"Search for Biomarkers and Pathogenesis Studies in Canine Epilepsy"

Thesis

Submitted in partial fulfillment of the requirements for the degree

Doctor of Philosophy (PhD)

awarded by the University of Veterinary Medicine Hannover

by

Draginja Kostic

Smederevo, Serbia

Hannover, Germany 2018
University of Veterinary Medicine Hannover
Department of Small Animal Medicine and Surgery
Center for Systems Neuroscience Hannover

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List of abbreviations

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ABC  avidin-biotin-peroxidase complex
AEA  anandamide, arachidonylethanolamide
AED  Antiepileptic drugs
BBB  blood brain barrier
BSA  bovine serum albumin
CA  Cornu Ammonis
CB1R  cannabinoid receptor type 1
CB2R  cannabinoid receptor type 2
CBD  Cannabidiol
CNS  central nervous system
CSF  cerebrospinal fluid
DAG  1.2-diacylglycerol
DSI  depolarization-induced suppression of inhibition
DSE  depolarization-induced suppression of excitation
ECs  endocannabinoids
ECS  endocannabinoid system
EDTA  ethylene diamine tetraacetic acid
ELISA  enzyme-linked immunosorbent assay
et al.  et alii
Fig.  figure
FAAH  fatty acid amide hydrolase
g  gram
GABA  gamma-aminobutyric acid
GFAP  glial fibrillary acidic protein
List of abbreviations

h       hour
H$_2$O$_2$  hydrogen peroxide
IE      idiopathic epilepsy
IF      immunofluorescence
IgA     immunoglobulin A
IHC     immunohistochemistry
IL      interleukin
IVDH    intervertebral disc herniation
IVETF   International Veterinary Epilepsy Task Force
Kg      kilogram
mAb     monoclonal antibody
MAP     mitogen-activated protein
mg      milligram
MGCS    modified Glasgow Coma Scale
min     minutes
mL      milliliter
MRI     magnetic resonance imaging
MUO     meningoencephalitis of unknown origin
µg      microgram
µl      microliter
NAPE-PLD N-acylphosphatidylethanolamine-hydrolyzing phospholipase D
ng      nanogram
nM      nanomolar
NME     necrotizing meningoencephalitis
OPC     oligodendrocyte progenitor cells
List of abbreviations

PBS          phosphate-buffered saline
PKA          protein kinase A
PNS          peripheral nervous system
PTE          post-traumatic epilepsy
rSpear       Spearman´s rank correlation coefficient
SCI          spinal cord injury
SUDEP        sudden unexpected death in epilepsy
Tab.         table
TBI          traumatic brain injury
THC          $\Delta^9$-tetrahydrocannabinol
TLE          temporal lobe epilepsy
TMB          3.3´.5.5´ Tetramethylbenzidine
T2WLR        T2-weighted hyperintensity length ratio
1AG          1-arachinodoyl glycerol
2AG          2-arachinodoyl glycerol
%            percent
°C            degree Celsius
List of abbreviations
1. Summary

Search for biomarkers and pathogenesis studies of canine epilepsy

Draginja Kostic

Epilepsy is the most common chronic neurological disease in dogs. The aim of treatment is seizure freedom but cannot be achieved in many cases. Successful treatment is considered a useful decrease in seizure frequency. To improve the outcome, a constant search for new treatment strategies is performed, influencing either the disease or epileptogenesis.

Biomarkers are needed in neuroscience to depict changes on cellular or molecular level within the brain’s specific microenvironment. Finding useful biomarkers in epilepsy could support the search for new therapeutic strategies.

In the current thesis, we have evaluated two potential prognostic biomarkers, glial fibrillary acidic protein (GFAP) and interleukin-1β (IL-1β) and one receptor possibly involved in pathogenesis of canine epilepsy, the cannabinoid receptor type-1 (CB1R).

In the first part of the study GFAP, the main intermediate filament protein in astrocytes, was evaluated as potential biomarker for intracranial disorders using an ELISA. Healthy beagles and dogs with the clinical diagnoses idiopathic epilepsy, brain tumor, inflammation, spinal cord injury (SCI) and traumatic brain injury (TBI) were included. Significant differences were found between GFAP CSF levels of dogs with tumor and epilepsy and between inflammatory diseases and epilepsy. Furthermore, in TBI patients, high serum GFAP levels had a strong correlation with the Glasgow Coma Scale score. In dogs with SCI no significant difference between chronic and acute cases and severity of clinical signs could be detected.

The second part of the project focused on the possible involvement of IL-1β, a potent proinflammatory cytokine, in neuroinflammation in epilepsy. Levels of IL-1β in serum
Summary

and CSF were measured using an ELISA and compared between healthy dogs and TBI and epilepsy patients. IL-1β concentrations in CSF were not detectable. Additionally, serum values were not elevated in dogs with TBI in comparison to healthy controls. However, dogs with epilepsy had increased levels of IL-1β in serum regardless of the underlying cause of the disease.

In the last part of the study, we investigated the expression of CB1R in hippocampus of epileptic dogs and quantitatively compared it to control animals. For that purpose, brain tissues of control dogs, dogs with idiopathic and structural epilepsy were CB1R immunolabeled using immunohistochemistry and double immunofluorescence staining. Expression of CB1R was qualitatively and quantitatively evaluated in several regions of hippocampus. Moreover, the number of CB1R positive astrocytes in dentate gyrus (DG) was compared between the groups of animals.

In epileptic dogs a disease associated reorganization of CB1R expression was observed. In dogs with idiopathic epilepsy the CB1R expression was significantly decreased in the CA1 region compared to controls. Conversely, hippocampus of dogs with structural epilepsy revealed a significant increase in CB1R staining intensity in comparison to controls. Comparison of idiopathic and structural tissue demonstrated that both, the immunopositive area and the optical density of the staining reached significantly higher levels in patients with structural epilepsy. In addition, about 50% of astrocytes displayed positive CB1R staining in the tissue examined.

In the current search for different biomarkers in canine epilepsy, the most important findings were that GFAP levels in CSF reflect severe structural changes in the brain parenchyma and GFAP serum levels in TBI may predict the outcome; IL-1β in serum is increased in epilepsy independent of the etiology of the seizures; CB1R expression in canine hippocampus was increased in structural epilepsy and downregulated in idiopathic epilepsy patients and more than 50% of astrocytes expressed CB1R. These
Summary

findings, especially the disease associated influences need to be considered, when further validating new treatment approaches for dogs with epilepsy.
2. Zusammenfassung

Suche nach Biomarkern und Pathogenese-Studien zur Epilepsie bei Hunden

Draginja Kostic

Epilepsie ist eine der häufigsten chronischen neurologischen Erkrankungen bei Hunden. Idealerweise würde deren Behandlung zur vollständigen Anfallsfreiheit führen, was in vielen Fällen nicht erreicht wird. Daher wird als erfolgreiche Behandlung eine klinisch relevante Verringerung der Anfallshäufigkeit angesehen. Um Epilepsie besser behandeln zu können, sind neue Behandlungsstrategien notwendig, die entweder die Krankheit selber oder die Epileptogenese modifizieren. Biomarker werden besonders in den Neurowissenschaften benötigt, um Veränderungen auf zellulärer oder molekularer Ebene in der spezifischen Mikroumgebung des Gehirns darzustellen. Zuverlässige Biomarker zur Prognose oder Pathogenese der Epilepsie können dabei wesentlich zur Entwicklung neuer therapeutischer Ansätze beitragen.

Auf der Suche nach Biomarkern und um die Pathogenese der Epilepsie besser zu verstehen, haben wir zwei mögliche prognostische Biomarker, Glial Fibrillary Acid Protein (GFAP, saures Gliafaszerprotein) und Interleukin-1β (IL-1β) und einen möglicherweise an der Pathogenese beteiligten Cannabinoid Rezeptor Typ-1 (CB1R) evaluiert.

Im ersten Teil der Studie wurde GFAP, das wichtigste intermediäre Filamentprotein in Astrozyten, als potenzieller Biomarker für intrakranielle Störungen evaluiert. Gesunde Beagle und Hunde mit den klinischen Diagnosen idiopathische Epilepsie, Gehirntumor, Gehirnentzündung, Rückenmarkstrauma (SCI) und Schädel-Hirn-Trauma (TBI) wurden eingeschlossen. Signifikante Unterschiede wurden zwischen GFAP-CSF-
Zusammenfassung


Bei Hunden mit Epilepsie kam es zu einer krankheitsabhängigen Reorganisation der CB1R Expression. Bei Hunden mit idiopathischer Epilepsie war die CB1R-Expression in der Cornu Ammonis 1-Region im Vergleich zu den Kontrollen signifikant verringert. Umgekehrt zeigte der Hippocampus von Hunden mit struktureller Epilepsie einen signifikanten Anstieg der Intensität der CB1R-Färbung im Vergleich zu den Kontrollen.
Zusammenfassung

Ein Vergleich von Gehirngewebe von Hunden mit idiopathischer und struktureller Epilepsie zeigte, dass sowohl der Bereich mit CB1R Expression als auch die optische Dichte der Färbung bei Patienten mit struktureller Epilepsie signifikant höhere Werte erreichten. Ca. 50% der Astrozyten wiesen in allen untersuchten Bereichen eine positive CB1R-Färbung auf.

In vorliegender Studie waren die wichtigsten Befunde, dass GFAP-Werte im Liquor cerebrospinalis die Schwere struktureller Veränderungen im Parenchym unabhängig der Ursache der Läsion widerspiegeln und GFAP-Serumspiegel in TBI die Prognose vorhersagen könnten; IL-1β war im Serum bei Hunden mit Epilepsie unabhängig der Ursache für die Krampfanfälle erhöht; Die CB1R-Expression im Hippocampus von Hunden war bei struktureller Epilepsie erhöht, bei idiopathischer dagegen herunterreguliert. Diese krankheitsabhängigen Veränderungen sollten bei der Weiterentwicklung neuer Behandlungsansätze für Hunde mit Epilepsie berücksichtigt werden.
Zusammenfassung
3. Aims of the study

In treatment of epilepsy, the main goal is seizure freedom, but it cannot be achieved in many cases. To improve the outcome, a constant search for new treatment strategies is performed, influencing either the disease or epileptogenesis. Biomarker with diagnostic-prognostic value could help in the development of new therapeutic approaches. Therefore, the main purpose of the current study was to evaluate potential biomarkers in canine epilepsy, in serum, cerebrospinal fluid and brain parenchyma.

In the first part of the study, the value of glial fibrillary acidic protein (GFAP) as a neurobiomarker in serum and cerebrospinal fluid in various canine CNS diseases was assessed. This part of the study should prove the hypothesis that severe CNS tissue destruction leads to measurable GFAP serum levels independent of the cause of the disease; it should be proven, that structural epilepsy is causing elevated serum GFAP levels and that GFAP serum levels might be a potential biomarker for the development of posttraumatic epilepsy. Therefore, a correlation of GFAP in serum and the outcome in traumatic brain injury should be evaluated.

Next, the concentration of IL-1β in peripheral blood of dogs with traumatic brain injury and epilepsy was investigated, as well as its presence in cerebrospinal fluid of dogs with epilepsy using an ELISA. We hypothesized that IL-1β elevation in serum of dogs with TBI and that in serum and CSF reflects chronic inflammation in naturally occurring canine epilepsy in a clinical setting.

The endocannabinoid system (ECS) and its cannabinoid receptor type 1 are thought to play an important role in the pathogenesis of epilepsy. A better understanding of the role of this system and disease associated influences need to be considered, when further validating new treatment approaches manipulating ECS. Therefore, the aim of
Aims of the study

this part of the study was to investigate the expression of CB1R in hippocampus of epileptic dogs and quantitatively compare it to control animals.
4. General introduction and literature overview

4.1. Epilepsy in dogs

4.1.1. Background

Epilepsy is the most common chronic neurological disorder in dogs, with an estimated prevalence 0.6–0.75 % in the general population (Heske et al. 2014); Kearsley-Fleet et al. (2013). It is characterized by abnormal neuronal activity and hyperexcitability manifesting in seizures (Fisher et al. 2005). Epilepsy is defined as a disease of the brain characterized by an enduring predisposition to generate two or more unprovoked epileptic seizures at least 24 h apart (Fisher et al. 2014).

The disease has multiple etiology. Its cause can be genetic and in other cases seizures are the result of an insult to the brain parenchyma, such as traumatic brain injury (TBI), inflammation, neoplasm, infection or vascular disorder (De Risio and Platt 2014). Genetic background of epilepsy in specific breeds has been discovered, with specific prevalence in Labrador retriever (3.1 %), Belgian shepherd (9.4 %) and petit Basset Griffon de Vendeen, but is also observed in breeds such as Vizsla, Bernese mountain dog, Standard poodle, Belgian shepherd, Border collie, Australian shepherd and many more (Hulsmeyer et al. 2015).

Treatment of epilepsy is focused on reducing seizure frequency and improving quality of life (Bhatti et al. 2015). Drugs that target the underlying cause of seizures are still in research phase. The multiple etiology of epilepsy presents the challenge in treatment development (Podell et al. 2016). Various antiepileptic drugs (AED) are used in dogs and may have an impact on the quality of life, with treatment interventions posing a fine balance of potential benefits and harms to the patient (Packer and Volk 2015).
4.1.2. Terminology and classification

The International Veterinary Epilepsy Task Force (IVETF) published collaborative consensus statement on definition and classification of epilepsy (Berendt et al. 2015). *Epilepsy* is defined as a disease of the brain characterized by an enduring predisposition to generate epileptic seizures (Bateman and Parent 1999).

*Epileptic seizure* is manifestation of excessive synchronous, usually self-limiting epileptic activity of neurons in the brain which may be characterized by short episodes with convulsions or focal motor, autonomic or behavioral features (LeCouteur and Child 1989).

*Reactive seizure* is a seizure occurring as a response from the normal brain after metabolic or toxic disturbance (De Risio 2014).

Based on etiology, epilepsy can be idiopathic, structural or of unknown origin.

*Idiopathic epilepsy* (IE) is classified in 3 groups: genetic epilepsy, suspected genetic epilepsy, epilepsy of unknown cause (Berendt et al. 2015)

*Structural epilepsy* (SE) is characterized by epileptic seizures which are provoked by cerebral pathology (vascular, inflammatory/infectious, traumatic, anomalous/developmental, neoplastic and degenerative) (Berendt et al. 2015).

*Epilepsy of unknown origin* – there is a suspected structural cause, which despite diagnostic attempts, remains obscure (Berendt et al. 2015)

**Seizure type classification:**

*Focal epileptic seizures* are characterized by lateralized (limited to one hemisphere) and/or regional signs (motor, autonomic or behavioral signs, alone or in combination) (Berendt et al. 2015). The abnormal electrical activity arises in a localized group of neurons or a network of neurons within one hemisphere. The clinical signs reflect the functions of the area or areas.
General introduction and literature overview

*Generalized epileptic seizures* are characterized by involvement of both cerebral hemispheres involving both sides of the body (Berendt et al. 2015). They may occur alone or evolve from a focal epileptic seizure (De Risio 2014). The seizures could present as tonic, clonic or tonic-clonic and myoclonic epileptic seizures. Convulsive seizures are additionally characterized by loss of consciousness, salivation, urination and/ or defecation (myoclonic seizures excluded). Non-convulsive generalized epileptic seizures are atonic, called ‘drop attacks’ with sudden and general loss of muscle tone (Berendt et al. 2015).

*Status epilepticus* can be defined as epileptic seizures that last longer than 5 minutes or there is incomplete recovery of consciousness between two or more seizures (Berendt et al. 2015).

*Cluster seizures* can be defined as two or more seizures within a 24-h period (Berendt et al. 2015).

Seizure itself, whether focal or generalized is called *ictus* (Berendt et al. 2015). It is followed by the *postictal phase*, when brain restores normal function (Berendt et al. 2015). It could last minutes, or days and the animal’s behavior maybe abnormal (De Risio 2014). Blindness or aggression can develop postictally.

4.1.3. Diagnosis

Diagnosis of epilepsy in dogs is a complex procedure. Firstly, it should be established from the patient’s history and complete clinical and neurological examination whether the observed seizures are in fact epileptic (Thomas 2010). The next step is to determine, if the seizures are caused by an intra- or extracranial (reactive seizures) disorder (De Risio 2014). Neurological examination can reveal problems consistent with focal, diffuse, bilateral and often symmetric forebrain involvement. Physical
examination and further special diagnostic methods can detect exogenous toxic, metabolic and structural forebrain disorders (Podell 1996).

**Idiopathic epilepsy**: no structural cause is detectable (Patterson 2014).

Diagnostics of IE are based on exclusion of all the differential diagnoses (Monteiro et al. 2012). The exclusion is made based on the age at epileptic seizure onset, unremarkable interictal physical and neurological examinations, and exclusion of metabolic, toxic and structural cerebral disorders (De Risio 2014).

In 2015 IVETF suggested certain levels of confidence for the implementation of the diagnostic procedures for IE (De Risio et al. 2015):

- **Tier I confidence level** - A history of two or more unprovoked epileptic seizures occurring at least 24 h apart, age at epileptic seizure onset between 6 months and 6 years, unremarkable interictal physical and neurological examination (except for antiepileptic drug (AED) induced neurologic abnormalities and post-ictal neurologic deficits), and no clinically significant abnormalities on blood tests and urinalysis (De Risio et al. 2015)

- **Tier II confidence level** - Unremarkable fasting and post-prandial bile acids, magnetic resonance imaging (MRI) of the brain and cerebrospinal fluid (CSF) analysis in addition to previous diagnostics listed in tier I (De Risio et al. 2015)

- **Tier III confidence level for the diagnosis of IE** - Identification of ictal or interictal EEG abnormalities characteristic for seizure disorders according to criteria validated in human medicine, in addition to factors listed in tier I and II (De Risio et al. 2015)

**Structural epilepsy** is presumed, when the seizures have a known cause, an identifiable structural change in the brain (De Risio and Platt 2014). Interictal neurological examination is often abnormal and may reveal asymmetric neurological deficits in dogs with lateralized brain pathology (De Risio et al. 2015). In combination
with age of the seizure onset (< 6 months and > 6 years old), abnormal pathohistological, MRI and CSF findings as well as type of seizures (usually rather focal than generalized) diagnosis of structural epilepsy can be set with high certainty (Pakozdy et al. 2008).

Reactive seizures can originate from systemic metabolic disorders (e.g., hypoglycemia, electrolyte disorders, portosystemic shunt resulting in hepatic encephalopathy) or from intoxications (e.g., carbamates, organophosphates, lead poisoning, ethylene glycol toxicity, strychnine) (De Risio et al. 2015). The history and clinical presentation may help with diagnosis; however, clinical presentations are as variable as intoxications (De Risio 2014). Toxic disorders are often acute, and sometimes accompanied with muscle tremors and fasciculations as initial clinical signs (Podell 1996). Metabolic disorders can present with an acute, subacute, or chronic onset (Brauer et al. 2011). Both disorders can be detected in physical examination, and neurological examination usually points to diffuse, bilateral and symmetrical forebrain involvement.

