be more sensitive and due to the possibility to collect CSF via port, it was decided to use this dog after a wash out time (12 weeks). The ECG was obtained via external telemetry (DSI™ JET) and the dog was habituated to wearing the jacket prior to the experiment. Baseline ECG was normal, with a mean heart rate of 125 bpm (calculated over 20 minutes of baseline recording, SD +/- 21.13).

Figure 79: ECG, recorded with external telemetry, during baseline recording (7 seconds, HR 77 bpm, sinusarrhthmia).

During the infusion (16.5 mg/kg over 10 minutes), the first signs were stiffness of the limb muscles and unstable gait. Hind legs showed muscle twitches and their severity increased throughout the infusion. A tonic contraction of the legs, paired with ophistotonus was observed towards the end of the infusion. Then, tonic-clonic movements, predominantly of the hind legs, appeared. Midazolam was administered intravenously within 20 seconds and stopped the involuntary muscle movements. When lifted up for blood sampling, the dog behaved aggressively and did not tolerate handling. This lasted for 20 minutes, after which he was calm again. Mean heart rate over 20 minutes from the start of infusion was 199 bpm (SD 10.73). This increase in heart rate was accompanied by vanishing of the sinusoidal arrhythmia (**Figure 80**). The ECG at the time point of convulsion could not be interpreted due to movement artefacts. One hour to one hour and twenty minutes after the infusion, mean heart rate was 155.9 bpm (SD 6.408). QTcv interval was not prolonged by administration of compound 3. Mean QTcv time in baseline was 237 msec (SD 8.952), within the twenty minutes after the start of the infusion the mean QTcv was 226.6 msec (SD 7.235) and one hour after start of the infusion it was 241.7msec (SD 5.669). All these values are within the reference ranges published for Marshall Beagle dogs (Hanton & Rabemampianina, 2006).

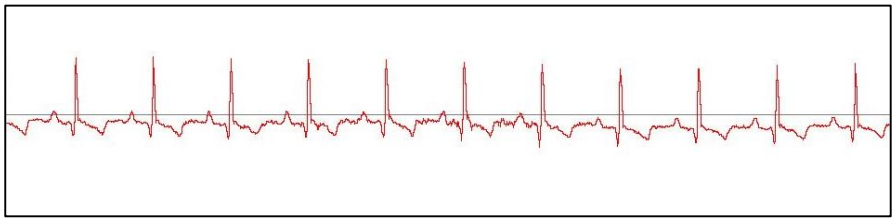


Figure 80*:* Increased heart rate and vanishing of sinusarrythmia 6 minutes after start of the first infusion.

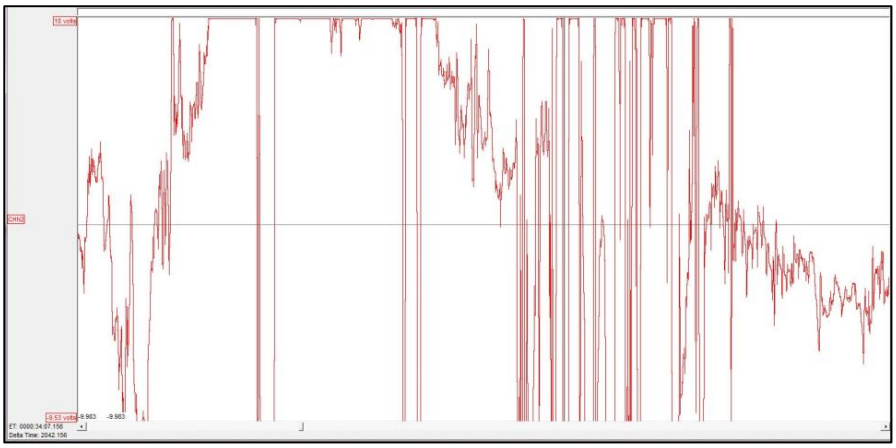


Figure 81: ECG Artefacts at the time of clinical convulsion with compound 3.

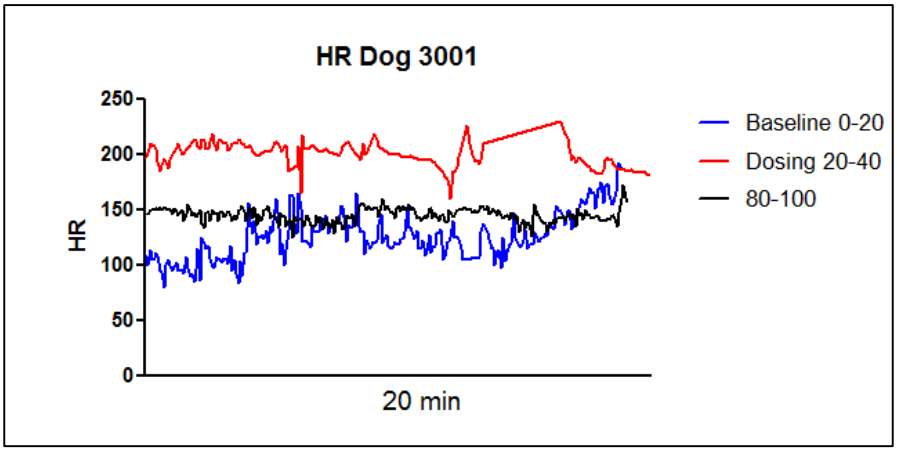


Figure 82: Heart rate change during 20 minutes of baseline (blue), at the beginning of the first infusion (red) and one hour after dosing start (black).

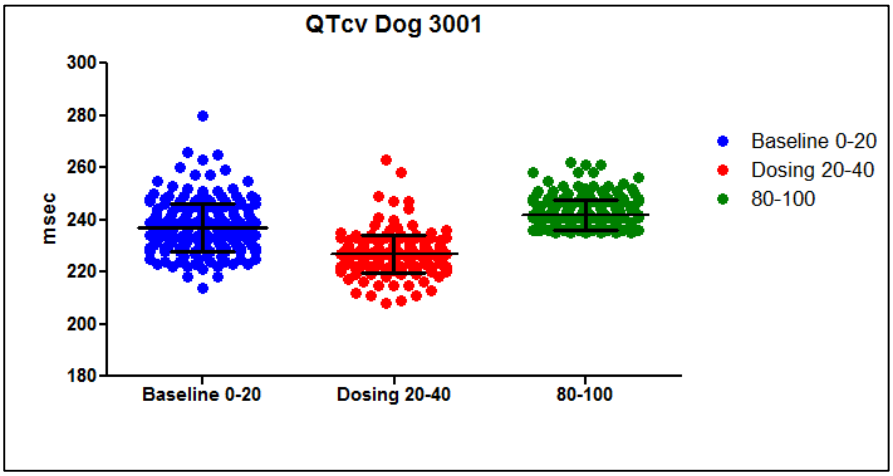


Figure 83: QTcv from the male dog in paseline, during 20 minutes starting with the infusion of compound 3 and one hour after start of the infusion (Median +/-SD).

Two and a half hours after the convulsion, a CSF sample was collected. The dog appeared to be sedated for a total of five hours. Exposure 20 minutes after the convulsion was 1600.7 ng/mL. He ate all food offered five and a half hours after compound administration. The drop in body temperature was observed again. Minimum temperature was 36.15°C, 7 hours after start of the infusion.

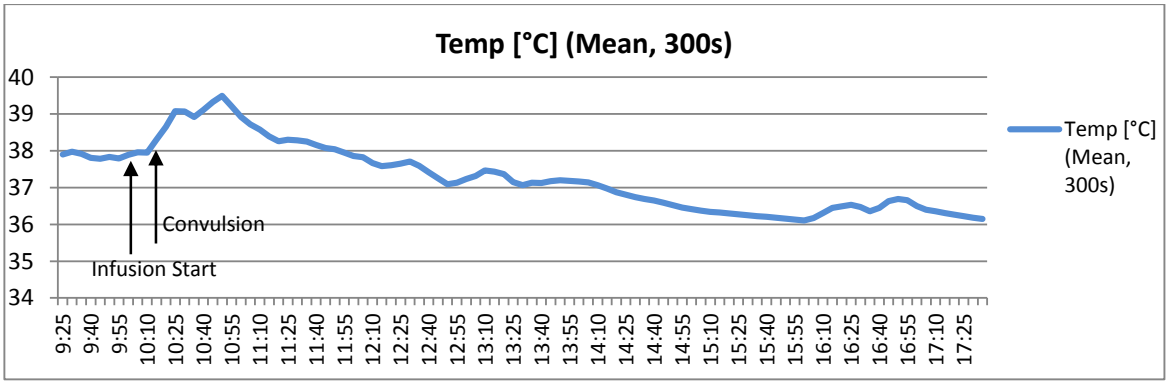


Figure 84: Temperature of the male dog after administration of compound 3 (measured by implant).

Clinical symptoms were compared to those observed in earlier NHP toxicology studies with a duration of more than one day (five days, two weeks). Route of administration in these studies was oral gavage. In NHPs, salivation and emesis were observed which were not seen in the dog. Also ocular signs (dilated pupils, bilateral nystagmus) were not present in dogs. As opposed to NHPs, dogs had an increased heart and respiration rate. Reddening of the mucosal membranes and increased pulsation of femoral vessels could indicate a rise in blood pressure, although this was not monitored specifically.

Symptoms in NHP toxicology studies	Symptoms present in EEG dog studies?	Comment
Activity Decreased	Yes	/
Convulsions	Yes	/
Dilated Pupils	No	/
Emesis	No	/
Jerks	Yes	Severity NHP study: mild Severity in dog study: mild, progressing to moderate and then convulsions
Nystagmus	No	Was considered a premonitory sign for convulsions in NHP
Salivation	No	Emesis was also present in NHP
Tremor (fine)	No	"Shivering" in NHP Decrease in body temperature in dog EEG study

Table 16: Symptoms in NHP toxicology studies and EEG dog studies.

Compound 3 had also been tested in a NHP primate study with intravenous infusions. Differences to the infusion scheme as opposed to the dog EEG study were that first a higher bolus was administered and then, infusion rate was held constant until convulsions

occurred. Free plasma concentration of compound 3 in this study is shown in **Figure 85**. Time points at which convulsions occurred are marked (*). In three out of six NHPs, convulsions were seen at administration of the initial dose. In one animal, no convulsion was seen despite very high plasma exposure. In two NHPs, time point of convulsions was during the infusion. Plasma exposure at which the dog in the EEG study had a convulsion is also indicated. As the sample was collected approximately 20 minutes after the symptom, the actual drug plasma concentration was higher. Still, the exposure in the dog is within the range of that in the NHPs.

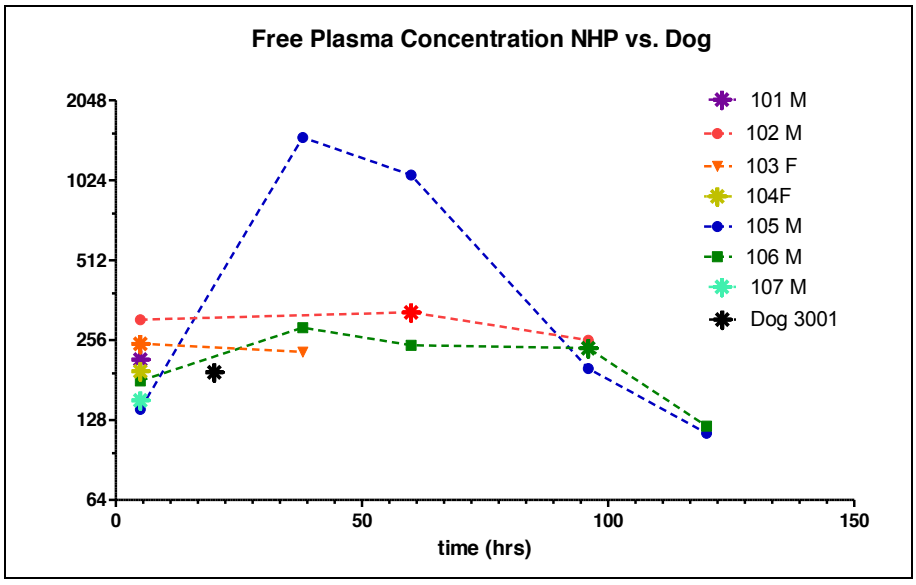


Figure 85: Exposure (C_{free}) NHP and dog. Time points marked with * indicate convulsions. Doses for NHP 101, 102 and 103 (not naïve) were determined by modeling to achieve a target of 2000 ng/mL total. Target exposure for NHP 105, 106 and 107 (naïve) was 1500 ng/mL total.

EEG Results: Visual Analysis (First Experiment)

EEG recordings of both dogs were normal in the baseline period (example female, **Figure 86**). During the first infusion of compound 3, a slowing of background activity was observed in both dogs. This effect was not persistent and normal background activity appeared regularly. Episodes of slow EEG activity, interrupted by normal background patterns, were observed during the both infusion steps and in the observation period.

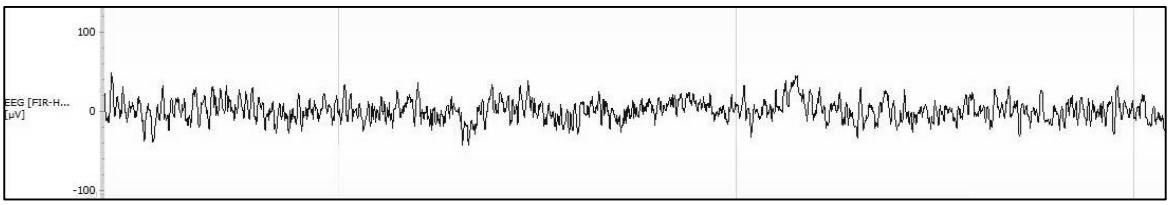


Figure 86*:* Baseline recording (example female dog).

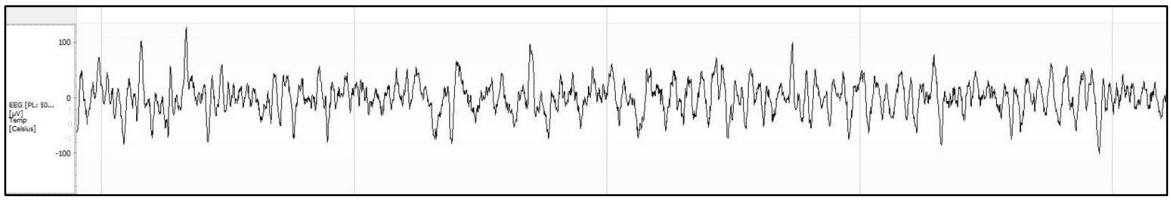


Figure 87*:* Slowing of background activity 8 minutes after the start of the first infusion of compound 3 (10 seconds, female dog).

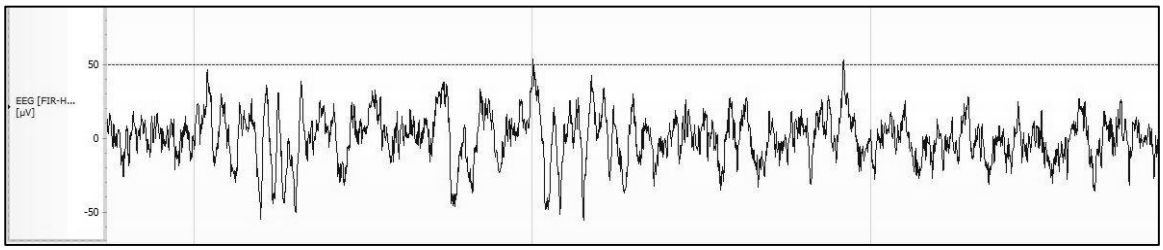


Figure 88: Example of the slowing of background activity during the 20 minutes observation period (8 seconds, female dog). The axis line is set to 50 µV, indicating the peak frequencies at that time.

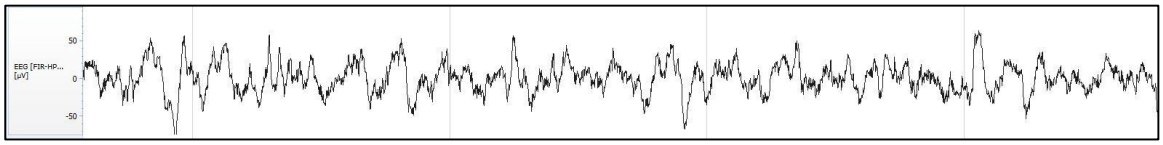


Figure 89*:* Slowing of background activity after administration of the 2nd dose of compound 3 (10 seconds, female dog).

After the second dose, spike-like patterns appeared in the EEGs of both dogs. In the female, amplitudes between 50 µV and 70 µV predominated. In two episodes 10 minutes and 15 minutes after termination of the infusion, amplitude reached 100 µV. In the female, the last such event in the EEG was 20 minutes after stop of the infusion. In the male dog, amplitudes consistently were higher than 100 µV. These episodes had a low incidence and disappeared within 10 minutes after completion of the second infusion. Video recordings showed no definite cause of artefact at that time but still, it cannot be ruled out that e.g. swallowing was the reason for this EEG sign.

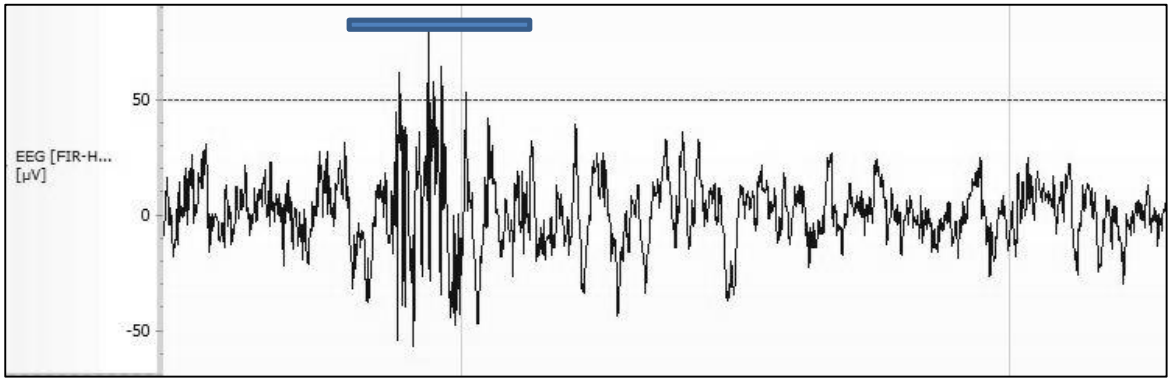


Figure 90*:* Spike-like patterns (marked blue) five minutes after completion of the 2nd infusion (5 seconds, female dog). The amplitude is low (60 µV).

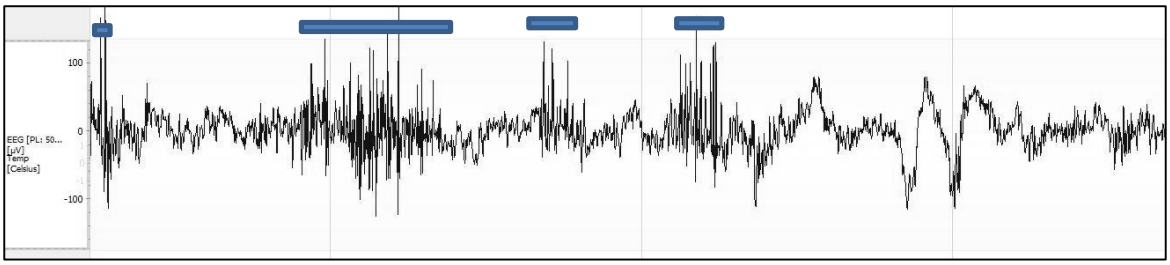


Figure 91*:* Spikes of above 100 µV amplitudes (marked blue) approximately five minutes after administration of the 2nd infusion of compound 3 (10 seconds, male dog).

EEG Results: Visual Analysis (Second Experiment)

Baseline EEG of the male was normal. After the start of the infusion, no distinct EEG changes were noticed. Artefact rate was higher than in other recordings, which might be due to the jacket touching the subcutaneous transmitter. Isolated spikes-like EEG signs and episodes with a higher degree of synchronization could be identified, but it was not clear if they were artefacts or real symptoms. Before and during clinic convulsion no paroxysmal activity was present in the EEG. High frequencies with peak amplitudes of 200 µV predominantly in a downward deflection appeared after start of clinical symptoms. Midazolam had already been administered at that time.

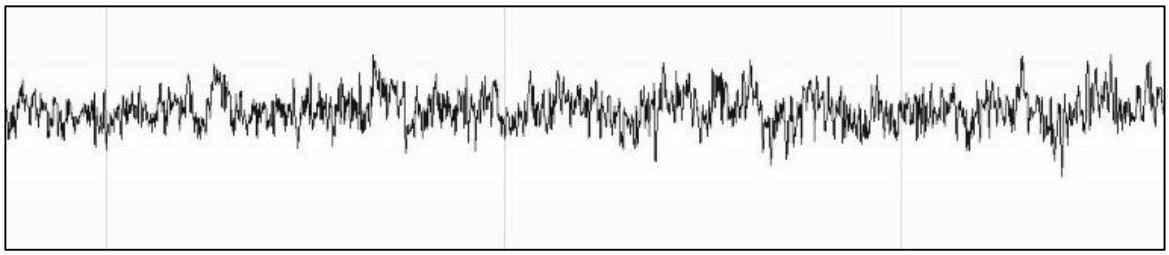


Figure 92*:* Normal EEG background activity, at the time point of clinic convulsion (10 seconds, male dog).

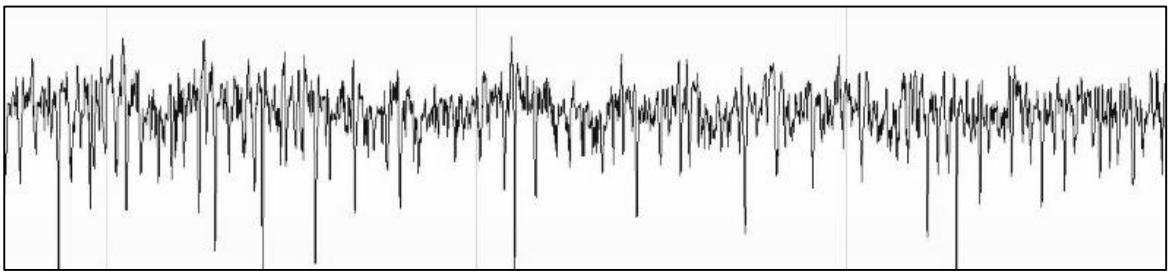


Figure 93: High-frequency synchronized EEG activity, one minute after midazolam treatment (10 seconds male dog).

EEG Results: Quantitative Analysis

The median relative power was calculated and for both dogs and it was found that increases in the delta and theta frequencies were induced by administration of compound 3. The other frequency bands were not affected in the same direction or degree in the two dogs. The

increase in the delta band was also observed in the second experiment, whereas the change in the theta band was not. All other medians values were not influenced by treatment in the second experiment.

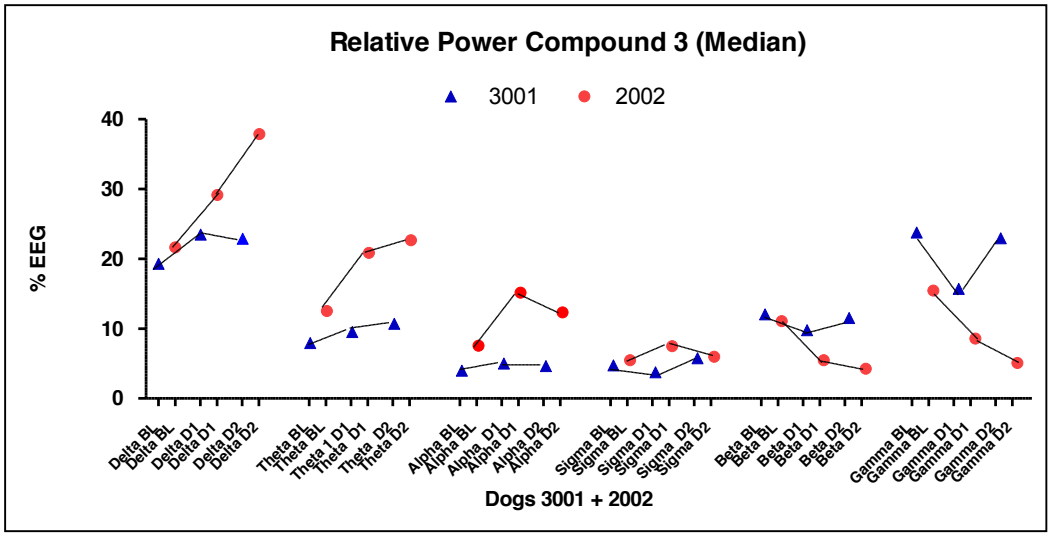


Figure 94: Changes of median relative power with compound 3.

