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Epilepsy in veterinary science

Edited by
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Epilepsy in veterinary science

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Editorial: Epilepsy in veterinary science

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Editorial on the Research Topic
Epilepsy in veterinary science

Epilepsy is a unique manifestation of abnormal network excitability of the brain of humans and animals, which remains poorly understood, and, therefore, challenging to treat, presenting with an ongoing and frequently life-long predisposition for epileptic seizures. Epilepsy occurs in dogs with a similar population prevalence to humans. Broad etiologic classes are largely shared between the two species. Etiologies range from a few monogenic inherited genetic epilepsies with onset in childhood and juveniles, the presence of genetic risk variants and risk haplotypes in association with epilepsies without identifiable lesions on brain MRI (unknown etiology), and structural epilepsies, e.g., from brain tumors, post-traumatic epilepsy, and malformations of cortical development (1, 2).

Autoimmune encephalitis and autoimmune-associated epilepsy form a new distinct etiologic group in humans offering new treatment approaches. In humans, autoimmune encephalitis is frequently associated with neural antibodies, presents with new onset of epileptic seizures, psychiatric and behavioral symptoms, and subtle inflammatory CSF and MRI changes, and may cause new-onset refractory status epilepticus (NORSE) (3). Huenerfauth et al. contribute evidence for the involvement of GABA-A receptor antibodies in new-onset refractory status epilepticus (NORSE) in dogs and parallels to humans (4). Five different neural antibodies have been described in dogs up to now including this case report (NMDAR1, GFAP, GAD65, IgLON5, GABA-A receptor antibodies). Yet their clinical impact is not always as clear as for LGI1 antibodies in cats with limbic encephalitis (5, 6), and more research is necessary to explore disease associations. This is especially important considering the frequent occurrence and the efforts and challenges of the treatment of status epilepticus in dogs (7), and the potential for treatment with anti-inflammatory or immunosuppressive drugs.

There is an urgent need to develop and evaluate better therapies for canine epilepsy, considering the high proportion of dogs with idiopathic epilepsy (IE) and to drug-resistant epilepsy (8–10). The need to step forward in canine epilepsy is reflected by the manuscripts submitted to the Research Topic "*Epilepsy in veterinary sciences*." Twenty-two manuscripts were published on this Research Topic, 19 focused on canine epilepsy, and 18 on treatment aspects of epilepsy. Overall, the type of manuscripts ranged widely from single case reports to observational and explorative cohort studies (phenotype, biomarkers, physiologic responses, seizure detection), to reviews and prospectively controlled treatment studies. All contributed significantly to knowledge in the field. Most manuscripts of the Research Topic collection, "*Epilepsy in veterinary science*" explored treatment strategies and potential biomarkers for treating epilepsy in dogs. These manuscripts highlight the relevance of difficult-to-treat epilepsy in dogs and the need to develop novel treatment strategies for client-owned dogs with IE.

The genetic background of many dog breeds appears to predispose them to epilepsy with a severe clinical course. The appearance of tonic-clonic seizure in clusters appears as a point-of-no-return across many breeds (9). Breed-specific epilepsies in Greater Swiss Mountain dogs and Border Collies were the focus of three manuscripts of this article collection. All of them considered treatment response, survival, and quality of life an important aspect of the epilepsy description. First, Ostermann et al. confirmed a severe manifestation of IE with frequent tonic-clonic seizures in a large cohort of Greater Swiss Mountain dogs. In addition, they proved the frequent occurrence of cluster seizures and status epilepticus, significantly impacting survival time. Their finding aligned with previous studies on the association of cluster seizures with drug refractoriness (9). Santifort et al. explored a cohort of 116 Border Collies with IE and confirmed the previously found association between a younger age of onset and a more severe manifestation of epilepsy. This finding raises the question, of whether the age of onset should be more closely explored in dogs as a characteristic feature of epilepsy syndromes (11). It is a concern that a significant percentage of owners perceived the quality of life of their dogs as heavily impacted. Finally, Mauri et al. explored the role of 3T single voxel magnetic resonance spectroscopy to track changes in brain metabolites in these two breed-specific epilepsies (Border Collies, Greater Swiss Mountain dog) in a prospective cohort study. N-acetyl aspartate (NAA) was used as a marker for neuronal degeneration and glutamate-glutamine for neuronal excitability. Their results showed decreased glutamate-glutamine in thalamic regions in treated dogs compared to untreated dogs and controls. There is a need for more functional neuroimaging studies in veterinary epilepsy (see also review provided by Szabo and Salinas in this Research Topic).

Large breed dogs show IE more frequently than small breed dogs offering the chance to apply neurostimulation devices and strategies designed for use in humans. Previously, Charalambous et al. showed the potential efficacy of repetitive transcranial magnetic brain stimulation (rTMS) in drug-resistant dogs with IE (12). Yet more data are necessary for a targeted approach e. g., to define the targeted brain areas and changes in the canine brain networks in space and time. Interestingly, 2-deoxy-2-[^{18}F] fluoro-D-glucose positron emission tomography (18F-FDG-PET) could demonstrate differential effects of precisely targeted high-frequency rTMS on glucose metabolism in the pre-frontal cortex and hippocampus of healthy Beagle dogs (13). The upcoming potential of neurostimulation for the non-pharmacologic treatment of seizures in dogs is the focus of three manuscripts within the Research Topic "*Epilepsy in veterinary science*": A review by Nowakowska et al. summarizes current knowledge on available neurostimulation methods for treatment of drug-resistant epilepsy in dogs, and highlights repetitive transcranial magnetic and transcutaneous vagal nerve stimulation. Castillo et al. documented. changes in electroencephalography (EEG) power spectra, increased heart rate variability, and decreased heart rate after transcutaneous vagal nerve stimulation in healthy Beagle dogs. EEG was recorded with subdermal wire electrodes for1 h before and 1 h after transcutaneous vagal stimulation for 120 s with a device marketed for dogs. Further exploration of this approach in controlled studies is warranted. Notably, Hirashima, Saito, Igarashi et al. reported on the successful use and long-term follow-up of an implanted vagal nerve stimulator in a dog with drug-resistant IE. There was a significant (> 50%) and long-lasting decrease in tonic-clonic seizures but less impact on focal seizures. No side effects other than coughing occurred. The owner was also provided with an external magnet that could induce a vagal pulse that could prevent focal seizures' evolution to tonic-clonic seizures.

A series of three manuscripts provide insights into metabolic alterations of current treatment strategies with phenobarbital or medium-chain triglycerides (MCT) in dogs with IE: Watanagura et al. investigated alterations in the microbiota-gut-brain axis and behavioral changes that occurred in phenobarbital-treated dogs in a prospective cohort study. Analysis of fatty acids with gas chromatography mass spectrometry (GC-MS) indicated an association between the short-chain fatty acid butyrate and drug responsiveness in new-onset epilepsy (Watanagura et al.). This opens exciting research perspectives on fecal short-chain acids as potential biomarkers of drug responsiveness). Berk et al. explored the metabolite and neurotransmitter profiles in a prospective well-controlled study with a cross-over design in dogs with IE and documented changes in amino acids, fatty acids, and neurotransmitters associated with dietary MCT oil supplements. Furthermore, the ß-hydroxybutyrate-triacylglyceride ratio showed an overall negative correlation with seizure frequency. In support of this study, Schmidt et al. further explored altered urinary neurotransmitter profiles in dogs with IE.

Lastly, the Research Topic "*Epilepsy in veterinary science*" includes also studies, which provide data on the potential efficacy of new treatment strategies in dogs with drug-resistant IE. Polidoro et al. investigated the pharmacokinetics of an intranasal, rectal and oral administration of a cannabidiol (CBD) formulation in six healthy Beagle dogs. Fischer et al. explored the efficacy of a cyclooxygenase-2 inhibitor (firocoxib) as an add-on treatment in dogs with phenobarbital-resistant epilepsy in a translational pilot study. The research hypothesis originated from rodent data on seizure-induced induction of p-glycoprotein at the blood-brain barrier. Considering, that only the two dogs with the highest seizure frequency were partial responders with a >50% decrease in seizures (18.2% per protocol, 11.8% intent-to-treat) argued against the overall efficacy of the add-on treatment (Fischer et al.). Subsequently, Kriechbaumer et al. introduced a time-to-event study design for dogs with drug-resistant epilepsy. Thereby dogs participated in the study only until their pre-defined clinical trial endpoint (n-th seizure). This protocol aimed to reduce the study duration of non-responders. The efficacy evaluation follows International Veterinary Epilepsy Task Force (IVETF) suggestions (14). The International League Against Epilepsy regulatory task force and pediatric commission proposes a similar approach where children participate until the same number of seizures occurs as in a predefined baseline period (15–17). Both canine studies demonstrate a lack of relevant placebo responses in drug-resistant dogs, contrary to previous findings (18).

Other manuscripts explored current medical treatment strategies in dogs with reactive (acute symptomatic) seizures or IE: efficacy of levetiracetam as first-line treatment of presumed reactive seizures from exogenous poisoning by Stabile et al., variables with a potential influence on treatment with bromides in 220 dogs from

the Netherlands by Lichtenauer et al.; aggressive behavior as an adverse event of zonisamide by Kanazono et al., the approach of primary care UK veterinarians toward the management of canine IE by Griffin et al..

The call for papers for the Research Topic "*Epilepsy in veterinary science*" addressed originally also other animal species than dogs. Spontaneous epilepsy has been reported in many other animal species, and animal models are widely used as research tools in epilepsy. Yet contributions from different species were limited to three manuscripts on cats and baboons. Hasegawa, Asada, Hamamoto et al. and Hasegawa, Asada, Mizuno et al. report on their efforts to cure drug-resistant epilepsy in two cats with epilepsy surgery. Their approaches were individualized to the cats' epilepsy type. Hippocampectomy was chosen in one cat, and corpus callosotomy in the other cat (Hasegawa, Asada, Hamamoto et al. and Hasegawa, Asada, Mizuno et al.). The manuscripts highlight the challenges of identifying the epileptogenic zone in chronic drug-resistant epilepsy, even if structural lesions are visible on MRI. Szabo and Salinas provide a review of neuroimaging in baboons. Baboons with spontaneous epileptic seizures are a large animal model for idiopathic generalized epilepsies in humans and photosensitivity. A similar genetically defined generalized idiopathic epilepsy has been described in Rhodesian Ridgebacks (19). The review provides insights into the perspectives of structural and functional neuroimaging in a large animal species. While structural imaging is usually standard in individual animals with idiopathic generalized epilepsies, statistical approaches can identify gray matter volume/concentration changes. Functional neuroimaging can map epileptic networks, altered functional connectivity of physiological networks, photoepileptic responses, and the effects of anti-seizure therapies (Szabo and Salinas).

Research for better treatment of epilepsy in dogs and cats is driven by the strong human-animal bond, the efforts, and the psychological and emotional stress owners face when caring for an animal with epilepsy beyond the financial issues involved. The power of this human-animal bond can drive efforts to better our treatment armory of epilepsy. Novel seizure detection devices will provide objective data on tonic-clonic seizure counts, which can supplement the reports of the owners in treatment studies in veterinary epilepsy. The data provided by Hirashima, Saito, Kuriyama et al. show the potential of this approach. Like in human epilepsy, in addition to drug treatment, the new era of epilepsy treatment in pets also includes dietary, neurostimulation, and surgical options. The hope is that the convergence of human and veterinary research needs will enhance awareness and funding of epilepsy research in dogs and cats (1).

Author contributions

AF drafted the work. HV provided review and advice. Both authors are responsible for the content. Both authors contributed to the article and approved the submitted version.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Focal Cortical Resection and Hippocampectomy in a Cat With Drug-Resistant Structural Epilepsy

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Epilepsy surgery is a common therapeutic option in humans with drug-resistant epilepsy. However, there are few reports of intracranial epilepsy surgery for naturally occurring epilepsy in veterinary medicine. A 12-year-old neutered male domestic shorthair cat with presumed congenital cortical abnormalities (atrophy) in the right temporo-occipital cortex and hippocampus had been affected with epilepsy from 3 months of age. In addition to recurrent epileptic seizures, the cat exhibited cognitive dysfunction, bilateral blindness, and right forebrain signs. Seizures had been partially controlled (approximately 0.3–0.7 seizures per month) by phenobarbital, zonisamide, diazepam, and gabapentin until 10 years of age; however, they gradually became uncontrollable (approximately 2–3 seizures per month). In order to plan epilepsy surgery, presurgical evaluations including advanced structural magnetic resonance imaging and long-term intracranial video-electroencephalography monitoring were conducted to identify the epileptogenic zone. The epileptogenic zone was suspected in the right atrophied temporo-occipital cortex and hippocampus. Two-step surgery was planned, and a focal cortical resection of that area was performed initially. After the first surgery, seizures were not observed for 2 months, but they then recurred. The second surgery was performed to remove the right atrophic hippocampus and extended area of the right cortex, which showed spikes on intraoperative electrocorticography. After the second operation, although epileptogenic spikes remained in the contralateral occipital lobe, which was suspected as the second epileptogenic focus, seizure frequency decreased to <0.3 seizure per month under treatment with antiseizure drugs at 1.5 years after surgery. There were no apparent complications associated with either operation, although the original neurological signs were unchanged. This is the first exploratory study of intracranial epilepsy surgery for naturally occurring epilepsy, with modern electroclinical and imaging evidence, in veterinary medicine. Along with the spread of advanced diagnostic modalities and neurosurgical devices in veterinary medicine, epilepsy surgery may be an alternative treatment option for drug-resistant epilepsy in cats.