4.1.4. Treatment and outcome

Treatment of epilepsy in dogs aims at suppression of seizures and is frequently not influencing the epileptogenesis or pathophysiological mechanisms of epilepsy (Bhatti et al. 2015). Antiepileptic drugs (AED) are used for seizure management in idiopathic epilepsy and as add on to treating underlying disease in structural epilepsy (Podell et al. 2016). Despite the goal of the treatment to eradicate seizures, that is rarely achieved. It is more likely to decrease frequency, duration and severity of seizures. However, there is a need for balancing the treatment goals with adverse effects of AED
and animal’s quality of life. The treatment plan should include the following (Bhatti et al. 2015):

- Decision on start of AED treatment: interictal period of ≤ 6 months, status epilepticus or cluster seizures, severe postictal signs, increased seizure frequency and duration
- Selection of the most appropriate AED and dosage, usually differs from case to case
- Monitoring serum AED concentrations and treatment adjustment accordingly
- Change or addition of different AED, when necessary

Selection and monitoring of the best-known AED in veterinary medicine is suggested as follows (Podell et al. 2016):

**Phenobarbital** has together with potassium bromide the longest history of use in veterinary medicine (Ravis et al. 1989). It should be monitored in serum after two and six weeks from the beginning of treatment and afterwards every six months or two weeks after dosage change; adequate range in serum is 15–35 ml µg/mL. Adverse effects are hepatotoxicity, idiosyncratic blood dyscrasia, necrolytic dermatitis. Initial dosage of phenobarbital is 2.5 mg/kg two times a day (Ravis et al. 1989). It is used as monotherapy and add-on treatment for all types of seizures and etiology.

**Potassium Bromide** is best known for its use as add-on therapy with phenobarbital but can also be prescribed as monotherapy in cases with liver disease. Controls of serum levels should be done three months after starting application, and then every 6 months or one month after the next change of dosage. Expected range of serum levels is 1000–3000 µg/mL, for monotherapy or 800–2500 µg/mL as add on with phenobarbital. Cautions and risks to anticipate with potassium bromide use are pancreatitis, sedation, ataxia. Recommended dosage is 40 mg/kg/day, as add on 30 mg/kg/day.
Imepitoin is a relatively new drug, available since 2013, approved for veterinary medicine use in Europe and has equivalent effect as phenobarbital. Recommended dosage is 10-30 mg/kg twice a day. Therapeutic drug monitoring is not needed, and adverse effects are rare (Tipold et al. 2015)

The most frequently used AED from human medicine are Levetiracetam, Gabapentin, Pregabalin and Zonisamide.

Seizure freedom is, of course, most favorable and therefore the primary outcome measurement of epilepsy treatment (fig.1) (Potschka et al. 2015a). However, it is not frequently achieved. A positive outcome is considered in response to AED treatment, when no seizures are observed “during a phase of at least three times the duration of their longest pre-intervention inter-seizure interval in the preceding 12 months or during 12 months, whichever is longer”( fig.1) (Kwan et al. 2010).

More realistic expectations, when starting with AED treatment in dogs, is achievement of a partial success, such as the prevention of cluster seizures or status epilepticus, reduction of seizure frequency and reduction in seizure severity. This is considered the secondary goal (fig. 1) in AED treatment and a certain success, since severe and prolonged seizures can lead to changes in brain parenchyma (Wilcox and Vezzani 2014) resulting in behavioral changes, drug resistance, reduced quality of life, as well as sudden unexpected death in epilepsy (SUDEP) (Ryvlin et al. 2013; Shankar et al. 2013). If the secondary goal is not achieved, the next therapeutic “trial” respectively drug is applied. Drug-resistant epilepsy is diagnosed, when seizure freedom or clinically relevant seizure reduction is not achieved with two therapeutic trials (Potschka et al. 2015a). However, there are cases with presumed drug resistant epilepsy responding to other treatment attempts (Callaghan et al. 2011). Therefore, the term “resistant to the drug used in a specific treatment trial” may be used (Potschka et al. 2015a).
Seizure free
> 3 times longest pre-treatment interictal interval and at least 3 months

Primary goal: seizure freedom

Continue and evaluate long-term success

Partial therapeutic success?
- prevention of cluster seizures or status epilepticus
- relevant reduction of seizure frequency considering pre-treatment seizure frequency
- reduction in seizure severity

Secondary goal

Continue and evaluate long-term success

Alternate AED schedule

Figure 1. Categorization of seizure control after treatment with antiepileptic drugs (AED).

Seizure freedom is the primary treatment goal, still the category of partial therapeutic success takes into account that the prevention of cluster seizures or status epilepticus, reduction in seizure frequency and severity can be of significant clinical relevance in veterinary patients.
4.1.5. Epilepsy biomarkers

There is a constantly growing need for biomarkers in epilepsy, searched by epidemiologists, physicians, and scientists. This is due to the above-mentioned lack of treatment of the pathophysiology of epilepsy, since mechanisms underlying the disease are not fully elucidated (Bhatti et al. 2015). “Biomarker is any substance, structure or process that can be measured in the body or its products, and influence or predict the incidence or outcome of disease” (WHO 2001). Development of biomarkers is a process of their analytical, clinical validation, and the demonstration of clinical utility (Frisoni et al. 2017).

Mechanisms underlying epilepsy can best be revealed studying the epileptogenesis (Pitkanen et al. 2015). Epileptogenesis refers to the development and extension of tissue capable of generating spontaneous seizures (Pitkänen and Engel 2014). It is a process of epilepsy development after an initial insult or a change is inflicted on brain parenchyma (Chandel et al. 2016). In over half cases of epilepsy, epileptogenesis is initiated by structural causes such as traumatic brain injury (TBI) or stroke (Scheffer et al. 2017).

Most research on biomarkers of epilepsy is focused on diagnostic, prognostic and predictive biomarkers (Engel et al. 2013). Challenges in the discovery of this type of biomarkers in epilepsy are recognized and suggested for further research (Pitkanen et al. 2018).

*Diagnostic biomarkers* are “used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease” (Biomarker 2016). This field of research is mainly focused on epileptogenesis. Difficult points in discovery of these biomarkers are the confirmation of epilepsy diagnosis after the 1st
seizure, status epilepticus (type, refractoriness, risk of mortality), epileptogenicity of the focal area (Pitkanen et al. 2018).

**Prognostic biomarkers** are used to “identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest” (Biomarker 2016). There is a need for prognostic biomarkers for epilepsy development after a given brain injury type or that identify the development of cognitive dysfunction in epilepsy, co-morbidogenesis, response to epilepsy surgery, cure, sudden unexpected death (SUDEP) (Pitkanen et al. 2018).

**Predictive biomarker** is “a biomarker used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent” (Biomarker 2016). Weak point in this part of biomarker search in epilepsy is the individual drug-responsiveness, drug-refractoriness and response to monotherapy (Pitkanen et al. 2018).

Search for biomarkers in epilepsy, usually leads two overlapping of biomarkers characteristics, for instance diagnostic and prognostic (Pitkanen et al. 2018). The biomarker can be plasma/serum/exosomal and cerebrospinal fluid molecular biomarkers, brain tissue molecular biomarkers, imaging biomarkers, electrophysiologic biomarkers, and behavioral/cognitive biomarkers.

In our study, we focused on diagnostic-prognostic biomarkers in epilepsy, looking into serum, CSF and brain tissue.

### 4.2. Epilepsy, astrocytes and glial fibrillary acidic protein

**Astrocytes** are star-shaped glial cells, second most abundant in CNS of vertebrates (von Bartheld et al. 2016). Morphologically, several types are distinguishable (Matyash and Kettenmann 2010).
Protoplasmic astrocytes are the most prevalent type of astrocytes and extend thick, short branches originating from the soma which branch out into secondary fine processes ending with characteristic end-feet (Sofroniew and Vinters 2010). Astrocytic end-feet encase blood vessels, but are also capable of ensheathing several thousand synapses (Khakh and Sofroniew 2015). Perivascular end-feet are involved in “glymphatic” drainage (Iliff et al. 2012) providing para-arterial influx of nutrients to neurons and para-venous clearance of toxic metabolites from the CNS (Khakh and Sofroniew 2015).

Fibrous astrocytes have longer, thinner branches and their end-feet envelop nodes of Ranvier and contact blood vessels (Khakh and Sofroniew 2015). They are involved in repair of damaged tissue especially in spinal cord, a process resulting in scar formation (Sofroniew and Vinters 2010).

Radial astrocytes are specialized astrocytes that are the first cells to develop during early embryogenesis (Khakh and Sofroniew 2015). After maturation, they transform into stellate astrocytes, although radial glial cells remain in the retina (Müller glia) and cerebellum (Bergmann glia).

Among many roles astrocytes play in CNS, most important ones are support and nutrition of neurons (Kacem et al. 1998), regulation of synaptic activity (Angulo et al. 2004; Fellin et al. 2004) and covering and supporting blood brain barrier (BBB) (Abbott 2002; Min and van der Knaap 2018; Tao-Cheng et al. 1987). They envelop almost all synapses and are conveniently involved in maintaining the fluid, ion, pH, and transmitter homeostasis of the synaptic interstitial fluid (Brown and Ransom 2007). Through they connection with blood vessels, they are involved in regulation of local CNS blood flow (Gordon et al. 2007). In addition to having direct effects on synaptic activity via the release of gliotransmitters, astrocytes have the potential to exert
powerful and long-term influences on synaptic function through the release of growth factors and related molecules (Brown and Ransom 2007).

During neuronal injury or disease, astrocytes undergo changes known as reactive astrogliosis (Yu et al. 1993). Considering complexity of the reactive astrogliosis features, there have been various definitions and descriptions of the process (Sofroniew and Vinters 2010). Sofroniew’s group suggest that reactive astrogliosis is every response of astrocytes to injury of the CNS that varies with nature and severity of the disease; the altered activity of astrocytes could be beneficial or harmful for surrounding tissue (Sofroniew and Vinters 2010) and is mirrored in hypertrophy and proliferation of astrocytes, leading to increase of astrocytic density (Liddelow and Barres 2017).

As mentioned, astrocytes are involved in synaptic function, actively controlling synaptic transmission. They do not propagate an action potential but undergo changes in intracellular calcium concentration which may be important in astrocyte-neuron and astrocyte-astrocyte communication (Moftakhar et al. 2010; Shi et al. 2017). This close structural and functional partnership of the perisynaptic astrocytic process with the neuronal pre- and postsynaptic structures is called “tripartite synapse” (Araque et al. 1999). This close connection to synapses allows conclusion that impaired astrocytic function and inability to maintain homeostasis play key roles in the pathogenesis of epilepsy (Coulter and Steinhauser 2015). For instance, increased levels of glycogen have been found in the hippocampus of temporal lobe epilepsy (TLE) patients (Dalsgaard et al. 2006). Since, astrocytes can store glycogen, they can potentially provide a significant supply of energy to sustain the high energy demands of epileptic neuronal networks (Coulter and Steinhauser 2015). Moreover, the uptake of glutamate is mainly mediated by transporters localized on astrocytic membranes, and altered activity of the astrocytic transporters, seems to be a common feature of epilepsy and
other brain disorders (Seifert et al. 2006). Excess of extracellular glutamate is present in human epileptic tissue and induces recurrent seizures and neuronal death (Glass and Dragunow 1995).

*Glial fibrillary acidic protein* (GFAP) is the main intermediate filament protein in astrocytes, commonly used as specific marker for mature astrocytes (Bignami et al. 1972) and plays a significant role in astrocytic function and morphology (Middeldorp and Hol 2011). In human medicine, GFAP is developed as biomarker, e.g. increased GFAP concentrations occur in cerebrospinal fluid (CSF) in patients with both acute (Yang and Wang 2015) and chronic (Ishiki et al. 2016) forms of brain injury. It has been suggested that rapid astroglial destruction as found in acute intracerebral hemorrhage is mandatory for increased GFAP levels in blood (Mayer et al. 2013). Furthermore, GFAP blood levels were shown to correlate with severity and outcome after traumatic brain injury (TBI) (Lei et al. 2015; Nylén et al. 2006).

Because of its role in astrocytic morphology and function, GFAP is subject of many ongoing research for biomarkers. In epileptic patients GFAP has been reported that repeated seizures lead to increase of GFAP expression in hippocampus. In epilepsy-associated lesions GFAP expression was relatively high compared to control brains (Stringer 1996). There was also upregulation of GFAP expression in different brain areas in animal models for epilepsy (Gramsbergen and van den Berg 1994; Steward et al. 1992). Moreover, elevation of GFAP in CSF was measured in pediatric seizure patients (Gurnett et al. 2003).

In the first part of our study, GFAP was evaluated as potential biomarker in CSF and serum in different CNS diseases and epilepsy. In the last part of this thesis, astrocytes were evaluated for their expression of cannabinoid receptor type-1 in canine epilepsy.
4.3. Epilepsy, neuroinflammation and interleukin-1 beta

Cytokines are important signaling molecules (Turner et al. 2014) involved in immunity, inflammation and hematopoiesis, but also in functional alteration of cells in the central nervous system (CNS) (Hofer and Campbell 2016). Cytokines are well known to strongly influence signaling processes in CNS during injury, inflammation or disease (Lucas et al. 2006; Rodriguez-Smith et al. 2017). They can be pro- or anti-inflammatory (Dinarello 2007).

Interleukin-1 beta (IL-1β) belongs to IL-1 family of pro-inflammatory cytokines and plays an essential role in injury and inflammation (Dinarello 1996). In CNS it is mainly produced by activated microglia (Yao et al. 1992), but also neurons (Watt and Hobbs 2000), astrocytes (Zhang et al. 2000) and oligodendrocytes (Blasi et al. 1999). In the healthy brain, IL-1β levels are low, but detectable (Vitkovic et al. 2000) suggesting a certain function in CNS physiology such as sleep (Jewett and Krueger 2012), learning and memory (Marin and Kipnis 2013), as well as neuromodulation on different levels of cell communication in CNS (Vezzani and Viviani 2015). In CNS diseases, involvement of IL-1β is described in neurodegeneration (Hesse et al. 2016), depression (Farooq et al. 2017), neuro-trauma (Allan et al. 2005) and epilepsy (Vezzani et al. 2011). In chronic and acute inflammatory processes in the CNS, it plays both, a beneficial and a harmful role (Hewett et al. 2012) and therefore could represent a target for drug development (Luheshi et al. 2009).

There is a new concept of inflammation, that occurs as interaction of innate immune system in brain and injured tissue and is called neuroinflammation (Graeber et al. 2011). Neuroinflammation is also present in epilepsy as consequence of neuronal activity (Xanthos and Sandkuhler 2014). It is thought to be a compensatory mechanism for maintaining the homeostasis during seizures. However, it can become maladaptive, when it is not properly resolved or when it spreads to remote sites, thus contributing to
the pathogenesis of the disease (van Vliet et al. 2018). In animal models of epilepsy mediators of inflammations, such as cytokines, can also act as neuromodulators by affecting neuronal excitability (Rijkers et al. 2009).

The potential IL-1β involvement in inflammatory reactions in epilepsy has attracted considerable attention and despite equivocal reports on its implication in seizures (Rijkers et al. 2009), it presents a possibility for characterizing new treatment options (Dey et al. 2016). For instance, in hippocampus of rats after status epilepticus, there was rapid and significant increase of IL-1β expression (De Simoni et al. 2000). IL-1β expression in tumoral and peritumoral brain tissue was positively associated with the preoperative seizure frequency and epilepsy duration (Prabowo et al. 2013). In focal cortical dysplasia, IL-1β correlated with seizures frequency and the expression of IL-1β was higher in epileptic tissue (Ravizza et al. 2006). IL-1β is strongly expressed on activated microglia and astrocytes during the acute phase of epilepsy and in the chronic phase of spontaneous seizures in brain areas involved in seizure generation and propagation in laboratory animals (Ravizza et al. 2008). There is no agreement in reports about biomarker features of IL-1β level in serum of epilepsy patients, probably due to instability and very short half-life of this cytokine (Aronica and Crino 2011).

Our study evaluated levels of IL-1β in serum and CSF of dogs with structural and idiopathic epilepsy to confirm the role of IL-1β in neuroinflammation in canine epilepsy.

4.4. Epilepsy and cannabinoid receptor type-1

The knowledge of potential medicinal use of the hemp plant Cannabis sativa dates as far back as 5000 years (Blair et al. 2015b). However, in-depth knowledge of the structure of its derivates and their exact effect on human physiology has been elucidated in the last 60 years (Blair et al. 2015a). The two most abundant derivatives of the plant, phytocannabinoids, are psychoactive Δ9- tetrahydrocannabinol (THC)
General introduction and literature overview

(Gaoni and Mechoulam 1971) and non-psychoactive cannabidiol (CBD) (Mechoulam and Shvo 1963). Cannabinoids act on receptors in organism known as endocannabinoid receptors. These receptors are part of the endocannabinoid system (ECS), which also contains their lipid ligands or endocannabinoids, and enzymes involved in their metabolism (Di Marzo et al. 1998; Mechoulam et al. 1998). Best clarified are two G protein-coupled receptors: cannabinoid receptor type-1 (CB1R) (Matsuda et al. 1990) expressed mainly in CNS and cannabinoid receptor type-2 (CB2R) (Devane et al. 1988), present primarily in the peripheral tissue. Most thoroughly investigated endocannabinoids (De Petrocellis and Di Marzo 2009) that interact with these receptors are anandamide (Devane et al. 1992) and 2-arachidonoylglycerol (2AG) (Mechoulam et al. 1995).

Interestingly, endogenous cannabinoids are synthesized “on demand” by the enzymes N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipases (DGL-α and DGL-β), respectively (Bisogno et al. 2003) to control neuronal depolarization and elevated intracellular Ca^{2+} levels (Kondo et al. 1998). Endocannabinoids cross the synapse in a retrograde manner to act on presynaptic CB1 receptors followed by reuptake (Di Marzo and Petrosino 2007) and enzyme degradation by fatty acid amide hydrolase (FAAH) (Deutsch et al. 2002) and monoacylglycerol lipase (Dinh et al. 2002), respectively. Activation of presynaptic CB1R results in activation of a few effector systems that include inhibition of adenylate cyclase-dependent cAMP accumulation and protein kinase A (PKA) activation, inhibition of voltage-gated Ca^{2+} channels, activation of G protein-coupled inwardly-rectifying K^{+} (GIRK) channels, and downstream activation of the mitogen-activated protein (MAP) kinase pathway (Howlett et al. 2004). Because of these responses after activation of CB1R, ECS is thought to be involved in fine-tuning of synaptic transmission via regulation of presynaptic neurotransmitter release mechanisms.
(Capasso 2017). After presynaptic release of neurotransmitters and postsynaptic membrane depolarization, endocannabinoids are synthesized, travel back over the synapse to activate presynaptic CB1R, and inhibit further release of neurotransmitters, a process that has been termed either depolarization-induced suppression of inhibition (DSI) or depolarization-induced suppression of excitation (DSE) when occurring at inhibitory or excitatory synapses, respectively (Castillo et al. 2012; Kano et al. 2009). This mechanism has revealed potential therapeutic targets for the control of neuronal excitability (Mechoulam and Parker 2013). In epilepsy, studies suggest that regulation of CB1 receptors expression might be a step toward new therapeutic approaches to reduce seizure frequency (Capasso 2017; Prandi et al. 2018). Wallace and colleagues report that ECS modulates seizure termination and duration through CB1 receptor activation (Wallace et al. 2003). In a study of several models of neuronal damage, CB1 receptor relates to protection against epilepsy through mechanisms that include CB1-mediated inhibition of glutamatergic transmission, inhibition of harmful cascade signals, and reduction of Ca\textsuperscript{2+} influx (van der Stelt et al. 2002). In animal models of seizures, functional and anatomical evidence was found that on hippocampal glutamatergic neurons CB1R are crucially involved in protection against acute excitotoxic events (Monory et al. 2006). Falenski et al. demonstrated a selective reorganization of hippocampal CB1R expression (Falenski et al. 2007). In TLE model of epilepsy, increased CB1R within the dentate gyrus stratum moleculare including a marked increase in the inner third molecular layer (IML) and preservation of interneuronal staining in the Cornu Ammonis 1 (CA1) and dentate gyrus regions was reported (Magloczky et al. 2010). CB1R-positive terminals innervating CA1 and the IML of the dentate gyrus were markedly reduced while increased innervations were observed on glutamatergic spines throughout the strata radiatum and oriens (Wyeth et al. 2010).
General introduction and literature overview

Strikingly, in canine epilepsy, Gesell et al. described elevation of endocannabinoids in cerebrospinal fluid awaking interest in the expression of CB1R in brain of dogs with this disease (Gesell et al. 2013).
5. Manuscript I: Evaluation of GFAP as a neurobiomarker in serum and CSF in dogs with CNS diseases

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Contribution of Draginja Kostic to this publication: 80%
D. Kostic performed the experiments, analyzed the data and wrote the manuscript. S. Weiss and J. Siedenburg helped performing the experiments and analyzing the data. Statistical analysis was done by K. Rohn. R. Carlson was involved in the concept and design of the study and helped performing the experiments. D. Henke was involved in the clinical evaluation of patients and collection of samples and critically revised the manuscript. A. Tipold was involved in the concept and design of the study, the coordination of experiments, analysis of the data, and critically revised the manuscript.