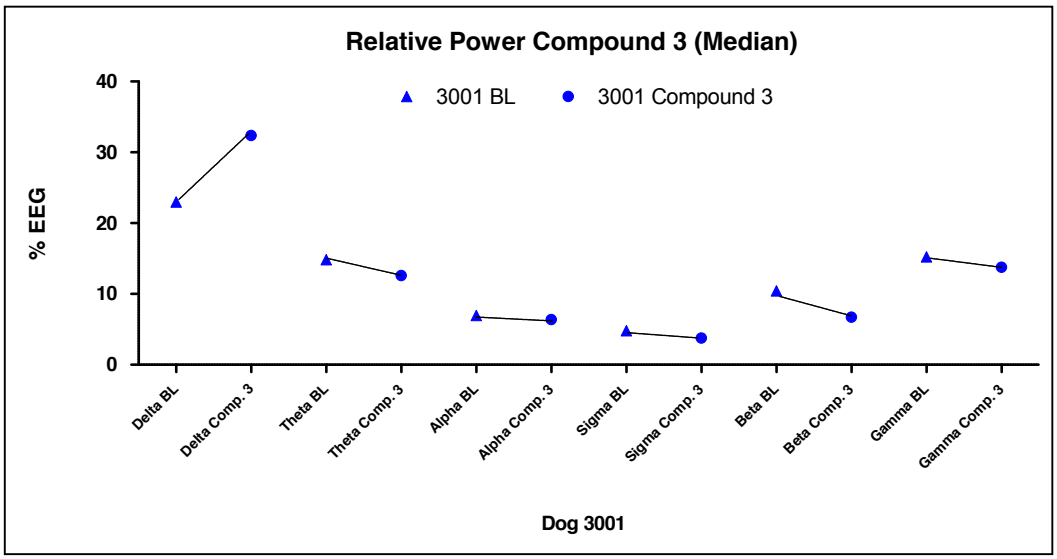


Figure 95*:* Changes of median relative power with compound 3 in the male dog.

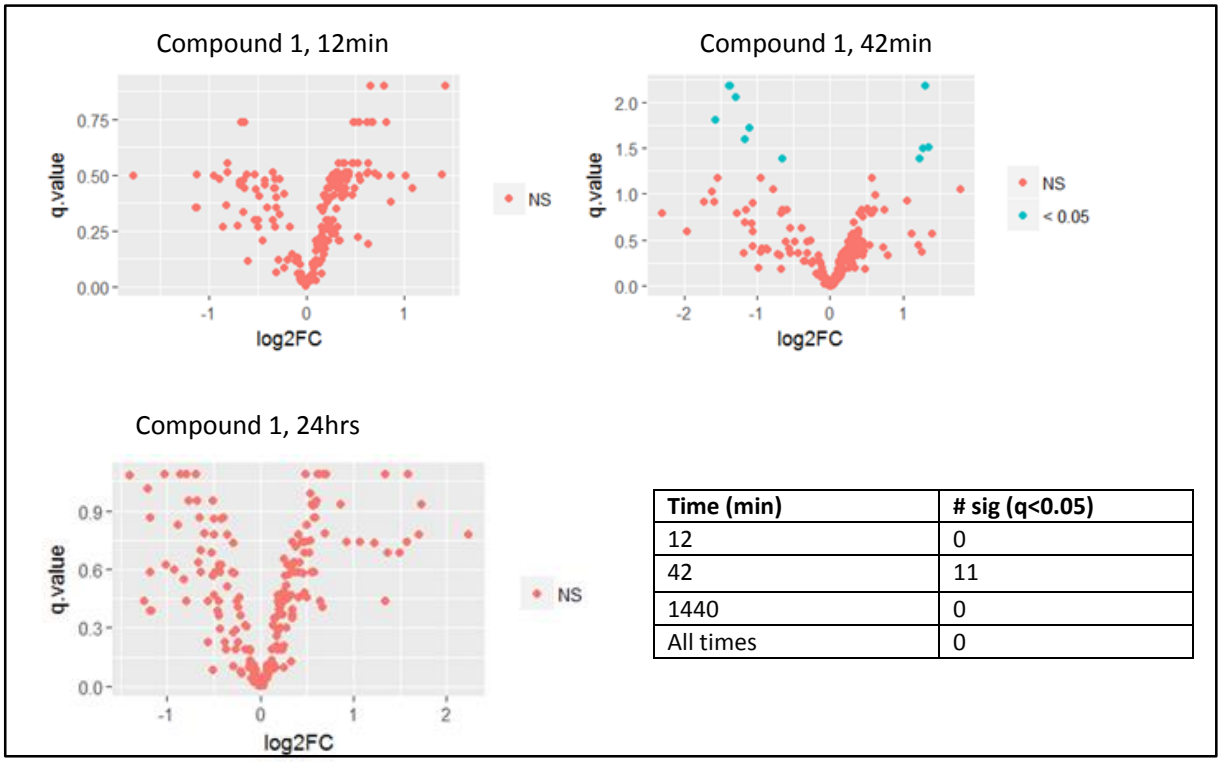
4 Biomarker Analysis

Raw data from the biocrates analysis (Biocrates Absolute IDQ® p180 Kit (Biocrates Life Sciences, Insbruck, Austria)) were summarized in an Excel Sheet by the department of DMPK, AbbVie GmbH & Co. KG, Ludwigshafen, and submitted to the Data and Statistical Science (DSS) department at AbbVie GmbH Co. KG, Ludwigshafen. For each treatment, the set of metabolites with $q < 0.05$ at all time-points is presented in **Figures 96** to **98**. From a

total of 178 metabolites and 43 pre-calculated ratios of interest that were detected with the kit, a total of 0, 14 and 0 metabolites were significantly changed at all time points for compounds 1, 2 and 3 respectively. Most significant changes at a q < 0.05 were detected with compound 2.

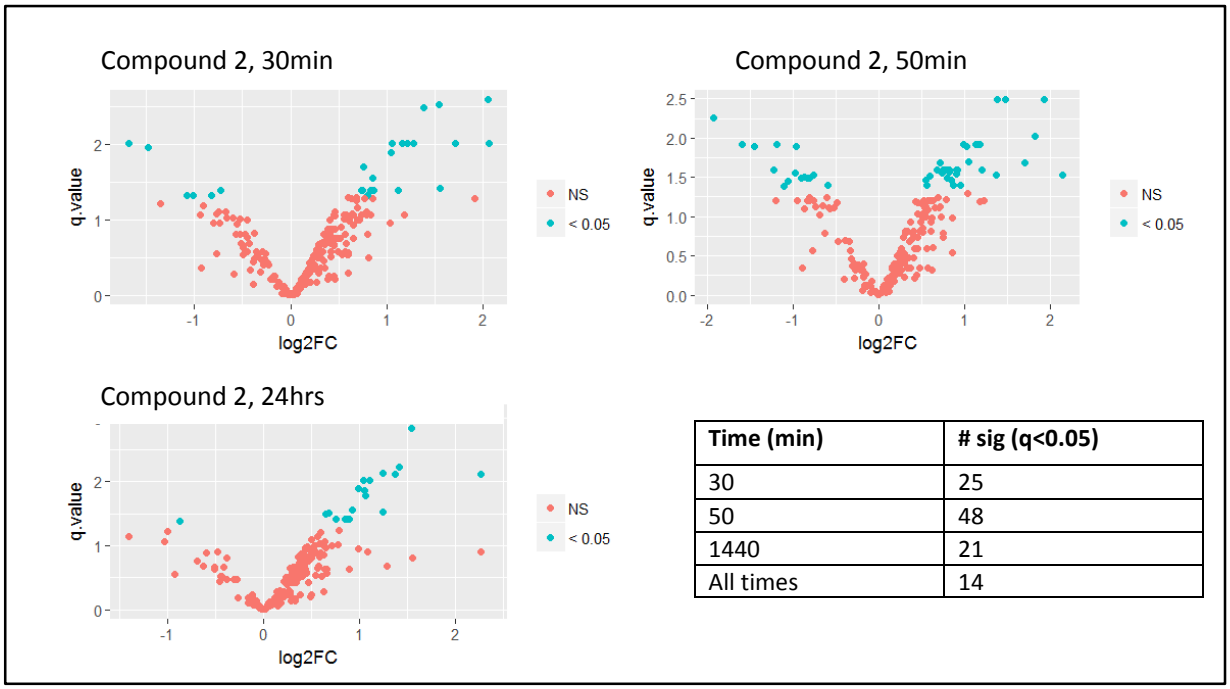
The Venn diagram (**Figure 99**) shows the overlap between treatments for all metabolites that changed with an FDR of q < 0.05 at any of the time points. One outcome was changed in all three experiments at q < 0.05, and this was the SDMA/ Arg ratio (**Figure 99**).

At a less stringent threshold of p < 0.05, 36 overlapping metabolites with an altered expression after treatment were detected (**Figure 100**). The 36 common metabolites that changed after all 3 treatments are listed in **Table 17**.



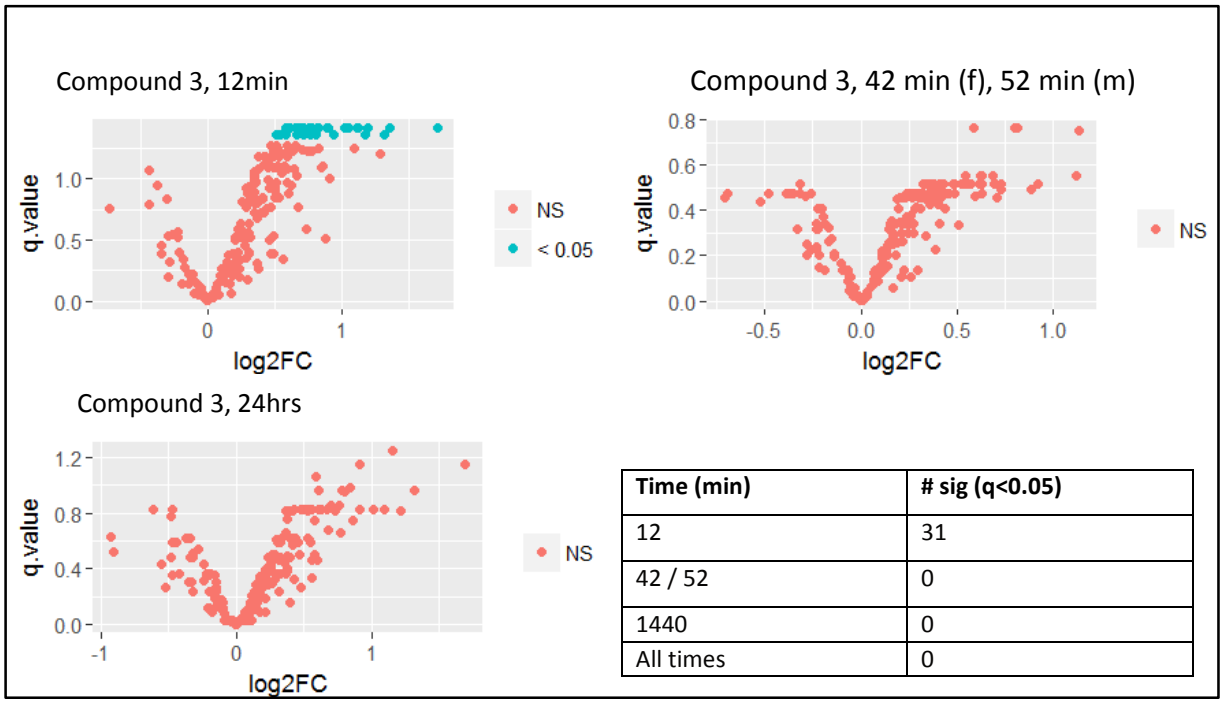
Time (min)	# sig (q<0.05)
12	0
42	11
1440	0
All times	0

Figure 96*:* Volcano plots showing the false discovery rate (y-axis) vs. the effect size (log base-2 of the FC) for each of the three time points after treatment with compound 1. Blue dots represent results with q < 0.05. The table shows the number of significantly changed metabolites at each time point, as well as the number of unique metabolites that were significantly changed at any time point.



Time (min)	# sig (q<0.05)
30	25
50	48
1440	21
All times	14

Figure 97: Volcano plots showing the false discovery rate (y-axis) vs the effect size (log base-2 of the FC) for each of the three time points after treatment with compound 2. Blue dots represent results with q < 0.05. The table shows the number of significantly changed metabolites at each time point, as well as the number of unique metabolites that were significantly changed at any time point.



Time (min)	# sig (q<0.05)
12	31
42 / 52	0
1440	0
All times	0

Figure 98: Volcano plots showing the false discovery rate (y-axis) vs the effect size (log base-2 of the FC) for each of the three time points after treatment with compound 3. Blue dots represent results with q < 0.05. The table shows the number of significantly changed metabolites at each time point, as well as the number of unique metabolites that were significantly changed at any time point.

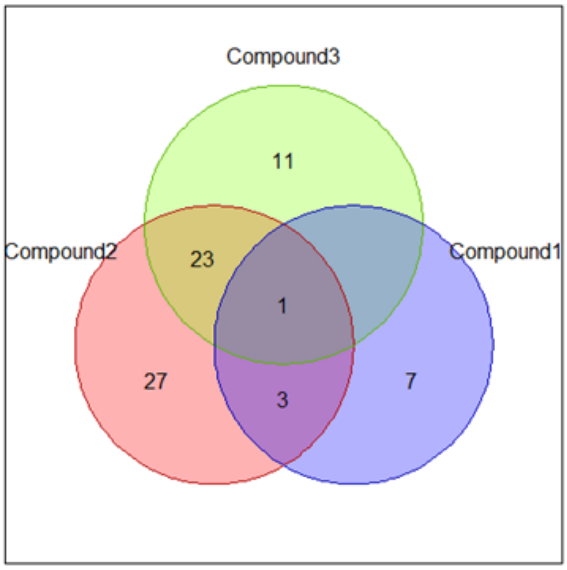


Figure 99: Number of overlapping metabolite changes (q < 0.05) at any time point

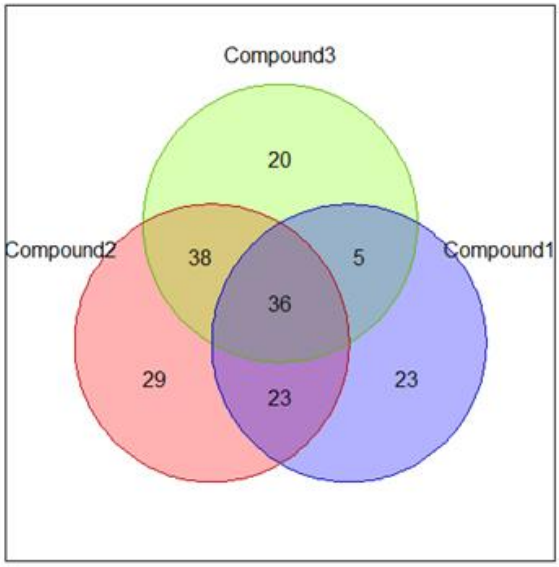


Figure 100: Number of overlapping metabolite changes (p < 0.05) at any time point

Metabolite Class/ Ratio	Metabolite	Time point 1 C1 – C2 – C3	Time point 2 C1 – C2 – C3	24hrs C1 – C2 – C3
Acylcarnithines (n=6)	C14.1.OH Hydroxytetradecenoylcarnitine	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ -↑ - ↑
	C14.2 Tetradecadienylcarnitine	↑ - ↑ - ↑	↑ - ↑ - ↑	↑- ↑ - ↑
	C16 Hexadecanoylcarnitine	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	C18 Octadecanoylcarnitine	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	C18.1 Octadecenoylcarnitine	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	C5.DC.(C6.OH) Glutarylcarnitine (Hydroxyhexanoylcarnitine)	↑- ↑ - ↑	↑ - ↑- ↑	↑ - ↑ - ↑
Amino Acids	Methionine (Met)	↓ - ↓ - ↓	↓ - ↓ - ↓	↓ - ↓- ↓
	Proline (Pro)	↓ - ↓ - ↓	↓ - ↓ - ↓	↓ - ↑ - ↓
	Nitro-Tyrosine	↓ - ↓ - ↓	↓ - ↓ - ↓	↓ - ↓ - ↓
Glycerophos-pholipids	lysoPC.a.C16.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	lysoPC.a.C26.0	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑

	lysoPC.a.C26.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C28.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C30.0	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C34.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C38.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C40.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C40.2	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C40.4	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.aa.C42.5	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.ae.C34.2	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.ae.C34.3	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	PC.ae.C42.2	↑ - ↑ - ↑	↑ - ↑ - ↑	↓ - ↑ - ↑
	PC.ae.C44.3	↓ - ↑ - ↑	↓ - ↑ - ↑	↓ - ↑ - ↑
Sphingolipids	SM..OH..C22.2	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	SM.C20.2	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	SM.C24.0	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	SM.C24.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	SM.C26.1	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
Amino Acids and biogenic Amines (ratios)	ADMA...Arg	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	MUFA..PC	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	SDMA...Arg	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↓ - ↑
	Total.DMA...Arg	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
	Tyr...Phe	↓ - ↓ - ↓	↓ - ↓ - ↓	↓ - ↓ - ↓
Sphingolipids	Total.SM	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑
Total non-OH SM	Total.SM.non.OH	↑ - ↑ - ↑	↑ - ↑ - ↑	↑ - ↑ - ↑

Table 17: Results of the metabolomic analysis: Altered metabolites with compound 1, 2 and 3, based on a unadjusted p-value < 0.05. Decreases (↓) or increases (↑) in metabolite level between baseline and the two post treatment time points are indicated; = means that metabolite level was similar to baseline (similar defined as a change less than). Red are non-significant changes, green are significant changes (p<0.05). "C x:y": C=Carbon atome, x=carbon number, y=number of double bonds; SM= Sphingomyelin, PC= Phosphatidylcholines; ADMA= Asymmetric dimethylarginine; SDMA= Symmetric dimethylarginine; Met= Methionine; Arg= Arginine; DMA=Dimethylarginine; aa= both moieties at the sn-1 and sn-2 position are fatty acids and bound to the glycerol backbone via ester bonds; ae= one of the moieties, either in the sn-1 or at sn-2 position is a fatty alcohol and bound via an ether bond 8: lyso-PC.a= Lyso – Phosphatidyl-cholines – acyl; PC.aa=Phosphatidyl-choline acyl-alkyl; PC.ae=Phosphatidyl-choline diacyl[2].

[2] (http://www.biocrates.com/images/List-of-Isobaric-and-Isomeric-Lipid-Species.pdf, accessed 07.Aug 2017) and (http://www.biocrates.com/products/research-products/absoluteidq-p180-kit, accessed 07.Aug 2017)

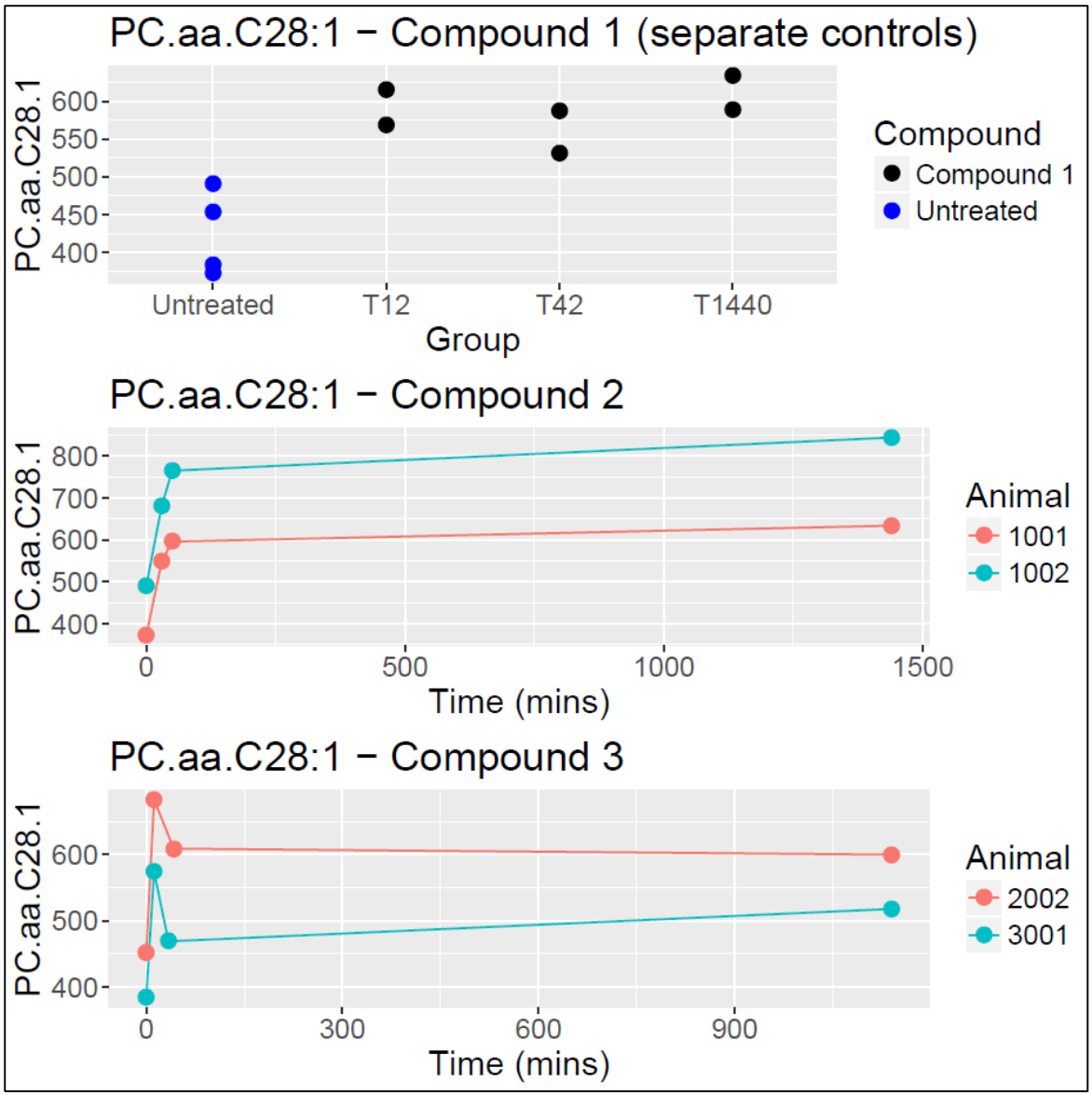


Figure 101: Example plot of the Biocrates results for the individual compounds. Results for the other overlapping metabolites are provided in the supplementary data. An increase in the level of phosphatidylcholine from baseline to time point 1, 2 and 3 (=24hrs) is evident for all treatments.

The 34 metabolites that were altered in all the three different treatments belonged to the class of acylcarnithines (n=6), glycerophospholipids (n=15); sphingolipids (n=5) and amino acids (n=3, methionine, nitro-tyrosine, proline). Also, the total DMA / arginine ratio, total sphingomyelin, total not-hydroxylated sphingomyelin and the tyrosine / phenylalanine ratio changed significantly on treatment.

VI.Discussion

The aim of this thesis was to evaluate use of EEG data for improved assessment of neurological symptoms, especially convulsion liability. Four reference and three in-house compounds with different modes of action were tested for this purpose. Several methods exist for evaluation of seizure liability, but the EEG has been identified as the most sensitive one (Easter *et al.*, 2009). Additional video recording supported correlation of symptoms to EEG, identification of artefacts and increased observation time. In addition, these experiments should generate new data to allow direct comparison of dog and NHP regarding their species specific sensitivity for neurological symptoms.

1 Question 1

By using the EEG, can premonitory signs of convulsions be identified that facilitate prophylactic anticonvulsive treatment and avoid the occurrence of convulsions in toxicology studies, thereby reducing the burden of animals?

The possibility to administer anticonvulsive treatment upon the first detection of paroxysmal EEG activity would potentially reduce the burden of seizure liability testing by preventing progression of a seizure into a convulsion. Authier *et al*. (2009) reported a possible treatment window of four minutes in NHPs in a study using PTZ as convulsive agent whereas Dürmüller *et al*. (2007) described a latency time of 60 seconds prior to the onset of PTZ – induced convulsions in dogs. These differences probably resulted from route and mode of application (Authier *et al.*, 2009). Whereas in the NHP study, PTZ was administered subcutaneously at fixed doses in 15-minutes intervals (Authier *et al.*, 2009), the dogs received a continuous intravenous infusion (Dürmüller *et al.*, 2007). With subcutaneous dosing the latency between paroxysmal activity and convulsions was prolonged by a factor 4 (Authier *et al.*, 2009). Both authors considered these periods sufficient to allow intravenous administration of anticonvulsive drugs (Dürmüller *et al.*, 2007; Authier *et al.*, 2009).

In our experiments, compound 1 and compound 3 induced convulsions. With compound 1, paroxysmal activity was present in the EEG of two dogs prior to onset of clinical convulsion as reported for PTZ induced convulsions (Dürmüller *et al.*, 2007; Authier *et al.*, 2014b). Compound 3 induced clinical convulsions in the second experiment in one dog without clear paroxysmal EEG activity prior to and during clinical convulsion. Spikes in the EEG could not definitely be related to either real EEG symptoms or artefacts. The EEG at onset and during most of the clinical convulsion was normal. An abnormal EEG was seen later, and lasted longer than the clinical symptom. DSI^{TM}'s technical support was contacted to identify a potential error in the synchronization between video and EEG. As all settings were correct, it was concluded that this was a real phenomenon. Fonck *et al.* (2015) point out that there are behaviors with a clinical presentation that can be mistaken for a seizure and recommend EEG for confirmation of the electroencephalographic origin (Fonck *et al.*, 2015). The dog

responded to midazolam treatment and autonomic signs were also present during muscle contractions. From a clinical point of view, the diagnosis of a convulsion was therefore clear.