Keywords: cat, drug-resistant epilepsy, electroencephalography, electrocoricography, epileptogenic zone, epilepsy surgery, magnetic resonance imaging, video-EEG

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INTRODUCTION

Epilepsy is a common functional cerebral disorder in cats and dogs. At present, treatment of canine and feline epilepsy is almost limited to antiseizure drug (ASD) therapy. Although most feline patients with epilepsy respond to ASD therapy and have a good prognosis, 20–39% of cases do not respond to ASDs and are referred to as drug-resistant epilepsy (DRE), which results in a poor outcome and quality of life (1–4). DRE is estimated to exist in approximately 30% of human and canine epilepsy patients (5, 6).

In human medicine, surgical treatment, so-called "epilepsy surgery," has been established as a common strategy for patients with DRE (7, 8). In order to plan for epilepsy surgery, various evaluations, so-called "presurgical evaluations," must be performed to localize the epileptogenic zone (historically known as the "focus"). The epileptogenic zone is conceptually defined as the minimum amount of cortex that must be resected or disconnected to produce seizure freedom, which is considered from the results of presurgical evaluations. Five abnormal zones, that is, symptomatogenic, irritative, seizure-onset, structurally abnormal, and functional deficit zones, are essential to presume the epileptogenic zone, and are investigated by specific modalities (9). Epilepsy surgery includes three methodologies, that is, resection, disconnection, and neuromodulation, such as vagus nerve stimulation and deep brain stimulation. Resection surgery is the most curative method, which involves the direct removal of the epileptogenic zone, and is applied for focal (localized-related) epilepsy. Especially, anterior temporal lobectomy or selective amygdalohippocampectomy is commonly performed for intractable mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis and shows a good postoperative outcome (10).

A recent survey of research priorities for idiopathic epilepsy in dogs revealed that non-ASD management, including epilepsy surgery, is highly expected by dog owners, general practice veterinarians, and veterinary neurologists (11). However, research on epilepsy surgery in the veterinary field is generally limited to experimental studies, with the majority concerning neuromodulation (12–17). There are some case reports regarding intracranial surgery in canine patients with seizures, but most of those lack modern imaging or electrodiagnostic evidence (18–21).

Feline temporal lobe epilepsy (FTLE), which resembles human MTLE, is the most commonly encountered epilepsy syndrome in cats (3, 22, 23). In fact, previous basic studies in human MTLE had progressed using feline seizure models such as kindling and kainic acid models (22). Under such a background, hippocampectomy, a type of resection surgery, would be naturally expected to be a surgical option for drug-resistant FTLE. Methodologies to detect the epileptogenic zone in FTLE using familial epileptic cats (24–29) and a cadaveric study of feline hippocampectomy (17) have been reported.

Recent technological advances, for example, digital electroencephalography (EEG) with video monitoring and high-field and advanced magnetic resonance imaging (MRI), and practical experiences of canine and feline brain surgery using a surgical microscope, ultrasonic aspirator, and neuronavigator, suggest that presurgical evaluations of the epileptogenic zone and epilepsy surgery could also be performed in veterinary medicine. On the basis of these backgrounds, we report a feline case with structural DRE that underwent intracranial epilepsy surgery with presurgical evaluations and its 1.5-year follow-up.

CASE PRESENTATION

A 12-year-old neutered male domestic shorthair cat had suffered from DRE for at least 2 years.

History

The cat was rescued by a local veterinarian at approximately 3 months of age. Since recurrent epileptic seizures and bilateral blindness were noticed, the cat was introduced to the authors' laboratory to investigate the underlying cause. At this time (when the cat was 3 months old), neurological examinations revealed walking toward the right (right circling in a large room), subtle hypermetric gait, decreased postural responses on the left side, and a bilateral deficit of the menace response with a normal pupillary light reflex, suggesting a forebrain lesion (more severe in the right hemisphere). Seizure type was focal seizures that consisted of behavioral arrest, star-gazing, facial twitching, mydriasis, salivation, circling, falling, and then (sometimes) evolving to generalized tonic-clonic seizures (GTCS) (**Supplementary Video 1**). Seizure duration was 30–60 s (focal seizures) to 2–3 min (if evolving to GTCS). General physical examinations, complete blood count, serum chemistry, blood gas, electrolytes, and urinalysis were within the reference intervals, and thoracic and abdominal radiographs were normal. Detection tests for FeLV (antigen), FIV (antibody), FCoV (PCR), and *Toxoplasma gondii* (PCR) were negative. Conventional MRI (1.5 Tesla) including T1-weighted (T1W) with/without contrast enhancement, T2-weighted (T2W), and fluid-attenuated inversion recovery (FLAIR) imaging of the brain revealed atrophy of the temporo-occipital cortex and hippocampus in the right hemisphere with dilation of the ipsilateral lateral ventricle and subtle dilation of the contralateral (left) lateral ventricle. However, the atrophic lesions had no obvious change in signal intensity on each sequence. There was no other structural abnormality or abnormal contrast enhancement in other regions. The results of cerebrospinal fluid analysis were also within the reference range. These clinicopathologic findings led to the diagnosis of structural epilepsy with right hippocampal and temporo-occipital cortical atrophy. As to the cause of the structural abnormalities, a perinatal cerebral vascular accident was suspected; however, it remains a matter of speculation. After diagnosis, the cat was transferred to our laboratory because it was difficult to keep it in an ordinary household.

The cat was accepted into our laboratory as a rare case for treatment and research with ethical approval from the Institutional Animal Care and Use Committee and Animal and Human Biology Research Ethics Committee of the Nippon Veterinary and Life Science University (accession numbers; 27S-60, 28K-6, 29K-2, and 2019K-1). After diagnosis, ASD

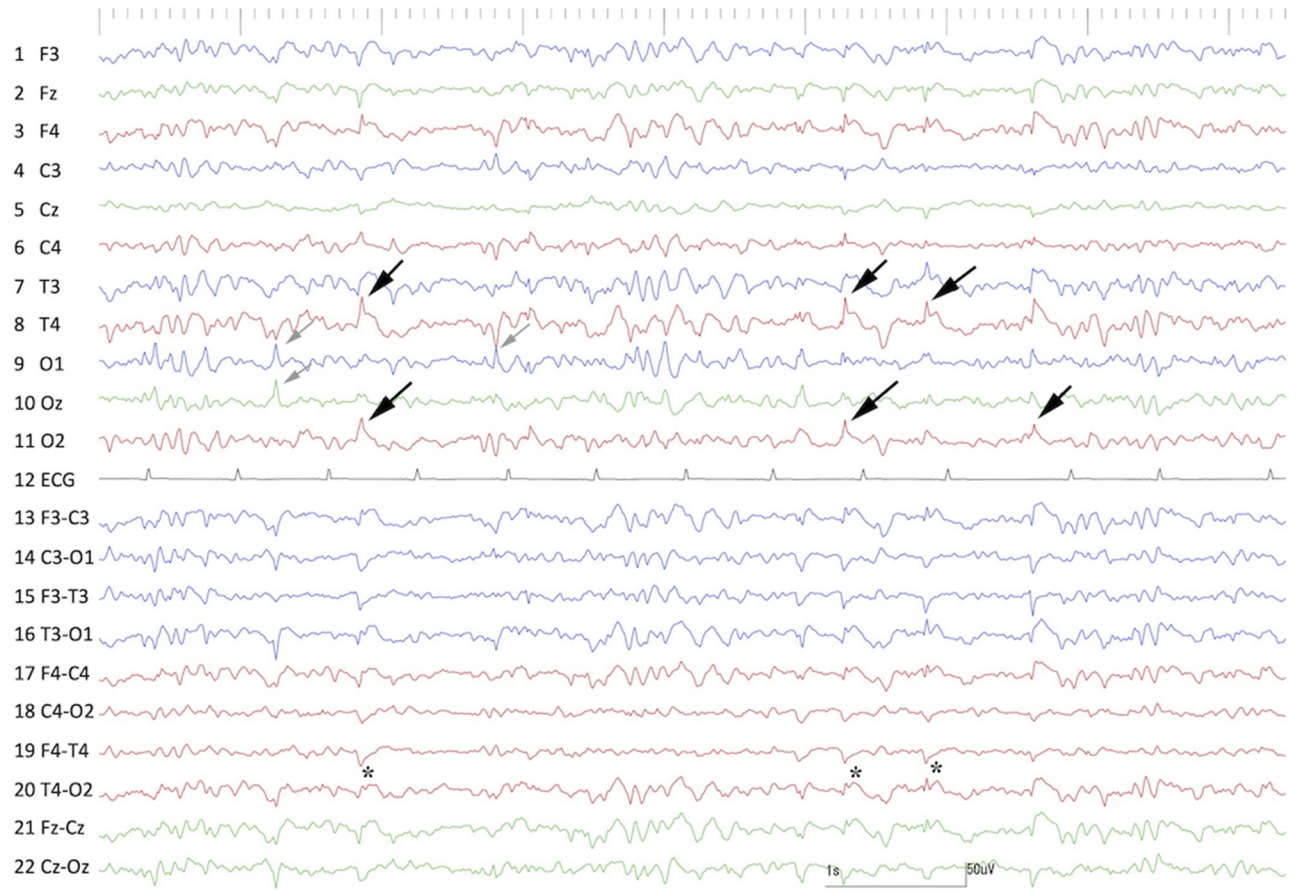

FIGURE 1 | Preoperative interictal scalp EEG under sedation with medetomidine. Remarkable spikes were observed frequently at T4 and O2 (black arrows) with phase reversal (*) at T4. Sporadic spikes were observed at O1 and Oz (gray arrows). Channels 1–11 were monopolar, and channels 13–22 were bipolar montages. Channel 12 was electrocardiography.

therapy with phenobarbital was started and maintained (2–4 mg/kg, q12h, PO; serum concentration 19–30.5 μg/ml) for 1 year with 1–2 seizures per 3 months (1–2 sz/3 m). Until the cat was 10 years old, seizure severity and frequency often deteriorated, and ASD therapy was maintained while dosing up and/or adding other drugs including diazepam, zonisamide, and gabapentin to control seizure frequency to <1 seizure per month (sz/m). However, after 10 years of age, the seizures could not be controlled (2–3 sz/m) even using multiple ASDs (finally, phenobarbital 2 mg/kg, q12h; diazepam 0.8 mg/kg, q12h; zonisamide 15 mg/kg, q12h; and gabapentin 15 mg/kg, q8h). The seizures were often clustered and/or developed to status epilepticus, and diazepam (1.0–2.0 mg/kg, IV) and levetiracetam (20 mg/kg, q8h, PO or IV) were also administered at that time. Most seizures were the same type as mentioned above, but sometimes they initiated from bilateral mydriasis and facial clonus (without behavioral arrest or star-gazing) and evolved to GTCS. The ratio of occurrence of this minor seizure type to the main seizure type was approximately 4:1. Furthermore, the cat developed severe cognitive dysfunction. During these years, physical and neurological examinations, blood tests, EEG examinations, and MRI scans were performed 1–2 times per year, but there was no apparent change, except for recurrent cystitis, which was treated adequately. Ovarian hysterectomy was performed at the time of MRI at 1 year of age. Combined vaccinations (Fel-O-Vax® 5; Zoetis Japan, Tokyo, Japan) were administered every 3 years.

When the cat reached the age of 12 years, we decided to perform epilepsy surgery due to the following reasons: (1) the seizures frequently clustered and developed into status epilepticus; (2) further ASDs might not be effective; (3) the cost for medications was increasing constantly; and (4) preparations to perform intracranial EEG and epilepsy surgery were made in our previous studies of epilepsy (24–29), and a video-EEG monitoring system and surgical microscope were installed in our laboratory.

Presurgical Evaluations

After the decision to perform epilepsy surgery was taken (2 weeks before surgery for intracranial electrode placement), neurological and physiological examinations, complete blood count, serum chemistry, electrocardiography, and indirect blood pressure were

reevaluated. There was no change in the neurological findings from those mentioned above and they suggested mainly a right forebrain lesion with bilateral blindness and severe cognitive dysfunction. Blood tests remained within the reference range. There was no specific change on electrocardiography or blood pressure measurement.

Preoperative scalp EEG (Neurofax EEG-1200; Nihon Kohden, Tokyo, Japan) with subdermal wire electrodes was performed for 30 min under sedation with medetomidine (20 μg/kg, IM). The EEG recording conditions are described in **Supplementary Data 1**. Isolated spikes or sharp-waves were observed on the right temporal (T4) and occipital (O2) regions dominantly and frequently, but sometimes they were also observed on the left occipital region (O1) (**Figure 1**).

Preoperative MRI was performed with a 3.0 T MRI system (Signa HDxt; GE Healthcare, Tokyo, Japan) under general anesthesia, which was induced by propofol and maintained with isoflurane. Obtained images included 3D T1W (spoiled gradient echo with IR preparation) with/without contrast enhancement (IV administration of gadodiamide), 3D T2W (3D T2 Cube), transverse plane FLAIR, T2*-weighted (T2*W), diffusion-weighted imaging (DWI), diffusion tensor imaging (15 axes), and dynamic susceptibility contrast perfusion MRI (27, 28). The MRI sequences are summarized in **Supplementary Data 1**. 3D T2W images were used for hippocampal volumetry by the semiautomatic region of interest method (26), and 3D T1W images were also used for statistical analysis using voxel-based morphometry (29). As described above, MRI revealed the right hippocampal and temporo-occipital atrophy without apparent signal changes on T1W, T2W, FLAIR, and contrast-enhanced T1W imaging (**Figure 2**). The volumes of the right and left hippocampi were measured by 3D volumetry using 3D T2W imaging as 0.119 and 0.24 cm^3, respectively (reference range of the feline unilateral hippocampus is 0.227 ± 0.02 cm^3) (**Figure 3A**) (26). Voxel-based morphometry analysis revealed a significantly decreased volume of the right hippocampus and temporo-occipital cortex (**Figure 3B**) and increased volume of the right lateral ventricle compared with reference feline brains (29). There was no evidence of hemorrhage on T2*W images. Isotropic DWI showed high signal intensity on both hippocampi and the right amygdala and lateral temporo-occipital cortex, but those apparent diffusion coefficient values were not lower than in the other cortex (**Figure 4**).