Submitted to Scientific Reports
5.1. Abstract

The value of glial fibrillary acidic protein (GFAP) as a neurobiomarker in serum and cerebrospinal fluid (CSF) in various canine CNS diseases was assessed. Hypotheses: (1) severe CNS tissue destruction leads to measurable GFAP serum levels independent of the cause of the disease; (2) GFAP in serum correlates with the outcome in traumatic brain injury (TBI); and (3) GFAP CSF levels reflect the role of astrocytes in the disease category.

Six controls and 212 dogs with idiopathic or structural epilepsy, spinal cord injury (SCI), or TBI were included. GFAP concentrations were determined by enzyme-linked immunosorbent assay (ELISA).

All CSF samples were positive for GFAP. Epileptic patients had higher CSF GFAP levels from those of healthy dogs (p<0.0001). There was also a significant difference between dogs with structural (brain inflammation and tumor) and idiopathic epilepsy (p = 0.0006, p = 0.0250, respectively). In dogs with SCI, CSF GFAP was higher than in healthy controls. No significant differences between chronic and acute cases and between different neurological grades were detected. In dogs with TBI, a correlation (r_s = -0.75) between serum GFAP and modified Glasgow Coma Scale (MGCS) score was found.

GFAP in CSF is not disease specific but could reflect severe structural changes in the parenchyma. Interestingly, GFAP serum levels in dogs with TBI were associated with the outcome and could be developed as a biomarker in TBI.
5.2. Introduction

There is a constantly growing need for biomarkers, which are sought by epidemiologists, physicians, and scientists. In neuroscience, biomarkers are especially needed for the specific microenvironment of the central nervous system (CNS), as such data are rarely obtainable in clinical settings (Garden and Campbell 2016). Very few biomarkers for CNS diseases in dogs are validated (Marrer and Dieterle 2007). A biomarker is “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (WHO 2001). Therefore, the ideal biomarker should have prognostic, pathogenic, and/or therapeutic value.

Astrocytes are mostly star-shaped glial cells and are the second numerous non-neuronal cells in the CNS (von Bartheld et al. 2016). Knowledge about their complex and multifaceted tasks in the CNS has rapidly expanded in the last years (Fiacco et al. 2009). Their most valuable roles in the CNS are the support and nutrition of neurons (Kacem et al. 1998), regulation of synaptic activity (Angulo et al. 2004; Fellin et al. 2004), and support of the blood-brain barrier (BBB) (Abbott 2002; Kacem et al. 1998; Min and van der Knaap 2018; Tao-Cheng et al. 1987). During neuronal injury or disease, astrocytes undergo changes known as reactive astrogliosis (Eddleston and Mucke 1993; Eng et al. 2000; Yu et al. 1993). Considering the complexity of the features of reactive astrogliosis, there have been various definitions and descriptions of the process (Sofroniew 2009). Sofroniew’s group suggests that reactive astrogliosis is every response of astrocytes to injury of the CNS, and that it varies with the nature and severity of the disease; the altered activity of astrocytes could be beneficial or harmful for surrounding tissue (Sofroniew 2009). The important role of reactive astrocytes in disease has been extensively described in neuroinflammation (Sofroniew
2009), neurodegeneration (Liddelow et al. 2017), tumors of the CNS (O'Brien et al. 2013), traumatic injuries (Chen and Swanson 2003), and epilepsy (Robel et al. 2015). Interestingly, Robel et al. proved that in epilepsy, reactive astrogliosis causes the development of spontaneous seizures. Also, in tumor-related seizures, dysfunctional astrocytes are considered to be involved in seizure development (You et al. 2012). In spinal cord injury (SCI), astrocytes are considered to have detrimental effects on tissue recovery, forming a glial scar on the injury site and, therefore, obstructing axonal growth (Lukovic et al. 2015; Rudge and Silver 1990). To evaluate changes in astrocytic function, glial fibrillary acidic protein (GFAP) was evaluated in a significant number of investigations (Eng et al. 2000; Hol and Pekny 2015a).

GFAP is the main intermediate filament protein in astrocytes, commonly used as specific marker for mature astrocytes (Bignami et al. 1972; Kimelberg 2004), and it plays a significant role in astrocytic function and morphology (Middeldorp and Hol 2011). In human medicine, GFAP has been developed as a biomarker [e.g., increased GFAP concentrations occur in the cerebrospinal fluid (CSF) of patients with both acute (Hol and Pekny 2015b; Yang and Wang 2015) and chronic (Ishiki et al. 2016) forms of brain injury]. It has been suggested that rapid astroglial destruction as found in acute intracerebral hemorrhage is mandatory for increased GFAP levels in blood (Mayer et al. 2013). Furthermore, GFAP blood levels were shown to correlate with the severity and outcome after traumatic brain injury (TBI) (Lei et al. 2015; Nylén et al. 2006). In epileptic patients, it has been reported that repeated seizures lead to an increase of GFAP expression in the hippocampus (Stringer 1996). Certain studies of acute SCI in human medicine state that serum GFAP levels were elevated for the first 72 hours and were predictive of the severity of the injury (Ahadi et al. 2015), as were CSF GFAP concentrations (Kwon et al. 2011). However, little is known about its neurobiomarker potential in veterinary medicine. Serum GFAP is described as a potential diagnostic
biomarker for progressive myelomalacia in dogs (Sato et al. 2013) and necrotizing meningoencephalitis (NME) in pug dogs (Miyake et al. 2013).

In the current study, the value of GFAP as a prognostic/diagnostic biomarker in various canine CNS diseases is assessed. Our hypotheses were as follows: (1) severe CNS tissue destruction leads to measurable GFAP serum levels independent of the cause of the disease; (2) GFAP in serum correlates with the outcome in TBI; (3) GFAP CSF levels reflect the role of astrocytes in seizure causation as well as disease category.

5.3. Materials and Methods

Study design and animals

In the current study, samples collected from dogs treated at the Department of Small Animal Medicine and Surgery of the University of Veterinary Medicine, Hannover, between 2009 and 2016, were evaluated. In addition, four patients were examined at the Department of Small Animal Medicine at the Vetsuisse Faculty, University of Bern, Switzerland. Patients were included in the study if they had presumed or confirmed idiopathic or structural epilepsy, TBI, or SCI.

The owners’ permission was granted for sampling and evaluation of the CSF and serum for all dogs. All procedures in healthy and diseased dogs were performed in accordance with the ethical guidelines of the University of Veterinary Medicine, Hannover, and for healthy dogs approved by the authority of Lower Saxony (Animal experiment number 33.42502/05-12.05 and 33.9-42502-05-14A453).

All animals included in the study (212 diseased and 6 healthy dogs) underwent clinical and neurological examinations, complete blood cell count, blood chemistry analysis,
CSF analysis, and magnetic resonance imaging (MRI), as well as other specific diagnostics necessary for the definitive or highly probable diagnosis (ultrasonography, radiography, computed tomography, histopathology).

Patients were classified into six groups according to the presumed or confirmed clinical diagnosis (Table 1).

Table 1: Number of patients grouped according to the presumed or confirmed clinical diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic epilepsy</td>
<td>97</td>
</tr>
<tr>
<td>Brain tumor (SE)</td>
<td>28</td>
</tr>
<tr>
<td>Brain inflammation (SE)</td>
<td>32</td>
</tr>
<tr>
<td>Traumatic brain injury</td>
<td>15</td>
</tr>
<tr>
<td>Spinal cord injury</td>
<td>40</td>
</tr>
<tr>
<td>Healthy</td>
<td>6</td>
</tr>
</tbody>
</table>

SE-structural epilepsy

Dogs were diagnosed with idiopathic epilepsy, tier 2 level of confidence (De Risio et al. 2015) on the following basis: they previously had two or more unprovoked epileptic seizures; age at onset of the disease was between 6 months and 6 years of age; interictal general and neurological examination was unremarkable; and the results of blood tests, urine analysis, ultrasound and radiographic examinations, as well as MRI and CSF analysis, were normal.

Patients with structural epilepsy were divided in two subgroups according to disease category (a tumor group and an inflammation group).
Brain tumor patients with a presumed diagnosis had clinical, neurological, blood, MRI, and CSF findings consistent with neoplasia of the CNS (Schwartz et al. 2011). Confirmed diagnosis was established in three patients by histopathological examination.

The inflammation group consisted of dogs with meningoencephalitis of unknown origin (MUO) with clinical, neurological, blood, MRI, and CSF findings corresponding to inflammatory changes in brain tissue (Coates and Jeffery 2014); one patient in this group had bacterial meningoencephalitis.

The diagnosis of traumatic brain injury (TBI) was suggestive from the patient’s history of recent head trauma, as well as clinical and neurological examination and imaging findings (Sande and West 2010b). Each TBI patient was graded using the modified Glasgow Coma Scale (MGCS) (Platt et al. 2001). The MGCS ranges from 3, which is assigned to the most severe cases, to 18, for the least severe cases. In these patients, follow-up data were collected to evaluate the outcome.

The patients with SCI were diagnosed using clinical, neurological, MRI, CSF, and radiological examinations (Sharp and Wheeler 2005). On the basis of the duration of clinical signs before presentation at the clinic, dogs with SCI were graded by acute cases (n = 29; mean number of days of clinical signs before presentation = 2.9) or chronic cases, if clinical signs lasted more than 21 days (n=11; mean number of months of clinical signs before presentation = 5.8 months). Regarding the severity of neurological signs, 40 dogs were categorized according to the Sharp and Wheeler (2005) classification system into five grades (Table 2).

Table 2: Classification of dogs with spinal cord injury (SCI) according to neurological signs
<table>
<thead>
<tr>
<th>Grade</th>
<th>Number of dogs</th>
<th>Neurological signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>pain only</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>ambulatory paraparesis</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>Non-ambulatory paraparesis</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>paraplegia with deep pain perception</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>paraplegia without deep pain perception</td>
</tr>
</tbody>
</table>

Grading of dogs with SCI according to Sharp and Wheeler (2005)

The six healthy dogs used as control subjects were hospital-owned beagles, aged between 2 and 5 years. They were considered healthy after physical and neurological examination, as well as blood, MRI, and CSF examinations.

Sample collection and analysis

CSF was obtained under general anesthesia through cerebellomedullary cisternal puncture. Blood was collected from the cephalic or saphenous vein and centrifuged at 14,000 rpm for 2 minutes. The GFAP in serum and the CSF of each patient and each healthy dog were evaluated, except for the TBI animals. In TBI patients, only serum was tested because of the contraindication of performing a CSF tap in trauma-induced intracranial diseases (Sande and West 2010b). Serum was taken from TBI patients 3 to 48 hours after the injury. The CSF and serum samples were immediately aliquoted, frozen, and stored at -20°C until measurement.
A commercial enzyme-linked immunosorbent assay (ELISA) kit for the measurement of human GFAP (Bio Vendor - Laboratorní Medicína a.s., Brno, Czech Republic) and validated for measurements in dogs (Toda et al. 2007) was used in the current study. In this sandwich ELISA kit, the 96-well plates are pre-coated with anti-human GFAP. The assays were performed following the manufacturer's instructions. Briefly, after adding samples, incubation, and washing, biotin labelled monoclonal anti-GFAP antibody was added and incubated. In the next step streptavidin-horseradish peroxidase conjugate was added, and after the last washing step, the remaining conjugate reacted with substrate solution [tetramethylbenzidine (TBM)]. Sulfuric acid solution (98%) was added to stop the reaction, and the optical density (OD) of the final product was measured using a plate reader (Synergy 2 multi-mode reader BioTek, Vermont, USA). A standard curve was made by plotting OD values against concentrations of standards, which was then used to determine the concentrations of the samples.

Statistical analysis

Data analysis was performed with the statistics program package SAS®, version 9.4 (SAS Institute, Cary, NC, USA). GraphPad Prizm 6 (GraphPad Software, Inc., La Jolla, USA) was used for the graphical presentation of the results. The assumption of normal distribution of quantitative parameters was examined using the Kolmogorov-Smirnov test and visual assessment of QQ plots of model residuals. Right-skewed variables (lognormally distributed) were logarithmized before the evaluation, and the results were presented on a logarithmic scale. All analyses were considered statistically significant if p values were less than 0.05.
In patients with epilepsy and SCI, the effect of different diagnoses on GFAP in CSF was calculated by one-way ANOVA with post-hoc Tukey test for multiple pairwise comparisons, concerning the experiment-wise error rate.

A Wilcoxon two-sample test was used to compare GFAP in CSF between acute and chronic SCI patients, considering the data were not normally distributed.

Furthermore, the Pearson ($r$) and Spearman (for nonparametric data) rank correlation coefficients ($r_s$) were calculated between different variables in patients with intracranial diseases or SCI. The direction of the correlation was evaluated as positive or negative (depending on whether the other variable increases or decreases). The associations were considered statistically significant if $p$ values were less than 0.05.

In idiopathic epilepsy patients, the relationship between GFAP in CSF and time points of sample collections and the last seizure event, seizure frequency, and seizure type (generalized or focal, cluster or status epilepticus) was analyzed with linear regression analysis. The effect of seizure type on GFAP concentrations was analyzed using one-way ANOVA with post-hoc Ryan-Einot-Gabriel-Welsch multiple Range test for multiple pairwise comparison.

GFAP concentrations in the CSF of dogs with inflammatory brain disease were correlated with the level of leucocytes in CSF using the Spearman correlation test. Logistic regression was used for calculation the effect of GFAP concentration in CSF to both the probability of the occurrence of multifocal or focal lesions in brain parenchyma and to the presence of a mass effect by the presumed tumor mass or peritumoral edema on the surrounding tissue visible on MRI images.

GFAP levels in the serum of TBI patients were correlated with the MGCS score using the Spearman correlation coefficient.

In SCI, patient Pearson correlation was calculated between concentrations of GFAP in CSF and T2-weighted hyperintensity length ratio (T2WLR), the ratio between the
length of a visible T2W hyperintense MRI signal in the spinal cord and the second lumbar vertebrae length (T2WLR), as described previously (Siedenburg et al. 2018).

5.4. Results

GFAP concentrations were evaluated in 212 patients and 6 healthy dogs. In all CSF samples including the controls (n = 203), GFAP was measurable. In contrast, all serum samples (n = 218) were negative for GFAP, with the exception of eight dogs with TBI, three dogs with SCI, and three dogs with brain inflammation.

GFAP in epilepsy patients

Of 218 dogs evaluated in the study, 157 dogs had epilepsy, structural and idiopathic. GFAP was measured using the described ELISA in paired CSF and serum samples. Only three serum samples were GFAP positive, and those were from patients with inflammatory brain lesions. These three dogs were diagnosed with presumed MUO, and one of them with presumed NME.

In all CSF samples of dogs with epilepsy, GFAP levels could be detected. In healthy controls, the mean value of the CSF GFAP was 0.3 ng/mL (range 0.1–0.5 ng/mL). The mean value of GFAP concentration in the CSF of patients with idiopathic epilepsy was 1.8 ng/mL (range: 0.2–26 ng/mL). In dogs with presumed or confirmed brain tumor, the mean of GFAP concentrations in CSF was 2.9 ng/mL (range: 0.3–10.4 ng/mL). For the group of dogs with inflammatory brain disease, the mean value of GFAP in CSF was 4.6 ng/mL (range: 0.1–24.3 ng/mL).
The least square (LS) means of the logarithmized GFAP concentrations in CSF were calculated for each group of dogs with epilepsy; the differences were compared, and the p values were estimated and adjusted to Tukey–Kramer. As shown in Figure 1 and Table 3, all three groups of epileptic patients had LS means of GFAP in CSF significantly different from those of healthy dogs (p<0.0001). When the LS means of GFAP concentrations in CSF were compared between the three groups of epilepsy patients, there was a significant difference between dogs with brain inflammation and dogs with idiopathic epilepsy (p = 0.0006) and brain tumor patients and the idiopathic epilepsy group (p = 0.0250). However, with a p value of 0.8686, there was no significant difference between LS means of GFAP concentrations in the CSF of patients with brain tumor and inflammation.

Table 3. Differences of the least square means of cerebrospinal fluid (CSF) glial fibrillary acidic protein (GFAP) levels between dogs with brain tumor, brain inflammation, idiopathic epilepsy, and healthy dogs

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Diagnosis</th>
<th>Standard Error</th>
<th>Adj. p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Idiopathic epilepsy</td>
<td>0.3365</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Healthy</td>
<td>Brain inflammation</td>
<td>0.3555</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Healthy</td>
<td>Brain tumor</td>
<td>0.3595</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Idiopathic epilepsy</td>
<td>Brain inflammation</td>
<td>0.1635</td>
<td>0.0006*</td>
</tr>
<tr>
<td>Idiopathic epilepsy</td>
<td>Brain tumor</td>
<td>0.1720</td>
<td>0.0250*</td>
</tr>
<tr>
<td>Brain inflammation</td>
<td>Brain tumor</td>
<td>0.2068</td>
<td>0.8686</td>
</tr>
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</table>

Adj. p - adjusted p value to Tukey-Kramer; Asterisk (*) – statistically significant difference
Figure 1. Logarithmic levels of glial fibrillary acidic protein (LGFAP) in cerebrospinal fluid (CSF) in healthy dogs compared with dogs with idiopathic and structural (inflammation, tumor) epilepsy; differences of the least square means of GFAP concentrations in CSF between patients with brain tumor (tumor), inflammatory brain disease (inflammation), idiopathic epilepsy, and healthy dogs. Boxes contain values from the first to third quartile and represent the least square means of CSF GFAP concentration in ng/mL. Lines inside of the box indicate median values, and ◊ represents mean values. Lines outside of the box indicate maximum (above the box) and minimum (beneath the box) observation. Outliers are marked with ○. Asterisk (*) marks the group with statistically significant difference to healthy dogs, plus (+) to idiopathic epilepsy patients.

Within each group of epileptic patients, association was estimated between variables of possible significance to the disease and GFAP concentration in CSF using different statistical analyses (Table 4). In the group of idiopathic epilepsy patients, linear regression analysis between levels of GFAP in CSF and the time point between sample
collection and the last seizure event was statistically insignificant ($R^2 = 0.0008$, $p = 0.8893$). The GFAP levels could not predict seizure frequency ($R^2 = 0.0591$, $p = 0.1659$). Similarly, multiple pairwise ANOVA comparison of GFAP concentration in the CSF of dogs with different seizure types showed no statistical significance ($p = 0.5421$).