The time needed for the treatment of a convulsion was below one minute, so in theory, EEG-based prophylactic treatment should be possible. After reviewing the literature it seems that this has not been done successfully in preclinical research so far.It has to be added that certain preparations need to take place prior to start of dosing: inserting an intravenous port, placing the anticonvulsant drug ready-to inject at the dose appropriate for the animal in a syringe within easy reach and preparing a second syringe with a flushing fluid.

One reason for the difficulty to detect epileptiform activity prior to the onset of convulsions is the quality of the raw, unprocessed, EEG signal, at least in the program used here (DSI™ Ponemah, Version 5.2). Although providing a live-view on the EEG, it did not facilitate meaningful interpretation of the unfiltered signal during the EEG studies. The EEG signal passes over the screen at high speed, so differentiation of real paroxysmal events from artefacts was not possible. For retrospective analysis, the first step was application of a high pass filter to stabilize the baseline. Also, unclear events were reviewed several times in relation with the video recording. A definite differentiation of true paroxysmal EEG activity from artefacts therefore only was possible after careful retrospective assessment, using filters and requiring more time than available in real-time analysis.

The absence of definite EEG symptoms before and during clinical convulsion induced by compound 3 is surprising. In an earlier rat EEG study, compound 3 induced spike-wave discharges (SWD) in two out of six rats from one hour post dose (60 mg/kg oral dose, 1170 ng/mL) without visible convulsions. These SWDs are shown in **Figure 102**. At 120 mg/kg, convulsions were observed in three out of six rats. In the EEG, two of the remaining three rats had SWD activity without behavioral changes. One rat did not demonstrate SWDs. Therefore, clinical symptoms without paroxysmal activity were not seen in the rat as in the dog EEG study. Paroxysmal events of increased amplitude and synchrony were seen in EEGs of both dogs after the second infusion during the first experiment without convulsion.

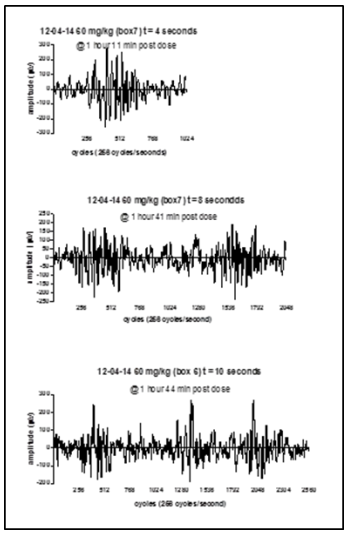


Figure 102*:* Rat EEG after 60 mg/kg oral dose of compound 3 Spike and wave discharges were not accompanied by clinical signs of convulsions.

In NHPs, nystagmus was seen prior to occurrence of convulsions. In dogs, this was not observed with any of the two compounds that induced convulsions in the EEG experiments. Also, in the NHP infusion study with compound 1, no prodromal nystagmus was described. The incidence of nystagmus in toxicology studies was found to be higher in NHPs than in dogs (Backes, 2016). Backes (2016) suspected that differences in handling practice can explain this as dogs can usually move freely in their kennels whereas NHPs are commonly restrained in a chair and therefore closer to human observers (Backes, 2016). In humans, Husain *et al*. (2003) observed that abnormal eye movements are a frequent sign in patients with non-convulsive status epilepticus and therefore, the nystagmus observed after administration of compound 3 could indicate the presence of a seizure prior to its generalization in the NHPs. In the rat the presence of non-convulsive seizures was confirmed with EEG, but assessment of nystagmus in rodents is difficult and presence of this symptom in rats could not be confirmed.

For dogs with idiopathic epilepsy, Berendt & Gram (1999) investigated clinical signs of non-convulsive seizures. They do not mention nystagmus and only note pupil dilation as ocular symptom (Berendt & Gram, 1999). However, their results are based largely upon owner descriptions and it can be suspected that an untrained person might miss this symptom. To sum up, nystagmus seems to be a potential sign of non-convulsive seizures, but its occurrence seems to depend on compound and species.

Instead of nystagmus, compound 3 induced clinical signs in dogs not described in rat and NHP. These symptoms were tachycardia and red mucous membranes. Reflex tachycardia can occur after infusion of high volumes ("Bainbridge effect", see (Vatner *et al.*, 1975)) but as infusion rate and volume were appropriate for dogs and this was not observed with infusion of compound 1, a compound-specific effect was suspected.

In the development of compound 3, cardiovascular safety was explored in the *in vitro* hERG (human ether-a-go-go related gene) and in the anesthetized dog model. hERG is a sodium channel on cardiomyocytes, responsible for the repolarization. The assay detects QT prolongation which is a measure of pro-arrhythmic drug effects. Results of the hERG assay corresponded well to *in vivo* testing in the anesthetized dog. There, the QT interval was only prolonged at a very high exposure level (2870 ng/mL). QT interval in our study was also not affected by administration of compound 3 at exposure levels up to 1600.7 ng/mL. A slight increase in QT length one hour after administration of the first dose could be related to the decrease in body temperature(Van der Linde *et al.*, 2008; El Amrani *et al.*, 2016).

In the anesthetized dog, a drop in mean arterial pressure and dP/dt was noted at 810 ng/mL. This exposure level is below the one achieved in the EEG study, and effects on the cardiovascular system were seen. Clinically, a rise in blood pressure was suspected but could not be objectively assessed as no blood pressure monitor was available. The role of central serotonin and related molecules on the cardiovascular system has been reviewed by Kuhn *et al.* (1980). Blood pressure effects seem to be species dependent and in dogs, a depressant

action has mostly been reported, especially in anesthetized animals (Kuhn *et al.*, 1980). One reference also reports blood pressure increases with 5-HTP in conscious dogs (Dunkley *et al.*, 1972). It is known that drugs can have opposite effects in anesthetized and conscious dogs, due to the absence of reflex responses in the anesthetized state (Sarazan *et al.*, 2011).

Decreased contractility, that has been detected in the safety pharmacology assessment of compound 3, leads to a reduction in heart performance and thereby to a reduced ability to adapt to stress (Sarazan *et al.*, 2012). A drop in mean arterial pressure (MAP) could be accompanied by a compensatory increase in heart rate which corresponds to the tachycardia observed in the EEG study. Therefore, both, a rise or a drop in blood pressure can explain the symptoms observed and objective pressure measurements would be needed for a final conclusion.

A cardiovascular symptom that has a clinical appearance that can be mistaken for seizures is syncope. EEG effects of syncope in humans have been described by Brenner (1997). However, they differ from the appearance of the EEG recorded during administration of compound 3: in syncope, an initial slowing of background activity is followed first by high-amplitude delta activity, progressing into subsequent flattening (Brenner, 1997). In our study, the EEG background activity slowed down during the first experiment with compound 3 in both dogs. This correlated to the clinic, as both dogs seemed to be slightly sedated. In the second experiment, no such EEG effect was observed. The ECG recorded via external telemetry simultaneous to the convulsion could not be interpreted, due to the high amount of movement artefacts. In conclusion, the possibility that the convulsions in NHP and dog were due to cardiovascular symptoms cannot be excluded.

It needs to be added that the metabolism of compound 3 varies between NHP and dog: in NHP, main metabolites are carbamoyl glucuronides, whereas in dog, aliphatic parts of the molecules are hydroxylated, meaning another metabolite is formed in the dog. Pharmacologic activity of this metabolite has not been further investigated and might explain differences in clinical symptoms.

Body temperature decreases are not typically regarded as premonitory sign of seizure (Markgraf *et al.*, 2014). In a rat study with the selective 5HT2c agonist Org 306039, a decrease in body temperature was also noted, but started only on dosing day three (Markgraf *et al.*, 2014). Another report describes that intrahypothalamic injection of serotonin causes hypothermia in rats (Cox & Lee, 1981). EEG changes accompanied by hypothermia were described by Ostojic *et al*. (2013) in models of absence seizures. He described that spike wave discharges were present at the same time as PTZ or gamma-hydroxybutyrate (GHB) induced hypothermia, but also in the period of spontaneous rewarming (Ostojic *et al.*, 2013). Therefore they concluded that mildly lowered core body temperature can cause non-convulsive seizures (Ostojic *et al.*, 2013). The hypothermia induced by compound 3 therefore could have contributed to the occurrence of non-convulsive seizures in the rat EEG.

Other premonitory signs of convulsions in the EEG studies were rather unspecific clinical neurological symptoms: ataxia, jerks, tremor, twitches of facial and/ or limb muscles, salivation, increased sensitivity to touch or noise, agitation and excitation. Twitches and myoclonic jerks were present prior to all compound induced convulsions described here and were accompanied by an abnormal EEG. Tremor was also observed, but Authier *et al.* (2017) reported that this symptom is not usually related with EEG paroxysmal activity and in the case of compound 3, could also be induced by hypothermia.

The premonitory symptoms of drug induced seizures in dogs overlap with reports on prodromal signs in dogs with idiopathic epilepsy. Prodromal signs in epileptic dogs, as described by Berendt & Gram (1999), were e.g.: anxiety, restlessness, attention-seeking, limb twitches, tremor or head tremor, tonic jaw opening, incoordination, autonomic signs (emesis, salivation) and dilation of pupils. Also, Fonck *et al.* (2015) noted that pre-convulsive symptoms are similar across preclinical species and also translatable to humans. However, Berendt & Gram (1999) report that occurrence of such symptoms does not necessarily mean a secondary generalization to convulsions. This is also true for toxicology studies, in which neurological symptoms are not always followed by convulsions.

EEG was not included in the study on epileptic seizure types and their respective clinical symptoms in dogs conducted by Berendt & Gram (1999). From EEG examinations in humans it is known that the prodromal state preceding seizures can, but must not, be accompanied by EEG changes (Berendt & Gram, 1999). The EEG is the accepted gold-standard for seizure detection, but its use for identification of the prodromal state is therefore limited.

The common term for the prodromal state is “aura”. This forewarning sensation is thought to originate from activation of a small group of neurons (Berendt & Gram, 1999). We used a bipolar montage with two electrodes. Depending on the location of the focal EEG changes relative to the electrodes, paroxysmal activity could be missed.

Technical limitations could also explain the absence of clear EEG symptoms prior to and at the beginning of the convulsion in the second experiment with compound 3: Given completely synchronous activity of both hemispheres, the differentially amplified signal would equal zero in recordings from bilateral electrodes. Inclusion of a reference lead would be beneficial in future EEG studies.

In conclusion, premonitory EEG signs are difficult to definitely diagnose during real-time viewing. They were only present within a short time frame prior to the convulsion. In one case, no clear premonitory EEG signs were identified prior to convulsion. Therefore, this method cannot help to prevent the occurrence of drug induced convulsions in animals that are monitored with EEG. Clinical prodromal signs vary between species and seem to not reliably forewarn of convulsions. Nevertheless, treatment decisions can rather be based on clinical symptoms than on EEG.

A dedicated study can, however, detect convulsion liability in a limited number of animals. As convulsions are a dose-limiting factor, early definition of the exposure levels correlated with convulsions could be a rationale for lower doses in long-term toxicology studies. Convulsive levels could be avoided in regulatory toxicological studies, thereby reducing the burden on these animals.

2 Question 2

Is combination of two procedures possible to enable simultaneous EEG recording and CSF sampling for analysis of potential biomarkers of seizure liability?

CSF ports in dogs are an established method in preclinical drug development (Wilsson-Rahmberg *et al.*, 1998). These implants enable repeated CSF sampling from conscious dogs. Samples are usually not contaminated by blood and of good quality (Wilsson-Rahmberg *et al.*, 1998). As implants are covered by skin, group housing is possible and natural behaviors of dogs are not impaired. Group housing is the recommended method for dog husbandry (Hawkins *et al.*, 2004). Also, it has been shown that the quality of measurements is better compared to single-housing conditions (Klumpp *et al.*, 2006). Next to the animal welfare perspective, group housing is also advantageous for detection of neurological symptoms: earlier evaluation of internal data showed that the detection rate for neurological symptoms is higher if an animal can move freely compared to restraint conditions (Backes, 2016). Therefore, completely internalized devices were also selected for EEG recording.

In this thesis, it was first investigated in a pilot study if EEG signals of good quality can be obtained from dogs with pre-implanted CSF ports. As results from intra-surgical evaluation were positive, dogs with and without CSF ports had EEG electrodes implanted. Neither in baseline recordings nor in experiments with CNS-active compounds, were differences in recording quality noticed between dogs with and without CSF ports.

For establishment of an assay, a set of reference compounds was selected as has been described in literature (Winter *et al.*, 2008; Easter *et al.*, 2009; Zhang *et al.*, 2011). For EEG recordings, especially the use of PTZ as a positive control agent has been described (Dürmüller *et al.*, 2007). This was not done due to the following reasons: first, for validation of equipment functioning, a positive control is not necessary, also not according to current guidelines (FDA, 2001). Also, testing PTZ as a positive control would mean an extra burden for the dogs and restrict their use in further experiments for ethical and legal reasons.

To allow for simultaneous implantation of CSF ports and EEG transmitters, bilateral coordinates were selected. This was based on external advice from a contract-research-organization (CRO). However, the transmitters provided by DSI^{TM} do not have a reference lead, so only a bipolar montage with one channel could be realized with the equipment available. Standard application of the M01 transmitter (DSI^{TM}) is measurement of

cardiovascular parameters; therefore they have a solid tip lead with a more rigid wire by default. During implantation, removal of the insulation and bending of the wire was more difficult with this lead. A query to the vendor showed that they had not been aware of this and it seemed this was the first time that this implant type was used for EEG recordings. Results show that baseline recordings at different vigilance states compared well to literature references so the transmitter and implantation scheme seem to be suitable for dog EEG recordings. In case of follow-up experiments with this transmitter, it is recommended to order it with two regular leads and, if possible, one reference lead.

In human and veterinary clinics, multiple electrodes are usually used to identify focal EEG changes and the use of only two electrodes is a limitation. Two electrodes have been shown to be a number sufficient for the detection of generalized EEG changes (Parmentier *et al.*, 2006; Dürmüller *et al.*, 2007). This was confirmed in our experiments as appearance of baseline and sleep patterns corresponded well to literature reports. However, EEG analysis is limited when only one channel is available: Focal EEG changes cannot be localized or even missed, connectivity analysis is not possible and artefact identification is more difficult.

The most common artefact in the EEG resulted from movement and muscle activity and this was also described by Authier *et al.* for NHPs (Authier *et al.*, 2009). In theory, the dental cement that covers the electrodes serves as insulation thereby preventing occurrence of artefacts in the EEG. However, the voltage of muscles is high enough to still be captured by implanted electrodes. This could be demonstrated by offering food. Artefacts appear simultaneously to jaw movements and can be distinguished from EEG activity due to their high frequency and amplitude. However, the relation between artefacts in the EEG and muscle activity is not always clear. Reasons can be video quality which was not sufficient to show subtle movements as from ears and eyelids. Also, some behaviors can be invisible for an external observer, such as movements of the tongue or increases in muscle tone.

Artefact prevention could have been done with the use of sedative agents, general or local anesthetics as it has been described by various clinical veterinarians (Fox & Stone, 1967; Klemm & Hall, 1970; Holliday & Williams, 1999). Local anesthetics were applied in the experiment evaluating the compatibility of subdermal needle electrodes with the DSI™ recording system. In the other experiments, use of accompanying pharmaceutics was not considered appropriate, as drug-drug interactions could occur and behavioral observations would be confounded. Facial twitches, for example, would have been concealed if the muscles were paralyzed.

Another approach in the veterinary clinic is the use of a poorly lit room with sound-attenuating walls for EEG recordings (Brass, 1959; Jones & Greufe, 1994; Holliday & Williams, 1999). This was not feasible in the EEG experiments, as poor light conditions reduce video quality and make detection of symptoms more difficult. More importantly, with convulsions being expected, poor light conditions would hinder administration of anticonvulsive treatment.

Taken together, the experiments showed that combination of CSF ports and EEG telemetry is possible and that the signal quality does not differ to that of dogs without pre-implanted CSF-ports. Both implant types do not influence normal animal behavior and therefore are acceptable methods with regards to animal welfare. As no reports on one-channel bilateral EEG recordings have been found in the literature, the impact of this is not clear. Comparison of recordings obtained with unilaterally placed electrodes would be necessary to confirm applicability of bilateral electrode placement for preclinical safety studies. The use of additional electrodes for EOG and EMG could increase validity of results, especially to assist artefact recognition and enable polysomnography in sleep studies.

2.1 Biomarker Analysis

Collection of samples for biomarker analysis at the time point of neurological symptoms was possible during the EEG experiments. 36 significant changes from baseline to post-treatment time points were observed with unadjusted p values < 0.05. Studies by Moser and Freeborn *et al.* (2015) investigated consequences of pesticide exposure (permethrin, deltamethrin, imidacloprid, carbaryl, triadimefon, fipronil) in rats on biomarker profiles (Moser *et al.*, 2015) and on the EEG (Freeborn *et al.*, 2015). They analysed rat plasma with different biomarker kits including the same biokrates kit selected for the EEG study (Absolute IDQ® p180 Kit Biocrates Life Sciences, Insbruck, Austria). Groups of rats each received a single dose of one of the treatments, and two different dose levels were selected from each pesticide (Freeborn *et al.*, 2015; Moser *et al.*, 2015). In addition, fipronil was also tested in repeated dosing conditions (Freeborn *et al.*, 2015; Moser *et al.*, 2015).

Overall, the amount of altered metabolites was similar in their study: 20% of the total 186 metabolites were significantly altered under their statistical criteria (Benjamini-Hochberg adjusted p-value < 0.05; absolute fold-change > 1.3) (Moser *et al.*, 2015). In the dog EEG studies, 36 metabolites (19.36%) were significantly altered in the three different treatments, using a q-value > 0.05.

Moser *et al.* (2015) observed a dose-dependency of metabolite changes, as “either only the high does showed changes or else, both low and high doses were significantly different from control” (Moser *et al.*, 2015). Dose dependent changes could be suspected in the dog study with most metabolites as well. Analysis of the 24 hour sample, which was not included in the study by Moser *et al.* (2015) however showed that many metabolites were altered for a prolonged time. Especially, some levels had further increased 24 hours after administration of the first dose. Therefore, changes in metabolite levels between the first and the second dose cannot be related to either a dose effect or a time effect after an initial insult.

Amongst the altered metabolites, three amino acids were affected in the dog EEG study and these were methionine, proline and nitro-tyrosine. A reduction indicates an increased turnover (Moser *et al.*, 2015). With increased formation of reactive oxygen species (ROS), methionine is oxidized to methionine sulfoxide (MetO, MeSOX, MetSO or MsX) (Hoshi & Heinemann, 2001). A reduction in methionine levels could therefore indicate increased

oxidative stress. Increase of nitrotyrosine is a marker for many diseases, including neurodegenerative diseases and neuronal injury (Greenacre & Ischiropoulos, 2001), so the meaning of a decrease is not clear. A decrease in plasma proline has been observed in rats after traumatic brain injury (Louin *et al.*, 2007). In their study, Louin *et al.* (2007) saw the decrease in proline at the 24 and the 72 hour time point and found that it correlated to reduced neurological scores of the animals. The fall in proline at the early time points was observed also in dogs. At the 24 hours' time point, the levels either showed an upward trend (compound 1), had returned to or were higher than baseline (compound 2), or were decreased (compound 3). The prolonged decrease in proline levels with compound 3 did not correlate with neurological symptoms in dogs, so the proposed use of proline as indicator of severity of neurological deficit (Louin *et al.*, 2007), is not supported here.

Lysophosphatidylcholines, phosphatidylcholines and sphingomyelines were altered with all three compounds. In the study by Moser *et al.* (2015) only Fipronil had an influence on these metabolites. Fipronil increased levels of diacyl and acyl-alkyls phosphatidylcholines and sphingomyelines but lowered lysophosphatidylcholines (Moser *et al.*, 2015). Phosphatidylcholines are important for membrane structure and signal transmission (Farooqui *et al.*, 2000; Lim & Wenk, 2009). From phosphatidylcholines, lysophosphatidylcholines are formed after partial hydrolysis (Farooqui *et al.*, 1997). Alterations in plasma levels of these glycerophospholipids have been associated with neurodegenerative diseases (Farooqui *et al.*, 2000; Klavins *et al.*, 2015; Li *et al.*, 2016). A relation between increased sphingomyelins and neurodegeneration in normal aging and disease has also been found (Farooqui *et al.*, 2000).

The overlap of the metabolite changes between the EEG study and the studies by Moser *et al.* (2015) and Louin *et al*. (2007) indicates that metabolomics profiling could detect neurologic insults. Further work would be needed to investigate which of the changes are specific for neurotoxicity.

3 Question 3

Can intravenous administration of escalating doses reduce inter-animal variability so that use of fewer animals is justified?

After oral dosing a significant variability of exposure levels between individual animals has been found (Backes, 2016) due to varying ADME (Absorption, Distribution, Metabolism, Excretion) properties. With intravenous dosing, there are no differences in absorption. In addition, emesis, which is frequently seen in toxicology studies, and gender differences influence exposure after oral dosing. This was seen with compound 2: Data from a previous toxicology study (PK data collected on day 1 after a single dose) with an oral dose of 10 mg/kg and from the EEG study with a dose of 14 mg/kg are shown in **Figure 103**. Plasma drug exposure is similar across studies, with the lowest levels achieved in the female in the

EEG study despite a higher dose. T_{max} in female dogs seems to be longer than in males with the same dose levels, and also C_{max} seems to be lower, especially in the EEG study. Compound 2 could not be administered intravenously as solubility was too poor to reach targeted exposure levels with reasonable infusion volume.

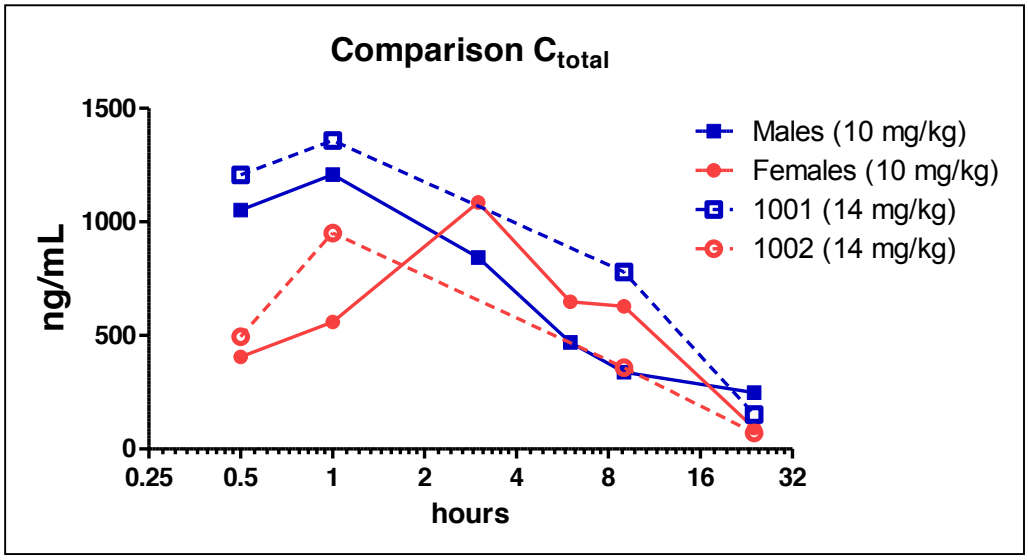


Figure 103*:* Comparison of plasma exposure in the EEG study to data from previous toxicology studies. Exposure levels are similar across studies and show a higher gender difference in the EEG study.

Compound 1 and compound 3 were administered intravenously. Variability was seen concerning the convulsive exposure level with compound 1. Plasma samples were collected within two to five minutes after clinical convulsion from both dogs. The total dose that induced convulsions in the male dog was 68 mg/kg, corresponding to a plasma concentration 7667.1 ng/mL. The female dog had convulsions eight minutes after the start of the second infusion while still being connected to the infusion line. Considering this, a total dose of 61.8 mg/kg corresponding to a plasma concentration of 13292.6 ng/mL induced convulsions in the female. The slightly higher compound need for the male dog is probably a result of the longer experimental time. This was 70 minutes for the male opposed to 38 minutes until clinical convulsion in the female, as dose level and infusion rate were higher in the second study. During the longer experimental time, some amount of compound 1 might have been already metabolized.

The lower total dose however resulted in higher plasma drug concentrations of 13292.2 ng/mL in the female as opposed to 7667.1 ng/mL in the male. In the male dog, the convulsion occurred during blood collection for TK analysis. The dog was lifted up and set on a table for this purpose. It is possible that in this case the convulsions were triggered by handling. In the female dog, an increased sensitivity to touch was also noted, but as opposed to the male dog, blood was collected directly within the recording kennel in this experiment and the female was handled less. A possible relation between sampling time points and occurrence of convulsions has also been reported elsewhere (Authier *et al.*, 2017). The

variability in convulsive exposure could in this case result from external manipulation. Therefore, all surrounding factors need to be well controlled and standardized amongst studies.