On the basis of the results of ictal symptoms, scalp EEG, and structural MRI, we suspected that the epileptogenic zone (symptomatogenic, irritative, and structurally abnormal zones) included, at least, the right atrophic hippocampus and temporo-occipital cortex. To confirm and investigate the "seizure-onset zone," we performed long-term intracranial EEG (iEEG) monitoring with synchronous video recording, so-called intracranial video-EEG (iVEEG).

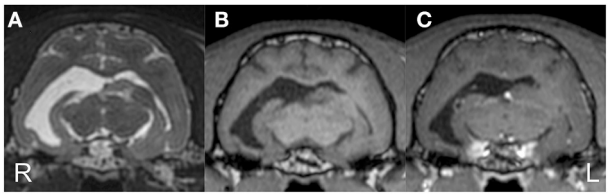

FIGURE 2 | Preoperative structural MRI. Transverse T2-weighted **(A)**, T1-weighted **(B)**, and contrast-enhanced T1-weighted **(C)** images at the level of the medial geniculate body. The right temporo-occipital cortex and hippocampus were markedly atrophied with an extended ipsilateral lateral ventricle. The contralateral (left) ventricle was also slightly dilated.

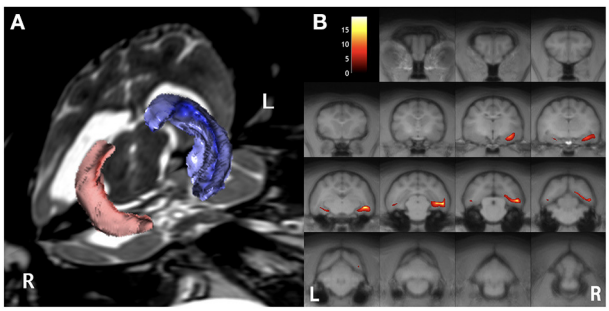

FIGURE 3 | Preoperative imaging analysis of structural MRI. Volume rendering for hippocampal volumetry **(A)** and the result of statistical voxel-based morphometry analysis for gray matter **(B)**. The volume of the right (red) hippocampus was 0.12 cm^3, whereas the left (blue) was 0.24 cm^3 **(A)**. On the map of voxel-based morphometry analysis **(B)**, the colored regions indicate significantly different areas compared with 12 healthy controls. Note, the left (L)/right (R) direction of voxel-based morphometry was different from the conventional direction.

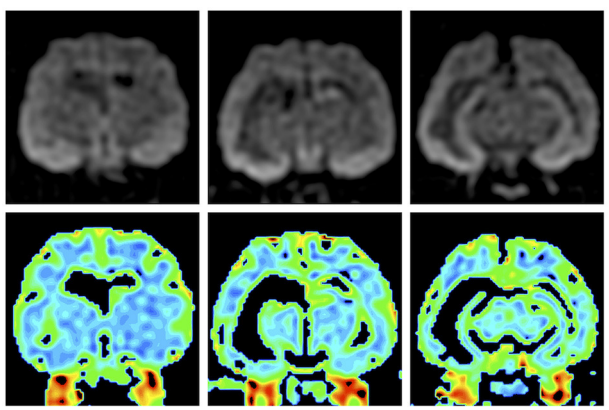

FIGURE 4 | Preoperative isotropic diffusion-weighted imaging (upper row) and colored apparent diffusion coefficient maps (lower row). Bilateral hippocampi and the right amygdala and temporo-occipital cortex showed hyperintensity, but there were no significant changes on the apparent diffusion coefficient map.

Preparation for iVEEG Monitoring

To place the intracranial electrodes, anesthesia was induced with propofol and maintained with isoflurane and oxygen inhalation. A constant rate of fentanyl infusion (0.1–0.3 μg/kg/min) was administered for analgesia during the operation. Under general anesthesia, the head of the cat was fixed in a stereotaxic frame (Model 1430M; David Kopf Instruments, Tujunga, CA, USA), and computed tomography (Aquilion PRIME; Canon Medical

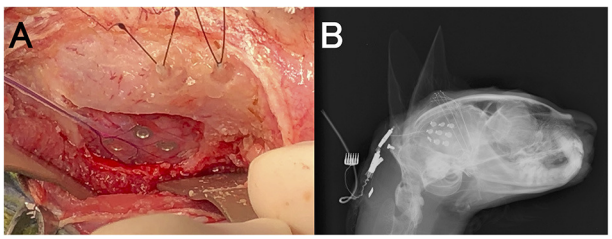

FIGURE 5 | Placement of intracranial electrodes. **(A)** Implantation of a 9-channel ECoG grid (on the dura covering the temporo-occipital cortex) and depth electrodes (into the hippocampus and amygdala). **(B)** Lateral radiograph after implantation of intracranial electrodes.

Systems, Tochigi, Japan) and MRI for stereotactic depth electrode insertion were performed. After those scans, the coordinates of the right and left amygdala and ventral portions of the hippocampi were measured, and the cat was moved and set on the operating table. After shaving and sterilization, a U-shaped incision beyond the midline was made on the right scalp and the right temporal muscle and a part of the left temporal muscle were detached from the skull. Small burr holes were made by marking the points on the skull over the extended line of the coordinates for the bilateral amygdala and hippocampus. Order-made epoxy-coated stainless depth bipolar electrodes (0.4 mm diameter and 25 mm length; Unique Medical, Tokyo, Japan) were inserted stereotactically into the bilateral amygdala and hippocampus according to those coordinates (**Supplementary Data 1**). Craniectomy was performed on the right temporal bone, and the dura on the temporo-occipital area was exposed. We placed order-made ECoG electrodes (1.5 cm × 1.5 cm grid-type silicon-sheet electrodes with 9 exploration electrodes, 5.0 mm interval; Unique Medical) on the dura mater (i.e., epidural space) overlying the atrophic temporo-occipital cortex (**Figure 5A**) (**Supplementary Data 1**). A reference screw electrode was placed in the external occipital protuberance and an internal ground was implanted subcutaneously in the back of the neck. The depth and ECoG electrodes were connected to the lead line, which was tunneled subcutaneously to the nape (**Figure 5B**). The temporal muscle and skin were sutured according to the usual method. The lead was connected to the EEG machine to confirm the connections and the ability for EEG measurement. After that, anesthesia was stopped, and the cat recovered normally. A fentanyl patch (2.5 mg/sheet/head) was used for 3 days, and cefazoline (25 mg/kg, BID) was administered for 14 days after surgery.

Long-Term iVEEG Monitoring

From 3 days after electrode implantation surgery, 24-h continuous iVEEG was monitored within an epilepsy monitoring cage (**Supplementary Data 1**) for 18 days. During this monitoring period, ASDs were limited to phenobarbital and diazepam (i.e., zonisamide and gabapentin were discontinued). Although the cat with the lead line was connected to the EEG machine via a slip-ring connector, it could move freely in the monitoring cage. Recorded iVEEG data were stored in the internal hard disk of the EEG machine.

Unfortunately, at the initiation of iVEEG monitoring, disconnection of the depth EEGs was noted. Therefore, iVEEG recording was limited to ECoG of the right temporo-occipital area. On interictal ECoG, interictal spikes were dominantly observed on electrodes #1, 3, and 9. During the monitoring period, we observed three spontaneous focal seizures evolving into GTCS. These three seizures and their EEG patterns were very similar. Typically, the cat suddenly stopped his behavior and/or gazed upward with low-voltage and high-frequency activity (suppression background activity) on all ECoG electrodes for approximately 20 s, and rhythmic (10–13 Hz) epileptiform activity with waxing amplitude started from electrode #1, which spread rapidly (<0.5 s) to the surrounding electrodes. At this time, the cat showed ventroflexion of the head, head turning and circling to the left, and facial twitching with more large amplitude discharges, which developed into GTCS (1–2 min). The amplitude and frequency of the epileptiform activity decreased gradually along with seizure termination; the cat and ECoG recovered to the interictal phase (**Figure 6** and **Supplementary Video 1**). We speculated that the first low-voltage and high-frequency activity on ECoG (arresting behavior and star-gazing) represented subcortical seizure activity, that is, hippocampal seizure, and the activity spread to the temporal cortical area, finally developing into a generalized seizure.

Identification of the Suspected Epileptogenic Zone and Surgical Strategy

Five conceptual abnormal zones (9), that is, symptomatogenic, irritative (EEG abnormal), seizure-onset, structurally abnormal, and functional deficit zones, all of which were suspected from the findings of each preoperative examination mentioned above, are summarized in **Table 1**. These results allowed us to identify the suspected epileptogenic zone in the right atrophic hippocampus and temporo-occipital cortex underlying ECoG electrodes #1, 3, and 9. According to the feline brain atlas (30), the temporo-occipital cortex showing interictal epileptiform discharges (electrodes #1, 3, and 9) is distributed in the lateral part of the ectosylvian and ectomarginal gyri.

Although our presurgical evaluations of the cat did not detect the epileptogenic zone as a single focus, it was suspected in the right atrophic hippocampus and/or temporo-occipital cortex. According to human epilepsy surgery strategy (8), if the epileptogenic zone of this cat was present in the right atrophic hippocampus, "MTLE" would be indicated and hippocampectomy would be applied. If the epileptogenic zone presented in the right atrophic temporo-occipital cortex, indicating "extra-temporal or temporal neocortical (lateral temporal lobe) epilepsy," the cat would be a candidate for focal cortical resection. If both, then both resection surgeries would be needed. Therefore, we planned a two-step surgical approach, that is, cortical resection of the temporo-occipital cortex at first, and then hippocampectomy if the seizures could not be controlled.

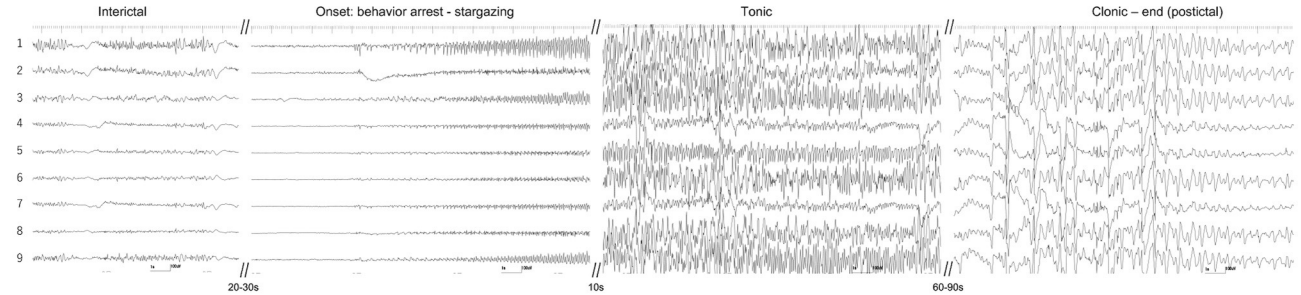

FIGURE 6 | Ictal ECoG of a spontaneous focal limbic seizure evolving into a generalized tonic-clonic seizure. Interictal spikes were mainly observed at electrodes #1 and 9. Seizure activity began as a low-voltage fast wave in all electrodes and the rhythmic discharge started from electrodes #1, 3, and 9. It evolved into a generalized tonic-clonic seizure. See also the movie of intracranial video-EEG (**Supplementary Video 1**).

TABLE 1 | Suspected epileptogenic zone of the present case.

Abnormal zone	Location	Modality
Symptomatogenic	Hippocampus	Ictal movie
Irritative (EEG abnormal)	Right temporal (T4), occipital (O2), and left occipital (O1)	Interictal scalp EEG
Seizure-onset	Hippocampus to right temporo-occipital cortex	Ictal video EEG (iVEEG monitoring)
Structurally abnormal	Right atrophic hippocampus and temporo-occipital cortex	Structural MRI
Functional deficit	Right forebrain, bilateral occipital lobes	Interictal neurological examination

EEG, electroencephalography; iVEEG, intracranial video-electroencephalography; MRI, magnetic resonance imaging.

The 1st Surgery: Focal Cortical Resection

At first, we planned to remove the focal temporo-occipital cortex to observe whether cortical resection alone would suppress the seizures. On the 19th day after the electrode-placing surgery, the cat was anesthetized and administered analgesics as in the previous operation. Besides, levetiracetam (20 mg/kg, IV) was administered preoperatively.

After aseptic treatment, the right temporal muscle was detached gently and the disconnected depth electrodes were removed from the brain (the thin leads had broken at the joint of the stainless electrodes). Then, the position of each ECoG electrode on the dura overlying the temporo-occipital region was pictured and the electrodes were removed. Using a surgical microscope (OPMI 6-CH; Carl Zeiss Meditec, Tokyo, Japan), a U-shaped dural incision was made, and a part of the temporo-occipital cortex was exposed. Matching the picture of the position of ECoG electrodes, focal cortical resection was performed carefully to aspirate only gray matter and leave white matter with bipolar cautery and suction (**Supplementary Video 2**). However, due to the atrophic and thin cortex, some white matter was also removed. The dura, temporal muscle, and skin were sutured. Then, the cat was awakened. Levetiracetam (20 mg/kg, TID, PO) was administered for 1 week postoperatively, and maintenance ASDs (phenobarbital and diazepam) were given continuously. Analgesics and antibiotics were also used postoperatively as in the previous surgical procedure.