In the group of dogs with inflammatory brain disease, a positive correlation between CSF GFAP levels and number of leukocytes in CSF ($r_s = 0.18$, $p = 0.3399$) was detected. The probability of focal or multifocal lesion detection on brain MRI did not depend on GFAP levels in CSF ($R^2 = 0.145$, $p = 0.206$).

Different variables in brain tumor patients and CSF GFAP levels were compared. Logistic regression analysis of the probability that multifocal or focal lesions appear on brain MRI with change of the GFAP levels found $R^2 = -0.0292$ with $p = 0.9038$. The same analysis between GFAP levels and appearance of mass effect in the brain parenchyma on MRI showed $R^2 = 0.6609$, but it was not statistically significant ($p = 0.1451$). In addition, linear regression calculated for effect of the time point between sample collection and the appearance of first clinical signs on the GFAP CSF levels, showed no statistically significant values ($R^2 = 0.0418$, $p = 0.6601$). Seizure types did not significantly influence GFAP CSF levels ($p = 0.2602$). The level of protein in CSF was positively associated with CSF GFAP concentration, but not significant ($r_s = 0.27$, $p = 0.3207$) (Table 4).
Table 4: Association of glial fibrillary acidic protein (GFAP) levels in cerebrospinal fluid (CSF) of epilepsy patients with different diagnoses and variables

<table>
<thead>
<tr>
<th>Table</th>
<th>Idiopathic Epilepsy</th>
<th>p, r$_s$, and R$^2$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time between sample collection and last seizure event</td>
<td>R$^2$ = 0.0008, p = 0.8893</td>
</tr>
<tr>
<td></td>
<td>Seizure frequency</td>
<td>R$^2$ = 0.0591, p = 0.1659</td>
</tr>
<tr>
<td></td>
<td>Seizure type</td>
<td>p = 0.5421</td>
</tr>
<tr>
<td>Brain inflammation</td>
<td>Multifocal/focal</td>
<td>R$^2$ = 0.1450, p = 0.2060</td>
</tr>
<tr>
<td></td>
<td>Number of leukocytes in CSF</td>
<td>r$_s$ = 0.1800, p = 0.3399</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>Multifocal/focal</td>
<td>R$^2$ = -0.0292, p = 0.9038</td>
</tr>
<tr>
<td></td>
<td>Mass effect or no mass effect</td>
<td>R$^2$ = 0.6609, p = 0.1451</td>
</tr>
<tr>
<td></td>
<td>Time between sample collection and first clinical signs</td>
<td>R$^2$ = 0.0418, p = 0.6601</td>
</tr>
<tr>
<td></td>
<td>Seizure type</td>
<td>p = 0.2602</td>
</tr>
<tr>
<td></td>
<td>Protein level in CSF</td>
<td>r$_s$ = 0.2700, p = 0.3207</td>
</tr>
</tbody>
</table>

R = Pearson correlation coefficient

r$_s$ = Spearman correlation coefficient

R$^2$ = Coefficient of determination

GFAP in TBI patients

In all TBI patients (n = 15), only serum samples were evaluated. We could detect GFAP in eight serum samples (Table 5). The mean concentration of GFAP in these serum samples was 5.9 ng/mL (range 1–26 ng/mL).

The Spearman correlation was evaluated between concentration of GFAP in serum of TBI patients and MGCS score (Fig 2), and a negative correlation (r = -0.75, p = 0.0011) was detected.
Table 5: Level of GFAP in serum and MGCS score of the patients with TBI

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP (ng/mL)</td>
<td>1.9</td>
<td>0</td>
<td>2</td>
<td>26.2</td>
<td>0</td>
<td>0</td>
<td>3.4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6.4</td>
<td>2.1</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td>MGCS score</td>
<td>12</td>
<td>17</td>
<td>13</td>
<td>3</td>
<td>18</td>
<td>8</td>
<td>9</td>
<td>15</td>
<td>14</td>
<td>18</td>
<td>13</td>
<td>9</td>
<td>10</td>
<td>13</td>
<td>15</td>
</tr>
</tbody>
</table>

GFAP-glial fibrillary acidic protein; TBI- traumatic brain injury; ID-identity; MGCS-modified Glasgow coma scale

Figure 2: Correlation between glial fibrillary acidic protein (GFAP) concentration in serum and modified Glasgow Coma Scale (MGCS) score; $r_s = \text{Spearman correlation coefficients}$.

GFAP in SCI patients
In 40 patients with SCI, GFAP concentrations were measured in paired CSF and serum samples. All serum samples were GFAP negative, except the serum of three patients (2 dogs with acute SCI and 1 dog with chronic SCI). One of the dogs with a positive serum GFAP value was classified with grade 5; the other two dogs were classified with grade 3.

In all 40 patients, GFAP was measurable in CSF samples. The mean concentrations of GFAP in SCI dogs with different neurological grades were as follows: grade 2: 4.4 ng/mL (range 1.1–12.6 ng/mL); grade 3: 1.3 ng/mL (range 0.6–2.4 ng/mL); grade 4: 6.5 ng/mL (range 0.3–26 ng/mL); grade 5: 6.1 ng/mL (range 0.2–26 ng/mL). To compare these groups of dogs with different neurological grades of SCI, the least square means of the logarithmized GFAP concentrations in CSF were calculated for each grade, and their differences were evaluated (Figure 3). There was a significant difference between the concentration of GFAP in CSF of healthy dogs and dogs with grade 2 (p = 0.0027), grade 4 (p = 0.0348), and grade 5 (p = 0.0070) SCI (Figure 3a).

The mean value of GFAP in CSF in the group of dogs with acute SCI was 2.5 ng/mL (range 0.3–12 ng/mL); and, in the dogs with chronic SCI, the mean concentration of GFAP in CSF was 6.3 ng/mL (range 0.2–26 ng/mL). The values did not differ statistically (p = 0.86) (Figure 3b).

The correlation was calculated between concentrations of GFAP in the CSF of dogs with SCI and T2WLR, and it was not statistically significant (r = 0.28, p = 0.4889).
Figure 3  a) Differences of the least square means of logarithmic glial fibrillary acidic protein (LGFAP) concentrations in cerebrospinal fluid (CSF) between different neurological grades according to Sharp and Wheeler (2005) of spinal cord injury (SCI). 0 on the x axis represents healthy dogs. b) Wilcoxon scores of GFAP in CSF of acute and chronic SCI; boxes contain values from the first to third quartile and represent least square means of CSF GFAP concentration in ng/mL; lines inside the box indicate median values, and ◊ represents mean values; lines outside of the box indicate maximum (above the box) and minimum (beneath the box) observation. Asterisk (*) indicates significant difference from healthy dogs.

5.5. Discussion

Considering the abundance of studies in human medicine on GFAP and its role in CNS diseases (Middeldorp and Hol 2011) and the vastly promising discoveries in the field, GFAP could be a valuable biomarker for veterinary neurology.

In the current study, concentrations of GFAP in serum and CSF in different neurological diseases in dogs were evaluated to establish GFAP as a valuable
neurobiomarker. Our interest in GFAP developed from the ever-increasing knowledge of the role of astrocytes in physiology and pathophysiology in the CNS, not only through mechanical but also functional changes in surrounding neuronal tissue (De Keyser et al. 2008). GFAP, being involved in astrocytic morphology and function (Middeldorp and Hol 2011), presents a perfect target for a biomarker search in CNS diseases.

In intracranial diseases, GFAP could be measured in all CSF samples. Similar findings have been described in human medicine, in both healthy and diseased CSF samples (Gurnett et al. 2003; Vagberg et al. 2015). GFAP is released in CSF after astrocytic mechanical injury or occurring astrogliosis (Petzold 2015). Low CSF GFAP values in healthy control dogs could be explained by regular metabolism of astrocytes (Vagberg et al. 2015). The first hypothesis that GFAP levels in CSF reflect a disease category of CNS diseases had to be rejected. GFAP values were elevated in epileptic patients (idiopathic and structural), as well as in SCI patients. We could, however, notice that levels of CSF GFAP were lower in idiopathic epilepsy patients than in brain tumors and inflammations. This could be explained by the fact that in idiopathic epilepsy, reactive gliosis might be observed, but the extent of tissue damage and astrocytic involvement is not as pronounced (Gurnett et al. 2003) as in brain tumor (Placone et al. 2016) or inflammation (Farina et al. 2007). Therefore, CSF GFAP levels seem to reflect structural changes in CNS tissue, rather than the very nature of the disease.

Increasing evidence suggests the involvement of astrocytes in seizure development (Robel et al. 2015; You et al. 2012). Furthermore, an increase of GFAP concentrations in the CSF of epileptic patients after seizure events was described (Gurnett et al. 2003). In this investigation, we introduced the hypothesis that GFAP CSF levels in dogs with epilepsy reflect the role of astrocytes in seizure causation. Therefore, the relationship between GFAP concentrations in the CSF of epileptic dogs and different variables associated with seizures in epilepsy was evaluated. However, a specific association
between GFAP levels and seizure occurrence and type could not be proved. Therefore, we could not support the hypothesis of an acute involvement of astrocytes in seizure generation in the dog population of the current study, as proposed in the hypothesis. There was a noticeable, although not statistically significant ($R^2 = 0.6609$, $p = 0.1451$) dependency between the mass effect of brain tumor and level of GFAP in CSF, suggesting an interaction between astrocyte function and tumor growth. This association could be either explained by the severity of tissue destruction or by Placone’s group’s statement that astrocytes around a brain tumor can enable the growth and spread of cancer (Placone et al. 2016).

All SCI patients in the current study had elevated GFAP in CSF compared with controls; however, the values of patients with grade 3 did not reach a level of significance. In this group, only five patients could be included and, therefore, these results should be acknowledged with caution. No statistically significant difference of GFAP CSF levels could be found between different grades of SCI patients reflecting the severity of the spinal cord dysfunction in dogs (Henke et al. 2013; Sharp and Wheeler 2005). Thus, it could be envisioned that GFAP CSF levels rather reflect glial injury in or reaction to SCI in general and not the severity of the disease in dogs with SCI. Similar results have been presented by Guez et al. who argue that CSF GFAP levels in SCI were elevated immediately after the injury due to extensive glial cell necrosis (Guez et al. 2003).

There was no difference between levels of GFAP in CSF in acute and chronic SCI patients. A possible explanation for these findings is the presence of secondary progressive tissue damage in SCI. It has been shown that astrocytes have beneficial and detrimental roles in spinal cord parenchyma after injury (Lukovic et al. 2015). Specifically, after first activation of the tissue defense seconds to hours after SCI, the secondary wave of progressive tissue damage starts (Hagg and Oudega 2006). This
secondary tissue degeneration can continue for weeks or even months (Hagg and Oudega 2006). In addition, astrocytes form glial scars, preventing axonal growth and containing reactive astrocytes. Thus, in chronic SCI, all processes of repair are present, including reactive astrogliosis (Faulkner et al. 2004). Moreover, this persistent astrogliosis leads to a constant level of GFAP in CSF (Petzold 2015) and could explain the lack of difference between levels of GFAP in dogs with either acute or chronic SCI.

Timely recognition of possible disease outcome after SCI could facilitate development of new treatment approaches (Raspa et al. 2016). T2WLR is known to help predict the outcome in dogs with SCI (Boekhoff et al. 2012; Levine et al. 2009; Siedenburg et al. 2018 accepted). By correlating T2WLR with the levels of GFAP in CSF of SCI patients, we have tried to indirectly associate GFAP with the outcome of the disease. Based on the result of the correlation (r = 0.28, p = 0.4889) CSF GFAP levels cannot be considered predictive of the outcome of dogs with SCI. Considering that CSF GFAP values were not different in chronic and acute SCI patients, as well as in dogs with different severity of the SCI, the lack of predictive value of GFAP in SCI is not surprising.

In the current study, it was also be evaluated whether the detection of GFAP in serum could have prognostic value, as shown in the literature (Nylen et al. 2006; Sato et al. 2013), or is describing the severity of the tissue damage (Miyake et al. 2013). Surprisingly, only 15 samples had positive GFAP levels in serum. However, the majority of the GFAP positive serum samples were detected in patients with acute traumatic injury of the CNS. This fact could support our hypothesis that mainly the severity of the tissue damage in the CNS leads to detectable GFAP in the serum of dogs. The presence of GFAP in serum in such trauma patients associated with mechanical tissue damage could be caused by damage of brain or spinal cord tissue.
leading to mechanical disruption of astrocytic integrity, followed by leakage of the BBB and release of GFAP and other metabolites into the blood circulation (Abbott 2002). The fact that, in three samples of the patients with brain inflammation, GFAP was detected in serum could actually be explained by extensive and severe reactive astrogliosis. This reactive astrogliosis might increase BBB permeability (Sofroniew 2009), which again leads to the leakage of metabolites and GFAP into blood.

In human medicine, serum GFAP was also found in cases of severe tissue damage, such as intracerebral hemorrhage (Foerch et al. 2012), TBI (Lei et al. 2015; Nylen et al. 2006), glioblastoma (Jung et al. 2007), bacterial meningitis, and subarachnoidal hemorrhage (Mayer et al. 2013). In dogs, GFAP was found in the serum of pug dogs with NME (Miyake et al. 2013) and progressive myelomalacia (Sato et al. 2013). Regarding NME in dogs, based on our results, we could not agree on Miyake’s group’s claim that GFAP in serum may be used as a specific marker for NME in pug dogs, since it was also detected in other inflammatory brain lesions. However, NME does include severe brain parenchyma damage, and our only patient with NME had positive serum GFAP values. Since serum GFAP-positive SCI patients of the current study did not have myelomalacia, we could not support Sato’s group’s statement that GFAP in blood has predictive value for progressive myelomalacia.

Being a disease with a high mortality rate, TBI is devastating for both humans and dogs (Sande and West 2010b) and presents a challenge for research in the biomarkers field (Yokobori et al. 2013). To better evaluate the neurological state of patients with head injury, the GCS was designed. It is a diagnostic tool to evaluate the level of consciousness and severity of the trauma. In dogs, Shores suggested a modified GCS that would be more applicable to animals (Shores 1983). Platt et al. suggested that MGCS scores between 3 and 8 have grave prognosis, 9 and 14 guarded, and 15 and 18 good prognosis (Platt et al. 2001). Moreover, MGCS is described to be predictive
of the survival in the first 48 hours after TBI (Platt et al. 2001). To show the association between GFAP in serum and the outcome of the disease, we have correlated serum GFAP values with the MGCS scores of dogs with TBI. Considering the correlation to MGCS was strongly negative ($r_s = -0.75$), we could suggest that GFAP in the serum of dogs with TBI is predictive of the outcome in the first 48 hours. Interestingly, the most severe case of TBI, according to MGCS score, had a level of GFAP one hundred times higher than in dogs with higher MGCS scores. This increase in GFAP level in serum in TBI reflects astrocytic involvement in severe acute injury. We are aware of our study design limits regarding the number of patients with TBI ($n = 15$) and the use of one breed (beagles) for reference values. However, similar results have been reported in human TBI, relating GFAP serum levels with the outcome of severe TBI (Nylen et al. 2006) and suggesting its usefulness in combination with other blood biomarkers (Diaz-Arrastia et al. 2014).

In summary, we have evaluated GFAP as a potential prognostic/diagnostic marker in various CNS diseases. Although GFAP in CSF did not reflect any specific disease category, it was increased in all patients with epilepsy, as well as in dogs with SCI, indicating structural changes in CNS parenchyma. In the CSF of the epileptic dogs, GFAP levels did not correlate with different seizure parameters, but were higher in structural than in idiopathic epilepsy, supporting the assumption of astrocytic involvement in the respective reaction to tissue destruction. In addition, GFAP was measurable in serum only in cases with severe mechanical tissue damage. Despite unspecific elevation of CSF GFAP levels, we have detected highly elevated serum concentrations of GFAP in dogs with TBI. Furthermore, GFAP serum levels in TBI dogs could be associated with the outcome of the disease in the first 48 hours, supporting its potential utility as a biomarker. The current study opens possibilities for further research of GFAP involvement in larger cohorts of dogs with TBI.
Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

5.6. References:


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**Author Contributions Statement**

Conceptualization, administration and writing-editing were done by A.T. Sample collection: D.K., S.W., D.H. and J.S. Sample analysis: D.K., S.W. R.C. J.S.
Investigation and writing were performed by D.K. Statistical analysis was done by K.R. and D.K. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests

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6. Manuscript II: Evaluation of IL-1β levels in epilepsy and traumatic brain injury in dogs

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Contribution of Draginja Kostic to this publication: 80%

D. Kostic was involved in the concept of the study, performed the experiments, analyzed the data and wrote the manuscript. R. Carlson helped performing the experiments and analyzing the data and critically revised the manuscript. D. Henke was involved in the clinical evaluation of patients and collection of samples and critically revised the manuscript. K. Rohn was involved in the statistical analysis and critically revised the manuscript. A. Tipold was involved in the concept and design of the study, the coordination of experiments, analysis of the data and critically revised the manuscript.

Submitted to BMC Neuroscience.
6.1. Abstract

Background

Epilepsy is a common neurological disease in dogs affecting approximately 0.6–0.75% of the canine population. There is much evidence of the presence of neuroinflammation in epilepsy creating new possibilities for the treatment of the disease. Increased expression of interleukin-1 beta (IL-1β) was reported in epileptogenic foci. We hypothesized that there is IL-1β elevation in serum of dogs with traumatic brain injury (TBI) and that in serum and cerebrospinal fluid (CSF) it reflects chronic inflammation in naturally occurring canine epilepsy in a clinical setting.

Method

Concentration of IL-1β in peripheral blood of dogs with TBI and epilepsy (idiopathic and structural) was investigated, as well as its presence in CSF of dogs with epilepsy using an ELISA. Levels of IL-1β were compared between healthy and diseased dogs. Nonparametric statistical tests were used and differences between all the groups were evaluated using Kruskal-Wallis test and Bonferroni’s post-hoc correction. To associate IL-1β concentrations in serum to important parameters in epilepsy, linear regression analysis was performed for the following variables: duration of the disease, seizure frequency, time point between sample collection and last seizure event.

Results

IL-1β levels were evaluated in CSF and serum of six healthy and 51 dogs with epilepsy (structural and idiopathic). In 16 dogs with TBI, only serum was tested. IL-1β concentrations in CSF were not detectable. Serum values were not elevated in dogs with TBI in comparison to healthy controls (p >0.05). However, dogs with epilepsy had increased levels of IL-1β in serum (p = 0.003) regardless of the underlying cause of the disease (p = 0.0045). There was no significant relationship between the variables and IL-1β levels. Statistically noticeable (p = 0.0630) was that approximately 10% of
patients with epilepsy \((R^2 = 0.105)\) had increased seizure frequency and IL-1\(\beta\) elevation.

**Conclusion**

Increased IL-1\(\beta\) levels were detected in peripheral blood in dogs with idiopathic and structural epilepsy leading to the assumption that an inflammatory reaction must be considered in search for new therapeutic approaches to treat seizures. However, to better understand the pathogenetic role of this cytokine in epilepsy, further evaluation of IL-1\(\beta\) in brain tissue is desired.

**Key words**

Interleukin-1 beta, epilepsy, traumatic brain injury, cerebrospinal fluid, serum, canine

6.2. Background

Cytokines are important signaling molecules (Turner et al. 2014) involved in immunity, inflammation and haematopoiesis, but also in functional alteration of cells in the central nervous system (CNS) (Hofer and Campbell 2016). Cytokines are well known to strongly influence signaling processes in CNS during injury, inflammation or disease (Lucas et al. 2006; Rodriguez-Smith et al. 2017). They can be pro- or anti-inflammatory (Dinarello 2007).