Infusion rate could also contribute to variability with intravenous infusions. In the experiment with compound 1, infusion rate for the female was 2.25 mL/min to reach the highest dose whereas for the male, it was 1.71 mL/min (31.6% higher). The relationship between infusion rate and exposure at the time point of clinical effect has been investigated by Ramzan & Levy (1985) with intravenous infusion of PTZ to rats: they run two experiments, with the endpoint in the first one being appearance of the first myoclonic twitch and in the second being generalized convulsions. They found that no significant difference existed between plasma, brain and CSF concentrations of PTZ as a consequence of infusion rate in rats (Ramzan & Levy, 1985). This was different to earlier results of a study investigating consequences of different infusion rates on exposure, dose required and latency to loss of righting reflex in rats (Danhof & Levy, 1984). In this experiment, phenobarbital levels in blood and brain at occurrence of the behavioral endpoint increased with increasing infusion rate in rats (Danhof & Levy, 1984). Also in humans, Marsch *et al*. (2001) found that a faster infusion rate of morphine led to significantly higher plasma levels". Ramzan & Levy mention that PTZ has only very little (2.5%) plasma protein binding (Ramzan & Levy, 1985). Opposed to this, phenobarbital was bound to plasma proteins at 36.2 ± 7.6% (Danhof & Levy, 1984) and morphine at 34% - 37.5% (Olsen, 1975). Differences in plasma protein binding could be a factor contributing to the differential influence of infusion rate on plasma exposure.

Plasma levels after intravenous infusion of compound 3 (8 mg/kg in the first infusion, 6 mg/kg in the second one), were very similar in the male and the female. Exposure in the male dog was higher (first time point: 1116.53 ng/mL, second time point: 1527.87 ng/mL) and clinical symptoms were more pronounced in this dog. Thus, exposure correlates to clinical symptoms. One cause could be the difference in body conditioning score (BCS) of both dogs: The male had a BCS of 3 and the female a score of 2. Due to the higher weight, a higher infusion rate was necessary to administer the complete dose to the male dog within the pre-defined 10 minutes of infusion time (55.91% faster in the first infusion step, 29.16% in the second infusion step). Therefore, with this compound, an influence of infusion rate on exposure is also possible.

Apart from reduced variability due to differences in absorption or metabolism, another advantage of an intravenous infusion study is the direct exposure and resulting shorter experimental time. In the EEG studies with intravenous infusion, plasma exposure levels at which symptoms occurred were reached within a maximum of two hours. The dogs were supervised through clinical examinations and video-EEG for the rest of the day after the experiment and neither were additional neurological symptoms observed nor did the severity of symptoms increase.

This was different in the oral study with compound 2: in this study, the male dog showed stereotyped behavior only in the afternoon and the symptoms lasted until the next morning, so overnight observation via video EEG was done. In conclusion, the study design using intravenous administration reduced the experimental burden for animals through shortened procedural time and shorter time during which neurological symptoms were present.

In the EEG study with compound 1, most neurological symptoms that were observed in longer-duration toxicology studies with oral administration in dogs (see results section, Table 12). Compound 3 was not tested in dogs, but in NHPs, and symptoms observed in oral toxicology NHP studies and in the NHP infusion studies overlap as well. Through the faster rise in exposure levels with intravenous dosing, active compound brain levels are also reached quicker. This could have been confirmed by measuring compound brain levels or analyzing CSF, as surrogate of brain tissue. Therefore, with the accelerated study design, relevant neurological symptoms can be detected and longer study durations do not alter the readout with regards to these symptoms.

With dedicated infusion studies, neurological symptoms could be induced in a short experimental time of two hours at maximum and determination of plasma substance levels was possible simultaneously to occurrence of symptoms. Exposure after intravenous administration of compound 1 and 3 was less variable than exposure after oral administration of compound 2. This is in line with previous findings from toxicology studies with compound 1 and other drug candidates (Backes, 2016). Some variability was still observed with intravenous dosing. Higher exposures were seen with increased infusion rates. The magnitude of this influence factor is probably compound related, as the effect was observed with morphine (Marsch *et al.*, 2001) and phenobarbital (Danhof & Levy, 1984) but not with PTZ (Ramzan & Levy, 1985). This study design therefore enables continuous observation for improved detection of neurological symptoms, sample-collection according to clinical signs and instant treatment of the animals if severe symptoms are observed.

Theoretically, lowered variability is a rationale for reducing animal numbers, but there are practical considerations: authorities require that a candidate drug's action is investigated in a study that uses the same route of administration that is intended for human use. In most cases, this is "*per os*" administration. Also, the design of toxicology studies in non-rodents is standardized. For example, a default number of three non-rodents per gender per dose group is usually used in 2-week dose range finding GLP studies. This is considered to be the minimum number necessary to appropriately investigate adverse effects.

Several authors have stressed the importance to identify neurological liabilities early in the drug development process (e.g. (Easter *et al.*, 2009)). Intravenous administration of escalating doses led to reduced exposure variability compared to toxicological studies thereby allowing the use of only two animals. Such studies can be included in early phase safety assessments when compound availability is usually limited. Recommendation therefore would be, in cases where seizure liability is a concern, to conduct such dedicated

studies prior to toxicological studies. In addition, the whole range of neurological symptoms that occurred in long term toxicology studies were also seen in the EEG studies. The possibility to collect samples for exposure analysis simultaneous to the occurrence of symptoms in such short studies can improve calculation of safety margins. Dose selection for toxicology studies with large animal numbers could be adapted accordingly to avoid reaching exposure levels at which severe neurological symptoms occur.

4 Question 4

Is the dog a relevant animal model for seizure liability assessments and how does the dog's sensitivity for neurological symptoms compare to other preclinical species?

Regulatory requirements for CNS testing of developmental drugs are limited to behavioral observations by Irwin test or FOB, which are usually done in rodents (FDA, 2001). As convulsions are often only observed in toxicology studies (Easter *et al.*, 2009), these experiments are not sufficient to detect seizure liability for all compounds. Multiple assays for seizure liability detection have been developed but none of them is regarded to be sufficiently sensitive and a combination of tests is usually selected. There is a general difference between these assays: some are considered as early screening tools for a medium to large amount of compounds, like computational methods (Zhang *et al.*, 2011), brain slices (Easter *et al.*, 2007) or the zebrafish locomotor assay (Winter *et al.*, 2008; Cassar *et al.*, 2017). Others are used to answer more specific questions about dose-relation or seizure type. Ethical reasons oppose to the use of the dog in the first category. For detailed assessments of seizure liability, this species has already been successfully used (Dürmüller *et al.*, 2007; van der Linde *et al.*, 2011b; Authier *et al.*, 2014b).

Dogs are regarded by some authors (Redman & Weir, 1969; Edmonds *et al.*, 1979; Easter *et al.*, 2009; Hasiwa *et al.*, 2011; Authier *et al.*, 2013; Authier *et al.*, 2014b) to be overly sensitive for convulsions, but retrospective analysis of internal data showed that this is not supported by exposure-based evidence (Backes, 2016). However, only few substances were tested in more than one non-rodent species and therefore, evaluation of species-specific sensitivity is limited (Backes, 2016). The choice of compound 1 and compound 3 for the EEG experiments enabled comparison with data previously collected in NHPs in a comparable study design and improved the data basis for evaluation of species sensitivity.

As previously reported (Backes, 2016), it is important to consider free plasma concentration for comparing exposure-related symptoms amongst species. The free concentration is the amount of substance unbound in plasma and is calculated by using species-specific plasma protein binding. The free concentration in plasma is the fraction of the total concentration that can reach its site of action within the brain. The example of compound 1 illustrates that the difference between NHP and dogs in convulsive threshold is reverted when free instead of total concentrations are considered. NHP had convulsions at a total exposure of 135000

ng/mL – 146000 ng/mL whereas dogs showed this symptom at 7667.1 ng/mL and 13292.6 ng/mL. Considering the unbound fraction in plasma in the dog (fu 0.3) that is higher than in the NHP (fu=0.012), convulsive exposure levels are very similar. C_{free} for the dog at the time of convulsion was 2300.13 ng/mL and 3987.78 ng/mL and for the NHP C_{free} was 1620-1752 ng/mL. Therefore, the dog is not more sensitive than the NHP with this compound.

The case of compound 1 is also an example how different outcomes can be seen in different assays: in the PTZ seizure threshold test this compound had anticonvulsive effects in rats, but actually it is convulsive at high doses in other species: first, convulsions occurred in toxicology studies in dogs and then in dedicated infusion studies in NHP and dog. Similar effects have been described for bupropion (Tutka *et al.*, 2004).

In the testing of compound 3, convulsions were observed in one dog. The corresponding sample was taken 20 minutes later due to post-ictal aggressive behavior of the animal. Therefore exact exposure at the time point of convulsions can only be estimated based on computational modeling. The difference between total and free exposure is not as marked as with compound 1, as the fraction unbound in plasma is more similar between dog and NHP (fu dog: 0.121 – fu NHP: 0.155). In NHP studies, there was one animal that had a very high exposure (9570 ng/mL) but did not show convulsions. The other NHPs convulsed at total plasma levels between 979 ng/mL and 1970 ng/mL (mean: 1588 ng/mL). Total plasma level in the dog 20 minutes after the convulsion was 1600.7 ng/mL, so the dog is close to the NHP mean. Considering free plasma concentration, the mean NHP exposure with convulsions is 238.6 ng/mL (range 151.7 ng/mL – 335.4 ng/mL) and the dog had a free plasma concentration of 193.68 ng/mL 20 minutes after the convulsion. The results from the testing of compound 3 therefore do also not confirm the hypothesis that the dog is more sensitive to convulsions than the NHP.

Concerning compound 2, the dog showed high sensitivity for head tremor. In NHPs, this symptom was not observed (compare results section). Oral bioavailability of compound 2 in NHP is only 12.6%, compared to 78.4% in the dog. A 10 mg/kg oral dose induced the head tremor in dogs and resulted in a C_{max} 1208 ng/mL (males) and 1086 ng/mL (females). In NHP, the same dose led to a C_{max} of 643 ng/mL in males and 653 ng/mL in females. Considering the plasma protein binding of 0.49 in dog and 0.41 in NHP, free concentrations at C_{max} (10 mg/kg oral dose) were 591.92 ng/mL (dog male), 532.14 ng/mL (dog female), 263.63 ng/mL (NHP male) and 267.73 ng/mL (NHP female). A dose of 30 mg/kg results in a free exposure in NHPs that is related with head tremor in dogs (582.2 ng/mL males; 619.1 ng/mL females). No head tremor was observed at this dose in NHP, but stereotypies, e.g. self-mutilation through chewing on body parts, circling, gnawing and picking at gums. A 5-fold higher CSF/ plasma ratio at C_{max} was observed in dogs compared to NHPs (0.2 in dogs; 0.04 in NHP after a 1 mg/kg oral dose) and can together with the higher bioavailability partially explain the higher sensitivity of this species. In rats, stereotyped behavior was also seen (walking on tip-toes).

The origin of the head tremor in dogs was not clear; partial seizures with motor signs were one explanation. This was not explored in more detail for to several reasons: the occurrence of partial seizures without generalization was considered rare; the finding had the same intensity over different dose ranges and midazolam was not effective in halting the tremors. In addition, the level of consciousness of dogs was not or only mildly impaired, they were responsive to external stimuli and no autonomic signs were observed. EEG recordings did not show definite seizure characteristics. The occurrence of triphasic waves in the male dog, has been described as a general sign of CNS toxicity that is not indicative for a single compound class or seizure (Blume, 2006). With compound 2, the dog EEG therefore served to exclude seizures as cause of the symptom.

In summary, comparison of exposure at the time point of neurological symptoms between two non-rodent species showed, that no difference existed between the dog and the NHP regarding sensibility for convulsions with compound 1 and compound 3. As opposed to NHP infusion studies, in dogs these studies can be performed with relatively freely moving animals and therefore, a broader range of symptoms can be detected. The dog is generally suited as an animal model for seizure liability testing and its use in seizure liability assessments in drug development has also been performed successfully by other researchers (Dürmüller *et al.*, 2007; van der Linde *et al.*, 2011b; Authier *et al.*, 2014b; Authier *et al.*, 2017). For development of antiepileptic drugs, use of the dog has been specifically proposed due to similarity of human and dog epilepsy (Potschka *et al.*, 2013). Studies using dogs have the advantage of a higher detection rate of neurological symptoms. One reason for this is larger body size compared to rodents which allows better identification of subtle symptoms. Also, the higher grade of domestication means the dog shows symptoms more readily and caretakers recognize them easier. Lastly, due to a larger blood volume, the possibility for repeated sample collection for exposure and biomarker analysis exists. In general, the use of the dog in seizure studies that have potentially a high burden for the animals must be carefully considered.

5 Question 5

Can quantitative and automated analysis of dog EEG support investigation of CNS effects of development drugs?

Quantitative EEG analysis has been recommended for detection of background EEG changes that are not evident upon visual analysis (Moore *et al.*, 1991). One advantage of quantitative over visual EEG analysis is its objective nature as a measure of drug effect (Jones & Greufe, 1994) and the possibility for statistical analysis (Eccles, 1988). Quantitative EEG analysis has found clinical application in anesthesia monitors in human (Kissin, 2000) and to some degree also in veterinary medicine (Otto & Short, 1991; March & Muir, 2005; Campagnol *et al.*, 2007; Ribeiro *et al.*, 2008). With regards to drug development, qEEG enables data management and data quality control suited to comply with GLP requirements (Jones &

Greufe, 1994). Also, as opposed to behavioral observations, qEEG effects have been considered to be better translatable from preclinical species to clinical trials in humans to assess adverse CNS effects (Fonck *et al.*, 2015). However, it has also been pointed out, that the psychological state has an influence on the EEG and that this factor might limit inter-species translatability (Eccles, 1988; Freeborn *et al.*, 2015).

Several researchers tried to find specific drug fingerprints on the EEG (reviewed by (Van Riezen & Glatt, 1993)). Quantitative EEG patterns have been investigated with drugs from the same indication but from different chemical classes and with several modes of action (Frankenheim, 1982; Dimpfel *et al.*, 1988; Dimpfel, 2003). Another approach was the analysis of pathway-specific drug effects using e.g. cholinergic (Dimpfel, 2005) or dopaminergic (Dimpfel, 2008) compounds. The promise of qEEG categories is that the efficacy of a development candidate in a certain indication can be determined and ideally translated to the clinic. However, according to Porsolt *et al.* (2002) "there is debate as to whether qEEG, by virtue of the different profiles observed, is capable of identifying specific classes of psychotropic agent (…)"(Porsolt *et al.*, 2002). Also, Wilson *et al.* (2014) mention that there is a paucity on reports that evaluate translatability of pharmacologically induced qEEG changes (Wilson *et al.*, 2014).

One difficulty with interpreting qEEG data is that a dose and time dependency of effects has been found with different compounds and different doses or time points have been selected leading to differential results. Also, comparison of qEEG results reported by different authors amongst each other and with data from the dog EEG studies was found to be limited. Direct comparison would first necessitate a consistent definition of frequency bands and their respective bandwidths. This premise is not given, for example the gamma band is not always considered (e.g.(Jones & Greufe, 1994; Dimpfel, 2003; Dimpfel, 2008)) and a differentiation of lower and upper alpha and beta bands has been proposed by some authors in human medicine (e.g. (Herrmann *et al.*, 1979)) and preclinical research (e.g. (Dimpfel, 2003), (Dimpfel, 2008)). Also definition of the sigma power band is commonly done for sleep studies (Authier *et al.*, 2014a). In addition, species and the respective recording and analysis techniques vary between experiments. For example, route of administration and pharmacologic properties of a compound should be considered when selecting time points to derive qEEG data. Some authors present their results with a focus on selected brain areas. Lastly, measures taken to control vigilance state and the behaviors induced by administration of different drugs are often not mentioned. To sum up, different experimental designs make it difficult to find a conclusion regarding a compound's qEEG fingerprint.

This is not only true for drug-induced qEEG effects, but also for reference values that have been published e.g. for the dog by Moore *et al.* (1991) and Jones & Greufe (1994): These reference values were established using scalp electrodes rather than implanted telemetry. It is known that the voltage of the electric brain activity is reduced by the skull and the overlying muscle layers. Thereby, absolute power values of different frequency bands vary

between methods, species and even dog breeds. Changes in relative power are therefore more suitable to compare results obtained in different studies.

One example, for which dose- and time dependent effects have been described are dopaminergic substances (Dimpfel, 2008; Wilson *et al.*, 2014). This dose-dependency affects behavior and concomitant on qEEG parameters (Dimpfel, 2008; Wilson *et al.*, 2014). Also, Dimpfel describes differential changes in hippocampus, striatum, reticular formation and frontal cortex (Dimpfel, 2008). Wilson *et al*. reported results of the effects on rat EEG after 1 mg/kg subcutaneously administered apomorphine (Wilson *et al.*, 2014). They reported an increase in the relative power in the gamma band, which they defined from 30-70Hz, 30 and 60 minutes after apomorphine administration in quiet waking conditions (Wilson *et al.*, 2014). Quantitative EEG changes in humans after a low dose of apomorphine (total 0.75mg s.c.; 0.01 mg/kg given an average weight of 70 kg) were increases in relative and absolute beta 3 (they differentiated beta 1, 2 and 3 with beta 3 ranging from 21 to 32 Hz) power localized over frontal-central scalp areas 30 minutes after apomorphine (Luthringer *et al.*, 1999). In general, an “EEG activating effect” is related with apomorphine. In the dog study, there were no significant changes in the gamma or beta power in both dogs, but the dog is also different to other species, regarding the emetic effects of apomorphine. A time point around 30 minuntes after apomorphine administration was selected to enable comparison to the results reported by Wilson *et al.* (2014). Quinpirole, which is an agonist at D2 receptors, increased gamma and reduced delta power. Compound 1 induced qEEG changes mainly consisted of a rise in delta and theta and a decrease in gamma in both dogs. This effect was not clear by visual EEG analysis only. To sum up, dopaminergic compounds induced differential changes in dog EEG. A relation to this specific pathway therefore cannot be made.

In drug safety testing, the specific EEG fingerprint of a dug class is of less interest than identification of potential patterns that indicate CNS liabilities. This application of EEG has been investigated (Benignus, 1983; Eccles, 1988; Jones & Greufe, 1994; Jones *et al.*, 1995). Fonck *et al.* (2015) mention that their own results show that qEEG has the potential to be used as a translatable safety biomarker. In humans, it has been observed that increases in delta and theta as well as triphasic waves, that were present with compound 2, are an unspecific sign of neurotoxicity. Interestingly, the increase is already obvious at the first time point, whereas visual EEG interpretation could only detect this pattern change during the late afternoon and the night. In the female dog, no remarkable qEEG changes were present, but a mild increase in theta at the first time point could also be identified. This difference between male and female dog was also present in their clinical behavior and explained by the lower exposure reached in the female dog despite being administered the same dose. These EEG symptoms have been identified in relation to drug toxicities or metabolic encephalopathy (Blume, 2006). In dog EEG, increase in theta and/ or delta were the most significant changes in qEEG. These parameters therefore could be used as identifiers of neurotoxicity.

However, administration of propofol also increased delta, theta, alpha and sigma band while beta and gamma showed a marked decrease. Similar effects have also been reported in literature: Bergamesco (2003) describes that propofol anesthesia significantly increased both, absolute and relative power of delta. This increase in delta was dose-related, whereas the other frequency bands were not affected by the amount of anesthetic administered (Bergamasco *et al.*, 2003). The increase in the lower frequency bands under general anesthesia was significant in several reports whereas changes in the higher frequency bands were not (Itamoto *et al.*, 2001; Jeserevics *et al.*, 2007). The influence of general anesthetics on the canine EEG could be confirmed, with the EEG telemetry setup, but opposed to the findings reported by Bergamesco (2003), all lower frequency bands were increased and higher frequencies were decreased.

Changes in qEEG therefore need to be interpreted in relation to behavior and cannot be used as an indicator of neurotoxicity alone. As clinical symptoms were present at the time of qEEG effects, the practical utility of these measures as predictor of neurotoxicity prior to induction of severe symptoms is limited. Also, for correct assessment of qEEG changes, artefacts have to be excluded and the vigilance state needs to be considered (Wilson *et al.*, 2014) as each state has been linked to a specific qEEG pattern (Freeborn *et al.*, 2015). In humans, the level of consciousness can be controlled by either recording resting state EEGs (eyes closed) or setting a task that ensures a stable level of wakefulness (Wilson *et al.*, 2014). The vigilance level in animals can be held constantly high by making them walk on a treadmill (Krijzer *et al.*, 1993; Porsolt *et al.*, 2002). Alternatively, episodes with reduced wakefulness need to be excluded from qEEG analysis. This approach was chosen in this thesis, as visual evaluation of recordings was also needed for exclusion of artefacts. In practice it was found that due to the testing conditions in which the dogs were constantly observed, decreases in their vigilance level were only observed in baseline and overnight recordings (with the exception of drug-induced sedation with compound 3).

In summary, qEEG levels were altered after compound administration, but only at the same time as neurological symptoms and did not enable detection of neurological symptoms prior to their clinical occurrence. Also, accurate qEEG analysis is time intensive due to the need to manually review the EEG traces. In conclusion, this parameter is not recommended as a useful safety biomarker for neurotoxicity.

6 Additional Outcomes

6.1 EEG Recording with Subcutaneous Needle Electrodes

A telemetry transmitter was connected to stainless-steel needles to test whether this would enable recording minimal-invasive EEGs with the same equipment used for the implanted telemetry. It was possible to record an EEG with this method, the quality however did not compare to EEGs recorded with implanted telemetry. First, electrode movements were an

additional source of artefact and second, the method failed to show subtle EEG transients like spindles under propofol anesthesia. In general, signals obtained with non-invasive electrodes were of lower voltage than those recorded with implanted electrodes. This difference in amplitude had already been observed by Redding & Colwell, who simultaneously recorded signals from implanted electrodes and alligator clamps to evaluate performance of the non-invasive clamps (Redding & Colwell, 1964). They concluded, that both recordings were generally comparable but that amplitudes differed significantly between cortical and scalp electrodes, with lower voltages at scalp electrodes, which could potentially dampen high frequencies (Redding & Colwell, 1964).

A limitation in our experiment was that no electrophysiology needle electrodes were used due to technical problems in connecting the cables to the electrodes. As the method has been successfully tested by Authier *et al*. (2015), it is possible that this is the reason for the poor performance of the subcutaneously recorded EEG compared to the EEG recorded from implanted electrodes.

The majority of reports on dog EEG in the veterinary clinic describes the use of subdermal or clip electrodes. The use of subcutaneous needle electrodes in dogs in a preclinical research setting has been described (Jones & Greufe, 1994; Authier *et al.*, 2015). Advantages of this approach are the relatively lower effort and reduced costs: the devices for EEG acquisition from subcutaneous electrodes can be reused and no surgery is needed. From an animal welfare perspective, the risk and stress associated with surgery are avoided using this method. However, so far, only acute studies with physical restraint have been described with the use of needle electrodes and telemetry remains so far the best option if long-term studies are planned. Experience from the veterinary clinic has shown that sedation or anesthesia often cannot be avoided in order to record high quality EEGs with needle electrodes. In safety studies, simultaneous application of other substances and the drug candidate is usually avoided due to possible drug-drug interactions or confounding effects on symptoms.

An EEG recording system utilizing 15 subcutaneous needle electrodes has recently been introduced to the veterinary clinic for monitoring epileptic dogs at home and in clinics (Wielaender *et al.*, 2017; Wielaender *et al.*, 2018). It needs to be evaluated, if this approach is suitable for use in drug development as well. The use of a larger montage permits visualization of the EEG signal in different montages. For shorter duration studies (up to 24 hours) it can be an alternative to implanted telemetry. Probably, animals need to be separated as long as the electrodes are inserted and this would discourage its use in longer duration studies. One general disadvantage of a jacket is the risk of concealing certain symptoms, like isolated muscle twitches on the trunk or piloerection.

Risk of non-invasive electrodes is, next to the higher artefact coverage that loss of electrodes can occur during a study. If unnoted or at a time point when replacement is not possible, e.g. due to symptoms, this would mean that an animal was dosed without getting the intended

readout. Repetition of experiments or use of additional animals is not in line with 3R principles; therefore the risk of data loss after equipment failure and the concomitant need to repeat experiments needs to be weighed against the burden (and the costs) of surgical implantation of telemetric devices.

One major application for long-term EEG in drug development is for sleep studies. The dog has been recognized as a model for sleep research with good translatability to humans: in the course of domestication, dogs adapted to human day- and night rhythms (Parmentier *et al.*, 2006; Kis *et al.*, 2014). Traditionally, rodents are used for sleep studies, but their sleep cycle is different to the human one to a greater degree than the dogs, that has adapted to humans during the domestication process. Therefore results from dog sleep studies have been proposed to have a higher degree of translatability to humans (Parmentier *et al.*, 2006). In addition, there are some sleeping disorders that naturally occur in both, humans and dogs, (narcolepsy) or that have been induced in dogs (obstructive sleep apnoe) and like with epilepsy, dogs have been discussed as a translational model for this disease (Horner *et al.*, 1998).

To conclude, the use of non-invasive electrodes for EEG studies can be an alternative to implanted telemetry in acute studies. In order to avoid restraint of dogs that can confound symptoms, training would be necessary. For long-term monitoring and especially sleep studies, use of implanted telemetry is advantageous due to higher signal quality, reduced risk of data loss, data quality and animal welfare.