Seizure Outcome After the 1st Surgery

Although the primary neurological deficits (right forebrain signs, bilateral blindness, and cognitive dysfunction) were unchanged, there were no significant postoperative adverse effects or complications. Moreover, for 66 days after surgery, seizure freedom was achieved under maintenance ASD therapy (phenobarbital 2 mg/kg, q12h and diazepam 0.7 mg/kg, q12h). At 1 month (30 days) after surgery, scalp EEG and MRI were performed. On scalp EEG, frequent spikes and polyspikes were still observed on the right occipital region (O2), and less frequent spikes were also observed on the left occipital region (O1) (**Figure 7**). There was no significant change around the resected cortex on MRI. However, at 67 days after surgery, focal seizures evolving into GTCS were clustered (3 sz/d), which were similar to those observed preoperatively, so it was considered that the seizures also originated from the remaining cortex and/or atrophic hippocampus. Therefore, we prepared for the second operation as initially planned. Finally, two more seizures were counted; therefore, a total of five seizures were observed between the first and second surgeries (5 sz/85 days, 1.8 sz/m on average).

The Second Surgery: Hippocampectomy and Additional Cortical Resection

At 86 days after the first surgery, the second surgery, consisting of additional cortical resection and total hippocampectomy of the right side, was performed. Anesthesia and presurgical and postsurgical medications were the same as in the first surgery.

After the skin incision, by detaching the temporal muscle and dural incision while paying attention to adhesions, the previous operation site, that is, partially resected temporo-occipital cortex, was explored. The site of the resected cortex was covered by pale and spiderweb-like pia and arachnoid

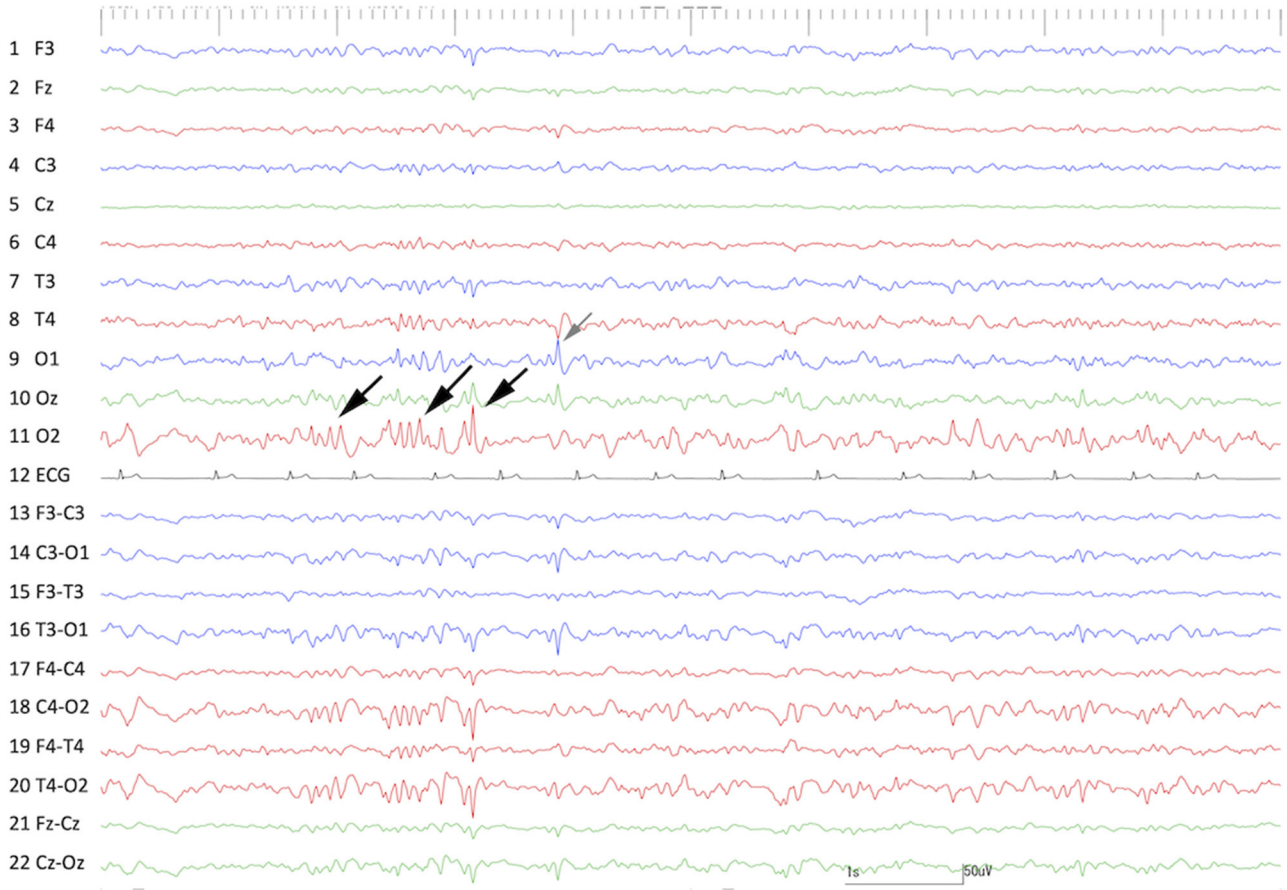

FIGURE 7 | Interictal scalp EEG at 30 days after the 1st surgery (cortical resection). Frequent spikes and polyspikes were observed at O2 (black arrows) and less frequent spikes were also recognized at O1 (gray arrow).

maters. After the abruption of those tissues, the underlying white matter was observed. By removing the white matter with suction, the extended lateral ventricle was opened, and the atrophic, slightly wrinkled, and faded hippocampus was observed (**Figure 8A** and **Supplementary Video 3**). As the hippocampus is a banana-shaped longitudinal structure extending dorsomedial to ventromedial along the inner aspect of the lateral ventricle, it was divided at the middle, and the dorsal and ventral portions were removed separately.

Initially, the ventral part was slightly rotated medially to visualize the hippocampal head. The border between the hippocampus and amygdala was unclear; therefore, the tip of the hippocampal formation, that is, hippocampal tuberculum, was cauterized. Then, the middle site of the hippocampal body was dissected by bipolar cautery, avoiding the choroid plexus. The ventral portion of the hippocampus was rotated laterally and the medial aspect, including fimbria, was detached from the inner arachnoid, choroid plexus, and blood vessels, and the ventral portion was removed from the lateral ventricle (**Supplementary Video 3**).

The dorsal end of the hippocampus was dissected by bipolar cautery proximally to the midline, that is, the dentate gyrus tubercle, and the dorsal part of the hippocampal body was peeled away from the medial structures along the fimbria and finally removed from the lateral ventricle (**Supplementary Video 3**). While the dorsal part was being removed, the dorsolateral aspect of the medial geniculate body and caudal cerebral artery were observed medially (**Figure 8B**).

After intraventricular handling, intraoperative ECoG was recorded on the residual temporo-occipital cortex to confirm the remaining spikes, and additional resections were performed. The resected cortex and hippocampus were submitted for histopathological evaluation. Finally, the intracranial surgical site was covered with artificial dura (GORE-TEX® ePTFE patch; W.L. Gore & Associates, Newark, DE, USA) after repeated confirmation of hemostasis. It was sealed with factor XII and fibrinogen adhesion (Beriplast P combi-set tissue adhesion; CSL Behring, Tokyo, Japan). The temporal muscle and scalp were closed as usual. The cat recovered without any problems, and postoperative levetiracetam was administered for 1 week and

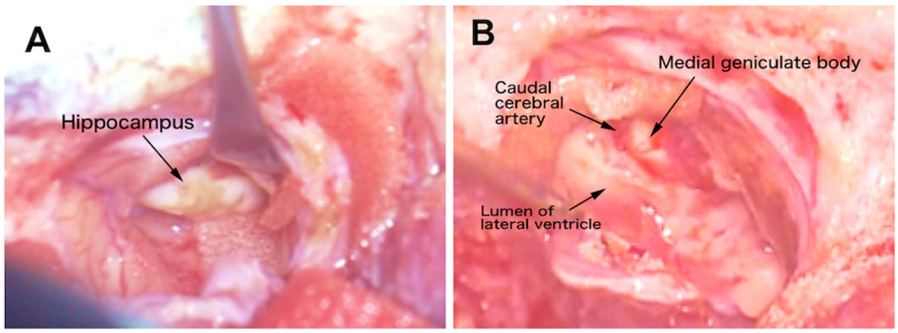

FIGURE 8 | Video captures of the intraoperative microsurgical operation site. **(A)** The appearance of the atrophic hippocampus. **(B)** After hippocampectomy. The caudal cerebral artery and medial geniculate body were seen medially. See also the operation movie shown in **Supplementary Video 3**.

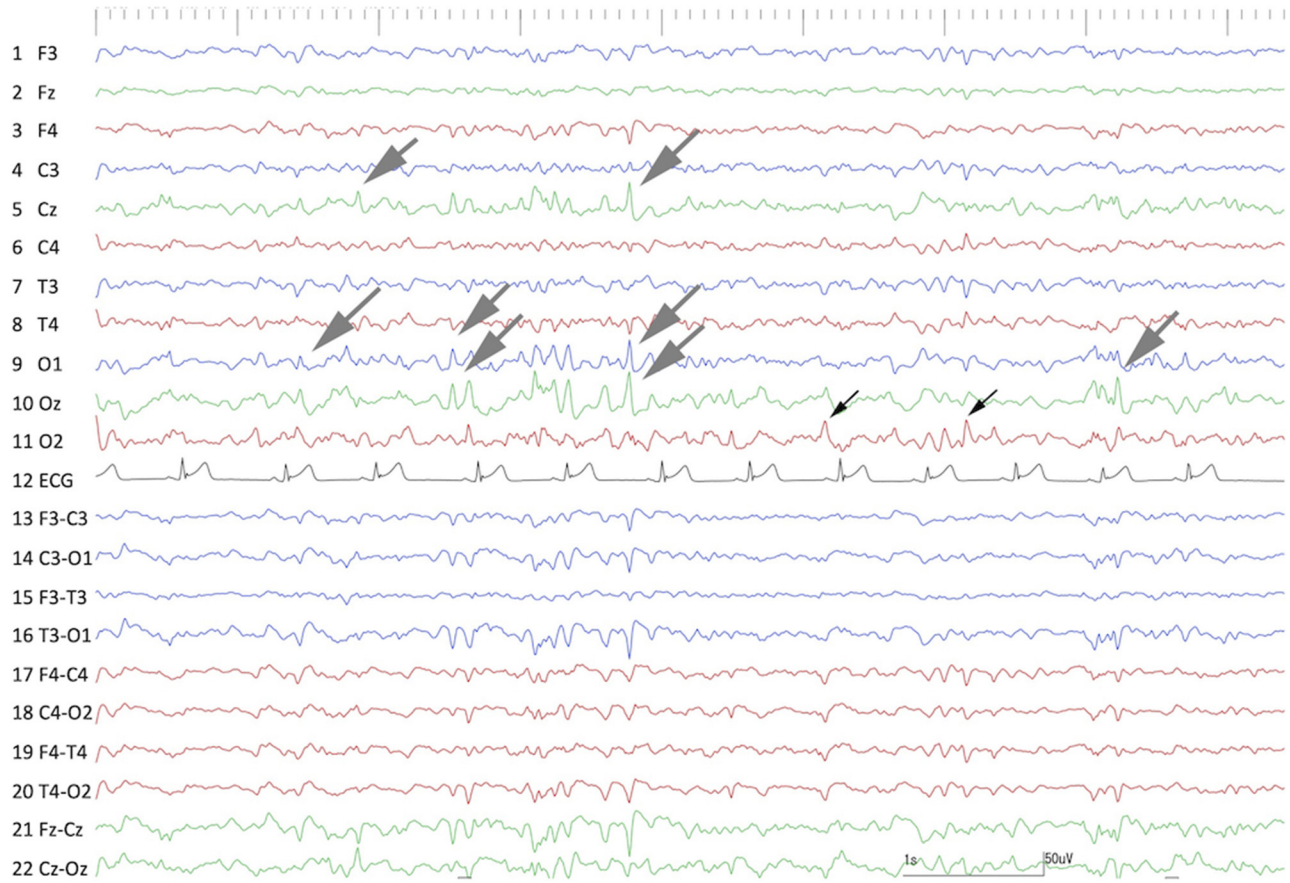

FIGURE 9 | Interictal scalp EEG at 60 days after the 2nd surgery (hippocampectomy). Frequent spikes and polyspikes were observed at O1, Oz, and Cz (gray arrows). Less frequent spikes were still observed at the operated region (O2) (black arrows). Compare to **Figure 1**.

maintenance ASDs were continued as in the postoperative management of the first surgery.