Interleukin-1 beta (IL-1\(\beta\)) belongs to IL-1 family of pro-inflammatory cytokines and plays an essential role in injury and inflammation (Dinarello 1996). In CNS it is mainly produced by activated microglia (Yao et al. 1992), but also neurons (Watt and Hobbs 2000), astrocytes (Zhang et al. 2000) and oligodendrocytes (Blasi et al. 1999). There is ever-growing knowledge of this interleukin’s activity in healthy as well as inflamed brain parenchyma (Hewett et al. 2012). In the healthy brain, IL-1\(\beta\) levels are low, but detectable (Vitkovic et al. 2000) suggesting a certain function in CNS physiology such as sleep (Jewett and Krueger 2012; Krueger et al. 1984), learning and memory (Marin
and Kipnis 2013), as well as neuromodulation on different levels of cells communication in CNS (Hewett et al. 2012; Srinivasan et al. 2004; Vezzani and Viviani 2015). In CNS diseases, involvement of IL-1β is described in neurodegeneration (Hesse et al. 2016; Kempuraj et al. 2016), depression (Farooq et al. 2017), neurotrauma (Allan et al. 2005) and epilepsy (Kauffman et al. 2008; Vezzani et al. 2011). In chronic and acute inflammatory processes in the CNS, it plays both, a beneficial and a harmful role (Hewett et al. 2012) and therefore could represent a target for drug development (Loddick and Rothwell 1996; Luheshi et al. 2009).

Epilepsy is a common neurological disease in dogs affecting approximately 0.6–0.75% of the canine population (Berendt et al. 2015). Current treatment options for the disease are limited and aim for reduction of seizure frequency not influencing the pathophysiology (Bhatti et al. 2015). There is much evidence of the presence of neuroinflammation in epilepsy (Vezzani et al. 2011). The potential IL-1β involvement in inflammatory reactions in epilepsy has attracted considerable attention and despite equivocal reports on its implication in seizures (Rijkers et al. 2009), it presents a possibility for characterizing new treatment options (Dey et al. 2016). For instance, blocking IL-1β signaling might prevent status epilepticus in epileptic patients (Auvin et al. 2010).

Traumatic brain injury (TBI) is considered a global health problem and is known to have detrimental consequences in human (Kim et al. 2018; Potts et al. 2006) and veterinary medicine (DiFazio and Fletcher 2013), such as cognitive impairment (Rodney et al. 2018) or development of post-traumatic epilepsy. TBI can cause inflammatory reactions in the brain (Cederberg and Siesjo 2010) and subsequently leads to epileptogenesis (Vezzani et al. 2011; Webster et al. 2017). It has been suggested that increased IL-1β levels during inflammation after TBI, have predictive value for development of post-traumatic epilepsy (PTE) (Diamond et al. 2014).
In veterinary medicine, IL-1β concentration in peripheral blood has been described as possible marker for early stages of inflammation in dogs (Prachar et al. 2013). Increased expression of IL-1β in CNS has been reported in dogs with acute spinal cord injury (SCI) in choroid plexus (Moore and Oglesbee 2012), in brain lesions of dogs with canine distemper virus infection (Grone et al. 2000) and brain parenchyma of dogs with TBI (Yu et al. 2011). In dogs with degenerative myelopathy, a decrease of IL-1β in plasma was recorded (Lovett et al. 2014).

Therefore, concentration of IL-1β in peripheral blood of dogs with traumatic brain injury and epilepsy was investigated, as well as its presence in cerebrospinal fluid (CSF) of dogs with epilepsy using an ELISA. We hypothesized that there is IL-1β elevation in serum of dogs with TBI and that in serum and CSF it reflects chronic inflammation in naturally occurring canine epilepsy in a clinical setting.

6.3. Method

Patient inclusion and sample collection

The study included paired serum and CSF samples of six healthy beagles, 51 epilepsy and 16 TBI patients presented between 2013-2016 at the Small Animal Clinic of the University of Veterinary Medicine Hannover, Germany. Six TBI samples were kindly provided by Dr Diana Henke, from Vetsuisse Faculty, University in Bern. Samples from diseased dogs were collected after owner’s written consent. The study design followed ethical guidelines of the University and procedures on healthy dogs were approved by the authorities of Lower Saxony (animal experiment number 33.9-42502-05-14A453). Dogs were selected based on history and available diagnostic data. All patients in the study underwent clinical and neurological examination, complete blood testing and different imaging studies. Depending on the presumed or confirmed diagnosis, dogs
were divided in the following groups: idiopathic epilepsy, structural epilepsy, TBI, healthy (table 1)

Table 1: Groups of the patients according to diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Findings and number of dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic epilepsy</td>
<td>Seizures and normal interictal general, neurological, MRI and CSF examinations; n = 30</td>
</tr>
<tr>
<td>Structural epilepsy</td>
<td>Seizures and presumed inflammatory brain disease; n = 9</td>
</tr>
<tr>
<td></td>
<td>Neoplastic brain diseases; n = 12</td>
</tr>
<tr>
<td>TBI</td>
<td>History of head injury, clinical, neurological and imaging</td>
</tr>
<tr>
<td></td>
<td>examinations; n = 16</td>
</tr>
<tr>
<td>Healthy</td>
<td>Normal physical and neurological examination; n = 6</td>
</tr>
</tbody>
</table>

MRI-magnetic resonance imaging; CSF-cerebrospinal fluid; TBI-traumatic brain injury

Control group consisted of healthy, clinic owned beagles with normal clinical and neurological examination, normal blood values as well as normal CSF analysis.

Dogs with epilepsy were classified as patients with presumed or confirmed structural or idiopathic epilepsy according to recommendations for standardized diagnosis by the International Veterinary Epilepsy Task Force (De Risio et al. 2015).

In order to diagnose idiopathic epilepsy (n = 30) in dogs tier 2 level of confidence was applied (De Risio et al. 2015): dogs had a history of two or more unprovoked epileptic seizures, age at onset of the disease was between 6 months and 6 years, inter-ictal general and neurological examination were unremarkable, results of blood tests, urine analysis, ultrasound and radiographic examination, as well as MRI and CSF analysis were normal. Clinical data such as duration of the disease, seizure frequency, seizure
severity (single generalized seizures, cluster seizures or status epilepticus), time point between sample collection and last seizure event were recorded.

The group of dogs with structural epilepsy consisted of patients with inflammatory CNS disease (eight patients with meningoencephalitis of unknown origin (MUO) and one patient with bacterial encephalitis) and neoplasm of brain tissue presumably causing the observed seizures.

All patients diagnosed with structural epilepsy (n = 21) underwent physical and neurological examination, blood work as well as MRI and CSF tests. The presumed diagnosis ensued following recommendations (De Risio and Platt 2014) to diagnose either brain tumors (Schwartz et al. 2011) or brain inflammation (Coates and Jeffery 2014) in dogs.

In the study, 16 patients with TBI were included. Traumatic brain injury was diagnosed when patient’s history indicated recent (3 – 48 hours) head injury, thorough physical and neurological examination and corresponding imaging findings (Sande and West 2010a). Level of consciousness, motor and brainstem function of each TBI patient was graded using modified Glasgow Coma Scale (MGCS) (Shores 1983). MGCS is a clinical coma scale for dogs. The least severe cases of TBI had the highest MGCS score (MGCS = 18) and the most severe cases had an assigned MGCS score of “3”.

In all epileptic patients and healthy beagles, CSF was obtained via suboccipital puncture in general anesthesia. Saphenous and cephalic vein blood was collected, centrifuged at 14 000rpm for 2 minutes and serum was separated. In patients with TBI, only serum was tested. In cases with head injury CSF tap could lead to deterioration of clinical signs and such procedures are therefore contraindicated (Sande and West 2010a). Blood sampling in TBI dogs occurred 3 – 48 hours after the injury and referral to the clinics. All samples were immediately aliquoted, frozen and stored at -20°C until measurement.
IL-1β determination

IL-1β was measured in paired CSF and serum samples of epileptic patients and healthy dogs, as well as in serum of TBI patients. Concentration of IL-1β was evaluated using a canine specific Enzyme-Linked Immunosorbent Assay (ELISA) test (Kit No. SEA563Ca; Cloud-Clone Corp, Houston TX, USA). The sandwich type ELISA was performed according to manufacturer’s instruction manual. Briefly, in the wells pre-coated with antibody specific to IL-1β, 100µl of sample was added, followed by biotin-conjugated antibody, specific to IL-β. Next, Avidin-conjugated to Horseradish Peroxidase is added and incubated. When 3,3',5,5’-Tetramethylbenzidine (TMB) substrate is added, only wells that contained biotin-conjugated antibody, IL-1β and enzyme conjugated avidin had changed color. After adding sulphuric acid, the color change of the final product was measured using plate reader (Synergy 2 multi-mode reader, BioTek, Vermont, USA). The measured optical density was then compared to standard curve values and expressed in pg/mL. Detection range of the test was 7.8 – 500pg/mL.

Statistical analysis

All statistical analyses were performed using statistics program package SAS®, version 9.2 (SAS Institute, Cary, NC, USA). IL-1β data did not follow a normal distribution. Therefore, nonparametric statistical tests were used and differences between all the groups were evaluated using Kruskal-Wallis test and Bonferroni’s post-hoc correction. First, all groups of patients were compared to healthy dogs and to each other. Afterwards all epileptic patients together were compared to TBI and healthy group. Mean values with standard deviation were calculated for each evaluated group.
The graphical presentation of the results was done using GraphPad Prizm 6 (GraphPad Software, Inc., La Jolla, USA) for.

To associate IL-1β concentrations in serum to important parameters in epilepsy, linear regression analysis was performed for the following variables: duration of the disease, seizure frequency, time point between sample collection and last seizure event. The relationship was described with the coefficient of determination, R-squared ($R^2$).

Levels of IL-1β were compared between groups with different seizure types resp. seizure severity (single generalized seizures, cluster seizures or status epilepticus) applying one-way ANOVA test.

### 6.4. Results

IL-1β concentration was evaluated in a total of 73 dogs included in the study. In all CSF (n=57) samples IL-1β could not be measured using the described ELISA.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Healthy</th>
<th>TBI</th>
<th>Idiopathic epilepsy</th>
<th>Structural epilepsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean values ± SD (range)</td>
<td>14.8</td>
<td>92.6 ± 75.1</td>
<td>118.6 ± 81.4</td>
<td>134.9 ± 79.2</td>
</tr>
<tr>
<td></td>
<td>(0.0–58.0)</td>
<td>(12.9–248.0)</td>
<td>(0.0–312.0)</td>
<td>(48.0–237.0)</td>
</tr>
</tbody>
</table>
| TBI – traumatic brain injury; SD – standard deviation

In healthy dogs (n=6), two serum samples had detectable IL-1β levels. Mean concentration and range of IL-1β in these control samples was 14.8 (0.0 – 58.0) pg/mL (Table 2).

In all TBI patients (n = 16) only serum samples were evaluated and in all samples IL-1β was measurable. Mean value, SD and range of IL-1β concentration in serum of TBI
patients was 92.6±75.1 (12.9 – 248.0) pg/mL (Table 2). However, after applying Kruskal-Wallis test to compare levels of IL-1β in serum of healthy dogs and TBI patients, it was concluded that there was no significant difference between these two groups (p>0.5) despite single elevated values.

Epilepsy patients were divided in idiopathic and structural epilepsy group. In all patients (n=51), IL-1β was measurable, except for two patients with idiopathic epilepsy. Dogs with idiopathic epilepsy had mean values, SD and range of serum IL-1β of 118.6 ± 81.4 (0.0 – 312.0) pg/mL (Table 2). IL-1β in serum of patients with inflammatory brain disease and brain tumor showed mean values, SD and range of 134.9 ± 79.2 (48.0 – 237.0) pg/mL and 154.2 ± 93.7 (37.5 – 308) pg/mL, respectively (Table 2).

In order to compare levels of IL-1β in serum samples of epileptic patients to healthy dogs, Kruskal-Wallis test for unequal sample size was performed and showed that both idiopathic as well as structural epilepsy patients had higher levels of IL-1β in serum then healthy dogs (p = 0.003; figure 1a). However, there was no statistical difference in the level of IL-1β in structural and idiopathic epilepsy groups (p>0.05; figure 1a). The same test was used to compare IL-1β serum levels in healthy patients and dogs with idiopathic epilepsy, inflammatory brain disease and brain tumors and each group had higher IL-1β levels than controls regardless of the underlying cause of seizures (p = 0.0045; figure 1b.) When compared to each other, there was no difference between dogs with idiopathic epilepsy, inflammatory brain disease or neoplasia regarding their levels of IL-1β in serum (p > 0.05; figure 1b).
Fig.1. a) Levels of interleukin-1 beta (IL-1β) in serum of healthy dogs compared to dogs with idiopathic and structural epilepsy and traumatic brain injury (TBI) b) Level of interleukin-1 beta (IL-1β) in serum of healthy dogs compared to dogs with idiopathic epilepsy and dogs with inflammation and tumor in the brain; Boxes contain values from the first to third quartile of serum IL-1β levels pg/ml. Lines inside of the box indicate median values, and (+) represents mean values; Lines outside of the box indicate maximum (above the box) and minimum (beneath the box) observation; The asterisk mark statistically significant difference after Bonferroni correction (* for p<0.05; ** for p < 0.01; *** for p<0.001).

Linear regression analysis of IL-1β levels relationship with time point between sample collection and the last seizure event (mean = 4.3 days) resulted in $R^2 = 0.0014$ and with p= 0.8963 was statistically not significant (Table 3). The same was concluded for the linear regression analysis between levels of IL-1β in epileptic patients and the duration of the disease (mean = 198 days, $R^2 = 0.0097$, p = 0.6112). Interestingly, the analysis of the linear regression of IL-1β levels and seizure frequency showed statistically noticeable, but not significant relationship, with $R^2 = 0.105$ and p = 0.0630 (Table 3). In patients with different type of seizures resp. different seizure severity no
statistically significant difference \( (p = 0.7164) \) was calculated using the one-way ANOVA test regarding levels of IL-1β.

Table 3. Analysis of IL-1β in serum of epileptic patients and different variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>( R^2 )</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time point between sample collection and last seizure</td>
<td>0.0014</td>
<td>0.8963</td>
</tr>
<tr>
<td>Duration of the disease</td>
<td>0.0097</td>
<td>0.6112</td>
</tr>
<tr>
<td>Seizure frequency</td>
<td>0.105</td>
<td>0.0630</td>
</tr>
<tr>
<td>Type of seizure (single generalized seizures, cluster</td>
<td>n/a</td>
<td>0.7164</td>
</tr>
<tr>
<td>seizures or status epileptic)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R\(^2\)- coefficient of determination

6.5. Discussion

In the current study IL-1β was measured in serum and CSF of dogs with epilepsy, which is to authors’ knowledge the first canine-based study. IL-1β levels should be evaluated to prove the occurrence of an inflammatory reaction in canine epilepsy. In addition, the concentration of the IL-1β was also measured in serum of TBI patients, as these patients tend to develop post traumatic epilepsy (Steinmetz et al. 2013).

In all patients with epilepsy, as well as in healthy dogs, IL-1β was not measurable in CSF using the described ELISA. Based on the origin of metabolites in the CSF, an association between the occurrence of IL-1β in CSF and brain tissue would have been highly plausible and could tell more about the role of IL-1β in epilepsy (Rijkers et al. 2009). However, in the current study as well as in human samples the low sensitivity of the ELISA tests, time between sample collection and the last seizure event and
different causes of the disease most probably prevented the detection of the cytokine in CSF samples (Rijkers et al. 2009). The time interval between last seizure event and sample collection (mean value = 4.3 days) seems not to have influenced values in canine serum, which is similar to recent report in human patients (Gao et al. 2017). Regarding veterinary research of other CNS diseases, another attempt to asses IL-1β in CSF of dogs with degenerative myelopathy failed since values were below the detection limit of the ELISA (Lovett et al. 2014).

Epileptogenesis is a subject of great scientific interest. Better understanding of this process offers numerous possibilities of revealing the disease cause and for new treatment approaches. After severe TBI, a high percentage of human and canine patients develop PTE (20% resp. 14.3%) (Englander et al. 2003; Steinmetz et al. 2013). During the process of disease development, an increase of IL-1β occurring in brain tissue after the injury was described (Lu et al. 2005; Webster et al. 2017). Thus, we were interested to measure IL-1β concentration in TBI in peripheral blood and tried to confirm that a spillover from the CNS occurs in the first 2 days after injury and can be evaluated in a clinical setting. However, there was no statistical difference between IL-1β serum values of healthy dogs and dogs with TBI values, even IL-1β was measurable in every sample in comparison to healthy controls with only 2 measurable samples. In human medicine intracranial levels of IL-1β are significantly higher than in plasma in TBI patients and the production of cytokines in CNS seems to be highly compartmentalized (Helmy et al. 2011). This could explain low levels of IL-1β in our serum samples, despite its increased production in the brain.

Pro-inflammatory cytokines and IL-1β are potentially involved in pathophysiology of epilepsy (Dey et al. 2016). Experimental research associated IL-1β production in epileptogenic brain areas with acute and subsequently, chronic neuroinflammation in epilepsy (Vezzani et al. 2011). With premise that it mirrors inflammation in epilepsy,
we have evaluated levels of IL-1β in CSF and serum of dogs with idiopathic and structural epilepsy. Even the cytokine was not detectable in CSF, in serum samples of dogs with epilepsy significantly elevated levels were detected when compared to healthy controls. Interestingly, there was no difference between idiopathic epilepsy and structural epilepsy patients. This remarkable result suggests that regardless of the cause of epilepsy, IL-1β is elevated in the blood. Also, increased IL-1β in blood of dogs with epilepsy, confirms the presence of neuroinflammation in the disease. The neuroinflammation in epilepsy presents inflammatory response of the brain tissue to neurogenic activity, i.e. seizures (Xanthos and Sandkühler 2013). Such an acute response includes release of IL-1β and other cytokines which in turn can help the brain to maintain homeostasis or harmfully, perpetuate and spread chronic inflammation, neuroexcitability and weaken the blood-brain barrier (van Vliet et al. 2007). The leakage of the BBB that occurs, could lead to the increase of the IL-1β in blood (van Vliet et al. 2018), which explains the cytokine detected in our patients with epilepsy.

Increased levels of IL-1β in serum were detected and especially single cases displayed high values, in both structural and idiopathic epilepsy. There have been reports in human medicine of presumed idiopathic epilepsy cases, that were in fact immune-mediated (Lancaster et al. 2010). This could explain high value of IL-1β in single case of idiopathic epilepsy. Similar results have been found evaluating IL-17 in serum and CSF of dogs with idiopathic epilepsy (Freundt-Revilla et al. 2017a). Nonetheless, further association with different variables in epilepsy was needed to better explain the role of the cytokine. Considering that the seizures present the common denominator for the three evaluated groups of epileptic patients, their association with IL-1β should be assessed and the following variables were evaluated: time point between sample collection and last seizure event, duration of the disease, seizure frequency, type of seizure resp. seizure severity. However, no significant
relationship between the variables and IL-1β levels could be calculated. Nevertheless, it was statistically noticeable (p = 0.0630) that approximately 10% of patients with epilepsy (R² = 0.105) had increased seizure frequency and IL-1β elevation. Similar results were described in human medicine and dependency of seizure frequency and IL-1β production occurred (Gao et al. 2017; Uludag et al. 2015). Regardless, there are still controversial reports on the exact role and the mechanism of the influence of IL-1β on seizures in epilepsy (Rijkers et al. 2009; van Vliet et al. 2018). The fact that there was no relationship between IL-1β and the time point of the sample collection or the duration of the disease could be explained by potential constant chronic inflammation without IL-1β level fluctuations. In addition, Gao et al. suggested that no interictal and postictal alteration of the cytokine’s level in peripheral blood in epilepsy occurs (Gao et al. 2017). No differences between seizure types regarding IL-1β levels could be proven, although those differences might be better evaluated in the first hour after the event (Aronica and Crino 2011).