6.2 Video-EEG Analysis

Toxicological studies are not ideally designed for the assessment of seizures: Clinical observations are usually only done at pre-defined time points (Backes, 2016), and as the average duration of a seizure was estimated to be less than three minutes (Metea *et al.*, 2015), occurrence of such an event can be missed. In such cases, safety margins would not be calculated correctly. Video observation can increase the probability of detection of convulsive episodes and allow detailed description of the event (Elander, 2013). Limitations of video recordings are that they do not allow detection of subtle changes in behavior (e.g. ocular symptoms) or partial seizures that do not lead to clear symptoms (Williams *et al.*, 2006). Also, large amounts of data are generated during several days of video recording and analysis takes long or requires large personnel efforts. There are software solutions that support video analysis for some species and would be a possibility to increase convulsion detection rate.

Blinded analysis, as it has been recommended by Jeserevics *et al*. (2007), was not possible in the exploratory pilot studies described here as the same person was responsible for study planning, study conduct and video - EEG interpretation. If such studies are performed in the context of regulatory studies in future, blinding of the EEG interpreter or EEG analysis by a second person would be necessary.

Recordings were acquired using DSI™ Ponemah. Synchronization with video was enabled with the Noldus Media Recorder. One major disadvantage of this software solution is that only one camera can be selected per animal at a time meaning that 360° views of a freely moving dog are not supported. The solution to include one not-implanted transmitter in each recording enabled saving a synchronized video. However, the program does not display this video in the same window as the one allocated to the animal. In practice, the second-angle videos were only used in rare cases to confirm suspected artefact sources.

Analysis of recordings was done using DSI™ NeuroScore software version 3.2. This program has been developed for preclinical applications and has been used by other authors in the field as well (Authier *et al.*, 2009). The default settings of the automated seizure detector had a high false positive detection rate (low specificity). This was reduced when a custom-made protocol was run, but visual analysis was still necessary to exclude false detected episodes. On the other hand, sensitivity was high, meaning that all real seizures were detected by the program. This has been observed by other authors as well (Authier *et al.*, 2009). The automatic seizure detection tool has one major disadvantage: it presupposes the presence of a seizure in a recording, in order to align the settings to the individual EEG traces. To my knowledge, there are no reported universally applicable identifiers of seizures recorded with implanted telemetry in dogs, so the customization of detection protocols also depends on the preliminary identification of a seizure. However, the protocol is still useful, especially when long recordings need to be analyzed, as a search function is available after the seizure detection protocol has been carried out. This allows the analyzer to jump from one detected episode to the next one, thereby reducing the time necessary for visual analysis.

VII. Abstract

Preclinical safety is an important part of drug development in animals and humans. In toxicology studies, seizure liability can be detected at high doses as convulsions. Non-convulsive seizures induce only subtle behavioral changes and their assessment in animals is challenging. Electroencephalography (EEG) is the only method to correlate animal behavior to seizure activity and video-EEG is the current gold-standard for preclinical seizure liability assessments (Authier *et al.*, 2014b). In most cases there are no clear premonitory signs that forewarn of convulsions but epileptiform EEG activity prior to clinical manifestation has been reported during a period potentially sufficient for prophylactic anticonvulsive treatment (Dürmüller *et al.*, 2007).

Aim of this thesis was investigation of a study design for assessment of neurological symptoms in dogs. This design should optimize detection of neurological signs while minimizing study duration and animal numbers. Video-EEG was used to increase symptom detection rate and to explore the possibility to refine seizure liability testing by enabling EEG-based anticonvulsive treatment. For establishment of the EEG system in our facility, reference substances were tested first. Then, three in-house drug candidates with different modes of action and known neurological side effects were chosen. Two telemetered beagle dogs were used per experiment. Substance effects on clinical symptoms and on the EEG were investigated. CSF and blood samples for analysis of drug exposure and biomarkers were collected simultaneous to symptoms. Results were compared to previous toxicological studies thereby enabling evaluation of non-rodent species differences in sensitivity for neurological symptoms.

Results showed that combination of implants for CSF collection and EEG recording is possible. In this study design, intravenous administration was superior to oral dosing as it led to a reduced variability in exposure levels. Also, experimental time was significantly reduced compared to standard toxicology studies while the same neurological symptoms were induced. This shortened duration enabled continuous clinical observations for a better evaluation of CNS effects and immediate veterinary assistance in the spirit of animal welfare. The EEG was not superior to clinical observations in forewarning of convulsion risk and did not enable convulsion prevention. This was due first to the short latency between onset of abnormal EEG activity and convulsions which was below one minute with in-house compounds. Secondly, accurate interpretation of the unfiltered EEG signal was limited, especially differentiation of artefacts and epileptiform activity.

In conclusion, a study design using intravenous infusions is suitable for the characterization of neurological symptoms. All the symptoms, which were already known from studies with a longer duration, were also seen. This allowed better correlation of neurological symptoms to exposure and immediate veterinarian treatments. For substances with a high risk to induce severe neurological symptoms, such studies can guide dose selection for longer regulatory toxicological studies to prevent occurrence of severe neurological symptoms.

VIII. Zusammenfassung

Im Rahmen der Entwicklung von Human- und Veterinärarzneimitteln wird die Anwendersicherheit neuer Medikamente in präklinischen Sicherheitsstudien erforscht. Zentralnervöse Nebenwirkungen werden häufig erst in toxikologischen Prüfungen erkannt, wenn bei hohen Dosierungen Krampfanfälle bei den Versuchstieren auftreten. Epileptische Anfälle können allerdings auch subtilere Symptome, deren Erkennen in Tieren schwierig ist, verursachen. Die Elektroenzephalographie (EEG) bietet in Tierstudien die einzige Möglichkeit, nicht-konvulsive Anfälle zu diagnostizieren. Daher ist die Kombination von Videoüberwachung und EEG in der präklinischen Arzneimittelentwicklung gegenwärtig der Goldstandard für die Sicherheitsbewertung einer Substanz im Hinblick auf ihr Risiko, Anfälle auszulösen (Authier *et al.*, 2014b). Meist gibt es keine klinischen Warnzeichen vor dem Auftreten von Krampfanfällen. Allerdings wurde das Auftreten epileptiformer EEG-Aktivität vor klinischen Symptomen beobachtet. Das beschriebene Zeitfenster ist potentiell ausreichend für prophylaktische antikonvulsive Behandlung (Dürmüller *et al.*, 2007).

Ziel dieser Arbeit war es, in Pilotstudien ein neues Studiendesign für die Charakterisierung neurologischer Nebenwirkungen zu evaluieren. Dieses Studiendesign sollte die Erkennungsrate neurologischer Nebenwirkungen optimieren und dabei gleichzeitig eine Reduktion der dazu nötigen Tiere und der Studiendauer ermöglichen. Der Einsatz von EEG und Videoüberwachung sollte es ermöglichen, Substanz-induzierte Anfälle im Frühstadium zu erkennen und ihr klinisches Auftreten zu verhindern. Um das EEG-System in der Forschungseinrichtung neu zu etablieren und um zu evaluieren, ob Implantate für Liquor-Entnahme und EEG-Aufzeichnung kompatibel sind, wurden zuerst Referenzsubstanzen getestet. Zur Beantwortung der eigentlichen Fragestellung wurden drei Arzneimittelkandidaten mit unterschiedlichen Wirkmechanismen ausgewählt, von denen bekannt war, dass sie neurologische Symptome verursachen. Je Substanztest wurden zwei Hunde mit implantierten EEG-Sendern verwendet. Zwei der Substanzen wurden in eskalierenden intravenösen Dosen verabreicht, die dritte wurde als einzelne orale Dosis gegeben. Effekte der Substanzen auf klinische Symptome und auf das EEG wurden evaluiert. Parallel wurden Blut- und Liquor-Proben zur Bestimmung der Substanzspiegel und potentieller Biomarker genommen. Die Auswahl der Substanzen bot zusätzlich die Möglichkeit, die Empfindlichkeit der beiden regelmäßig in Arzneimittelprüfungen verwendeten Nicht-Nager Spezies Hund und Affe für neurologische Symptome vergleichend zu bewerten.

Die Ergebnisse zeigen, dass die Kombination von Implantaten für EEG-Aufzeichnung und CSF-Probennahme möglich ist. Die intravenöse Applikation war der oralen Substanzgabe vorzuziehen, da die Variabilität der Substanz-Plasmaspiegel geringer war. Alle Symptome, die aus früheren toxikologischen Studien mit längerer Dauer bekannt waren, wurden ebenso beobachtet. Durch das Dosierungsschema war ihr Auftreten allerdings auf eine verkürzte Zeitspanne reduziert. Die kurze Studiendauer ermöglichte durchgehende klinische Beobachtung, somit die Erkennung aller Symptome und zeitnahe veterinärmedizinische

Behandlungen, was im Sinne des Tierschutzes einen Vorteil darstellt. Für eine frühzeitige Erkennung von Krampfanfällen war das EEG nicht besser geeignet als klinische Beobachtung, da die Interpretation des ungefilterten EEG Signals durch das Auftreten von Artefakten erschwert war.

Das Studiendesign, in dem das EEG angewendet wurde, ist zur Charakterisierung neurologischer Nebenwirkungen geeignet, da alle Symptome, die aus Studien mit längerer Dauer bekannt waren, ebenso beobachtet wurden. Durch die verkürzte Dauer wurde ermöglicht, Symptome und Substanzplasmaspiegel zu korrelieren und zeitnahe tierärztliche Behandlungen durchzuführen. Bei Substanzen, die ein hohes Risiko für neurologische Nebenwirkungen haben, kann dieses Studiendesign genutzt werden um im Vorfeld von behördlich geforderten toxikologischen Studien Dosierungen zu bestimmen, bei denen keine schweren neurologischen Nebenwirkungen zu erwarten sind.

IX. Appendices

1 Medication: Surgery

Active Agent	Trade Name	Vendor	Dose	Administration	Route
Enrofloxacin	Baytril® Flavour 50 mg/ 15mg	Bayer Vital GmbH	5 mg/kg	Starting 1 day prior to surgery, daily for a total of 7 days	p.o.
Enrofloxacin	Baytril® - 2,5% Injektionslösung	Bayer Vital GmbH	5 mg/kg	On the day of surgery	s.c.
Caprofen	Rimadyl®20mg Tabletten	Zoetis Deutschland GmbH	4 mg/kg	Starting 1 day prior to surgery, daily for a total of 7 days	p.o.
Caprofen	Carprieve®	Zoetis Deutschland GmbH	4 mg/kg	On the day of surgery instead of the oral dose	s.c.
Buprenor-phine	Buprenovet®	Intervet Deutschland GmbH	0.02(mg/ kg)	30 min prior to midazolam; every 6 hours for 3-4 days	i.m.
Midazolam	Midazolam-ratiopharm® 15mg/3ml	Ratiopharm GmbH	0.2 (mg/kg)	On the day of surgery, premedication	i.v.
Propofol	Propovet™ Multidose	Abbott Laboratories Ltd	initial: 6.6 mg/kg then after effect	prior to intubation	i.v.
Dexpanthenol	Bepanthen® Augen- und Nasensalbe 5 %	Bayer Vital GmbH	to cover both eyes	after loss of consciousness, repeated if needed	corneal
Isofluran	Isofluran CP	CP Pharma GmbH	approx. 2.0-3.5%	during surgery	per inhala-tionem
Lidocaine	Xylocain® Pumpspray	AstraZeneca GmbH	One pump spray	Prior to insertion of ear bars	ear canals
Bupivacain - hydrochloride	Bupivacain 0.25% Jenapharm®	Mibe GmbH Arzneimittel	Approx. 2-5mL	during surgery	on muscles
Dexametha-sone	Dexamethasone Injektionslösung ad us. Vet. 2mg/mL	CP Pharma GmbH	1 mg/kg	during surgery, prior to drilling	i.v.
Ringerlactat	Ringer Lactat Hartmann B. Braun Vet Care	B. Braun Melsungen AG	8-10 ml/kg/h	during surgery	i.v.
Povidon-Iod	Braunovidon® Salbe 10%	B. Braun Melsungen AG	to cover wound	after wound closure	topic

2 Materials for Implantation of Electroencephalography Transmitters in Dogs

Instruments	Specifications
Adson-Forceps, straight, serrated	n=2
Adson-Brown tissue forceps, straight, with teeth	n=2
Allis tissue clamps	n=2
Dental elevator	n=1
Medium/large gelpi retractors (fine)	n=2
Weitlaner retractor 2 x 3 with wide spread	n=1
Kelly hemostat forceps, curved	n=1-2
Kelly hemostat forceps, straight	n=1-2
Halsted mosquito forceps, curved	n=2
Halsted mosquito forceps, straight	n=2
Debakey forceps	n=2
Mayo-scissors straight, 15cm	n=1-2
Metzenbaum scissors, curved, 14.5 cm	n=1-2
Olsen-Hegar needle holder/ Webster needle holder	n=1
Backhaus towl clamps	n=6
Trocar	n=1
Screwdriver	n=1
Drill	handpiece and motor; motor on i.v. stand; with footpiece
Drill bits	1.18mm
Drill holder for manual drilling	n=1
Bur	1.4mm, n=1-2
10 blade with scalpel handle	n=1
Screw-holding forceps, curved	n=1
Skin stapler	n=1
Plate	to mix dental cement, n=1
Spatula	to mix dental cement, n=1
Lineal	n=1
Allen Wrenches	n=3 (5/32", 2", 3")
Others	**Specifications**
Stereotaxic with ear bars	n=1, e.g. Stoelting Co.
Screws	n=2; Plastics One®, Size 0-80 (Shaft 3.2mm/ head diameter 2.5mm/ shaft diameter 1.57mm)
Sterile covers	MTP- Medical Technologies GmbH; camera cover folded with paper insertic aid, 13x242cm, sterile; to cover the drill
Cidex® OPA	Advanced Sterilization Products®, Johnson + Johnson
Cidex® OPA Test Strips	Advanced Sterilization Products®, Johnson + Johnson
Large sterilization bin with lid	n=1-2

Bowl	to rinse the transmitter in sterile saline prior to implantation
Cotton tip applicators	Several sterilized in surgical pack, additional packs
Gauze	Several sterilized in surgical pack, additional packs
small towels	n=5-6; to place around stereotaxic
Skin marker	Mediware Skin Marker, servoprax GmbH
Electro-cautery	If necessary, use only prior to implantation of transmitter
Bone cement	Smartset GHV, DePuy CMW, Johnson+Johnson
Electric razor	Aesculap® Vega/ Isis B. Braun Melsungen AG
Sterile gloves	KruTex Vet-Gel surgical gloves
Endotrachealtubus	Rüsch® Super Safety Clear Trachealtubus, size appropriate for the respective dog
SMI-Spoon Resorbierbarer Gelatineschwamm	SMI AG
Surgical Glue	3M Vetbond™
Sutures	3/0 Dafilon®, B. Braun Melsungen AG 3/0 Monosyn®, B. Braun Melsungen AG
Syringes	different sizes; e.g. 1mL, 5mL, 10mL
Sterile cannulas	Different diameters
Sterile bag-in-bag	In case of multiple days of surgery: to sterile pack the drill handpiece

3 Medication: Experiments

Active Agent	Trade Name	Vendor	Dose (mg/kg)	Route
Midazolam	Midazolam-ratiopharm® 15mg/3ml Injektionslösung	Ratiopharm GmbH	0.2	i.v.
Apomorphine	Emedog 1 mg/mL	Laboratoire TVM	0.8	s.c.
Propofol	Propovet™ Multidose	Abbott Laboratories Ltd	initial: 6.6; after effect	i.v.
Metamizol Natrium 500mg/ml	Metamizol WDT	Wirtschaftsgenossenschaft deutscher Tierärzte eG	20-50	i.v.
Metoclopramide	Emeprid® Injection	Cewa	1	s.c.
Maropitant	Cerenia® ad us. vet	Zoetis GmbH	1	s.c.
Lidocainehydrochloride 20mg/mL	Lidocainehydrochloride 2%	Bela-Pharm Arzneimittelfabrik	1mL per cm skin field	s.c.

4 Supplementary Data

4.1 Biomarker Analysis

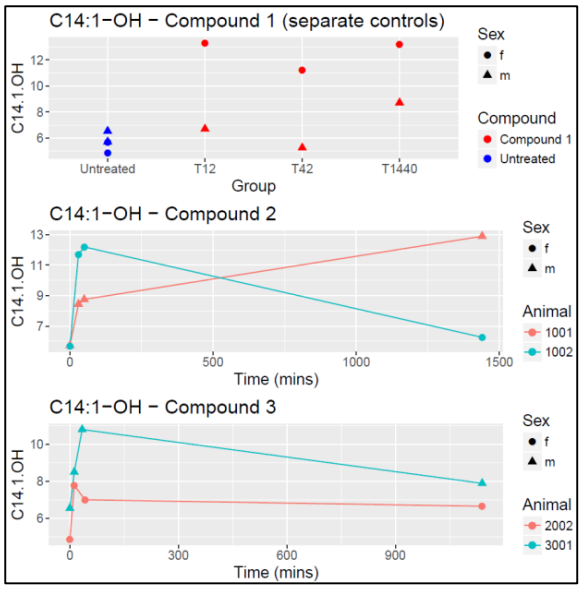


Figure 104*:* Hydroxytetradecenoylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

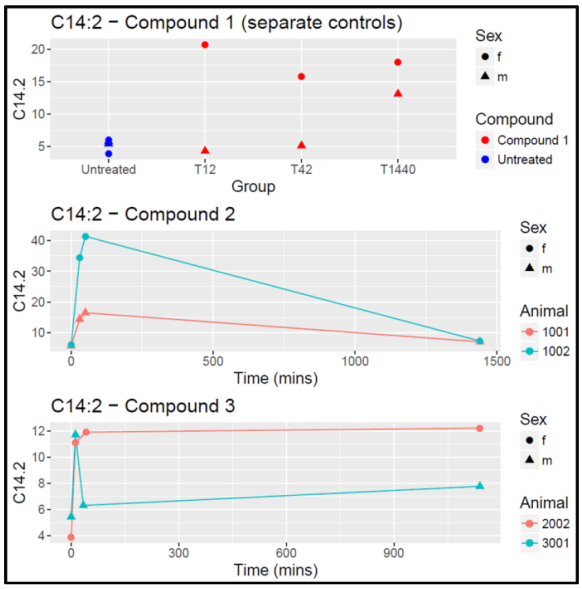


Figure 105*:* Tetradecadienylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

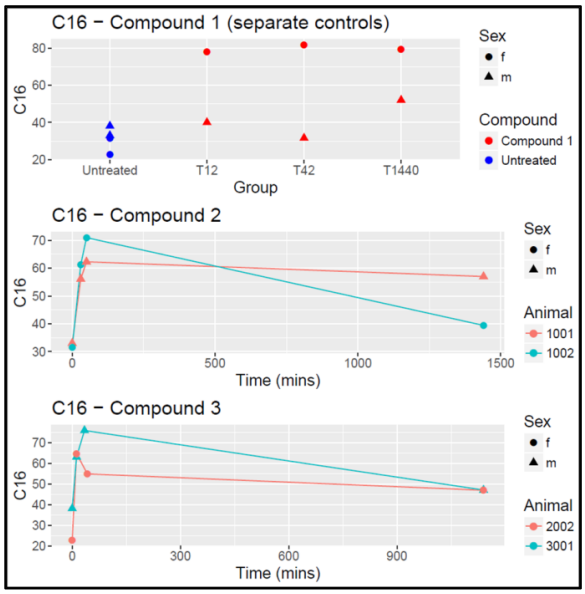


Figure 106*:* Hexadecanoylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

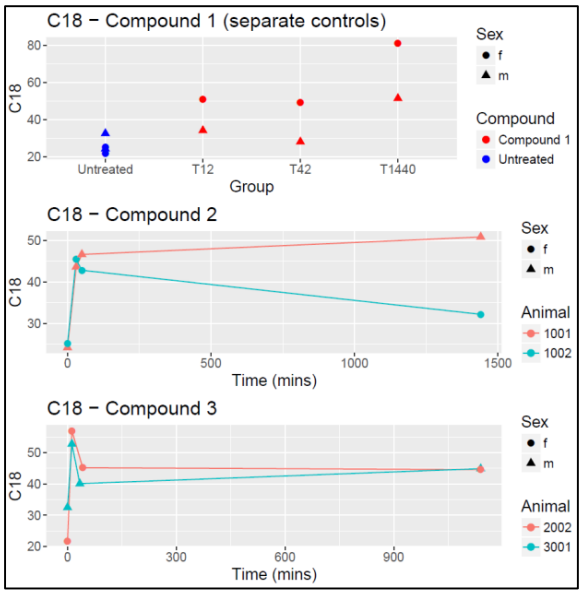


Figure 107: Octadecanoylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

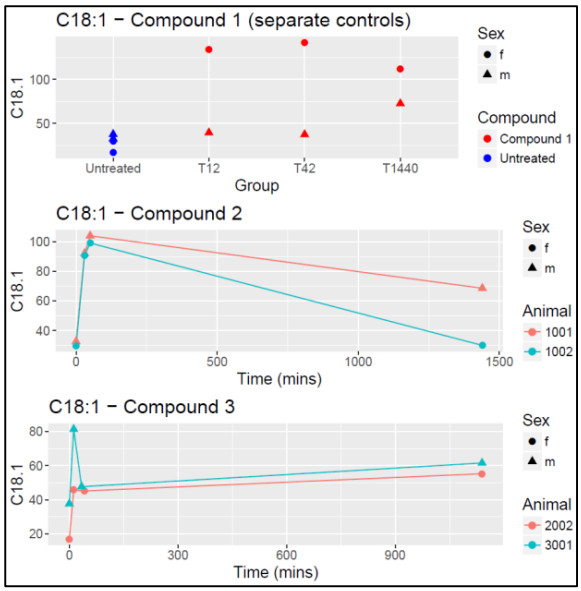


Figure 108*:* Octadecenoylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

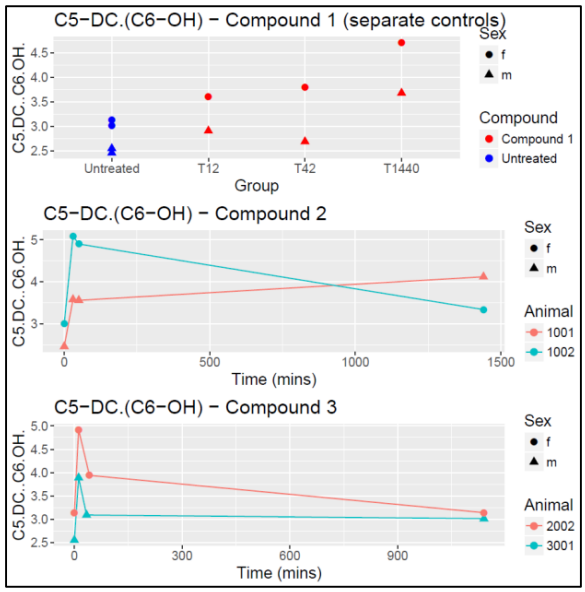


Figure 109*:* Glutarylcarnitine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

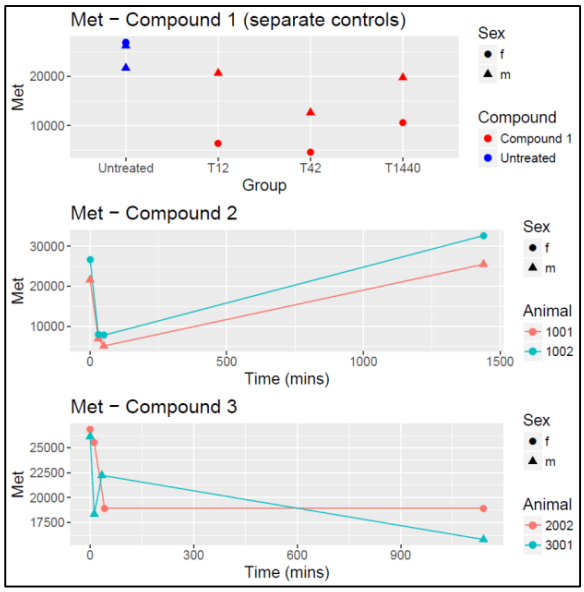


Figure 110: Methionine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

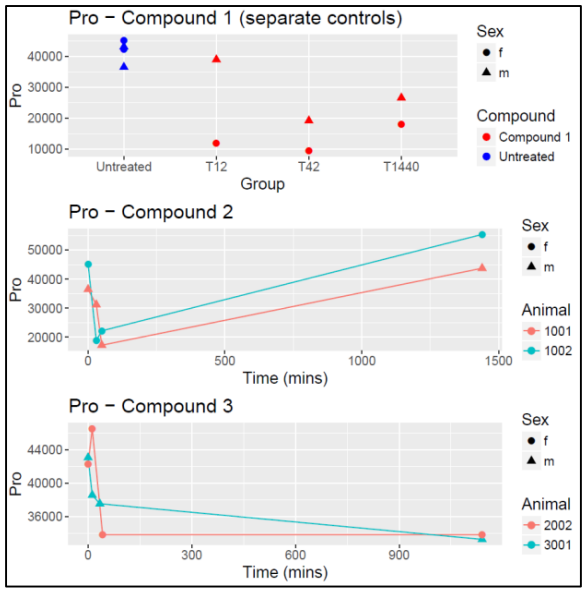


Figure 111*:* Proline changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

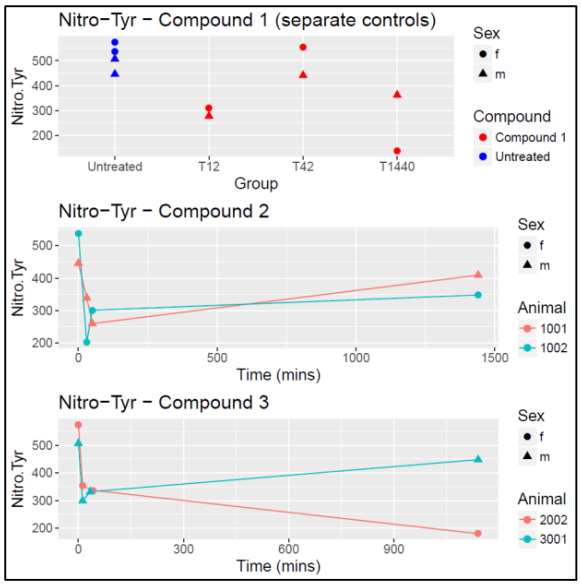


Figure 112*:* Nitro-Tyrosine changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