Seizure Outcome After the Second Surgery

As in the first surgery, there were no adverse effects or complications after the second surgery, and neurological status was unchanged from the preoperative state. After the second surgery, no seizures were observed for 91 days; however, frequent spikes on O1 and Oz (left and center of the occipital region) and sporadic spikes in O2 (occipital region on the operated side) were still observed on postoperative scalp EEG, which was performed at 60 days after the second surgery

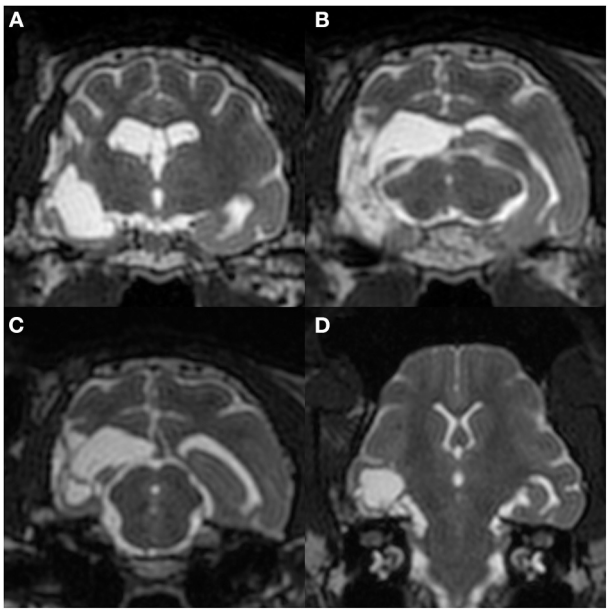

FIGURE 10 | Postoperative MRI at 60 days after the 2nd surgery. Transverse T2-weighted images at the hippocampal head **(A)**, medial geniculate body (**B**; matching **Figure 2**), and rostral colliculus **(C)**, and a dorsal image perpendicular to the long axis of the hippocampus **(D)**. The right hippocampus and a part of the temporo-occipital cortex had been removed without apparent surrounding signal changes.

(**Figure 9**). Concurrent MRI revealed complete resection of the right hippocampus and partially resected temporo-occipital cortex (**Figure 10**). At 92 days after the second surgery, facial myoclonus with mydriasis followed by GTCS, which had been recognized as a minor seizure type preoperatively, occurred. The same type of seizure was observed four times (at 122, 150, 270, and 401 days after the second surgery) until the cat was euthanized at 1.5 years (597 days) after the second surgery due to the reason described below. Therefore, the final seizure frequency was 0.25 sz/m (<1 sz/3 m) on average, that is, a >85% reduction compared with the preoperative seizure frequency, although ASD therapy (phenobarbital 2 mg/kg, q12h and diazepam 0.7 mg/kg, q12h) had been continued.

At ∼1 year and 5 months after the second surgery, the cat developed subacute decreased appetite and spontaneous water intake, leading to dehydration and hypothermia, which required continuous fluid therapy, forced feeding, and warming. Clinicopathological examinations, including complete blood count and serum chemistry, thoracic X-ray, and abdominal ultrasound, were conducted, but they were unremarkable. Despite palliative treatment, the cat gradually weakened and developed to be bedridden and lethargy, resulting in a severe dermal burn by the electric warmer. Finally, the cat was euthanized at 597 days after the second surgery, and necropsy and histopathology of the whole body were performed.

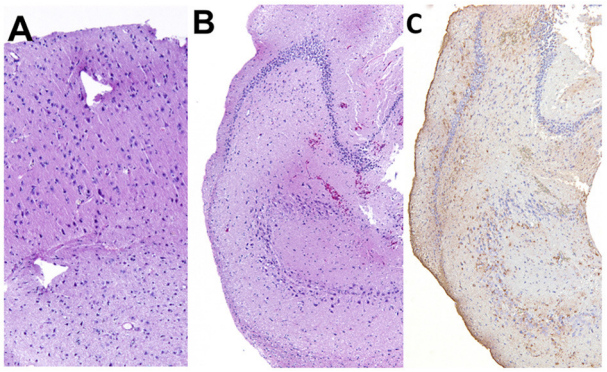

FIGURE 11 | Histopathology of the resected cortex **(A)** and hippocampus **(B,C)**. Shrunken neurons with pyknosis were generally observed in both specimens. In the hippocampus, mild gliosis was observed. **(A,B)** Hematoxylin and eosin staining. **(C)** Anti-glial fibrillary acidic protein immunohistochemistry.

Histopathology

A few parts of the resected lateral temporal cortex and hippocampus were submitted for histopathological evaluations. As the samples were surgically resected tissues, it was difficult to make an appropriate sectional angle for the correct evaluation of the cortex and hippocampus. On the resected cortical and hippocampal specimens, ischemic changes, that is, neuronal shrinking with pyknosis, were observed (**Figures 11A,B**). However, two board-certified veterinary pathologists judged that these changes were acute or artifactual changes by surgical removal or fixation rather than epileptic changes. Although it was difficult to evaluate the severity of neuronal loss due to the angle of the section and the whole hippocampal structure could not be evaluated, astrocytic glial proliferation was observed in the hippocampus by anti-glial fibrillary acidic protein immunohistochemistry (**Figure 11C**). A minor hemorrhage and slight infiltration of inflammatory cells around small vessels were also observed in the hippocampus. Other epileptogenic or pathological changes such as focal cortical dysplasia were not observed within the resected sample.

Histopathology of the whole brain collected at necropsy revealed the complete loss of the right hippocampus and a part of the temporo-occipital cortex and the presence of dural adhesions and granulomatous inflammation on the operated site. Additionally, old necrotic lesions with minimum gliosis were observed along the tracts of the depth electrodes. No changes related to epilepsy or death were found in other brain regions. In addition, no evidential findings suggesting the cause of the atrophic changes in the right hippocampus and temporo-occipital cortex were observed; therefore, a congenital or perinatal disorder, such as a focal vascular event, that affected the development of those areas was speculated.

In organs other than the brain, severe suppurative dermatitis, mild interstitial nephritis, chronic cystitis with mild pyelitis, mild lymphocytic cholangitis, and mild extramedullary hematopoiesis in the spleen were observed. Excepting severe suppurative dermatitis, which was the direct cause of euthanasia, there was

no lesion that might be related to the cause of dehydration and hypothermia at the end of life.

DISCUSSION

This is the first exploratory case study of intracranial epilepsy surgery with electroclinical and imaging data in modern veterinary medicine. In the present report, although complete freedom from seizures was not achieved, we demonstrated that epilepsy surgery is feasible in a feline patient with epilepsy and could be an alternative therapy for veterinary patients with DRE.

To date, research reports of epilepsy surgery for dogs or cats are limited to experimental studies for corpus callosotomy on normal dogs (12) or for amygdalohippocampectomy on a feline limbic seizure model induced with kainic acid (31), and experimental studies (14, 32) or a clinical trial (13) of neurostimulation (vagus nerve stimulation and deep brain stimulation) in dogs. However, a few, but very precious, case reports have been published in the veterinary field (18–21, 33).

To our knowledge, the oldest report was published by Oliver in 1965 (19). In this report, an 11-year-old boxer with frequent clonic seizures was operated on without any evidence of the epileptogenic focus. This dog had multiple necrotic lesions on the right occipital and frontal cortexes (i.e., structural epilepsy) that were suctioned, and seizures did not recur for several weeks. However, seizures recurred severely, and the dog died at ∼4 months after surgery. Although a histological evaluation was not performed, this was the first report of surgery aimed to prevent seizures in veterinary medicine. In 1971, Parker and Cunningham also reported surgical removal of an epileptogenic focus detected by 8-channel scalp EEG in a dog with structural epilepsy originating from fungal meningoencephalitis (18). In this case, a lateral transfrontal craniotomy was performed to approach the left frontal area, which showed frequent spikes on EEG. The epileptogenic lesion on the surface of the frontal lobe was a mass that was resected and diagnosed histologically as a "mycelioma" of fungal hyphae. Medications for infection were applied continuously, and this case survived for approximately 3 months after surgery without any seizures; however, it died unexpectedly from an unknown cause. This case report proved the utility of scalp EEG to detect an epileptogenic focus, and the lesion was found in the location indicated by the EEG findings. However, at that time, presurgical information using neuroimaging, which would reveal the characteristics, extension, or multiple occurrences of lesions, was not available. After that, as the use of advanced imaging modalities such as computed tomography and MRI has spread in the veterinary field, intracranial surgery for structural lesions that may cause recurrent seizures has become common for mass lesions. In contrast, surgery combined with electrodiagnostic evidence has not been performed (20, 21, 33).

In the present case, the seizure semiology observed from the first presentation until before surgery was consistent with FTLE, which was characterized by focal limbic seizures consisting of arrest, staring, facial twitching, and mydriasis, then contralateral head turning, circling, and hemi-limb clonus, and finally evolving into GTCS (22, 24, 25). These signs, especially for the first observed behavioral arrest, that is, staring and facial twitching, suggested that the symptomatogenic zone was located in the hippocampus or amygdala-hippocampus complex. In addition to seizure semiology, hippocampal sclerosis, atrophy, or necrosis is a well-known imaging and pathological change as a cause or result of TLE in humans and cats (6–8, 26, 29, 34–36). Although the MRI and histological findings of the resected hippocampus in this case did not correspond to typical hippocampal sclerosis and the hippocampal abnormality might have been congenital, there was an apparent loss of right hippocampal volume on structural MRI (volumetry and voxel-based morphometry) and gliosis was confirmed histologically. These imaging and pathological results, that is, structurally abnormal zone, and seizure outcome, as well as the fact that the seizure signs had changed postoperatively, suggested that the right atrophic hippocampus, at least, was included in the epileptogenic zone. Similar to the hippocampus, the right temporo-occipital cortex also showed atrophy and ischemic changes; however, cortical resection of these area performed in the first operation did not prevent seizures, even though seizure freedom was achieved for 2 months. Therefore, we thought retrospectively that, as also described below, this atrophic cortex was not the true or primary epileptogenic zone.

It is considered that chronic iEEG, that is, ECoG and depth EEG, which is direct recording from the cortex, with video monitoring (iVEEG), which allows us to observe seizure symptoms, is the best approach to suggest the true epileptogenic zone [6, 7, 19, 33]. However, iEEG requires invasive procedures and its recording site is limited to a small area, which makes it possible to miss the true seizure-onset zone. In fact, we placed a 9-channel ECoG grid on the atrophic temporo-occipital cortex in the present case, and ictal ECoG showed low-amplitude and high-frequency activity at the onset of seizure symptoms (behavioral arrest; early phase), which then gradually became rhythmic discharges from specific electrodes (#1, 3, and 9) with increasing amplitude as the seizure symptoms changed (drop and head turning, hemi-clonic, and GTCS; middle to late phase) (**Figure 6** and **Supplementary Video 1**). As the depth electrodes inserted into the hippocampus were broken before monitoring, we had recorded ECoG only and decided to resect the focal cortex showing ECoG abnormalities at the first surgery. As a result, the same seizure type as observed before surgery reoccurred; namely, we could not determine the true epileptogenic zone from ECoG. The observed low-amplitude and high-frequency activity at the early phase is well-known in human and experimental epilepsy (37–39). This EEG pattern is classified as a type 1 or 3 seizure pattern, which is most commonly observed in human patients with hippocampal-onset TLE (37, 39). This low-amplitude high-frequency activity on ictal iEEG is considered an indicator of the seizure-onset zone, although its mechanism of generation is still controversial (38). However, in current epileptology, this concept of low-amplitude high-frequency activity is incorporated into the concept of "high frequency oscillations," which are recognized as a specific biomarker of an epileptogenic zone (38, 40). Unfortunately, because the EEG system we used could not record the wide frequency band, we could not assess high-frequency oscillations. In addition, if the depth electrodes had not

TABLE 2 | Classification systems of surgical seizure outcome.

	Engel's Classification (1992)	ILAE Classification (2001)
I (1)	**Free of disabling seizures**	
	A: Completely seizure-free since surgery	Completely seizure-free: no aura
	B: Non-disabling simple partial seizures only since surgery	
	C: Some disabling seizures after surgery, but free of disabling seizures for at least 2 years	
	D: Generalized convulsion with ASD discontinuation only	
II (2)	**Rare disabling seizures ("almost seizure free")**	
	A: Initially free of disabling seizures, but now has rare seizures	Only aura; no other seizure
	B: Rare disabling seizures since surgery	
	C: More rare disabling seizures since surgery, but rare seizures for the last 2 years	
	D: Nocturnal seizures only	
III (3)	Worthwhile improvement	1–3 seizure days per year; ± aura
	A: Worthwhile seizure reduction*	
	B: Prolonged seizure-free intervals amounting to greater than half the followed-up period, but not <2 years	
IV (4)	**No worthwhile improvement**	
	A: Significant seizure reduction	4 seizure days per year to 50% reduction of baseline seizure days*; ± aura
	B: No appreciable change	
	C: Worse seizures	
V (5)		<50% reduction of baseline seizure days to 100% increase of baseline seizure days; ± aura
VI (6)		>100% increase of baseline seizure days; ± aura

**Classification of the present case. ILAE, international league against epilepsy.*

been broken and could record hippocampal EEG or if we placed the ECoG grid more ventromedially to cover the pyriform cortex, we might have been able to detect the true epileptogenic zone, that is, the hippocampus, and to perform hippocampectomy in a single surgery.

Regarding the functional deficit zone in the present case, the right forebrain dysfunction and bilateral cortical blindness were recognized by routine neurological examinations and were consistent with the structural MRI lesions, except for the left visual (occipital) cortex, which did not have an apparent lesion. To detect the functional deficit zone related to the seizure-onset zone more accurately, functional imaging such as diffusion and perfusion MRI, EEG-functional MRI, and nuclear imaging would be essential (9). However, in this case, we could only obtain interictal DWI, which was not informative. We tried to identify interictal perfusion MRI and/or interictal-early postictal changes using diffusion and perfusion MRI preoperatively, which is suggested to be useful for the detection of the functional deficit zone in feline epilepsy (27, 28). Still, we failed due to various factors such as the failure of a bolus injection of contrast agent or missing the timing of the scanning. Similar to the abovementioned depth-EEG, if we could have performed those techniques, the functional deficit zone might have been detected more accurately. Conversely, the dysfunction of the left occipital cortex (blindness of the right eye) was MRI-negative. However, this site showed EEG abnormalities, even though they were less frequent than in the right hemisphere, and might be another epileptogenic focus for the seizures that remained after the second surgery, as discussed later.