6.6. Conclusion

In this study, we have detected increased IL-1β in serum of dogs with epilepsy regardless of the cause. There is a constant challenge in finding new treatment options for epilepsy, considering multiple etiology and interindividual differences (Potschka et al. 2015b). This fact could be used for further therapy attempts. However, we could not detect IL-1β in CSF or make a connection between serum levels and seizures. Also, there was no change in serum level of IL-1β in dogs with TBI. Bearing that in mind, we suggest direct measurement of the IL-1β in brain parenchyma, to better understand its role in seizures and epilepsy. Also, among presumed idiopathic epilepsy cases, single dogs with very high levels of IL-1β, could in fact have immune-mediated epilepsy which needs more in-depth research.
List of abbreviations

**ELISA**: Enzyme-Linked Immunosorbent Assay **IL-1β**: interleukin-1 beta

**CNS**: central nervous system **CSF**: cerebrospinal fluid

**PTE**: post-traumatic epilepsy **SCI**: spinal cord injury

**TBI**: traumatic brain injury **TMB**: 3,3',5,5'-Tetramethylbenzidine

**MGCS**: modified Glasgow Coma Scale **MRI**: magnetic resonance imaging

**MUO**: meningoencephalitis of unknown origin

6.7. Declarations

**Ethics approval**

The study design followed ethical guidelines of the University and procedures on healthy dogs were approved by the authorities of Lower Saxony (animal experiment number 33.9-42502-05-14A453).

**Consent for publication**

Not applicable

**Availability of data and material**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests

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**Authors’ contributions**

Conceptualization, administration and writing-editing were done by A.T. Sample collection: D.K, D.H. Sample analysis: D.K, R.C. Investigation and writing were performed by D.K. Statistical analysis was done by K.R. and D.K. All authors reviewed the manuscript.

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7. Manuscript III: Hippocampal expression of the cannabinoid receptor type 1 in canine epilepsy

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Contribution of Draginja Kostic to this publication: 70%

D. Kostic was involved in the concept of the study, performed the experiments, analysed the data and wrote the manuscript. M. Nowakowska helped analysing the data. J. Freundt Revilla was involved in the design of the study and helped analyzing the data and critically revised the manuscript. F. Attig provided technical support for performing experiments and data analysis. W. Baumgärtner was involved in the design of the study, supervised the experiments and critically revised the manuscript. K. Rohn was involved in the statistical analysis and critically revised the manuscript. F. Gualtieri supervised the experiments. H. Potschka supervised the experiments and critically revised the manuscript. A. Tipold was involved in the concept and design of the study, the coordination of experiments, analysis of the data, and critically revised the manuscript.
7.1. Abstract

Epilepsy, as a common chronic neurological disease, is a great challenge for therapeutic management in both, human and dogs. A possible explanation for differing treatment response is the distinct etiology and the lack of thorough understanding of the epileptogenesis. For patients with drug-refractory epilepsy novel therapeutic approaches are required. The endocannabinoid system (ECS) and its cannabinoid receptor type 1 (CB1R) are thought to play an important role in the pathogenesis of epilepsy. A better understanding of the role of this system and disease associated influences need to be considered, when further validating new treatment approaches manipulating ECS. Therefore, the aim of this study was to investigate the expression of CB1R in hippocampus of epileptic dogs and quantitatively compare it to control animals.

Paraffin embedded brain tissues of seven control dogs, five dogs with idiopathic epilepsy (IE) and seven with structural epilepsy (SE) were CB1R immunolabeled using immunohistochemistry (IHC) and double immunofluorescence(IF) (double staining: CB1R and glial fibrillary acidic protein (GFAP), anti-beta-tubulin III (TUBB3), anti-neuronal nuclei (NeuN), and anti-synaptophysin (SYP)). Expression of CB1R was qualitatively and quantitatively evaluated in the regions of dentate gyrus (DG), hilus, Cornu Ammonis (CA) 1 and CA3 and compared between the three groups of dogs. Number of CB1R positive astrocytes in DG was compared between IE, SE, and control animals.

In dogs with IE the CB1R expression was significantly decreased in the CA1 region compared to controls \((p \leq 0.01)\). The hippocampus of dogs with SE revealed a significant increase in CB1R staining intensity in comparison to controls (CA3: \(p \leq 0.0001\), DG: \(p \leq 0.01\), hilus: \(p \leq 0.05\)). Comparison of IE and SE tissue demonstrated
that both, the immunopositive area and the optical density of the staining reached significantly higher levels in patients with structural epilepsy (area: CA1 \( p \leq 0.001 \), CA3 \( p \leq 0.01 \), DG \( p \leq 0.05 \), hilus \( p \leq 0.01 \); optical density: CA1 \( p \leq 0.01 \), CA3 \( p \leq 0.0001 \), DG \( p \leq 0.0001 \), hilus \( p \leq 0.01 \)). Double IF showed no colocation of CB1R with NeuN, TUBB3 and SYP. However, about 50% of astrocytes displayed positive CB1R staining in all slides with no difference between IE, SE and controls.

In summary, CB1R expression in canine hippocampus was increased in structural epilepsy and downregulated in idiopathic epilepsy patients. The distinct disease-associated CB1R expression has to be considered in further development of indirect new treatment approaches for dogs with epilepsy.

7.2. Introduction

Epilepsy is the most common chronic neurological disease with a prevalence of 0.6-0.75% in the canine (Heske et al. 2014) and 1-3% in the human (Houser 1990) population. A chronic state of neuronal hyperexcitability in the brain results in seizures (Berendt et al. 2015). The main challenge in its treatment is the multiple etiology and interindividual differences in manifestations of the disease (Potschka et al. 2015a). Moreover, the exact pathogenesis of epilepsy is still not completely understood (Pitkanen et al. 2018; Stafstrom and Carmant 2015). Therefore, there is a necessity of better understanding underlying mechanism of the disease.

The knowledge of potential medicinal use of the hemp plant Cannabis sativa dates as far back as 5000 years (Blair et al. 2015b). However, in-depth knowledge of the structure of its derivates and their exact effect on human physiology has been elucidated only in the last 60 years (Blair et al. 2015a). The two most abundant derivatives of the plant, phytocannabinoids, are psychoactive \( \Delta 9- \)
tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1971) and non-psychoactive cannabidiol (CBD) (Mechoulam and Shvo 1963). Cannabinoids act on receptors in organisms, known as endocannabinoid receptors. These receptors are part of the endocannabinoid system (ECS) encompassing their lipid ligands or endocannabinoids, and enzymes involved in the metabolism of the endocannabinoids (Di Marzo et al. 1998; Mechoulam et al. 1998). Best clarified are two G protein-coupled receptors: cannabinoid receptor 1 (CB1R) (Matsuda et al. 1990) expressed mainly in CNS and cannabinoid receptor 2 (CB2R) (Devane et al. 1988; Munro et al. 1993), present primarily in the peripheral tissue. Most thoroughly investigated endocannabinoids (De Petrocellis and Di Marzo 2009) that interact with these receptors are anandamide (Devane et al. 1992) and 2-arachidonoylglycerol (2AG) (Mechoulam et al. 1995).

The endocannabinoid system is known to influence neuronal activity on the synaptic level through CB1R (Di Marzo and Petrosino 2007). CB1R is detected in all brain structures (Herkenham et al. 1991; Howlett et al. 2004) mostly in presynaptic terminals of both glutamatergic and gamma aminobutyric acid (GABA)-ergic neurons (Marsicano and Lutz 2008; Straiker and Mackie 2005). This receptor is especially expressed in fibers of interneurons and axonal terminals (Straiker and Mackie 2005), sympathetic nerves (Ishac et al. 1996), but is also described on non-neuronal cells, like astrocytes potentially controlling their activity (Sagan et al. 1999).

Apart from CNS, CB1R is expressed on circulating immune cells (Bouaboula et al. 1993), adipocytes (Bensaid et al. 2003), hepatocytes (Jeong et al. 2008), and adrenal cortex (Ziegler et al. 2010). In human research, CB1 receptors signaling is reported to be important for regulation of stress and mood responses (Hillard 2014), pain perception (Iversen and Chapman 2002), cardiovascular (Randall et al. 2002) and gastrointestinal function (Di Carlo and Izzo 2003). In CNS disease, changes in
expression of CB1 receptors are observed in animal models of multiple sclerosis (Baker et al. 2000), traumatic brain injury (Shohami et al. 2011), Huntington’s disease (McCaw et al. 2004), Alzheimer’s disease (Mulder et al. 2011). Experimental models of epilepsy demonstrated a potential protective role of CB1 receptors in epilepsy, based on mechanism that acts against excessive glutamatergic transmission (Kawamura et al. 2006; Sugaya and Kano 2018; Takahashi and Castillo 2006). Therefore, studies suggest that regulation of CB1 receptor expression might be a step toward new therapeutic approaches (Capasso 2017; Prandi et al. 2018; Rosenberg et al. 2017).

In dogs, CB1R expression is described in healthy dogs in salivary glands (Dall’Aglio et al. 2010), hair follicles (Mercati et al. 2012), skin (Campora et al. 2012) and in central nervous system in hippocampus (Campora et al. 2012), midbrain, cerebellum, medulla oblongata and grey matter of the spinal cord (Freundt-Revilla et al. 2017b). A stronger expression of CB1R was reported in the skin of dogs with atopic dermatitis than in healthy skin (Campora et al. 2012). Strikingly, in canine epilepsy, Gesell et al. described an elevation of endocannabinoids in cerebrospinal fluid awaking interest in the evaluation of CB1R expression in the brain of dogs with this disease (Gesell et al. 2013).

Dogs have been identified as a translational model for human epilepsy (Potschka 2012) and the response to manipulation of the ECS could be studied in this species. Especially, for patients with drug-refractory epilepsy novel therapeutic approaches are required. A better understanding of the role of the ECS and disease associated influences need to be considered, when further validating new treatment approaches manipulating ECS. However, the spatial distribution of CB1R in brain parenchyma in canine epilepsy has not been described. Therefore, the aim of this study was to
investigate the expression of CB1R in hippocampus of epileptic dogs and quantitatively compare it to control animals.

7.3. Materials and methods

Study design and animals

Brain tissue of 19 dogs was selected from the archive of the Department of Pathology, University of Veterinary Medicine Hannover. For the current study, brain samples were grouped according to diagnosis in 3 groups: controls (n = 7; age: range 2 to 120 months, mean ± SEM 36.78 ± 12.32), idiopathic epilepsy (n = 5; age: range 5 to 96 months, mean ± SEM 30.2 ± 16.7) and structural epilepsy (n = 7, age range 3 to 132 months, mean ± SEM 73.71 ± 20.53). The tissue of all dogs was evaluated, and the diagnosis established by a board-certified pathologist. In dogs with idiopathic epilepsy histopathological changes in brain parenchyma were not evident. Samples of dogs with structural epilepsy had underlining changes in parenchyma, presumably causing the clinically observed seizures: one dog with hydrocephalus, one with cerebellar infarct, three dogs with brain tumor, one dog with encephalitis and one with periventricular vacuolization. In all dogs naturally occurring epilepsy was diagnosed during clinical workup. The tissue was collected and the study performed respecting German Animal Welfare law, as well as Universities ethical regulations. The control brain tissue was also selected from the archive and belonged to animals without any CNS disease. Two of these control samples originated from healthy beagles, euthanized for another study (Kegler et al. 2015) with the animal experiment number 33.9-42502-05-13A346. No animal was euthanized for the current study.
Tissue preparation

The selected brain tissue from the archive was prepared for histological evaluation immediately after necropsy. The tissue was fixed in non-buffered formalin (10%), embedded in paraffin and cut at serial sections of 3-µm thickness. For further analysis, the sections were mounted on SuperFrost-Plus slides (Menzel Gläser, Braunschweig, Germany) and stained with hematoxylin and eosin (HE). Transversal cuts of brain tissue at the level of hippocampus were processed for further immunohistochemistry and immunofluorescence evaluation.

Antibodies

Immunohistochemistry (IHC) and immunofluorescence (IF) of the selected tissue was performed using a polyclonal antibody against cannabinoid receptor 1 (CB1 Abcam Cat# ab23703, RRID: AB_447623, 1:200 IHC, 1:20 IF), the immunogen corresponding to C terminal amino acids 461±472 of Human Cannabinoid receptor. For double immunofluorescence staining, the following monoclonal antibodies were additionally used: anti-glial fibrillary acidic protein (GFAP, Sigma-Aldrich Cat# G-A-5, RRID:AB_2314539, 1:500 IF), anti-beta-tubulin III (TUBB3, Sigma-Aldrich Cat# T8660, RRID:AB_477590, 1:200 IF), anti-neuronal nuclei (NeuN, Millipore Cat# MAB377, RRID:AB_2298772; 1:800 IF), anti-synaptophysin (SYP, Dako Cat# M0776, RRID:AB_2199013; 1:250 IF).
Immunohistochemistry

In order to evaluate CB1R expression, brain tissue at the level of hippocampus of seven controls, five dogs with idiopathic and seven dogs with structural epilepsy was immunohistochemically stained using the previously established avidin-biotin-peroxidase complex (ABC) method (Freundt-Revilla et al. 2017b; Seehusen et al. 2007). Briefly, after being dewaxed and rehydrated through different grades of alcohol, 3-μm thick sections were treated with 0.5% H$_2$O$_2$ in methanol to block endogenous peroxidase. For antigen retrieval, the sections were transferred into sodium-citrate buffer (pH 6.0-6.5) and heated for 20 minutes in the microwave at 800w. After incubation with 20% normal goat serum to block unspecific protein binding, the tissue was placed in phosphate-buffered saline (PBS) for 20 minutes and subsequently incubated with the CB1 primary antibody overnight at 4°C. Slide cut at the level of cerebellum was used as a positive control. Positive and negative controls were stained using the same protocol with the exception that for the negative control the primary antibody was substituted with rabbit serum (1:3000; R4505; Sigma Aldrich, Taufkirchen, Germany). Biotin-labeled goat-anti-rabbit IgG (dilution 1:200; BA-1000; Vector Laboratories, Burlingame, CA, USA), was added as secondary antibody and incubated for 45 minutes at room temperature. Subsequently, ABC complex was used (VECTASTAIN-ABC Kit Standard, PK 6100, Vector Laboratories, Burlingame, California, USA). The reaction was presented by color development with 3,3'-diaminobenzidine tetrahydrochloride (0.05% solution, DAB, Sigma Aldrich, Taufkirchen, Germany) with H$_2$O$_2$ (0.03%, pH 7.2). In the last steps, the slides were counterstained with Mayer’s hematoxylin, dehydrated and cleared in acetic acid-n-butyl ester (EBE, Roth, Karlsruhe, Germany), and mounted using Roti-Histokit (Roth, Karlsruhe, Germany). Light microscopy was used for the histological evaluation of the
receptor’s expression (BX51, Olympus Optical CO., Tokyo, Japan) and images of stained tissue were acquired using software (DP72, Olympus Optical CO., Tokyo, Japan).

Double immunofluorescence

The slides were checked for co-localization of CB1R and GFAP, NeuN, TUBB3 and SYP using a double immunofluorescence method as previously described (Freundt-Revilla et al. 2017b; Seehusen et al. 2007). All slides were incubated with the respective primary antibodies for 90 min. Cy3-labeled goat anti-mouse (red, 1:200, Alexa Fluor 555 dye, Life Technologies) and Cy2-labeled goat anti-rabbit (green, 1:200, Alexa Fluor 488 dye, Life Technologies) secondary antibodies were used to visualize the respective antigens. Nuclear counterstaining was performed with 0.01% bisbenzimide (H33258, Sigma Aldrich, Taufkirchen, Germany) and slides were mounted with Dako Fluorescent Mounting medium (DakoCytomation, Hamburg, Germany). The double labelling CB1R/GFAP, CB1R/NeuN, CB1R/TUBB3 and CB1R/SYP was visualized using an inverted fluorescence microscope (BZ-9000E, Keyence GmbH, Neu-Iserenburg, Germany) and examined through the BZ-II Analyzer software. The same software was applied for scanning the stained slides for later evaluation, to preserve immunofluorescence. Each staining was observed with the same microscope settings under which negative control sections showed no signal.

Qualitative and quantitative tissue analysis

In each tissue sample of control dogs, dogs with idiopathic or structural epilepsy the evaluation of immunohistochemistry and immunofluorescence was performed at the
level of the hippocampus. Quantitative evaluation of immunohistochemical staining was performed in dentate gyrus (DG), hilus, Cornu Ammonis 1 (CA) and CA3. Due to limited availability of archived tissue material double immunofluorescence staining could only be performed in DG.

Quantitative analysis of immunostaining in control and epilepsy dogs was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, [https://imagej.nih.gov/ij/](https://imagej.nih.gov/ij/)). Firstly, images were uploaded and converted in black and white. Spatial calibration and signal threshold value were set for each region (hilus, DG, CA1, CA3) to eliminate background signal. In each region, frames were randomly chosen for analysis of CB1 expression, using 3–5 regions of interest (66280 µm² each) per each segment of hippocampus of each animal belonging to either control, idiopathic or structural epilepsy dogs. Mean value of threshold of signals in each region was calculated. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels. Value of pixels was afterwards calibrated in optical density (O.D.) and used to express the intensity of CB1R immunoreactivity. Intensity of the signal as well as the area expressing this signal was calculated per region of hippocampus and compared between the three described groups.

Double immunofluorescence stained tissue was evaluated quantitatively using the computer-assisted image analysis software (ImageJ). The cell-counter tool of the software was applied on acquired images of different DG layers: polymorphic, molecular, granule cell layer and hilus in controls and dogs with idiopathic and structural epilepsy. CB1R/GFAP double positive cells were counted on the computer monitor by two investigators unaware of the sample identity in up to five counting frames for each layer. The number of CB1R positive astrocytes was compared in dentate gyrus of controls, idiopathic and structural epileptic dogs as well as among different layers.
Statistical analysis

Statistical analysis was performed using the SAS® program package, version 9.2 (SAS Institute, Cary, NC, USA) for repeated measures analysis of variance (ANOVA), the pairwise Tukey test and the Kruskal-Wallis test. GraphPad Prizm 6 (GraphPad Software, Inc., La Jolla, USA) was used for the graphical presentation of the results. The assumption of normal distribution of quantitative parameters was examined using the Kolmogorov-Smirnov test and visual assessment of QQ plots of model residuals. Inter-observer agreement for quantitative evaluation of double immunofluorescence staining was tested using interclass correlation test (ICC). All analyses were considered statistically significant, if p values were lower than 0.05.

7.4. Results

Immunohistochemistry

CB1R immunoreactivity was observed in hippocampi of all dogs examined. CB1R expression was preserved among the groups in all regions evaluated with major differences in CB1R intensity and areas between the groups.

Dense CB1 positive staining was visible in pyramidal cell layer in fibers surrounding pyramidal cells, the neuron itself remained unstained (fig.1d). Polymorphic and molecular layer of hippocampus expressed slightly lower CB1R than the pyramidal cell layer (fig.1 d, f).