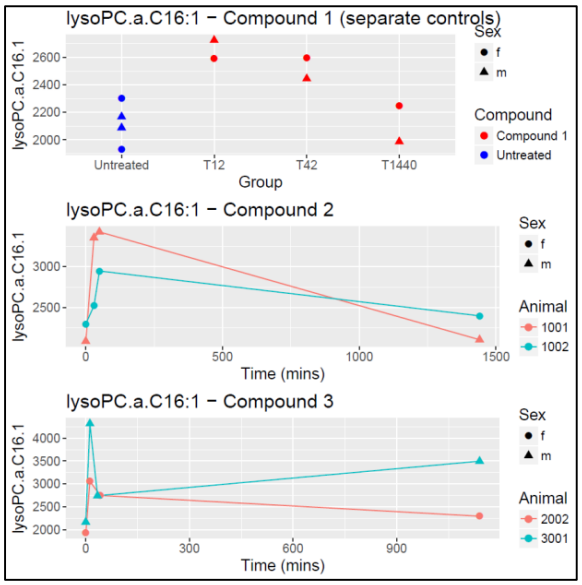


Figure 113: Lyso-Phosphatidylcholine a.C16:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

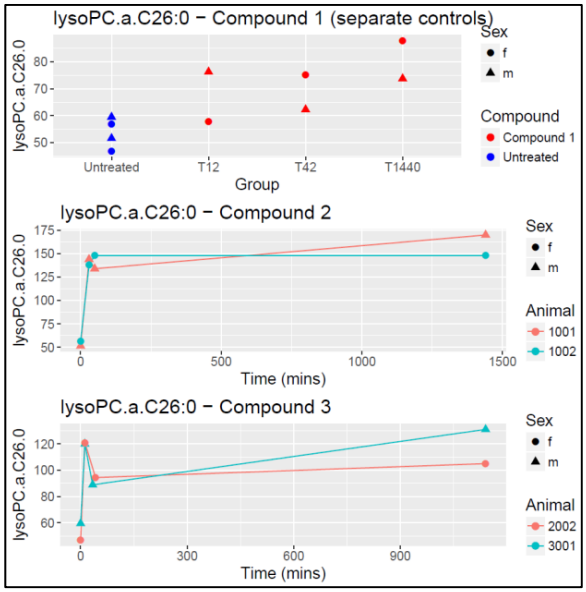


Figure 114: Lyso-Phosphatidylcholine a.C26:0 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

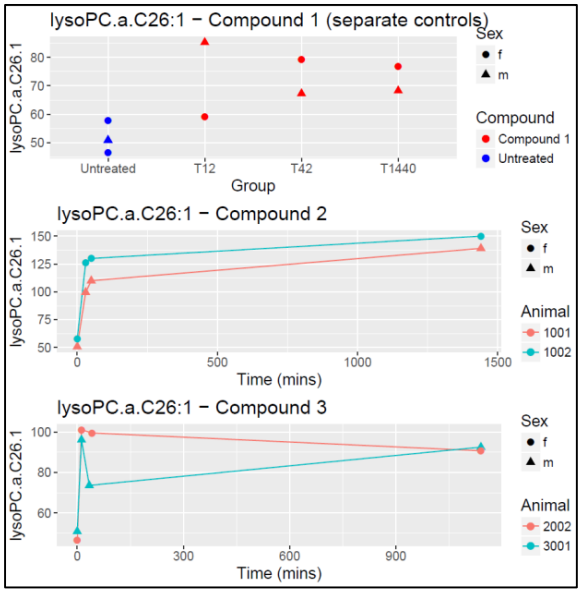


Figure 115: Lyso-Phosphatidylcholine a.C26:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

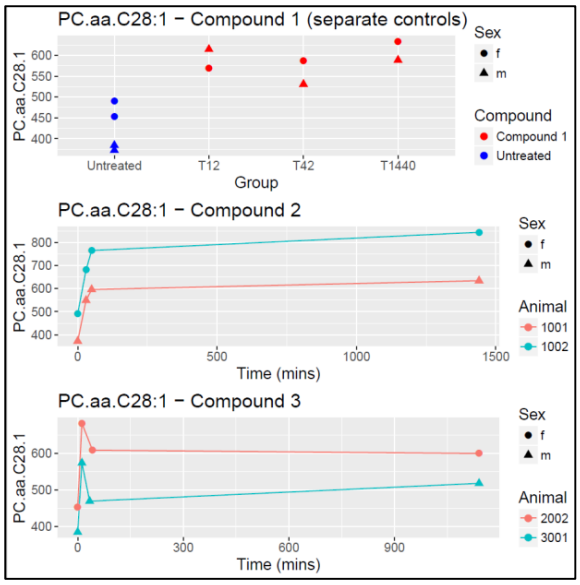


Figure 116: Phosphatidylcholine a.aC28:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

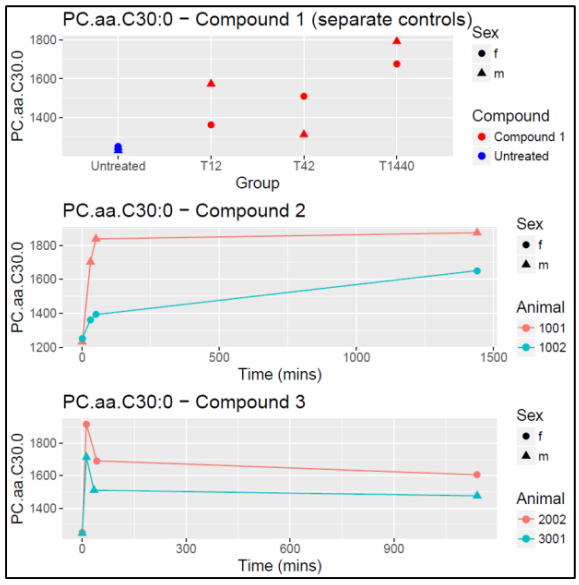


Figure 117: Phosphatidylcholine a.aC30:0 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

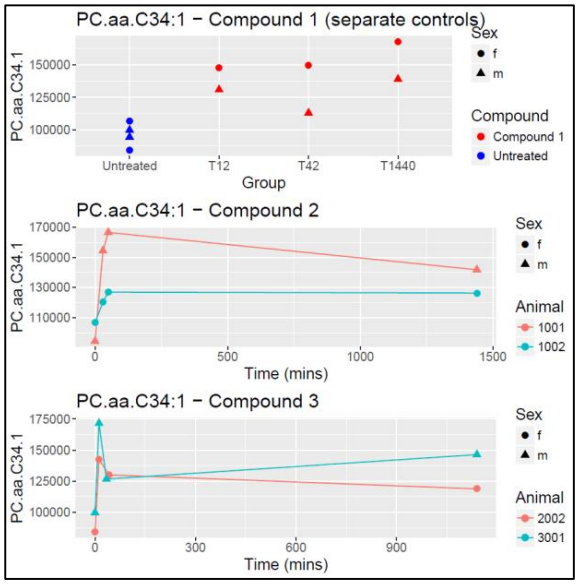


Figure 118: Phosphatidylcholine a.aC34:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

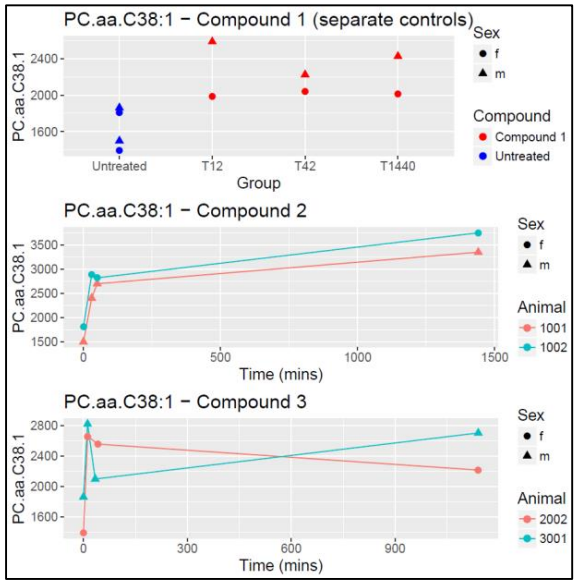


Figure 119: Phosphatidylcholine a.aC38:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

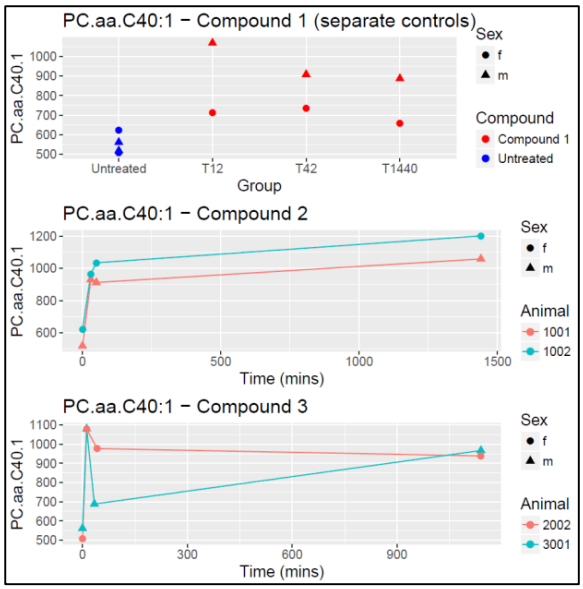


Figure 120: Phosphatidylcholine a.aC40:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

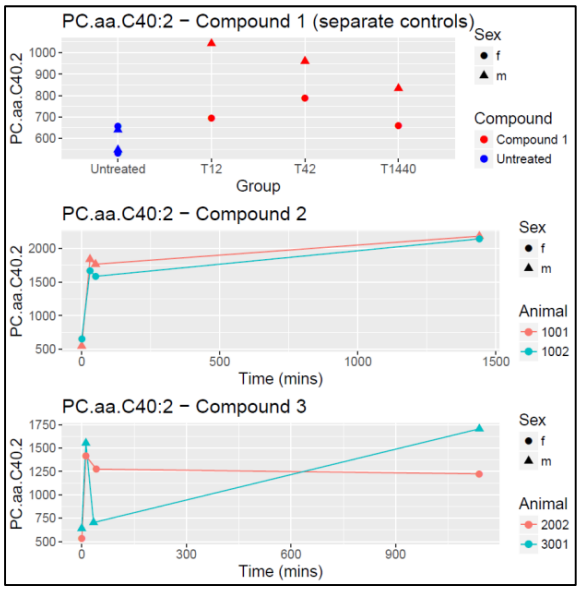


Figure 121: Phosphatidylcholine a.aC40:2 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

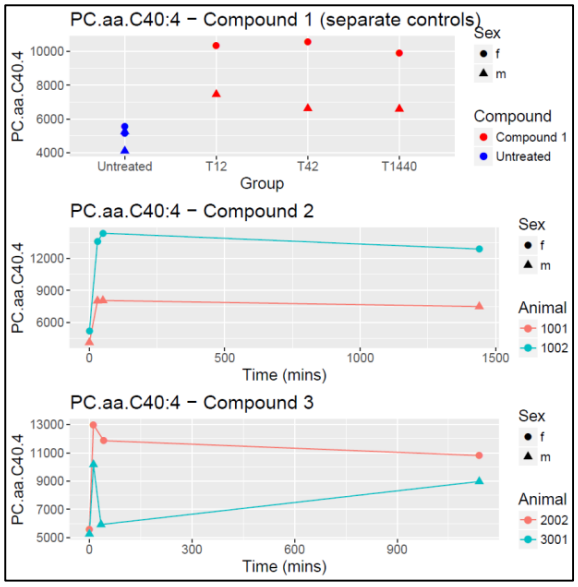


Figure 122: Phosphatidylcholine a.aC40:4 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

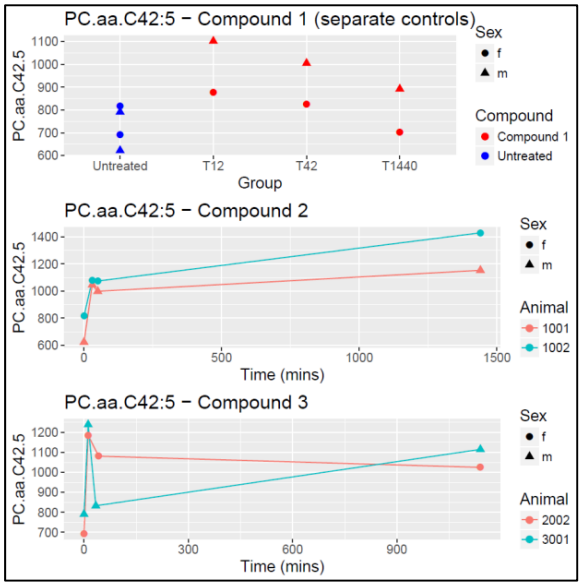


Figure 123: Phosphatidylcholine a.aC42:5 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

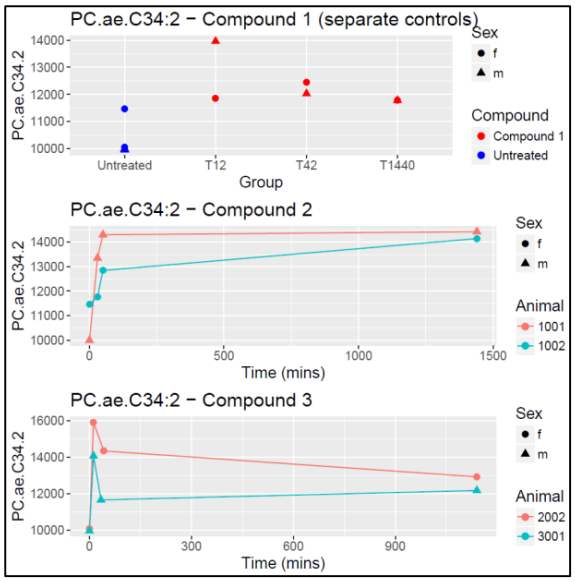


Figure 124: Phosphatidylcholine a.aeC34:2 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

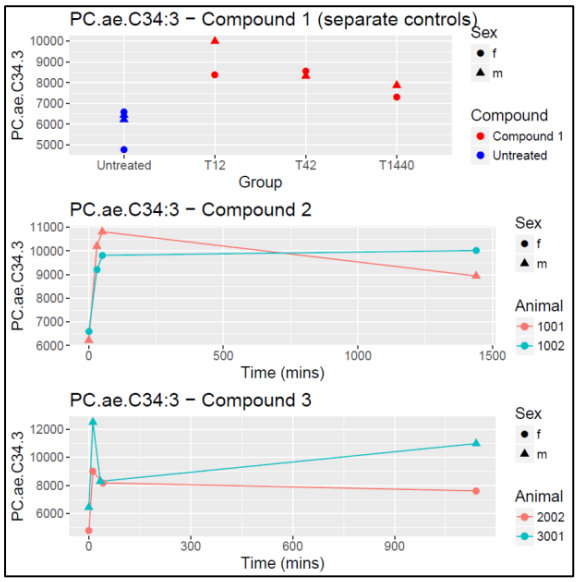


Figure 125: Phosphatidylcholine a.aeC34:3 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

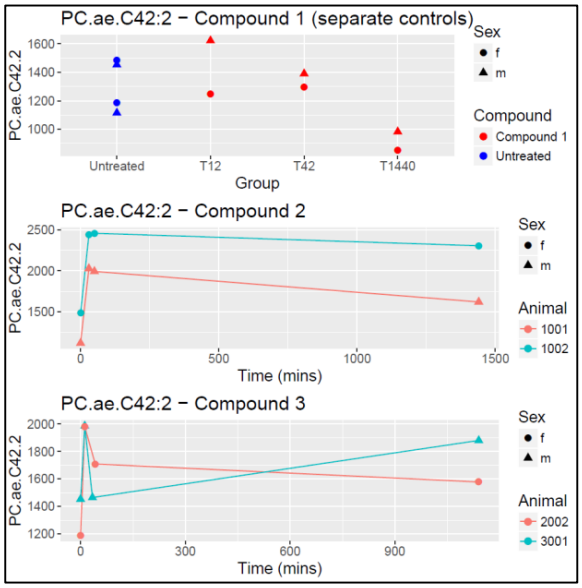


Figure 126: Phosphatidylcholine a.aeC42:2 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

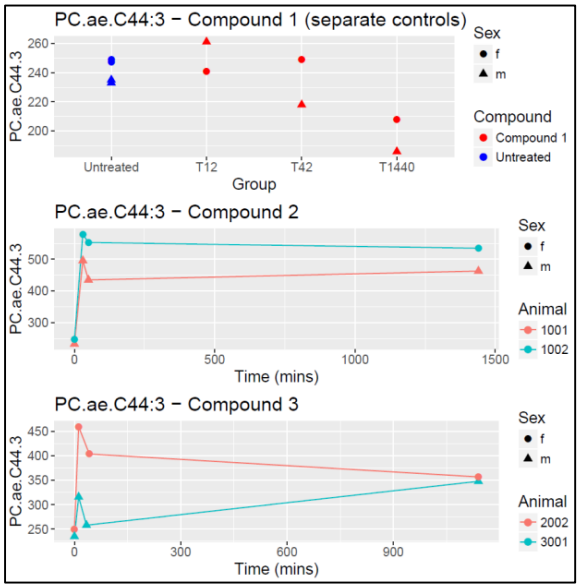


Figure 127: Phosphatidylcholine a.aeC44:3 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

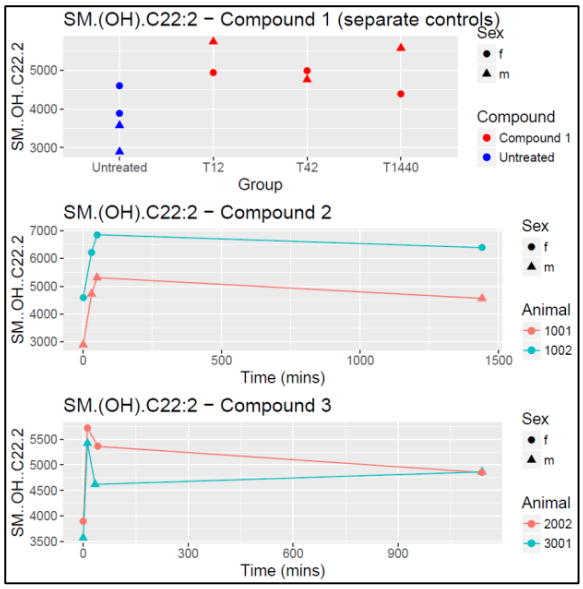


Figure 128: Sphingomyelin-OH-C22:2 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

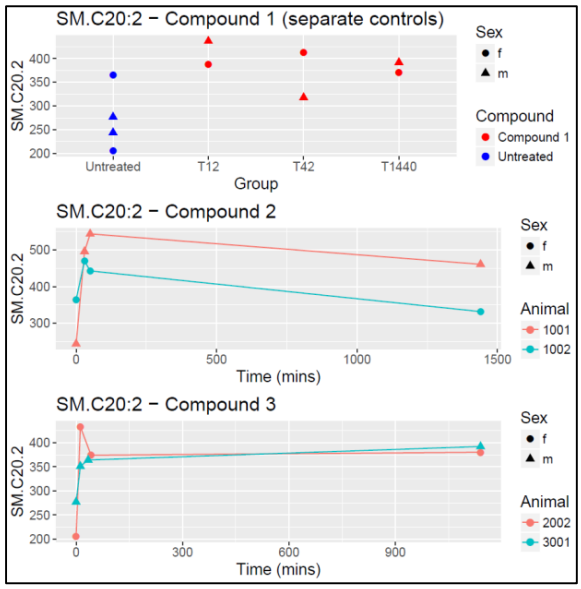


Figure 129*:* Sphingomyelin C20:2 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

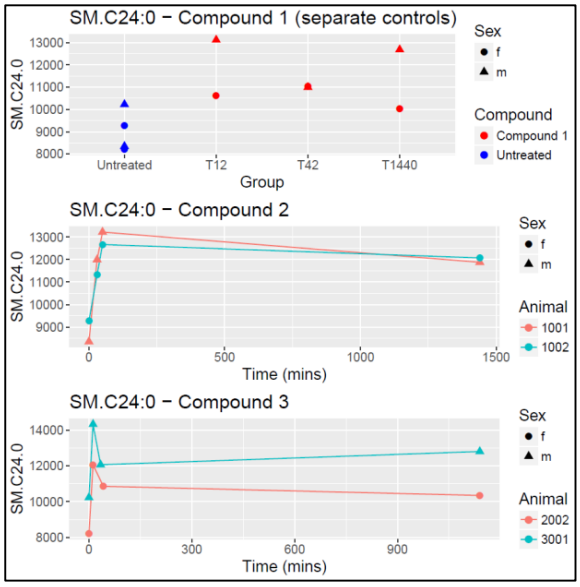


Figure 130: Sphingomyelin C24:0 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

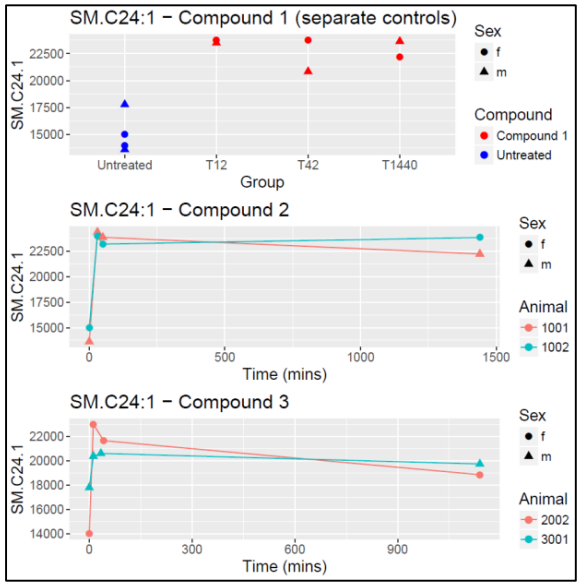


Figure 131: Sphingomyelin C24:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

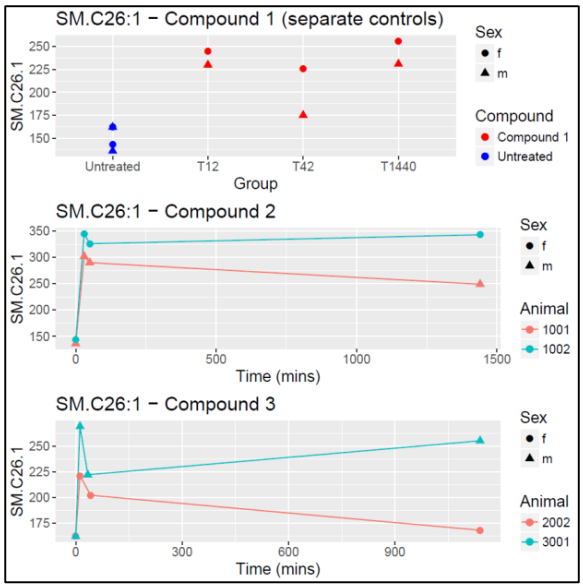


Figure 132: Sphingomyelin C26:1 changes from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

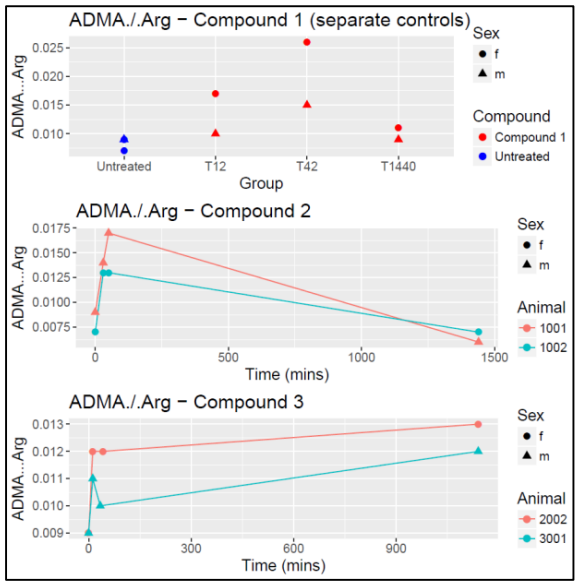


Figure 133: Changes in the ADMA/Arg ratio from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