The present case was diagnosed as drug-resistant FTLE with atypical atrophy of the hippocampus and temporo-occipital cortex, although the cause of that was suspected to be a congenital or perinatal disorder. In human medicine, MTLE is the most common form of DRE and most patients with MTLE are suitable for epilepsy surgery (7, 8, 41–43). Many studies, including systematic reviews, meta-analyses, and randomized controlled studies, have shown that surgical treatment for MTLE is superior to medical management and 60–80% patients achieve postoperative seizure freedom (41–43). In human patients with MTLE and hippocampal sclerosis, the rate of seizure freedom is increased to 80–90% (44). Although we could not determine whether the present case had typical hippocampal sclerosis, the right hippocampus was apparently atrophic, and its surgical removal resulted in a remarkable reduction of seizures. In human medicine, there are two main surgical methods for resecting mesial temporal structures, that is, anterior temporal lobectomy and selective amygdalohippocampectomy *via* several different approaches; however, there is no statistically significant difference in seizure outcome between these techniques (42). Anterior temporal lobectomy and selective amygdalohippocampectomy in human MTLE both involve hippocampal resection; however, it does not

represent a total resection, but a resection of <1.5 cm from the tip of the hippocampal head for the dominant hemisphere or <3 cm for the non-dominant side. Conversely, there is no report regarding surgical resection of the hippocampus in veterinary medicine. However, the approach to temporal lobectomy for hemangioma removal at the level of the hippocampal head in one dog has been reported (20). Experimentally, selective amygdalohippocampectomy (resection of the amygdala and ventral portion of the hippocampus) has been reported in a chronic feline epilepsy model with kainic acid microinjection into the unilateral amygdala (31). This study reported that selective amygdalohippocampectomy could inhibit seizures from the original site (unilateral kainate-injected amygdala); however, seizures from the contralateral amygdala and/or hippocampus occurred at 2 weeks after surgery. Furthermore, there was no detailed description of behavioral or clinical signs and postoperative complications in that report. In the present case, we performed total hippocampectomy with a partial cortical resection of the temporo-occipital region, but the cat did not show any additional behavioral or neurological changes or complications. There is a possibility that we could not notice subtle neurological or functional changes because the present case already had chronic neurological deficits including cognitive dysfunction, blindness, and right forebrain signs before surgery. In human studies, the complications of anterior temporal lobectomy or selective amygdalohippocampectomy with or without preoperative iVEEG monitoring are relatively rare (∼5%) (45). However, they include memory impairment (especially in the dominant hemisphere), dysphasia or speech error (in the dominant hemisphere), contralateral visual field defect, depression, ipsilateral cranial neuropathy (mainly for cranial nerves III or IV), and hemiplegia or hemiparesis, in addition to surgical complications such as infection, meningitis, cerebrospinal fluid leak, hemorrhage, hematoma, vasospasm and stroke, and death (7, 8, 41, 45). To date, there has been no report regarding the technique and complications for hippocampectomy in veterinary patients.

Notably, Zilli et al. reported a cadaveric study of cortico-hippocampectomy in non-epileptic cats to develop epilepsy surgery for FTLE (17). The surgical procedure described in that study was highly similar to that performed in the present case; however, the extent of the surgical field in the present case was more widespread than that in the cadaveric study. Therefore, both papers suggest that the ectosylvian gyrus for cortical incision and visual confirmation of the caudal cerebral artery and lateral aspect of the medial geniculate body after hippocampal removement are surgical landmarks when approaching and removing the feline hippocampus, respectively. In the non-epileptic cadaveric study, the authors could not remove the hippocampus totally, especially because of the difficulty to remove part of its head and tail, while we performed total hippocampectomy in the present case. As the reason for this, in the present case, the hippocampus and lateral cortex were extremely atrophied, resulting in an extended lateral ventricle, which made it easy for us to maneuver within the lateral ventricle for hippocampal resection. Therefore, the difficulty/ease of total hippocampectomy (with or without removal of the amygdala) may depend on the space of the surgical field in each case. In addition, it is speculated that a wider cortical resection and/or different approaches are needed in a case with normal hippocampal and lateral ventricular size. Conversely, another argument is whether total resection of the hippocampus is required to achieve seizure control in feline patients with DRE. In fact, hippocampectomy in anterior temporal lobectomy and selective amygdalohippocampectomy in humans is limited to the hippocampal head, as mentioned above. In order to discuss the procedure and efficacy of hippocampectomy in veterinary patients with epilepsy, further experimental studies and the accumulation of clinical cases are needed.

As mentioned above, surgery for MTLE in human medicine results in a good outcome as mentioned above (41–43). Seizure outcome following epilepsy surgery is commonly evaluated according to an outcome classification system. The most widely used system is "Engel's classification," which was suggested by Engel in 1992, but a new classification has recently been suggested by the International League Against Epilepsy (ILAE) (**Table 2**) (46). In the present case, postoperative seizure frequency (4 sz/year) was improved significantly (80–90% reduction) from the preoperative baseline (2–3 sz/m). When judged with the above human classification systems, the surgical seizure outcome of the present case could be classified as Engel Class IIIA and ILAE Class 4. However, in our case, we consider that the recurrent seizures observed after surgery were the minor seizure type from the contralateral occipital lobe that had been observed since the cat was 10 years of age (described below), while the original (major) type was inhibited by surgery. Therefore, if the classification systems were adopted to the original seizure type only, the outcome would correspond to Engel Class IA or ILAE Class 1.

Regarding the postoperative seizures, we considered that they originated from the left occipital lobe, that is, the mirror focus against the original (the right) temporo-occipital cortex, although we could neither detect those minor seizures during the iVEEG monitoring period nor place ECoG electrodes on the left hemisphere. Due to our delayed decision and preparations for epilepsy surgery, these minor but remaining seizures may have been produced by secondary epileptogenesis by the long-term duration of DRE from the original (primary) focus. This point is crucial in determining the timing of epilepsy surgery, especially for focal epilepsy. Although there is reportedly no significant relationship between the duration of epilepsy and postoperative seizure outcome in human TLE, there is a slight tendency that patients with a shorter duration have a better outcome than those with a longer duration (47). However, the accumulated evidence suggests that early surgical therapy is recommended for patients with DRE, especially with structural epilepsy such as MTLE with hippocampal sclerosis, cavernous malformations, or a benign tumor (44, 48–50). Furthermore, long-term unilateral TLE reportedly induces bilateral epileptogenicity (secondary epileptogenesis) and an unfavorable postsurgical outcome (51). Therefore, if we could have performed epilepsy surgery on the present case at a much earlier stage, for example, before the age of 10 years when the cat exhibited the minor seizure type, it

might have been possible to inhibit secondary epileptogenicity and provide a better postoperative seizure outcome.

In conclusion, we reported our experience of resection epilepsy surgery and its 1.5-year follow-up in a feline case with drug-resistant structural epilepsy. Although some recurrent seizures that might have originated from the hemisphere contralateral to the surgical side remained after surgery, focal cortical resection and hippocampectomy of the presumed primary epileptogenic zone by presurgical evaluations resulted in a remarkable reduction of seizure frequency without further complications. Although additional basic and clinical studies for presurgical evaluations, techniques for epilepsy surgery, and other ways to treat feline DRE are needed, this case report shows the possibility that intracranial epilepsy surgery with advanced neuromodalities may be a treatment option for DRE in veterinary medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee and Animal and Human Biology Research Ethics Committee of the Nippon Veterinary and Life Science University (accession numbers; 27S-60, 28K-6, 29K-2, and 2019K-1).

AUTHOR CONTRIBUTIONS

DH, RA, YH, YY, TK, and SM: conception, design, and data acquisition. DH, RA, YH, JC, and KU: analysis and interpretation of data and preparing figures. DH and YY: drafting the manuscript. All authors: revising and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.719455/full#supplementary-material

Supplementary Data 1 | Materials and methods for EEG, MRI, intracranial electrodes and epilepsy monitoring cage.

Supplementary Video 1 | Intracranial video-EEG of a focal limbic seizure evolving into a generalized tonic-clonic seizure.

Supplementary Video 2 | Movie of the first surgery: focal cortical resection in the ectosylvian gyrus.

Supplementary Video 3 | Movie of the second surgery: hippocampectomy.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
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Corrigendum: Focal Cortical Resection and Hippocampectomy in a Cat With Drug-Resistant Structural Epilepsy

Daisuke Hasegawa[1,2]*, *Rikako Asada*[1], *Yuji Hamamoto*[3], *Yoshihiko Yu*[1], *Takayuki Kuwabara*[1], *Shunta Mizoguchi*[1], *James K. Chambers*[4] and *Kazuyuki Uchida*[4]

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Keywords: cat, drug-resistant epilepsy, electroencephalography, electrocoricography, epileptogenic zone, epilepsy surgery, magnetic resonance imaging, video-EEG

A Corrigendum on

Focal Cortical Resection and Hippocampectomy in a Cat With Drug-Resistant Structural Epilepsy

by Hasegawa, D., Asada, R., Hamamoto, Y., Yu, Y., Kuwabara, T., Mizoguchi, S., Chambers, J. K., and Uchida, K. (2021). Front. Vet. Sci. 8:719455. doi: 10.3389/fvets.2021.719455

In the original article, there was an error, the sex of the case cat was incorrect.

A correction has been made to Abstract and Case Presentation, and Long-Term iVEEG monitoring sections. The term "female" has been corrected to "male," and also "her" was changed into "his," respectively.

In the original article, the names of the authors were spelt incorrectly in reference 11. Michael G, Jones C, Mary R, Packer A, Royal T, Volk HA, et al. should be Jones GMC, Volk HA and Packer RMA. The corrected reference appears below.

Jones GMC, Volk HA, Packer RMA. Research priorities for idiopathic epilepsy in dogs : viewpoints of owners, general practice veterinarians, and neurology specialists. *J Vet Intern Med.* (2021) 35:1–14. doi: 10.1111/jvim.16144

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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CASE REPORT
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Case Report: 1-Year Follow-Up of Vagus Nerve Stimulation in a Dog With Drug-Resistant Epilepsy

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A vagus nerve stimulation (VNS) system was surgically implanted to treat drug-resistant epilepsy in a 5-year-old male Shetland Sheepdog. At regular visits during a 1-year follow-up, treatment efficacy and adverse effects were assessed, and programmable stimulation parameters were adjusted to optimize stimulation intensity while avoiding adverse effects. The frequency of generalized tonic–clonic seizures was reduced by 87% after the initiation of VNS. The owner reported that the dog regained his personality, and the quality of life of both the dog and owner improved. The only adverse effect of VNS was a cough that was controlled by adjusting stimulation parameters. There were no surgical complications or other issues with the VNS device. This is the first long-term evaluation of VNS therapy in a dog, and the results obtained suggest that gradual adjustments of VNS parameters facilitate optimum VNS dosing.

Keywords: drug-resistant epilepsy, vagus nerve stimulation, dog, case report, neurostimulation, epilepsy surgery

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INTRODUCTION

Although epilepsy is a common neurological disorder in dogs, it is not controlled in approximately 30% of cases despite a correct diagnosis and appropriate medical treatment; and, thus, in those cases, it is called refractory epilepsy (1). Recently, the term drug-resistant epilepsy (DRE) has also been used. Surgery and neurostimulation therapy are clinically important treatment options for human epilepsy patients (1). Vagus nerve stimulation (VNS) is a type of neurostimulation that is becoming more widespread as adjunctive therapy for human epilepsy (2) because it reduces seizure frequency and improves quality of life (QOL) (2, 3).

In humans, the efficacy of VNS for epilepsy patients improves over time, with adjustments of stimulation parameters preventing adverse effects and increasing treatment efficacy. A long-term follow-up study on epilepsy patients for whom VNS parameters were gradually adjusted reported gradual reductions in median seizure frequency of 25, 40, and 53% after 3, 6, and 12 months of VNS, respectively (4). In veterinary medicine, two clinical studies on surgically implantable VNS and transcutaneous non-invasive VNS indicated the potential of VNS as adjunctive therapy for dogs with DRE (5, 6); mean reductions in seizure frequency of 34.4% and 25.9% were reported after 13 and 16 weeks of VNS, respectively. However, the long-term efficacy and safety of VNS in dogs and adjustments of VNS stimulus dosing remain unclear.

A duration of at least 24 weeks has been proposed to assess the outcomes of therapeutic interventions for canine epilepsy (7). We herein describe the 1-year clinical course of VNS therapy in a dog with DRE, in which stimulus parameters were gradually adjusted during the year.