The strongest immunoreactivity of CB1R was observed in DG, particularly in its molecular cell layer and hilus (fig. 1 a, c, e). Neuronal cells of hilus were not CB1R positive (fig. 1 a, c, e, f). However, strong, dense, dot-like CB1R staining was noticed surrounding the cells (fig.1). Granule cell layer expressed no CB1R immunoreactivity
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(fig. 1 a, c, e, f). Astrocytes, on the other hand, appeared to show the strongest CB1R immunostaining (fig. 1 b).

In samples of dogs with structural epilepsy, CB1R immunostaining appeared to have stronger intensity than controls (fig. 1). To support this observation quantitatively, ImageJ software was used. The O.D. values of CB1R immunoreactivity signal were used to express intensity in the respective area of the hippocampus. Examined areas expressing the signal were evaluated in µm². In the CA1 region of hippocampus the area expressing CB1 receptor was smaller in idiopathic than in structural epilepsy patients (p < 0.001) and controls (p < 0.01) (fig. 2). Correspondingly, the intensity of the CB1 receptors expression in the same area was stronger in dogs with structural than idiopathic epilepsy (p < 0.01) (fig. 3). Similar findings occurred in the other regions of the hippocampus and in idiopathic epilepsy patients the area of CB1R expression was smaller than in structural epilepsy, in DG (p < 0.05), CA3 (p < 0.01) and hilus (p < 0.01) (fig. 2). On the other hand, the intensity of the CB1R expression in hippocampus (fig. 3) was stronger in all structural epileptic patients than in tissue of the controls in DG (p < 0.01), CA3 (p < 0.0001) and hilus (p < 0.05). In the same regions, hippocampal tissue from dogs with idiopathic epilepsy displayed lower intensity (fig. 3) of CB1 receptor expression than the tissue of dogs with structural epilepsy DG (p < 0.0001), CA3 (p < .0001) and hilus (p < 0.01).
Figure 1: CB1R immunostaining of hippocampus in (a, b, d, f) control dog, (c) dog with structural epilepsy and (e) dog with idiopathic epilepsy demonstrating the increased CB1R immunoreactivity in tissue of the dog with structural epilepsy; there is an intense dot-like CB1R immunoreactivity of fibers surrounding unstained neuronal bodies marked with arrow (d); (b) the circle shows strong CB1R labeling of astrocytes; the dentate gyrus of the hippocampus depicts strong dot-like CB1R immunoreactivity in the molecular layer (m.l.) and hilus (h); the granule cell layer (g.c.l.) appears to be devoid of CB1R immunoreactivity; (d, f) pyramidal cell layer (p.c.l.) expresses strongly CB1R labeled fibers surrounding unstained soma; p.l.- polymorphic layer; h.m.l.- hippocampal molecular layer;
Figure 2. Area of CB1R expression in the hippocampus displays a) significantly decreased CB1R expressing area in the CA1 region in idiopathic epilepsy in comparison to structural epilepsy and controls; in DG (b), CA3 (c) and hilus (d) the area expressing CB1R in idiopathic epilepsy was significantly smaller in comparison to structural epilepsy, but did not differ significantly to controls; Error bars indicate SEM and asterisks in the figures indicate significant differences, *p < 0.05, **p < 0.01, ***p < 0.001; CA : Cornu Ammonis; DG : dentate gyrus.
Figure 3. Optical density of CB1R expression in the hippocampus was significantly higher in structural than in idiopathic epilepsy in the regions CA1 (a), DG (b), CA3 (c) and hilus (d); hippocampal regions DG (b), CA3 (c) and hilus (d) express CB1 receptor with significantly higher intensity in dogs with structural and idiopathic epilepsy compared to control animals; error bars indicate SEM and asterisks in the figures indicate significant differences, *p < 0.05, **p < 0.01, ****p < 0.0001; CA: Cornu Ammonis; DG: dentate gyrus; O.D: optical density
Immunofluorescence

Double immunofluorescence staining was performed to identify the cells expressing CB1R in hippocampus. Co-localization of CB1 with NeuN was evaluated to detect neurons expressing the receptor, however, no co-expression was found (fig. 4d). The same findings occurred evaluating double immunofluorescence staining with SYP (fig. 4c), the marker for most neuronal synapses and TUBB3 (fig. 4a), the marker for neuronal microtubule.

However, a marked co-expression of CB1R with GFAP was observed (fig. 4b), which was previously described in astrocytes of healthy dogs (Freundt-Revilla et al. 2017b). The co-localization was present in control dogs and epileptic patients, in all hippocampal regions, especially emphasized in DG.

To quantitatively describe the co-expression of CB1R and GFAP, CB1R positive astrocytes were counted in DG of control dogs, dogs with idiopathic epilepsy and structural epilepsy by two examiners (DK, JF) (fig. 5) resulting in 88% of interrater agreement. In all 3 groups together, the sum of counted CB1R positive astrocytes presented more than half of total number of astrocytes counted in DG. Interestingly, there was no statistical difference between the number of CB1R positive astrocytes in control group and idiopathic epilepsy group (p = 0.9308), control and structural epilepsy (p = 0.1189) and idiopathic and structural epilepsy group of animals (p = 0.1811) (fig. 6a). In different layers of DG there was no statistically significant difference between numbers of CB1R positive astrocytes in structural epilepsy (p = 0.5253) and idiopathic epilepsy (p = 0.4118). An exception was detected in control dogs and more positive CB1R astrocytes were found in hilus than in molecular layer of DG (p = 0.0044; fig. 6b).
Figure 4: Double immunofluorescence staining of the hippocampus of a healthy dog: (a) staining CB1R (green A2, B2, C2 and D2) and TUBB3 (red, A3) reveals no co-localization (A4). (c) Staining with SYP (red, C3) also resulted in no co-localization CB1R/SYP (C4). The same was found staining NeuN (d) (red, D3) and CB1R/NeuN (D4) suggesting a lack of expression of CB1 receptors in neuronal and part of synaptic vesicles; (A1-D1) nuclear staining (blue) with bisbenzimide; (b) staining GFAP (red, B3) and clear co-localization of GFAP and CB1R (B4) ; Scale bar = 200 μm (A1-A4) and 50 μm (B1-D4). CB1R: Cannabinoid receptor type 1; GFAP: glial fibrillary acidic protein; TUBB3: beta-tubulin III; NeuN: neuronal nuclei; SYP: synaptophysin.
Figure 5: Automatically scanned images of double immunofluorescence staining used for counting of CB1R positive astrocytes; slides of DG of a healthy dog (Control), a dog with idiopathic epilepsy (Idiopathic) and a dog with structural epilepsy (Structural); Astrocytes were counted in molecular layer (m.l.), hilus (h); the granule cell layer (g.l.) and polymorphic layer (p.l.). High numbers of CB1R/GFAP double positive cells are visible, especially in hilus of all 3 groups. CB1R: Cannabinoid receptor type 1; GFAP: glial fibrillary acidic protein.

Figure 6: Difference in the number of CB1R positive astrocytes in a) different diagnoses and b) different layers; Boxes contain values from the first to third quartile and represent the number of CB1R positive astrocytes. Lines inside of the box indicate
median values, and ◊ represents mean values. Lines outside of the box indicate maximum (above the box) and minimum (beneath the box) observation; Outliers are marked with ○. Asterisk (*) marks statistically significant difference between number of CB1+ astrocytes in hilus and molecular layer of dentate gyrus; CB1+: cannabinoid receptor type 1 positive; controls: tissue of control dogs; Idiopathic: tissue of dogs with idiopathic epilepsy; Structural: tissue of dogs with structural epilepsy; Granular: granule cell layer; Molecular: molecular layer; Polymorphic: polymorphic layer.

7.5. Discussion

In the current study, we described for the first time hippocampal expression of CB1R in canine idiopathic and structural epilepsy. CB1R is part of endocannabinoid system mostly expressed in CNS and known for its involvement in epilepsy (Rosenberg et al. 2015). Hippocampus is strongly connected to epileptic activities in brain parenchyma, hence, it has been the focus of epilepsy research for decades (Schwartzkroin 1994). Additionally, influences of pathophysiological mechanisms on the endocannabinoid system need to be considered, when further validating respective new treatment approaches. Therefore, we analysed CB1 receptor expression in different subregions of the canine hippocampus. The knowledge of CB1R expression in hippocampus of epileptic dogs could lead to better understanding of underlying mechanisms of seizure development and give a hint to treatment options manipulating the endocannabinoid system.

Qualitative evaluation of immunohistological distribution of CB1R immunoreactivity in hippocampus of controls and epileptic dogs demonstrated strong immunolabeling of fibers in pyramidal cell layer (without staining the soma of neuron) and slightly weaker staining in hippocampal polymorphic and molecular layer. Also, strong CB1R staining occurred in all samples in DG, especially in molecular layer and hilus. These findings
are consistent with previously described CB1R expression in healthy dogs (Campora et al. 2012; Freundt-Revilla et al. 2017b), as well as in healthy and epileptic experimental animals (Falenski et al. 2007; Kawamura et al. 2006; von Ruden et al. 2015). Functional implication of these findings was explained by Tsou et al., suggesting that the CB1R immune positive fibers are of GABA-ergic origin, enabling cannabinoid modulation or neurotransmission at perisomatic synapses of pyramidal neurons (Tsou et al. 1999). In dentate gyrus, the strong CB1R immunoreactivity seems to originate from dense CB1R labeling of mossy fibers (Kawamura et al. 2006). The exact role of the receptor in these fibers is not fully elucidated, although it has been suggested that persistently active cannabinoid receptors turn off the output of mossy fiber associated interneurons in the hippocampal CA3 area (Losonczy et al. 2004).

In hippocampus of controls and dogs with idiopathic and structural epilepsy difference in the intensity of CB1R immunostaining was observed. Optical density and area of CB1R immunoreactivity was compared in all three groups in CA1, CA3, hilus and DG. Indeed, a significant difference was found between hippocampus in idiopathic and structural epilepsy. Firstly, the area with positive CB1R immunostaining was smaller in dogs with idiopathic epilepsy in comparison to the other two groups. Surprisingly, the area of hippocampus of dogs with structural epilepsy expressing CB1R was larger and with significantly higher intensity of CB1R expression compared to idiopathic epilepsy and controls. This finding is similar to reports in experimental animals. Seizures itself can increase the density of CB1R in pyramidal cell layer (Karlocai et al. 2011; Magloczky et al. 2010; von Ruden et al. 2015). Reason for detecting high expression of the receptors in structural epilepsy could be the sprouting of new fibers of interneurons expressing CB1R (Karlocai et al. 2011). Another explanation would be that the ECS accounts for compensatory mechanisms by increased expression of receptors in certain subpopulations of cells in hippocampus (Chen et al. 2003). Both
mechanisms might be involved in increased CB1R expression in dogs with structural epilepsy. However, there are contradictory explanations on how CB1 receptor expression in certain regions of hippocampus influences epilepsy and seizures. Namely, CB1 receptors can influence neuroexcitability via two mechanisms. Endocannabinoids could act on receptors at excitatory synapses and suppress seizures by inhibiting glutamate release (Bhaskaran and Smith 2010). Conversely, endocannabinoid signaling could promote seizures by inhibiting GABA release at inhibitory synapses (Katona et al. 1999). It is likely that this dichotomous behavior of CB1R serves ECS for “fine-tuning” of synapses during development of epilepsy and seizures, but is also considered to be an anticonvulsive mechanism (Blair et al. 2015b).

The current study reveals interesting differences in intensity of CB1R expression between idiopathic and structural epilepsy with higher expression in CA3, hilus and DG in structural and downregulation in CA1 in idiopathic epilepsy patients. Difference in expression of CB1R has been also detected in experimental animals depending on the used model of epilepsy suggesting different mechanisms of seizure pathogenesis (Falenski et al. 2009; von Ruden et al. 2015; Wittner et al. 2017). In the present study, CB1R expression in CA1 is downregulated in dogs with idiopathic epilepsy. Similar trends are seen in animal models and humans with temporal lobe epilepsy (TLE) (Falenski et al. 2009). Specifically, research groups described reorganization of the CB1R in this type of epilepsy (Falenski et al. 2009) suggesting another defensive neuroplasticity mechanism of ECS to compensate for cell loss during hippocampal sclerosis present in TLE (Thom 2014). A limitation of our study was that due to quality of the archived tissue not all sub-regions of the hippocampus could be evaluated in detail, especially CA2. A possible reorganization of the receptors in idiopathic epilepsy group cannot be completely excluded. However, it was reported that in human TLE an overall downregulation of CB1R in hippocampus occurs (Ludanyi et al. 2008), which is
supporting the current finding in dogs with idiopathic epilepsy. This research group also considered the type of fibers and concluded that the decrease of CB1R is mainly on the glutamatergic terminals maintaining hyperexcitability in TLE (Ludanyi et al. 2008). Similarities between epileptic dogs and TLE in humans were also described regarding their cognitive deficit (Packer et al. 2018) and hippocampal asymmetry (Estey et al. 2017). These reports and the findings of CB1R downregulation in dogs with idiopathic epilepsy support the suggestion that dogs with idiopathic epilepsy mimic well human TLE.

CB1R presence is described on the axon terminals, interneuron fibers, on synapses and peri-somatic (Straiker and Mackie 2005). To examine its expression in dogs co-expression of CB1R in neurons using the marker NeuN, on astrocytes characterized by GFAP staining, microtubules in axons stained with anti-TUBB3, and synaptic vesicles using SYP marker in dentate gyrus was evaluated. Firstly, healthy tissue was double labeled with CB1R and the described markers. However, no co-locations were found, apart from CB1R/GFAP leading to the assumption that detection of CB1R on neurons, microtubules and synaptic vesicles in hippocampus is not feasible in dogs using the described markers. On the other hand, more than half of astrocytes in DG expressed CB1R in healthy and epileptic dogs. Considering that astrocytes play an important role in epilepsy (Tian et al. 2005), the high number of astrocytes expressing CB1R in DG in idiopathic and structural epilepsy and in the control group of animals presents an interesting starting point for further evaluation. However, no significant difference was detected in the number of CB1 positive astrocytes in DG between epileptic dogs and the control group. However, a difference in number of CB1R positive astrocytes between hilus and molecular layer was detected in controls, a finding which could not be shown in epileptic dogs. Therefore, a certain reorganization of CB1R positive astrocytes is possible in epileptic dogs. Nevertheless, it is becoming clear that
ECS plays an important role in the communication of the astrocytes with the surrounding neurons (Oliveira da Cruz et al. 2016). Communication was shown between presynaptic terminals, postsynaptic targets and associated astrocytes, which is called tripartite synapse (Araque et al. 1999) and endocannabinoids seem to be the key signal for that communication (Oliveira da Cruz et al. 2016). Astrocytes respond to endocannabinoids through activation of CB1R increasing intracellular calcium and stimulating the release of glutamate that modulates synaptic transmission and plasticity (Navarrete et al. 2014). In epilepsy, it is suggested that astrocytic glutamate release may play an epileptogenic role in initiation of epileptic seizures (Kang et al. 2005) and maintenance of epileptiform activity (Coiret et al. 2012). Regardless, very high expression of CB1R on astrocytes in canine DG represent an exciting possibility for further research and could provide more insight into mechanisms of epilepsy.

7.6. Conclusion

In summary, CB1R expression in canine hippocampus was increased in structural epilepsy and downregulated in canine idiopathic epilepsy patients. The distinct disease-associated CB1R expression is an important new aspect in the elucidation of epileptogenesis and has to be considered in further development of indirect new treatment approaches for dogs with epilepsy. Indeed, high expression of CB1R in astrocytes offers new consideration for treatment approach by modulation of astrocytic activity.
7.7. References

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8. General discussion

Diagnosis and treatment of epilepsy is challenging and the development of reliable biomarkers for epileptogenesis is definitely needed. In addition, biomarkers, which might predict the development of an epilepsy condition, identify the presence and severity of tissue capable of generating spontaneous seizures, measure progression after the condition is established and determine pharmacoresistance are searched for (Engel et al. 2013). During epilepsy, brain tissue is source of biomarkers, which can be evaluated in serum and CSF, but also directly in the tissue with the help of imaging methods (Pitkanen et al. 2018). The focus of the current project was the search for such biomarkers in canine epilepsy. Key findings of the study were: GFAP in serum may serve as possible neurobiomarker for severity of tissue destruction and has predictive value for the outcome of TBI; increased concentrations of IL-1β in epilepsy confirm presence of neuroinflammation in the disease; CB1R expression in hippocampus maybe involved in pathophysiology of canine epilepsy.

In the first part of the study, concentrations of GFAP in serum and CSF in different neurological diseases in dogs were evaluated to establish GFAP as a valuable neurobiomarker. Our interest in GFAP developed from the ever-increasing knowledge of the role of astrocytes in physiology and pathophysiology in the CNS and epilepsy specifically, not only through mechanical but also functional changes in neuronal tissue (De Keyser et al. 2008). GFAP, being involved in astrocytic morphology and function (Middeldorp and Hol 2011), presents a perfect target for a biomarker search in CNS diseases.

We proposed that if detected in CSF, GFAP levels are disease specific. In intracranial diseases, GFAP could be measured in all canine CSF samples. Similar findings have been described in human medicine (Gurnett et al. 2003; Vagberg et al. 2015). GFAP is released in CSF after astrocytic mechanical injury or occurring astrogliosis (Petzold
Low CSF GFAP values in healthy control dogs could be explained by regular metabolism of astrocytes (Vagberg et al. 2015). The hypothesis that GFAP levels in CSF reflect a disease category of CNS diseases had to be rejected, because it was elevated in multiple diseases examined. However, the levels of CSF GFAP were lower in idiopathic epilepsy patients than in brain tumors and inflammations. This could be explained by the fact that in idiopathic epilepsy, reactive gliosis might be observed, but the extent of tissue damage and astrocytic involvement is not as pronounced (Gurnett et al. 2003) as in brain tumor (Placone et al. 2016) or inflammation (Farina et al. 2007). Therefore, CSF GFAP levels seem to reflect structural changes in CNS tissue, rather than the nature of the disease.

Increasing evidence suggests the involvement of astrocytes in seizure development (Robel et al. 2015; You et al. 2012). Furthermore, an increase of GFAP concentrations in the CSF of epileptic patients after seizure events was described (Gurnett et al. 2003). In this investigation, we introduced the hypothesis that GFAP CSF levels in dogs with epilepsy reflect the role of astrocytes in seizure causation. Therefore, the relationship between GFAP concentrations in the CSF of epileptic dogs and different variables associated with seizures in epilepsy was evaluated. However, a specific association between GFAP levels and seizure occurrence and type could not be proved. Therefore, we could not support the hypothesis of an acute involvement of astrocytes in seizure generation in the dog population of the current study, as proposed in the hypothesis. There was a noticeable, although not statistically significant ($R^2 = 0.6609$, $p = 0.1451$) dependency between the mass effect of brain tumor and level of GFAP in CSF, suggesting an interaction between astrocyte function and tumor growth. This association could be either explained by the severity of tissue destruction or by Placone’s group’s statement that astrocytes around a brain tumor can enable the growth and spread of cancer (Placone et al. 2016).
All SCI patients in the current study had elevated GFAP in CSF compared with controls; however, the values of patients with grade 3 did not reach a level of significance. In this group, only five patients could be included and, therefore, these results should be acknowledged with caution. No statistically significant difference of GFAP CSF levels could be found between different grades of SCI patients reflecting the severity of the spinal cord dysfunction in dogs (Henke et al. 2013; Sharp and Wheeler 2005). Thus, it could be envisioned that GFAP CSF levels rather reflect glial injury in or reaction to SCI in general and not the severity of the disease in dogs with SCI. Similar results have been presented by Guez et al. who argue that CSF GFAP levels in SCI were elevated immediately after the injury due to extensive glial cell necrosis (Guez et al. 2003).