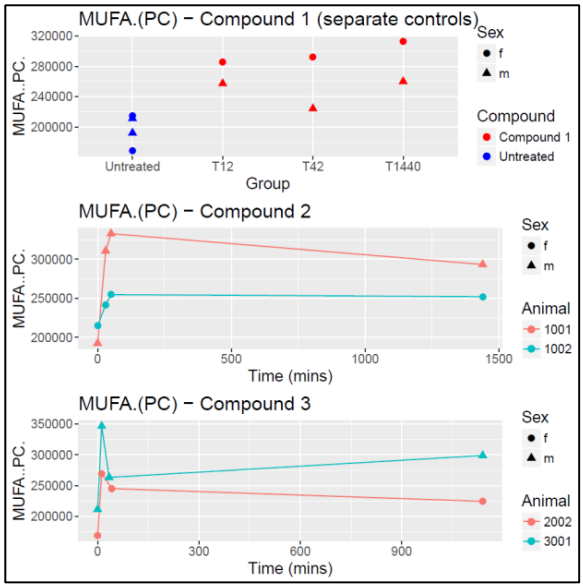


Figure 134: Changes in MUFA.(PC) from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

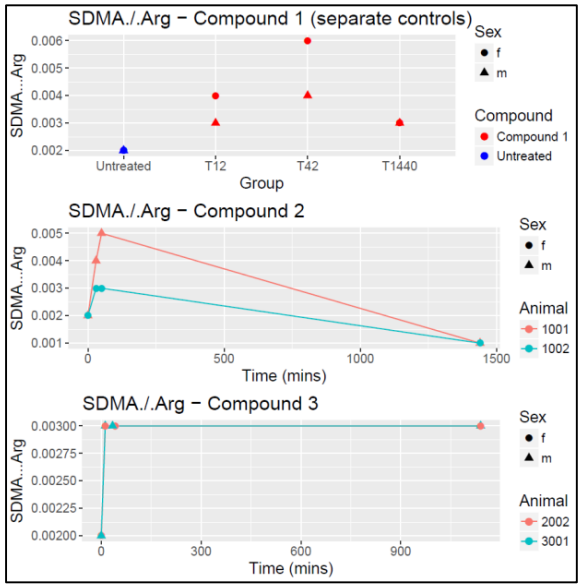


Figure 135: Changes in the SDMA/Arg ratio from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

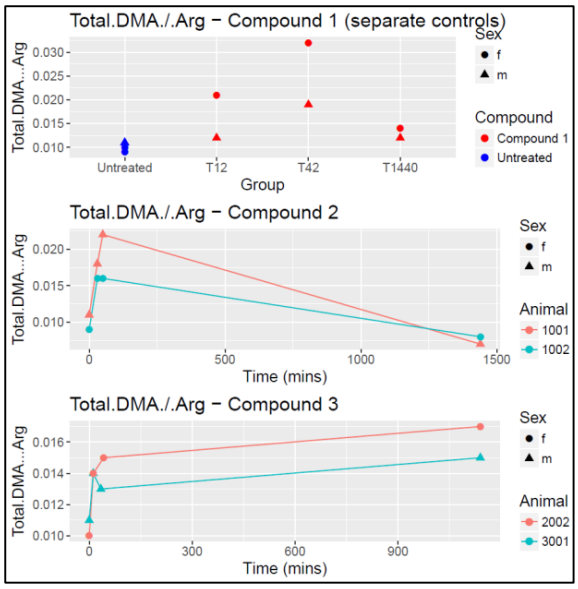


Figure 136: Changes in the total DMA/Arg ratio from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

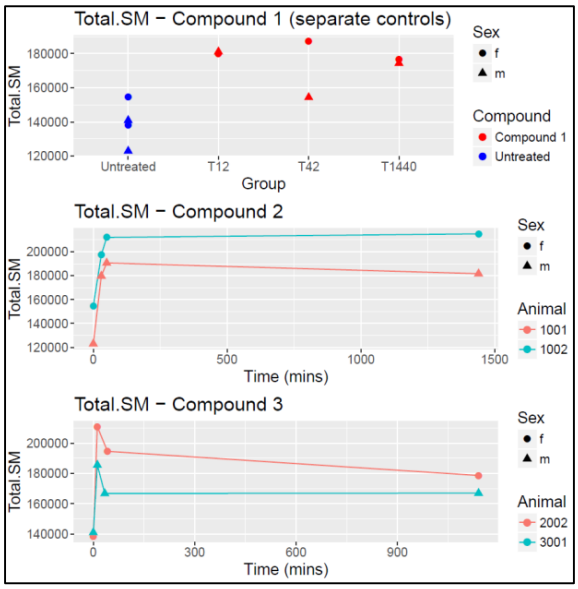


Figure 137: Changes in total sphingomyelins from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

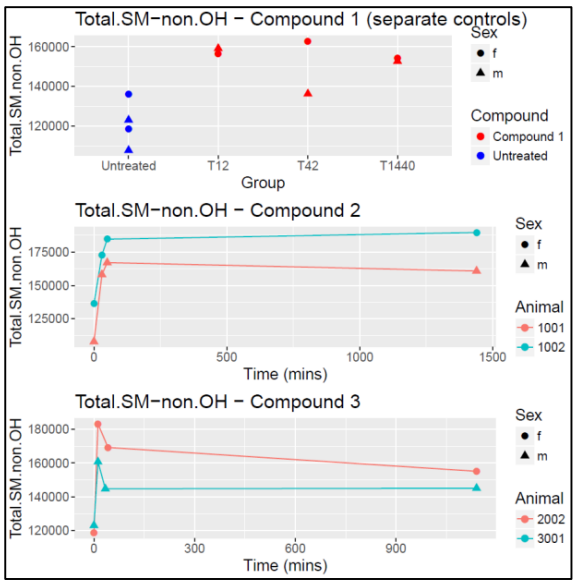


Figure 138: Changes in total non-OH sphingomyelins from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

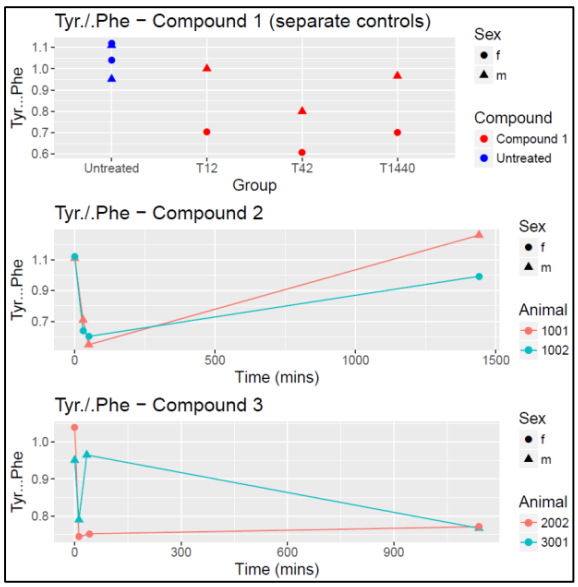


Figure 139: Changes in the Tyrosin/ Phenylalanin ratio from baseline to time points 1, 2 and 3 after administration of compound 1 (above), 2 (middle) and 3 (bottom).

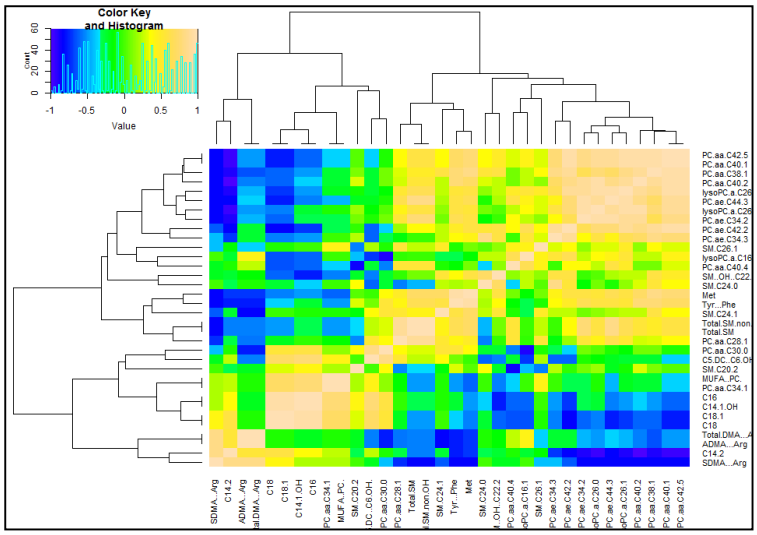


Figure 140: Correlations between the 34 detected metabolites (final time point, n=6).

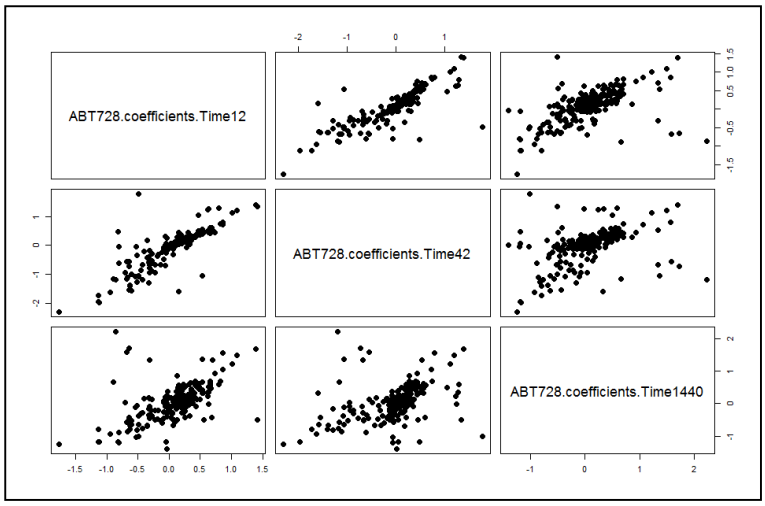


Figure 141: fold changes across time points for compound 1.

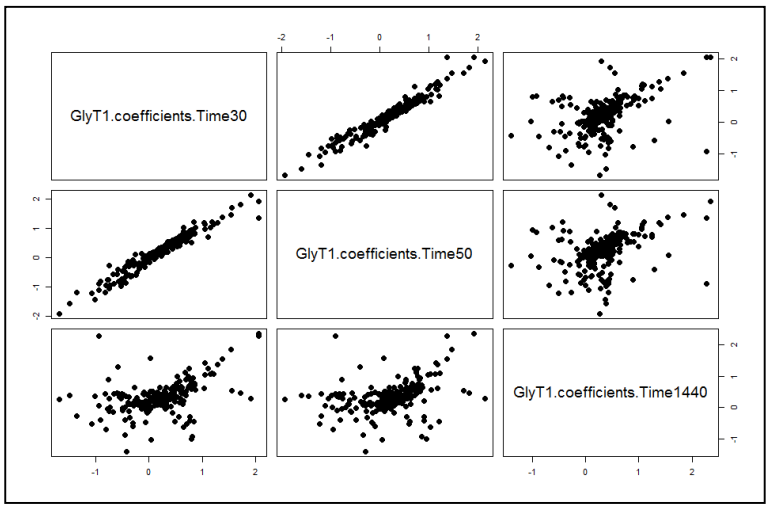


Figure 142: fold changes across time points for compound 2.

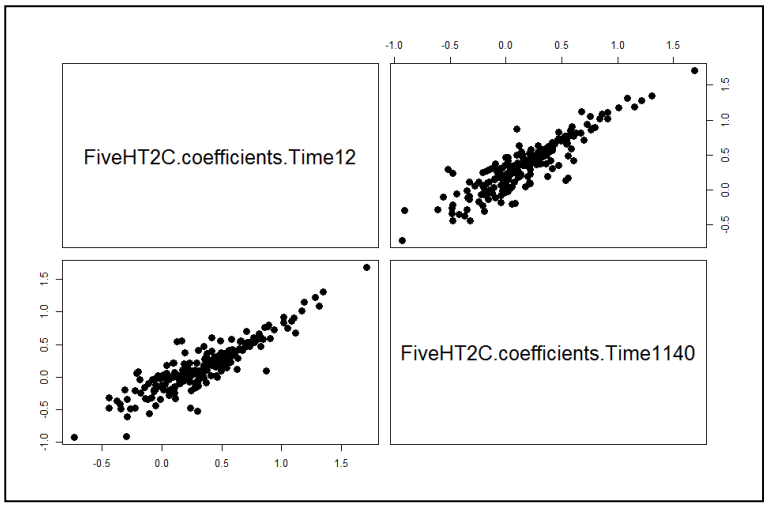


Figure 143: fold changes across time points for compound 3.

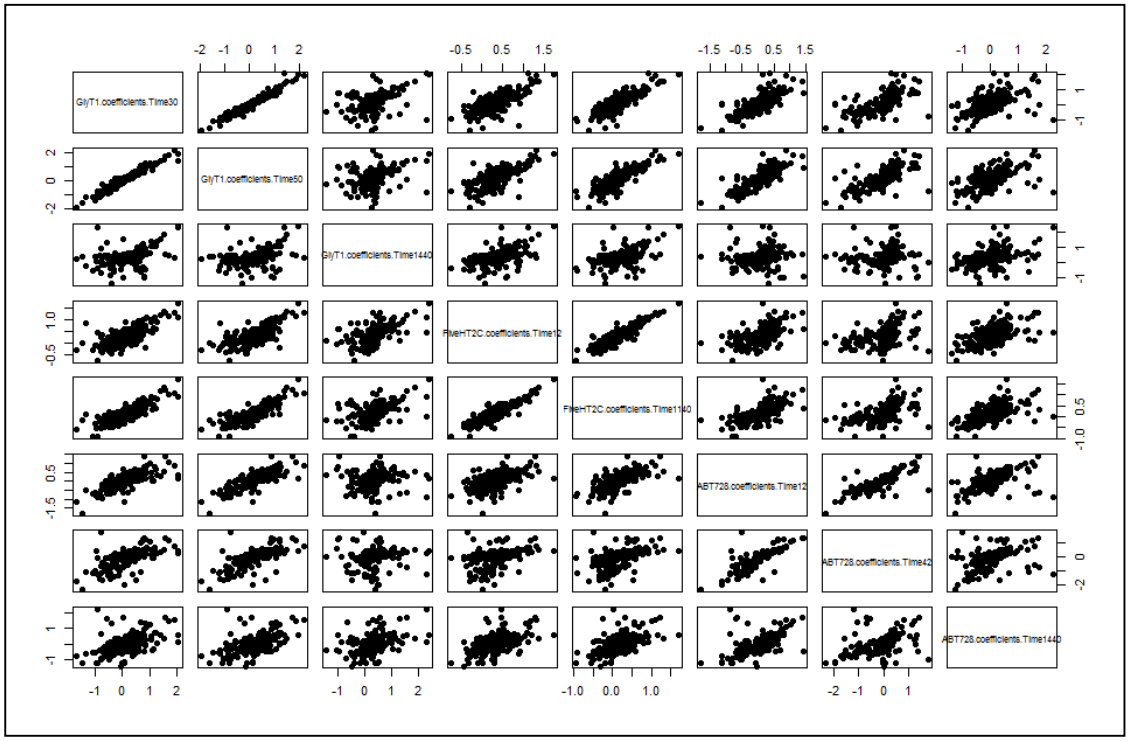


Figure 144: Fold changes across time points for all 3 compounds.

4.2 qEEG Data

The following scatter plots show the changes from baseline to post treatment values in each frequency band for each dog respectively. Mean +/-SD are displayed. The medians that were calculated for comparison of both dogs are close to mean levels.

6.2.1 *Midazolam*

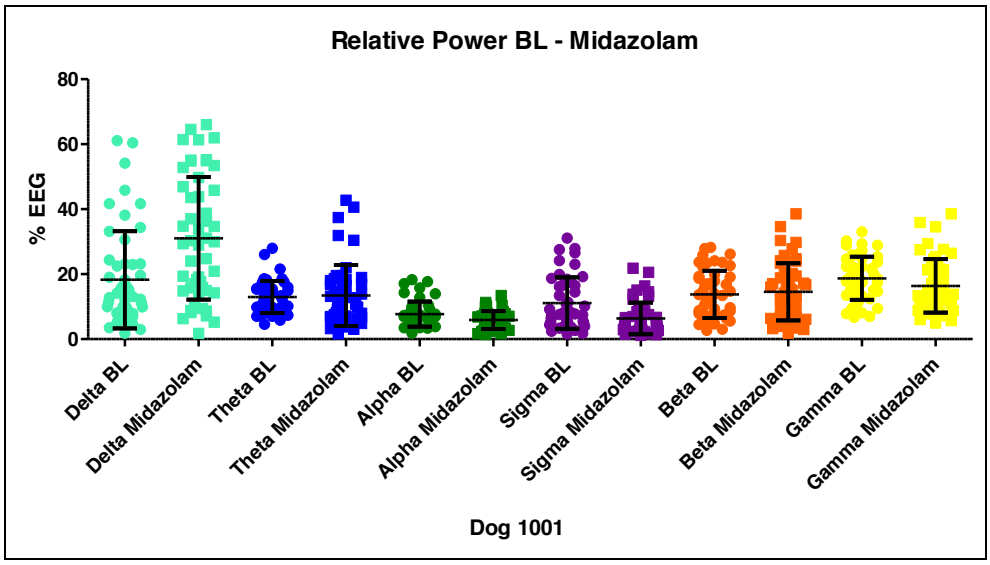


Figure 145: Changes in relative EEG power after midazola treatment (dog 1001).

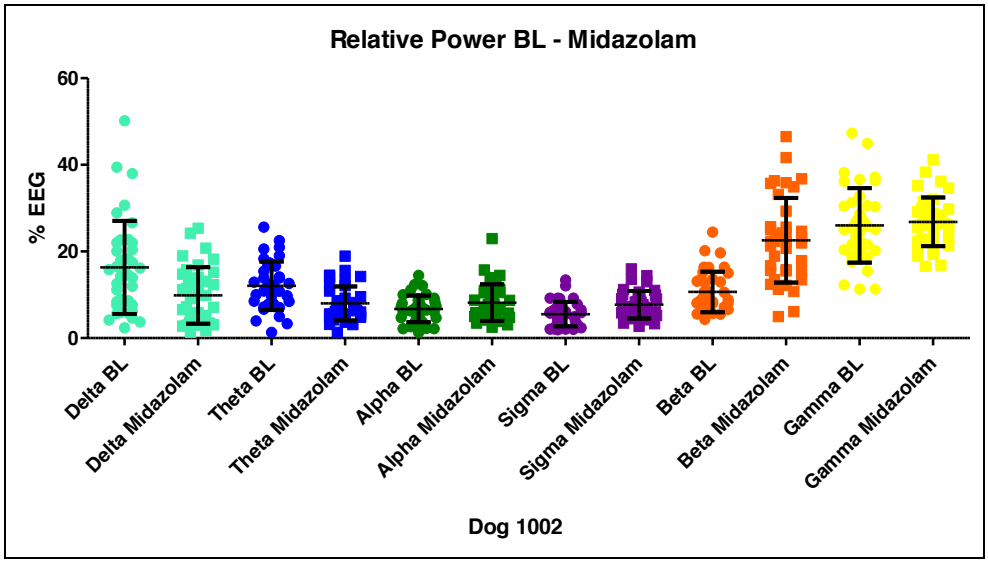


Figure 146: Changes in relative EEG power after midazola treatment (dog 1002).

6.2.2 *Propofol*

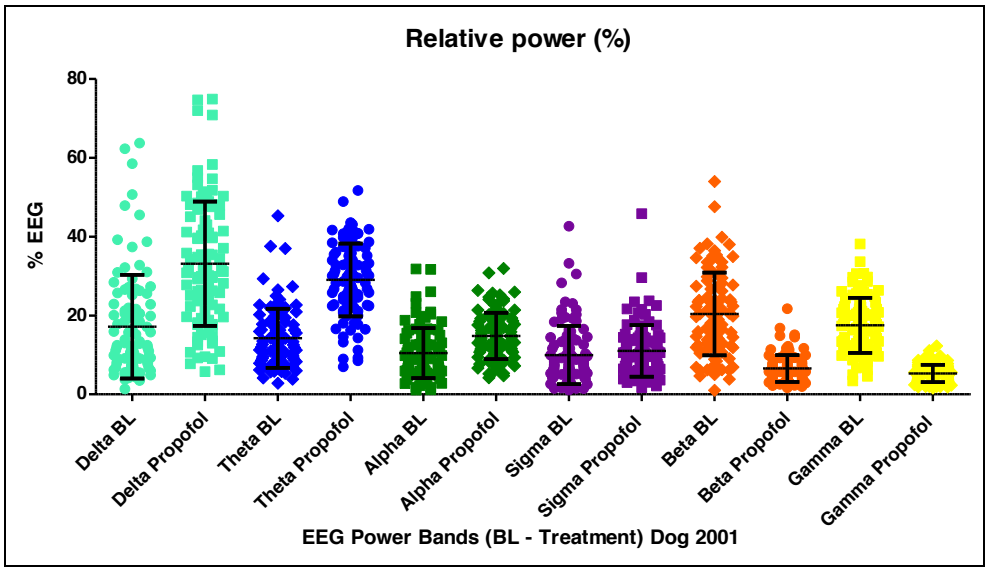


Figure 147: Changes in relative EEG power after propofol treatment (dog 2001).

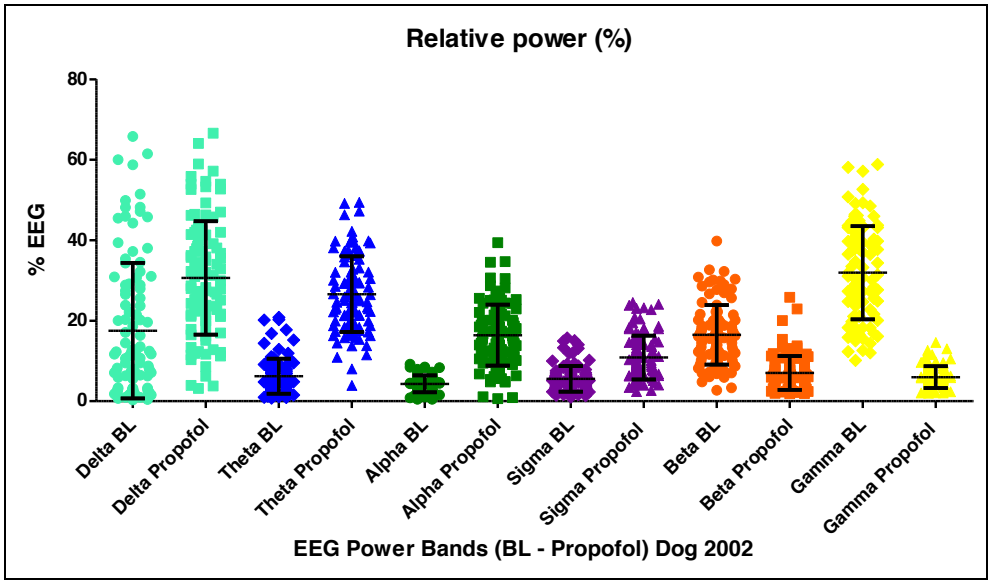


Figure 148*:* Changes in relative EEG power after propofol treatment (dog 2002).

6.2.3 *Apomorphine*

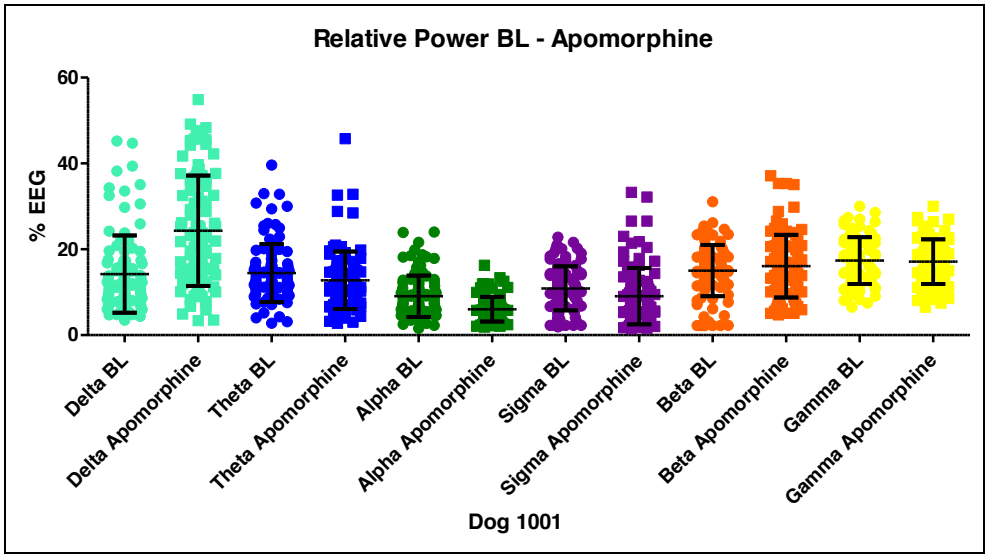


Figure 149: Changes in relative EEG power after apomorphine treatment (dog 1001).