CASE PRESENTATION

A 5-year-old non-castrated male Shetland Sheepdog with DRE was referred to Azabu University Veterinary Teaching Hospital (AUVTH) to evaluate the indication for VNS surgery. Frequent recurrent focal seizures (FS) and FS evolving into generalized tonic–clonic seizures (FS-GTCS) had occurred for 4 years. FS started with bilateral facial twitching or eyelid blinking, mastication, running without purpose, and immobilization and typically lasted for a few seconds to 1 min. FS evolving into GTCS started with the same motor activity before immediately developing into GTCS, which generally lasted 1–2 min. FS evolving into GTCS never continued for more than 5 min. The dog had been treated with various antiseizure drugs (ASDs), including zonisamide for 4 years, potassium bromide (KBr) for 3 years, phenobarbital for 2 years, levetiracetam for 6 months, and gabapentin for 5 months, with adequate dosing and serum concentrations (zonisamide, KBr, and phenobarbital) without good seizure control. The current ASD regimen consisted of zonisamide (8 mg/kg, BID, serum concentration: 56.8 μg/ml), gabapentin (15 mg/kg, TID), levetiracetam (40 mg/kg, TID), and KBr (20 mg/kg, BID, serum concentration: 1.2 mg/ml). Ursodeoxycholic acid and glycyrrhizinic acid were also administered due to slightly elevated liver enzymes. Despite strenuous attempts, seizure frequency progressively increased with countless FS every day and FS-GTCS clusters five to 14 times monthly for the last 6 months.

On presentation to AUVTH, the dog was alert and responsive, but restless. Physical and neurological examinations revealed no abnormalities. A complete blood count and serum biochemistry were unremarkable with the exception of a slight elevation of liver enzymes. X-ray showed microhepatica, and abdominal ultrasound revealed gallbladder mucocele. Fasting and postprandial total serum bile acid concentrations were 3.9 and 119 μmol/L, respectively. Computed tomography (CT) by the referring veterinarian showed no evidence of a portosystemic shunt or nodular lesions in the liver.

The Veterinary Medical Teaching Hospital of Nippon Veterinary and Life Science University diagnosed idiopathic epilepsy based on the Tier III level of the International Veterinary Epilepsy Task Force (IVETF) consensus proposal (8) 3 months prior to presentation to AUVTH. Magnetic resonance imaging (MRI) of the brain [3T, IVETF recommended protocol (9)] and a cerebrospinal fluid (CSF) analysis (proteins, cell counts, and cytology) revealed no abnormalities. Scalp electroencephalogram (EEG) under dexmedetomidine sedation was recorded using the recommended method (10). Despite semiology indicating FS, generally synchronized spike or spike-wave complexes were frequently observed in inter-ictal EEG (**Supplementary Figure 1A**). During EEG recordings, generalized electroencephalographic seizure activities without convulsions, that is, subclinical ictal EEG, were also noted (**Supplementary Video**). Based on EEG findings, the irritative zone remained unclear.

DRE was diagnosed based on these results and history, and liver cirrhosis was suspected to be caused by multiple ASD treatments, including phenobarbital. VNS was selected as adjunctive therapy because the epileptogenic zone was not identified, and less invasive surgery was preferable due to suspected cirrhosis. The owner also requested VNS therapy.

A VNS device was implanted at AUVTH following ultrasound-guided Tru-Cut liver biopsy. The dog was premedicated with butorphanol and atropine. Anesthesia was induced with propofol and maintained with isoflurane. The VNS device comprised a pulse generator (Demipulse™ Model 103; LivaNova USA, Inc., Houston, TX, USA) and electrode lead with two helical electrodes at the tip (VNS lead M304, LivaNova USA, Inc., Houston, TX, USA) (**Figures 1A,B**). The method to implant the VNS device was previously described (5). Briefly, the two helical electrodes and anchor tether were wrapped around the left vagosympathetic trunk in the cervical area (**Figure 1C**), and the electrode lead was connected to the pulse generator implanted in the subcutaneous space cranial to the left scapula. Surgery was uneventful. After confirmation by system diagnostics of proper functioning, the device was maintained at 0 current.

A bandage for cast padding and a self-adherent bandage were applied around the neck to prevent postoperative seroma. During hospitalization, bile peritonitis occurred due to gallbladder rupture, and cholecystectomy was performed 6 days after VNS implantation. Recovery was uneventful. The liver and gallbladder were histologically diagnosed with chronic hepatitis progressing to cirrhosis and mucocele, respectively. The dog was hospitalized for 9 days. Ampicillin sodium/sulbactam sodium was intravenously administered during VNS surgery. Amoxicillin/clavulanate potassium was orally administered for 18 days after VNS implantation.

The device was activated after the neck wound had healed, in accordance with the manufacturer's instructions (11). The owner continued to record the number of FS, FS-GTCS, and any other seizures daily in the diary. The owner answered a visual analog scale (VAS) created for epilepsy surgery (**Supplementary Data Sheet**) in the day the dog was discharged. The VAS was completed again at the end of the 1-year follow-up. This survey was performed to investigate a subjective assessment of the owner to the treatment outcome of VNS therapy such as the QOL of both the dog and the owner.

Nine days after discharge, the dog presented to AUVTH for the first evaluation after VNS implantation. Physical and neurological examinations revealed no abnormalities. There was no seroma, and the wound had healed (**Supplementary Figure 2A**). The device was activated using an external programming system with stimulation parameters output current 0.25 mA, pulse width 250 μs, frequency 20 Hz, on-time 30 s, and off-time 5 min, which are initial settings commonly recommended for the treatment of epilepsy in humans (2, 11) (**Supplementary Figure 3**). There was no cough,

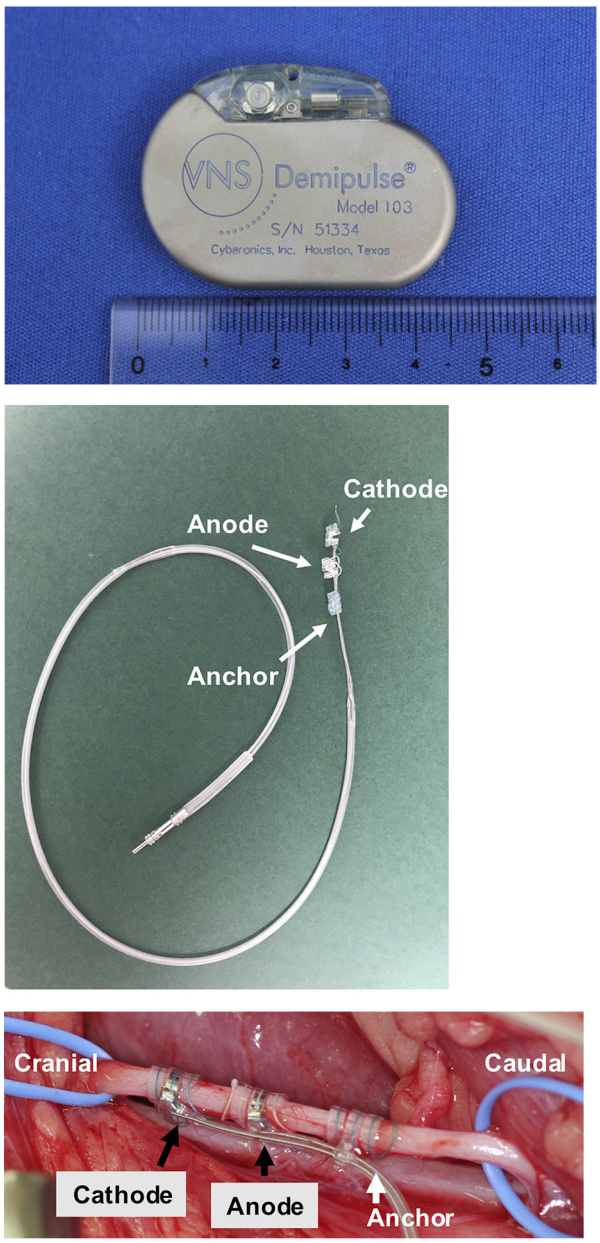

FIGURE 1 | The VNS device and an intraoperative photograph of the left vagosympathetic trunk. The pulse generator (width 45 mm, height 32 mm, depth 6.9 mm, and weight 16 g) **(A)**. The electrode **(B)**. The left vagosympathetic trunk wrapped by the two helical electrodes and anchor tether **(C)**. VNS, vagus nerve stimulation.

voice change, bradycardia, or Horner's syndrome. The dog did not seem to take notice of the region around the left side of the neck where the vagosympathetic trunk was stimulated. The ASD regimen was unchanged from 3 months before VNS implantation to the 1-year follow-up.

Reevaluations were conducted approximately every 2 weeks in the initial post-implant period until the maximum tolerated stimulus current was reached and then every 4 weeks until the 1-year follow-up to screen for any adverse effects, check device function, and assess VNS efficacy. The output current was gradually increased by 0.25 in each visit based on tolerability and treatment efficacy (11). Other VNS parameters were adjusted to optimize and minimize adverse effects when required.

The owner was provided with a VNS external magnet, which may be used to stop seizures by swiping over the pulse generator to instantaneously provide an extra temporary stimulation. The output current of the magnet-induced stimulation (i.e., magnet mode) was configured at 0.25 mA above the cycling stimulation (i.e., normal mode). The other parameters of the magnet mode included a pulse width of 250 μs, frequency of 20 Hz, and on-time of 60 s.

In the second reevaluation after implantation (day 14 after VNS initiation), the general condition of the dog as well as seizure frequency and intensity remained unchanged. When the output current increased from 0.25 to 0.50 mA, the dog began to cough during a 30-s cycle of stimulation. Therefore, the pulse width was decreased to 130 μs, which prevented coughing while maintaining the output current.

During the 1-year follow-up, stimulation parameters were adjusted in each visit based on the dog's responses (**Figure 2**). FS evolving into GTCS frequency began to decrease after day 14 but increased between days 42 and 63. There are strong fumes from exterior wall painting at a nearby apartment on days 53–62, and the dog became very nervous during this period. There was also a typhoon, which typically induced seizures in the dog, on days 46, 47, 54, and 55. During days 53–62, seizure frequency increased. A routine reevaluation was performed on day 63. It was then difficult to increase the output current without coughing. Therefore, to increase the stimulation intensity and not the output current, we increased the pulse width and decreased the output current while avoiding coughing.

After the output current was set to 0.5 mA and pulse width to 250 μs on day 63, it was not possible to increase the current to more than 0.5 mA without coughing. Since seizure frequency remained reduced and the owner was satisfied with the dog's condition, initial optimal dosing (i.e., highest efficient parameter setting without adverse effects) was attained on day 63, and a reevaluation was re-scheduled for every 4 weeks after day 84. The owner reported that the dog had regained his original character and slept well on day 112. The output current was not increased further due to coughing; therefore, the stimulation off-time was shortened at 3 and 1.8 min on days 112 and 287, respectively, according to a method described for humans (11, 12). On days 243 and 377, X-ray showed neither twisting of the electrode lead nor subcutaneous migration of the pulse generator (**Supplementary Figure 4**).

Throughout the 1-year follow-up, an 87% reduction was observed in the frequency of FS-GTCS (373 seizures for 6 months before VNS vs. 97 seizures in 1 year after VNS initiation). An 89% reduction in the frequency of FS-GTCS clusters was also achieved (55 clusters for 6 months vs. 12 clusters in 1 year) (**Figure 3A**). The number of FS-GTCS days also had 76% reduction (93 seizure days for 6 months vs. 45 seizure days in 1 year) (**Figure 3C**). Due to their very high frequency, the number of FS was not counted before VNS therapy, and, thus, there were no baseline

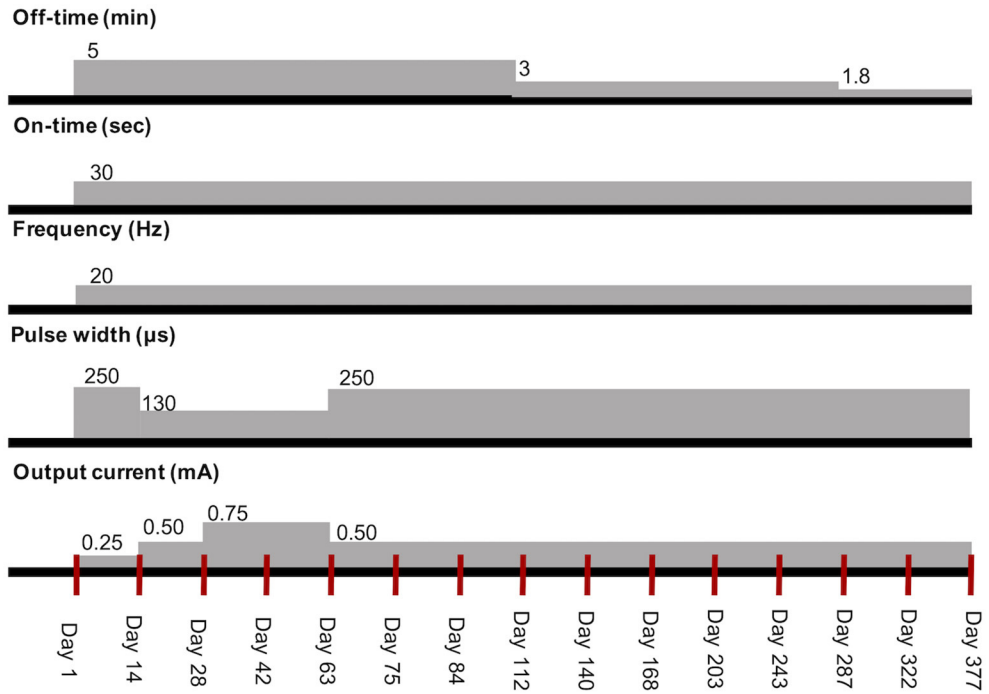

FIGURE 2 | Transition of stimulation parameters of VNS therapy in the dog. The red vertical lines mean each visit. VNS, vagus nerve stimulation.

data. The owner was instructed to count the number of FS after VNS initiation, and no decreases were noted during the 1-year follow-up (**Figures 3B,C**). However, VAS showed that the duration of FS shortened with measurement decreases from 100 to 15 mm. VAS also showed improvements in the QOL of the dog and owner with measurement decreases from 100 to 17 mm and from 93 to 13 mm, respectively (**Table 1**). Status epilepticus was not observed in the 6-month pretreatment or 1-year VNS treatment period.