In acute and chronic SCI patients no difference was found between levels of GFAP in CSF. Presumed cause for this phenomenon could be the presence of secondary progressive tissue damage in SCI. It has been shown that astrocytes have beneficial and detrimental roles in spinal cord parenchyma after injury (Lukovic et al. 2015). Specifically, after first activation of the tissue defense seconds to hours after SCI, the secondary wave of progressive tissue damage starts (Hagg and Oudega 2006). This secondary tissue degeneration can continue for weeks or even months (Hagg and Oudega 2006). In addition, astrocytes form glial scars, preventing axonal growth and consisting of reactive astrocytes might add to elevated GFAP levels. In chronic SCI all processes of repair are present, including reactive astrogliosis (Faulkner et al. 2004). Moreover, persistent astrogliosis leads to a constant level of GFAP in CSF (Petzold 2015) and could explain the lack of difference between levels of GFAP in dogs with either acute or chronic SCI.

Timely recognition of possible disease outcome after SCI could facilitate development of new treatment approaches (Raspa et al. 2016). T2WLR is known to help predict the
outcome in dogs with SCI (Boekhoff et al. 2012; Levine et al. 2009; Siedenburg et al. 2018 accepted). By correlating T2WLR with the levels of GFAP in CSF of SCI patients, we have tried to indirectly associate GFAP with the outcome of the disease. Based on the result of the correlation ($r = 0.28, p = 0.4889$) CSF GFAP levels cannot be considered predictive of the outcome of dogs with SCI. Considering that CSF GFAP values were not different in chronic and acute SCI patients, as well as in dogs with different severity of the SCI, the lack of predictive value of GFAP in SCI is not surprising.

The detection of GFAP in serum could have prognostic value, as shown in the literature (Nylen et al. 2006; Sato et al. 2013), or is describing the severity of the tissue damage (Miyake et al. 2013). Surprisingly, only 15 samples had positive GFAP levels in serum. However, the majority of the GFAP positive serum samples were detected in patients with acute traumatic injury of the CNS. This supports our hypothesis that mainly the severity of the tissue damage in the CNS leads to detectable GFAP in the serum of dogs. In trauma patients GFAP in serum could be associated with mechanical tissue damage caused by insult of brain or spinal cord leading to mechanical disruption of astrocytic integrity, followed by leakage of the BBB and release of GFAP and other metabolites into the blood circulation (Abbott 2002).

The fact that, in three samples of the patients with brain inflammation, GFAP was detected in serum could be explained by extensive and severe reactive astrogliosis. This reactive astrogliosis might increase BBB permeability (Sofroniew 2009), which again leads to the leakage of metabolites and GFAP into blood. In human medicine, serum GFAP was also found in cases of severe tissue damage, such as intracerebral hemorrhage (Foerch et al. 2012), TBI (Lei et al. 2015; Nylen et al. 2006), glioblastoma (Jung et al. 2007), bacterial meningitis, and subarachnoidal hemorrhage (Mayer et al. 2013). In dogs, GFAP was found in the serum of pug dogs with NME (Miyake et al.
and progressive myelomalacia (Sato et al. 2013). Regarding NME in dogs, based on our results, we could not agree on Miyake’s group’s claim that GFAP in serum may be used as a specific marker for NME in pug dogs, since it was also detected in other inflammatory brain lesions in our cases. However, NME does include severe brain parenchyma damage, and our only patient with NME had positive serum GFAP values. Since serum GFAP-positive SCI patients of the current study did not have myelomalacia, we could not support Sato’s group’s statement that GFAP in blood has predictive value for progressive myelomalacia (Sato et al. 2013).

TBI is devastating for both humans and dogs (Sande and West 2010b) and presents a challenge for research in the biomarkers field (Yokobori et al. 2013). To evaluate the neurological state of patients with head injury, the GCS was designed. It is a diagnostic tool to evaluate the level of consciousness and severity of the trauma. In dogs, Shores suggested a modified GCS that would be more applicable to animals (Shores 1983). Platt et al. suggested that MGCS scores between 3 and 8 have grave prognosis, 9 and 14 guarded, and 15 and 18 good prognosis (Platt et al. 2001). Moreover, MGCS is described to be predictive of the survival in the first 48 hours after TBI (Platt et al. 2001). To show the association between GFAP in serum and the outcome of the disease, we have correlated serum GFAP values with the MGCS scores of dogs with TBI. Considering the correlation to MGCS was strongly negative ($r_s = -0.75$), we could suggest that GFAP in the serum of dogs with TBI is predictive of the outcome in the first 48 hours. Interestingly, the most severe case of TBI, according to MGCS score, had a level of GFAP one hundred times higher than in dogs with higher MGCS scores. This increase in GFAP level in serum in TBI reflects astrocytic involvement in severe acute injury. We are aware of our study design limits regarding the number of patients with TBI ($n = 15$) and the use of one breed (beagles) for reference values. However, similar results have been reported in human TBI, relating GFAP serum levels with the
outcome of severe TBI (Nylen et al. 2006) and suggesting its usefulness in combination with other blood biomarkers (Diaz-Arrastia et al. 2014).

In the second part of the study, main point of interest was to show neuroinflammation in epilepsy and IL-1β’s involvement. Therefore, IL-1β was measured in serum and CSF of dogs with epilepsy. Additionally, concentration of IL-1β was also measured in serum of dogs with TBI, as these patients tend to develop post-traumatic epilepsy (Steinmetz et al. 2013).

In all patients with epilepsy, as well as in healthy dogs, IL-1β was not measurable in CSF using the described ELISA. Based on the origin of metabolites in the CSF, an association between the occurrence of IL-1β in CSF and brain tissue would have been highly plausible and could tell more about the role of IL-1β in epilepsy (Rijkers et al. 2009). However, in the current study as well as in human studies the low sensitivity of the ELISA tests, time between sample collection and the last seizure event and different causes of the disease most probably prevented the detection of the cytokine in CSF samples (Rijkers et al. 2009). The time interval between last seizure event and sample collection (mean value = 4.3 days) seems not to have influenced values in canine serum, which is similar to recent report in human patients (Gao et al. 2017). Regarding veterinary research of other CNS diseases, another attempt to asses IL-1β in CSF of dogs with degenerative myelopathy failed since values were below the detection limit of the ELISA (Lovett et al. 2014).

After severe TBI, a high percentage of human and canine patients develop PTE (20% resp. 14.3%) (Englander et al. 2003; Steinmetz et al. 2013). During the process of disease development, an increase of IL-1β occurring in brain tissue after the injury was described (Lu et al. 2005; Webster et al. 2017). Thus, we were interested to measure IL-1β concentration in TBI in peripheral blood and tried to confirm that a spillover from the CNS occurs in the first 2 days after injury and can be evaluated in a clinical setting.
However, there was no statistical difference between IL-1β serum values of healthy dogs and dogs with TBI values, even IL-1β was measurable in every sample in comparison to healthy controls with only 2 measurable samples. In human medicine intracranial levels of IL-1β are significantly higher than in plasma in TBI patients and the production of cytokines in CNS seems to be highly compartmentalized (Helmy et al. 2011). This could explain low levels of IL-1β in our serum samples, despite its increased production in the brain.

Pro-inflammatory cytokines and IL-1β are potentially involved in pathophysiology of epilepsy (Dey et al. 2016). Experimental research associated IL-1β production in epileptogenic brain areas with acute and subsequently, chronic neuroinflammation in epilepsy (Vezzani et al. 2011). With premise that it mirrors inflammation in epilepsy, we have evaluated levels of IL-1β in CSF and serum of dogs with idiopathic and structural epilepsy. Although the cytokine was not detectable in CSF, in serum samples of dogs with epilepsy significantly elevated levels were detected when compared to healthy controls. Interestingly, there was no difference between idiopathic epilepsy and structural epilepsy patients. This remarkable result suggests that regardless of the cause of epilepsy, IL-1β is elevated in the blood. Also, increased IL-1β in blood of dogs with epilepsy, confirms the presence of neuroinflammation in the disease. The neuroinflammation in epilepsy presents inflammatory response of the brain tissue to neurogenic activity, i.e. seizures (Xanthos and Sandkühler 2013). Such an acute response includes release of IL-1β and other cytokines which in turn can help the brain to maintain homeostasis or harmfully, perpetuate and spread chronic inflammation, neuroexcitability and weaken the blood-brain barrier (van Vliet et al. 2007). Occurring leakage of the BBB could lead to the increase of the IL-1β in blood (van Vliet et al. 2018), which explains the cytokine detected in our patients with epilepsy.
Increased levels of IL-1β in serum were detected and especially single cases displayed high values, in both structural and idiopathic epilepsy. There have been reports in human medicine of presumed idiopathic epilepsy cases, that were in fact immune-mediated (Lancaster et al. 2010). This could also explain high value of IL-1β in single case of idiopathic epilepsy in the current study. Similar results have been found evaluating IL-17 in serum and CSF of dogs with idiopathic epilepsy (Freundt-Revilla et al. 2017a). Nonetheless, further association with different variables in epilepsy was needed to better explain the role of the cytokine. Considering that the seizures present the common denominator for the three evaluated groups of epileptic patients, their association with IL-1β should be assessed and the following variables were evaluated: time point between sample collection and last seizure event, duration of the disease, seizure frequency, type of seizure resp. seizure severity. However, no significant relationship between the variables and IL-1β levels could be calculated. Nevertheless, it was statistically noticeable ($p = 0.0630$) that approximately 10% of patients with epilepsy ($R^2 = 0.105$) had increased seizure frequency and IL-1β elevation. Similar results were described in human medicine and dependency of seizure frequency and IL-1β production occurred (Gao et al. 2017; Uludag et al. 2015). Regardless, there are still controversial reports on the exact role and the mechanism of the influence of IL-1β on seizures in epilepsy (Rijkers et al. 2009; van Vliet et al. 2018). The fact that there was no relationship between IL-1β and the time point of the sample collection or the duration of the disease could be explained by potential constant chronic inflammation without IL-1β level fluctuations. In addition, Gao et al. suggested that no interictal and postictal alteration of the cytokine’s level in peripheral blood in epilepsy occurs (Gao et al. 2017). No differences between seizure types regarding IL-1β levels could be proven in the current study, although those differences might be better evaluated in the first hour after the event (Aronica and Crino 2011).
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The last part of the study described for the first-time hippocampal expression of CB1R in canine idiopathic and structural epilepsy. CB1R is part of endocannabinoid system, mostly expressed in CNS and known for its involvement in epilepsy (Rosenberg et al. 2015). Hippocampus is strongly connected to epileptic activities in brain parenchyma, hence, it has been the focus of epilepsy research for decades (Schwartzkroin 1994). Additionally, influences of pathophysiological mechanisms on the endocannabinoid system need to be considered, when further validating respective new treatment approaches. Therefore, we analyzed CB1 receptor expression in different subregions of the canine hippocampus. The knowledge of CB1R expression in hippocampus of epileptic dogs could lead to better understanding of underlying mechanisms of seizure development and give a hint to treatment options manipulating the endocannabinoid system.

Qualitative evaluation of immunohistological distribution of CB1R immunoreactivity in hippocampus of controls and epileptic dogs demonstrated strong immunolabeling of fibers in pyramidal cell layer without staining the soma of neuron and slightly weaker staining in hippocampal polymorphic and molecular layer. Also, strong CB1R staining occurred in all samples in DG, especially in molecular layer and hilus. These findings are consistent with previously described CB1R expression in healthy dogs (Campora et al. 2012; Freundt-Revilla et al. 2017b), as well as in healthy and epileptic experimental animals (Falenski et al. 2007; Kawamura et al. 2006; von Ruden et al. 2015). Functional implication of these findings was explained by Tsou et al., suggesting that the CB1R immune positive fibers are of GABA-ergic origin, enabling cannabinoid modulation or neurotransmission at perisomatic synapses of pyramidal neurons (Tsou et al. 1999). In dentate gyrus, the strong CB1R immunoreactivity seems to originate from dense CB1R labeling of mossy fibers (Kawamura et al. 2006). The exact role of the receptor in these fibers is not fully elucidated, although it has been suggested that
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Persistently active cannabinoid receptors turn off the output of mossy fiber associated interneurons in the hippocampal CA3 area (Losonczy et al. 2004).

In hippocampus of controls and dogs with idiopathic and structural epilepsy difference in the intensity of CB1R immunostaining was observed. Optical density and area of CB1R immunoreactivity was compared in all three groups in CA1, CA3, hilus and DG. Indeed, a significant difference was found between hippocampus in idiopathic and structural epilepsy. Firstly, the area with positive CB1R immunostaining was smaller in dogs with idiopathic epilepsy in comparison to the other two groups. Surprisingly, the area of hippocampus of dogs with structural epilepsy expressing CB1R was larger and with significantly higher intensity of CB1R expression compared to idiopathic epilepsy and controls. This finding is similar to reports in experimental animals. Seizures itself can increase the density of CB1R in pyramidal cell layer (Karlocai et al. 2011; Magloczky et al. 2010; von Ruden et al. 2015). Reason for detecting high expression of the receptors in structural epilepsy could be the sprouting of new fibers of interneurons expressing CB1R (Karlocai et al. 2011). Another explanation would be that the ECS accounts for compensatory mechanisms by increased expression of receptors in certain subpopulations of cells in hippocampus (Chen et al. 2003). Both mechanisms might be involved in increased CB1R expression in dogs with structural epilepsy. However, there are contradictory explanations on how CB1 receptor expression in certain regions of hippocampus influence epilepsy and seizures. Namely, CB1 receptors can influence neuroexcitability via two mechanisms. Endocannabinoids could act on receptors at excitatory synapses and suppress seizures by inhibiting glutamate release (Bhaskaran and Smith 2010). Conversely, endocannabinoid signaling could promote seizures by inhibiting GABA release at inhibitory synapses (Katona et al. 1999). It is likely that this dichotomous behavior of CB1R serves ECS for
“fine-tuning” of synapses during development of epilepsy and seizures, but is also considered to be an anticonvulsive mechanism (Blair et al. 2015b).

The current study reveals interesting differences in intensity of CB1R expression between idiopathic and structural epilepsy with higher expression in CA3, hilus and DG in structural and downregulation in CA1 in idiopathic epilepsy patients. Difference in expression of CB1R has also been detected in experimental animals depending on the used model of epilepsy suggesting different mechanisms of seizure pathogenesis (Falenski et al. 2009; von Ruden et al. 2015; Wittner et al. 2017). In the present study, CB1R expression in CA1 is downregulated in dogs with idiopathic epilepsy. Similar trends are seen in animal models and humans with temporal lobe epilepsy (TLE) (Falenski et al. 2007). Specifically, research groups described reorganization of the CB1R in this type of epilepsy (Falenski et al. 2009) suggesting another defensive neuroplasticity mechanism of ECS to compensate for cell loss during hippocampal sclerosis present in TLE (Thom 2014). A limitation of our study was that due to quality of the archived tissue not all sub-regions of the hippocampus could be evaluated in detail, especially CA2. A possible reorganization of the receptors in idiopathic epilepsy group cannot be completely excluded. However, it was reported that in human TLE an overall downregulation of CB1R in hippocampus occurs (Ludanyi et al. 2008), which is supporting the current finding in dogs with idiopathic epilepsy. This research group also considered the type of fibers and concluded that the decrease of CB1R is mainly on the glutamatergic terminals maintaining hyperexcitability in TLE (Ludanyi et al. 2008).

Similarities between epileptic dogs and TLE in humans were also described regarding their cognitive deficit (Packer et al. 2018) and hippocampal asymmetry (Estey et al. 2017). These reports and the findings of CB1R downregulation in dogs with idiopathic epilepsy support the suggestion that dogs with idiopathic epilepsy mimic well human TLE.
CB1R presence is described on the axon terminals, interneuron fibers, on synapses and peri-somatic (Straiker and Mackie 2005). To examine its expression in dogs, co-expression of CB1R in neurons using the marker NeuN, on astrocytes characterized by GFAP staining, microtubules in axons stained with anti-TUBB3, and synaptic vesicles using SYP marker in dentate gyrus was evaluated. Firstly, healthy tissue was double labeled with CB1R and the described markers. However, no co-locations were found, apart from CB1R/GFAP leading to the assumption that detection of CB1R on neurons, microtubules and synaptic vesicles in hippocampus is not feasible in dogs using the described markers. On the other hand, more than half of astrocytes in DG expressed CB1R in healthy and epileptic dogs. Considering that astrocytes play an important role in epilepsy (Tian et al. 2005), the high number of astrocytes expressing CB1R in DG in idiopathic and structural epilepsy and in the control group of animals presents an interesting starting point for further evaluation. However, no significant difference was detected in the number of CB1 positive astrocytes in DG between epileptic dogs and the control group. However, a difference in number of CB1R positive astrocytes between hilus and molecular layer was detected in controls, a finding which could not be shown in epileptic dogs. Therefore, a certain reorganization of CB1R positive astrocytes is possible in epileptic dogs. Nevertheless, it is becoming clear that ECS plays an important role in the communication of the astrocytes with the surrounding neurons (Oliveira da Cruz et al. 2016). Communication was shown between presynaptic terminals, postsynaptic targets and associated astrocytes, which is called tripartite synapse (Araque et al. 1999) and endocannabinoids seem to be the key signal for that communication (Oliveira da Cruz et al. 2016). Astrocytes respond to endocannabinoids through activation of CB1R increasing intracellular calcium and stimulating the release of glutamate that modulates synaptic transmission and plasticity (Navarrete et al. 2014). In epilepsy, it is suggested that astrocytic glutamate
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release may play an epileptogenic role in initiation of epileptic seizures (Kang et al. 2005) and maintenance of epileptiform activity (Coiret et al. 2012). Regardless, very high expression of CB1R on astrocytes in canine DG represent an exciting possibility for further research and could provide more insight into mechanisms of epilepsy.

To summarize, we evaluated GFAP, IL-1β and CB1R as different types of biomarkers in epilepsy. Although GFAP in CSF did not reflect any specific disease category, it was increased in all patients with epilepsy, as well as in dogs with SCI, indicating structural changes in CNS parenchyma. In the CSF of the epileptic dogs, GFAP levels were higher in structural than in idiopathic epilepsy, supporting the assumption of astrocytic involvement in the respective reaction to tissue destruction. In addition, GFAP was measurable in serum only in cases with severe mechanical tissue damage. Despite unspecific elevation of CSF GFAP levels, we have detected highly elevated serum concentrations of GFAP in dogs with TBI. Furthermore, GFAP serum levels in TBI dogs could be associated with the outcome of the disease in the first 48 hours, supporting its potential utility as a biomarker.

IL-1β in serum of dogs with epilepsy was increased regardless of the cause of seizures, a finding which could be used for further therapy attempts. However, we could not detect IL-1β in CSF or make a connection between serum levels and seizures. Bearing that in mind, we suggest direct measurement of the IL-1β in brain parenchyma, to better understand its role in seizures and epilepsy. Also, among presumed idiopathic epilepsy cases, single dogs with very high levels of IL-1β, could in fact have immune-mediated epilepsy which needs more in-depth research. CB1R expression in canine hippocampus was increased in structural epilepsy and downregulated in canine idiopathic epilepsy patients. The distinct disease-associated CB1R expression is an important new aspect in the elucidation of epileptogenesis and has to be considered in further development of indirect new treatment approaches for dogs with epilepsy.
Indeed, high expression of CB1R in astrocytes offers new consideration for treatment approach by modulation of astrocytic activity.
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