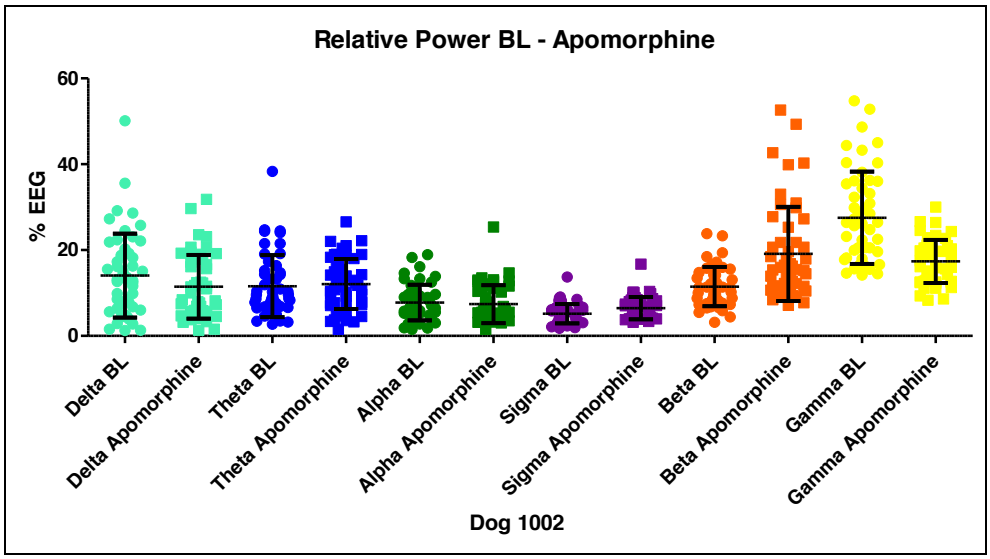


Figure 150: Changes in relative EEG power after propofol treatment (dog 2002).

6.2.4 *Quinpirole*

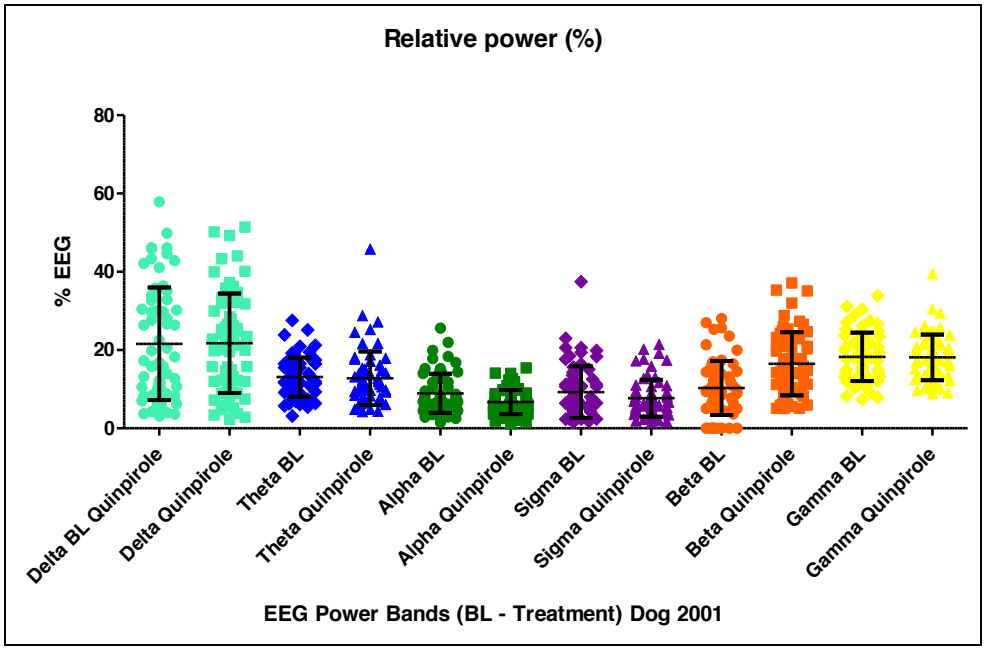


Figure 151: Changes in relative EEG power after quinpirole treatment (dog 2001).

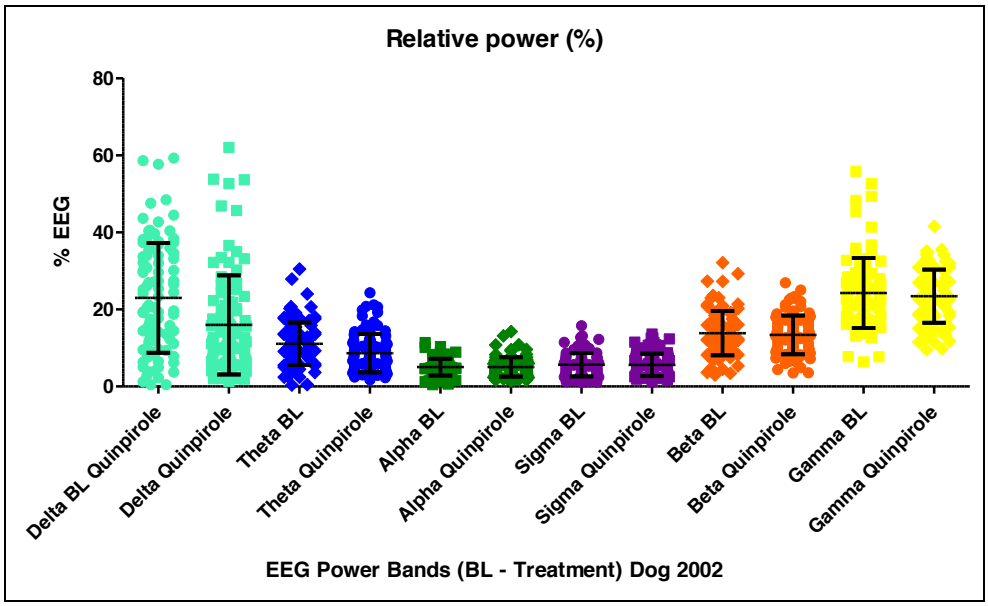


Figure 152: Changes in relative EEG power after quinpirole treatment (dog 2002).

6.2.5 *Compound 1*

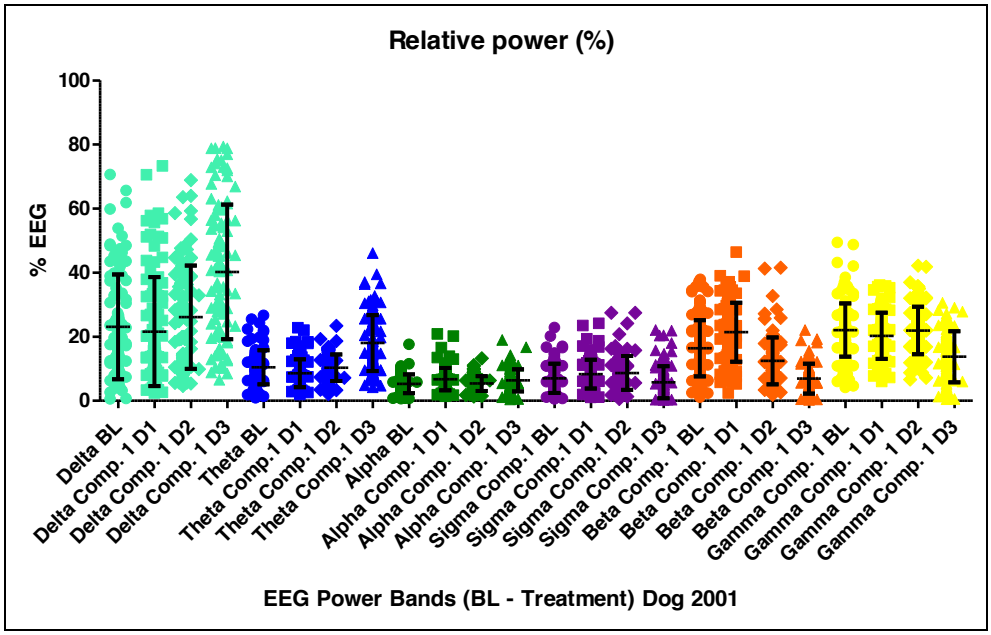


Figure 153: Changes in relative EEG power after compound 1 (dog 2001).

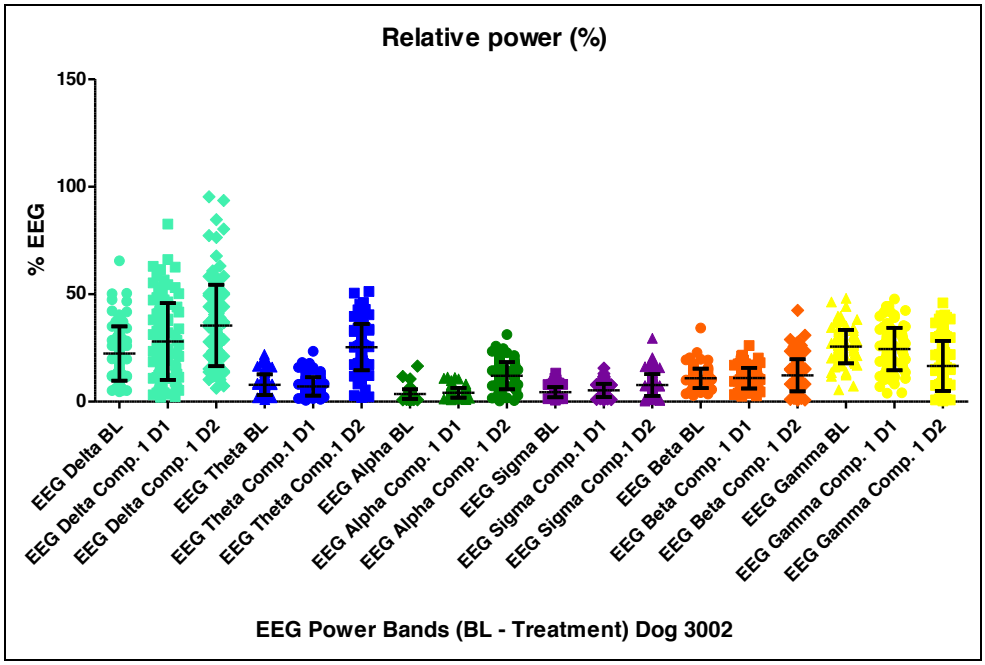


Figure 154: Changes in relative EEG power after compound 1 (dog 3002).

6.2.6 *Compound 2*

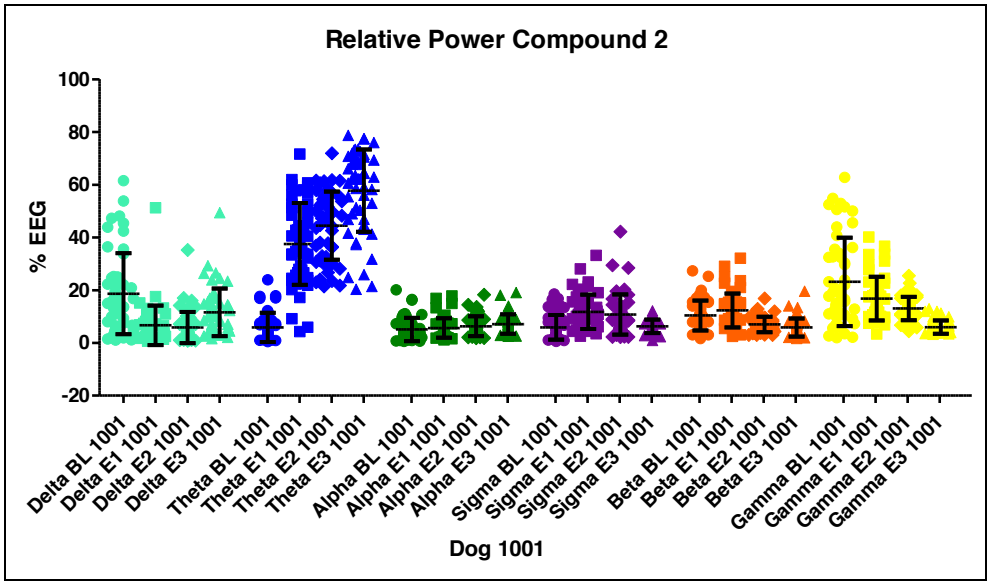


Figure 155: Changes in relative EEG power after compound 2 (dog 1001).

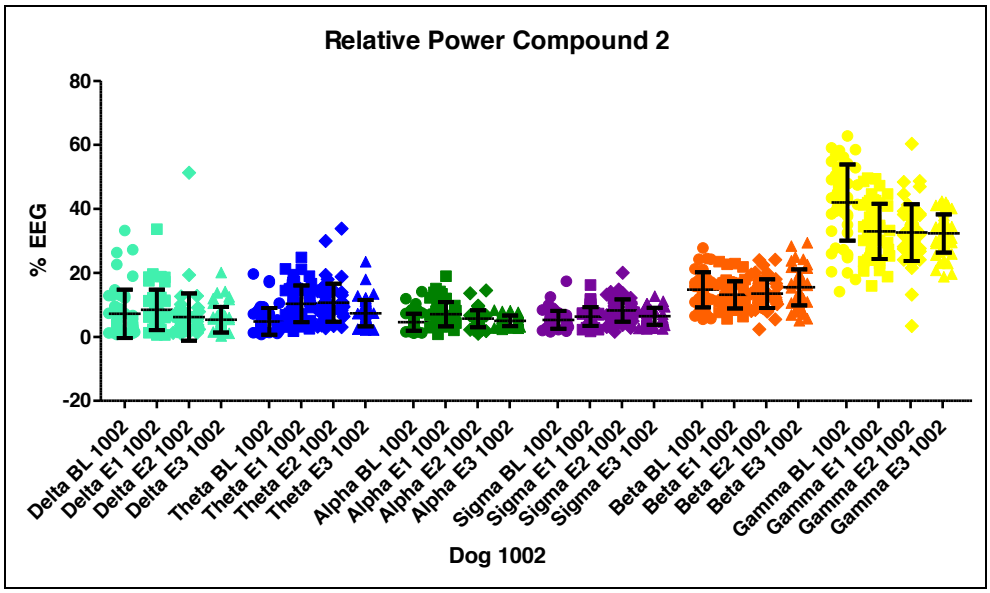


Figure 156: Changes in relative EEG power after compound 2 (dog 1002).

6.2.7 *Compound 3*

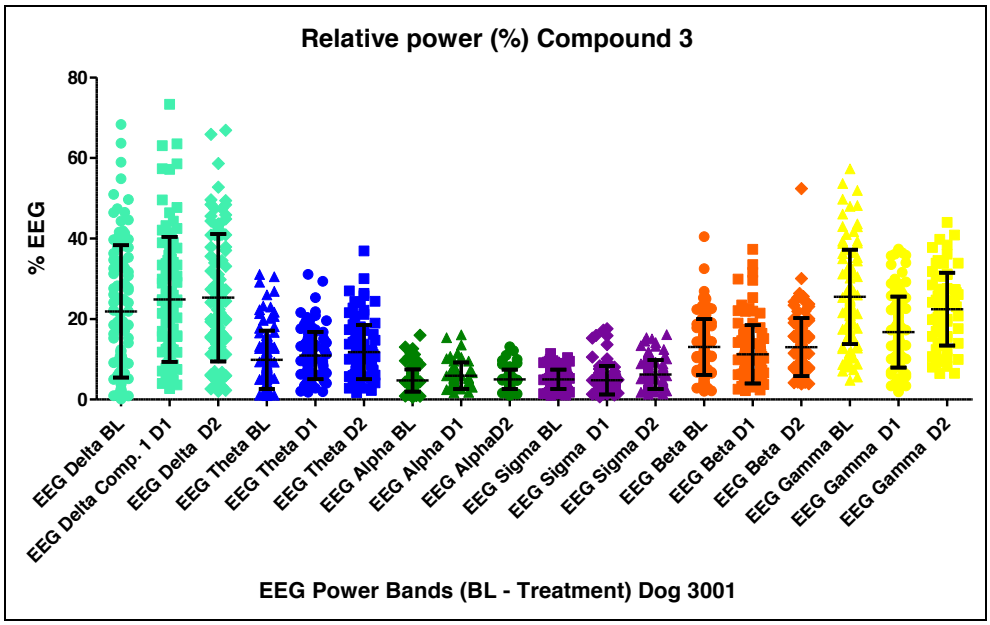


Figure 157: Changes in relative EEG power after compound 3 (dog 3001).

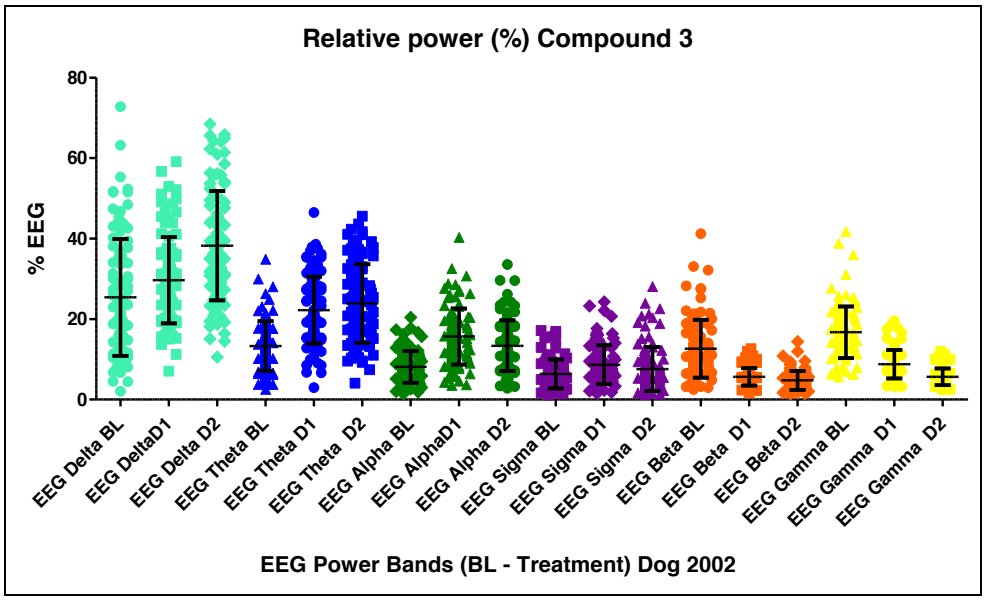


Figure 158: Changes in relative EEG power after compound 3 (dog 2002).

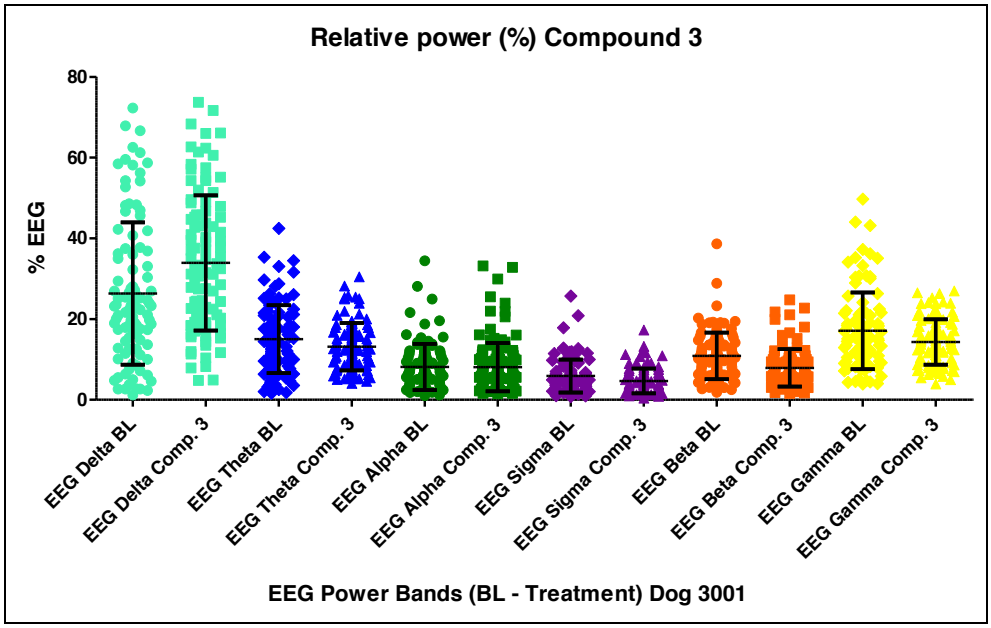


Figure 159: Changes in relative EEG power after compound 3, second experiment.

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XIII. Acknowledgements

An dieser Stelle möchte ich allen meine Dankbarkeit ausdrücken, die mir bei der Erstellung dieser Arbeit zur Seite standen.

Ein großer Dank geht zunächst an Frau Univ.-Prof. Dr. Heidrun Potschka, für die Bereitschaft, mich als externe Doktorandin zu betreuen und mir somit die Promotion an der Ludwigs-Maximilains Universität zu ermöglichen. Vielen Dank vor allem auch für die schnelle und sorgfältige Korrektur der Arbeit.

Frau Priv.-Dozentin Dr. Andrea Fischer, vielen Dank für die Ermöglichung eines Praktikums in der neurologischen Abteilung der Kleintierklinik, wodurch ich die ersten Erfahrungen mit dem EEG im Praxisgebrauch sammeln konnte. Vielen Dank auch für die Kommentare zu meiner Arbeit.

Mein größter Dank gilt Katja Hempel, für die Bereitstellung des Themas, für die vielen Möglichkeiten, mich auch jenseits der Doktorarbeit einzubringen und für die rundum sehr gute Betreuung, auf die ich mich immer vollkommen verlassen konnte. Ein sehr großes Dankeschön auch für die Korrektur der Arbeit und die offene und ehrliche Zusammenarbeit, aus der ich viel über mich und für meine Zukunft gelernt habe.

Gedankt sei auch Raimund Geil, Kim von der Weppen, Markus Wörner, Thomas Menrath, Klaus Winkelbauer und allen anderen Kollegen von PCS, die mich bei der praktischen Durchführung der Studien unterstützt haben. A special thanks also to my US colleagues, Amanda Wilsey and Kuldip Mirakhur, for coming over to support!

Besonders dankend erwähnt seien die Tierärzte, Kuldip Mirakhur, Florian Hofmaier, Susanne Rensing, Helga Lorenz und Marie-Christine Klein. Bei Marie-Christine Klein möchte ich mich, außer für die ständige Bereitschaft, mir bei allen praktischen tierärztlichen Fragestellungen uneingeschränkt zu helfen, auch für ihr offenes Ohr für alle privaten und sonstigen Sorgen danken. Helga Lorenz danke ich ebenso nicht nur für ihre Unterstützung bei der Durchführung der Studien, sondern vor allem auch für ihre stets große Hilfsbereitschaft bei jeglichen Fragen. Florian Hofmaier, vielen Dank für die große Unterstützung am Anfang meiner Arbeit!

Diana Clausnitzer, Volker Nimmrich und Robert Carr möchte ich vielmals danken, dass sie das pharmakokinetische Modeling übernommen haben!

Awwad Khader and Jennifer Mollon, thanks a lot for analysis and statistical evaluation of the Biocrates biomarker data. Herzliches Dankeschön auch an Martina Kron für die Beantwortung meiner statistischen Fragen!

Lynda Nolan, thanks a lot for always finding the perfect English wording for what I actually intended to say. Vielen Dank auch an Kai Schäfer, für die Korrektur der klinischen Beschreibungen!

Acknowledgements

Peter Veselcic, Karsten Wicke und Maria Vasileva möchte ich dafür danken, dass sie mir die Mitarbeit in ihrem Labor ermöglicht haben. Besonderer Dank vor allem an Peter Veselcic für den geduldigen Unterricht im Lesen von EEGs.

Christina Baicu (DSI), special thanks to you for the long hours solving all technical problems!

Herzlichen Dank auch an die Mitarbeiter von Pharmaceutics, Beate Reeder-Hilz, Daniela Schwöbel, Takeo Riepen und allen anderen, für die Unterstützung bei den Bestellungen und Formulierungen! Den Mitarbeitern der DMPK, Klaus Diry, Martina Förster, Klaus Magin und allen anderen, vielen Dank für die Messung der Substanz-Plasmaspiegel!

Paul Germann and Eric Blomme thank you for giving me the possibility to write my thesis at AbbVie and for the continuous support!

Ein weiterer Kollege, dem ich meinen Dank ausdrücken möchte, ist zunächst Andreas Popp, dessen zufällige Bekanntschaft mich zu AbbVie gebracht hat. Auch Günter Blaich, Sascha Höning, Andreas Holler, Manuel Douglas und Ulrich Bentz: Vielen Dank für eure Unterstützung!

Nicht zuletzt danke ich meiner Familie, meinen Eltern Kirsten und Josef, meine Großeltern Lisi und Dieter und meiner Schwester Marie für die liebevolle Unterstützung, jeden Tag und in allen Lebenslagen. Ebenso möchte ich meinem Freund Tobias für seine Liebe und die Motivation, vor allem zum Ende der Arbeit, von Herzen danken.