The owner used the handheld magnet at the onset of FS or the development of GTCS from FS. When the magnet was used during FS, subsequent GTCS did not occur; however, ongoing GTCS was not stopped. Once the duration of FS shortened, it was difficult to effectively use the magnet because FS had already stopped before the magnet was used.

The only adverse effect of VNS was a cough when the stimulation intensity increased. The owner reported that the dog tolerated VNS. There were no complications of surgery to implant the VNS device.

DISCUSSION

Adjustments of stimulation parameters to achieve optimum settings for each patient are important in VNS therapy. In humans, a higher stimulation intensity, particularly the output current, is generally more efficacious than a lower intensity (13, 14) but may not be necessary for some patients to achieve maximal anti-seizure effects, while a higher output current (>2.25 mA) may result in smaller reductions in seizure frequency (15). Parameter settings need to be adjusted to maximize efficacy and minimize complications. An initial low-intensity stimulus achieves better tolerance followed by gradual increases for accommodation to the stimulation (11, 13). In veterinary medicine, since there are currently no suggested methods for optimizing settings, we followed the initial settings recommended for the treatment of human patients and attempted incremental increases in each visit (2, 11, 13). Consequently, initial optimal dosing was successfully attained without persistent adverse effects within approximately 2 months in our dog.

As a tolerability strategy, if an increase in the output current is not tolerated, other stimulation parameters may be modified to accommodate tolerability. The dog began to cough after the initial increase in the output current to 0.5 mA. Therefore, the pulse width was reduced from 250 to 130 μs at the output current of 0.5 mA, which stopped the cough. After several weeks, the pulse width was reverted to 250 μs, and the cough was not elicited; therefore, the dog had habituated to the stimulation.

We shortened the stimulation off-time on days 112 and 287 to increase the duty cycle. A higher duty cycle increased VNS efficacy in some human patients (12). However, increasing the duty cycle was not beneficial in our dog (**Figure 3**). Further studies are needed to clarify the importance of the duty cycle in dogs.

It was difficult to increase the output current to more than 0.75 mA with a pulse width of 250 μs without coughing during the remaining treatment period. Consistent with the present results, a previous VNS study reported that it was not possible to

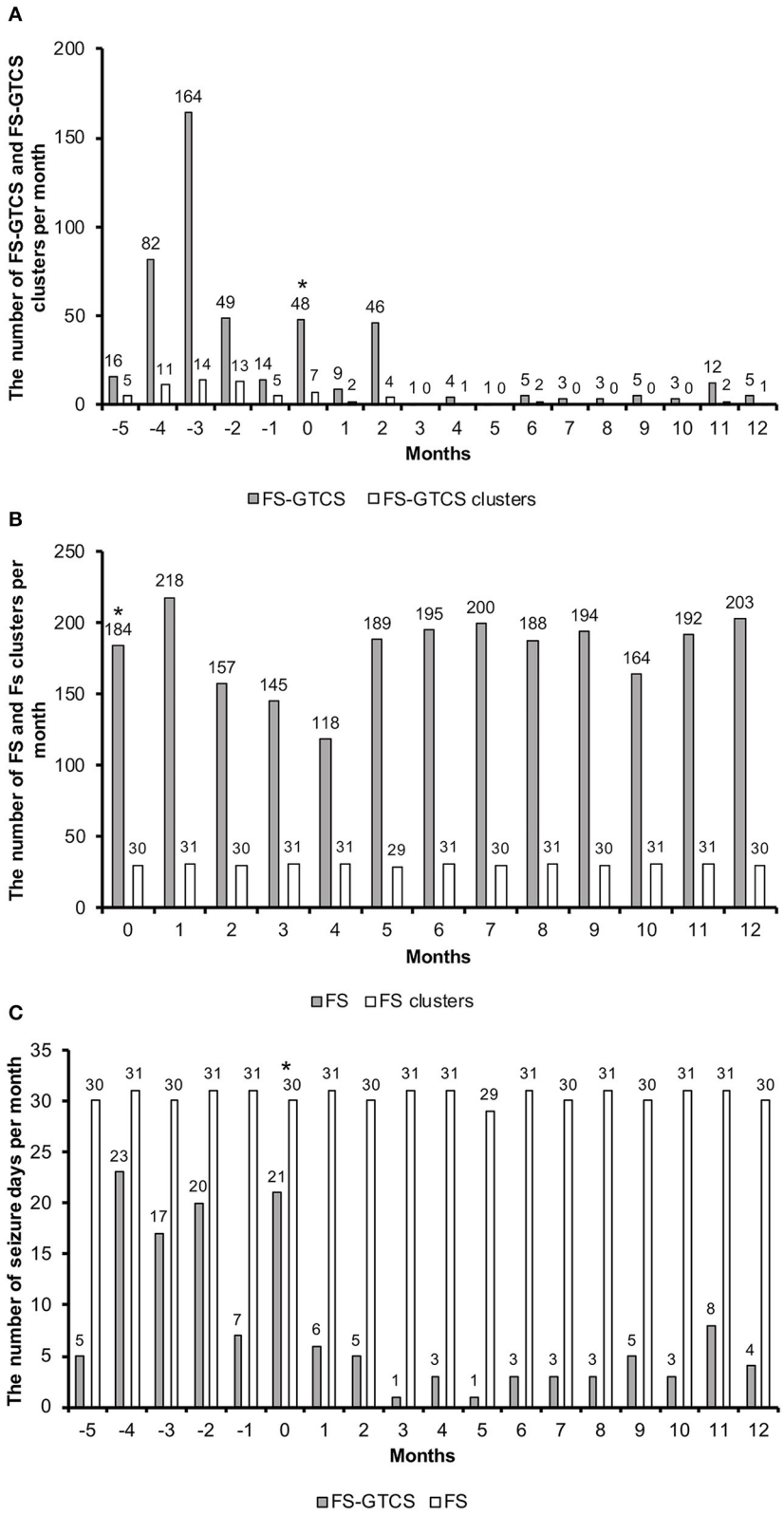

FIGURE 3 | The number of FS-GTCS and FS-GTCS clusters per month **(A)**. The number of FS and FS clusters per month **(B)**. The number of seizure days (FS-GTCS and FS) per month **(C)**. The asterisk indicates that VNS therapy was initiated in this month. Months −5 to 0 indicate the retrospective period; months 1 to 12 indicate the follow-up period. FS-GTCS, focal seizures evolving into generalized tonic–clonic seizures; VNS, vagus nerve stimulation.

TABLE 1 | VAS scale before VNS initiation and at the end of the 1-year follow-up of VNS therapy.

Questions	Before the initiation of VNS (mm)	At the end of the 1-year follow-up (mm)
Frequency of focal seizures	100	70
Frequency of generalized seizures	100	57
Frequency of cluster seizures	100	54
Frequency of status epilepticus	0	0
Duration of focal seizures	100	15
Activity level of the dog or cat	100	41
Burden of medication on the dog or cat	100	81
Appetite of the dog and cat	100	55
Ataxia in the dog and cat	100	8
Consciousness level in the dog or cat	100	11
QOL of the dog or cat	100	17
QOL of the owner	92	13
Satisfaction with surgery		23.3
Frequency of seizure days		

Details of VAS are shown in a supplementary data sheet. Measurements in millimeters are described in the table. Briefly, there are two descriptors representing extremes of answers for each question at the beginning and end (e.g., No FS and high frequency of FS). A longer measurement indicates a worse condition (a length of 100 mm is the worst). The question item of "Frequency of seizure days" was added in the VAS after the end of the 1-year follow-up (the supplementary data sheet used for this owner did not include this question). Thus, there were no answers from the owner. We determined to add this question because this information would help investigate the outcome of therapeutic investigations in canine epilepsy.

VNS, vagus nerve stimulation; QOL, quality of life; VAS, visual analog scale; FS, focal seizures.

increase the output current to more than 0.75 mA with a pulse width of 500 μs in nine out of 10 dogs without coughing (5). Despite a lower output current than that suggested for humans (2, 11), the anti-epileptic effects of VNS were confirmed in the dog of the present case report and the dogs reported by Muñana et al. (5). The vagus nerve comprises A-, B-, and C-fibers (16, 17). A study on rats showed that C-fibers were not associated with the anti-epileptic effects of VNS (18). A-fibers are myelinated somatic afferent and efferent fibers. B-fibers are myelinated efferent preganglionic autonomic fibers. A study on the activation threshold of each fiber type in anesthetized dogs reported that the thresholds of A- and B-fibers with a pulse width of 300 μs were 0.37 ± 0.18 and 1.6 ± 0.35 mA, respectively (16). The output current needed to treat our dog and the dogs reported by Muñana et al. (5) was markedly lower than the threshold of B-fibers. Although it currently remains unclear whether A- or B-fibers are more important for the anti-epileptic effects of VNS, even in humans, the outcomes of VNS in our dog and the dogs in the study by Muñana et al. (5) suggest that A-fibers contributed more to the anti-epileptic effects of VNS than B-fibers. Based on the present results and previous findings, an output current in the range of 0.25–0.75 mA is tolerated well by and effective for dogs and is the suggested setting for dogs with epilepsy.

VNS reduced not only seizure frequency but also seizure duration in humans (19). In the present study, the frequency of FS-GTCS decreased by 87% and that of FS-GTCS clusters by 89% in the 1-year treatment period. The number of FS-GTCS days also decreased by 76%. The duration of FS was also shortened with an 85% reduction in the VAS scale. These findings indicate that our dog achieved IVETF-defined partial therapeutic success for epilepsy (7).

The only adverse effect of VNS was coughing during the stimulation, which was successfully controlled by adjusting stimulation parameters. There were no surgical complications, such as a seroma at the incision site of the subcutaneous pocket for the pulse generator, which was a common complication in other VNS studies on dogs (5, 20). The surgical procedure in the study of Martlé et al. (20) reported the electrode lead twisting in 50% of the dogs. However, the VNS device operated well for 1 year with our surgical method that referred to the way reported in Muñana et al. (5). Therefore, the surgical procedure in the present and previous (5) studies and our postoperative treatment, that is, the neck bandage, may be preferable to prevent troubles of the electrode lead and a seroma.

There are several reasons why we selected non-pharmacological therapy for the dog in this case report. Few human patients control seizures with a third ASD when the first two ASD have failed (21), and, thus, non-pharmacological therapy is recommended for those cases. Although this has not yet been examined in the field of veterinary medicine, previous studies indicated many similarities in epilepsy between humans and dogs, such as its etiology and prevalence (22, 23). Therefore, from the negative result of humans (21) and similarities between humans and canine epilepsy, we determined increasing ASD dose was not beneficial for our dog. Moreover, in the present study, a hepatic disorder was suspected and chronic hepatitis was diagnosed. This hepatic problem also indicated increasing ASD was not appropriate. Therefore, we selected non-pharmacological therapy for the dog.

Among non-pharmacological therapies, VNS was selected for the dog in this case report for a number of reasons. The epileptogenic zone was hard to detect on preoperative examinations (i.e., ictal and interictal EEG, MRI, and seizure semiology). Human candidates for VNS are patients who are not appropriate for epilepsy surgery with craniotomy due to, for example, an unclear epileptogenic zone or a high risk of complications (2). The epileptogenic zone of the dog was not identified, and the suspected hepatic disorder indicated that less invasive surgery was preferable. Moreover, VNS is effective for a wide range of patients regardless of the seizure type (2) and was beneficial for the dog, which had several seizure types.

Previous VNS clinical trials in veterinary medicine did not use the VNS external magnet (5, 6). The termination of seizure activity and shortening of seizure durations and severity using a magnet-induced stimulation have been reported in humans (24). The owner used the magnet at the onset of FS activity to stop the development of GTCS. This immediate effect is an additional benefit of VNS therapy. Therefore, further studies are preferable to investigate the effects of the VNS external magnet in dogs.

The VAS showed considerable improvements in the QOL of both the dog and owner, with high satisfaction with VNS by the owner (**Table 1**). The QOL of the dog improved with VNS

therapy based on the restoration of his gentle character, improved sleep quality, and enjoyment of walking with other dogs. The QOL of the owner also improved because the dog looked happy and she also began to sleep well. Although the dog is not free of seizures, VNS therapy is an effective adjunctive treatment for this dog because of the markedly reduced frequency of FS-GTCS and improved QOL.

Our dog in this case report was nice to handle in the clinic. However, even if a dog is not so fond of clinics, it does not become a reason for excluding VNS therapy from treatment options because the stimulation parameters could be adjusted easily and quickly just by externally holding the programing wand over the dog's shoulder where the generator is located.

We reevaluated the dog every 2 weeks until the stimulation parameters were optimized for the dog. Such frequent visits could be difficult for some owners. We anticipate that a monthly visit is also appropriate, although more frequent visits during the titration phase may allow for faster achievement to the optimal stimulus setting.

To the best of our knowledge, this is the first long-term evaluation of VNS therapy in which stimulus parameters were adjusted during follow-ups to optimize VNS dosing in the dog. The present results suggest the potential benefits of VNS as adjunctive non-pharmacological therapy and the benefit of gradual adjustments of stimulation parameters while avoiding adverse effects in dogs with DRE. The suggested settings and protocol for adjustments of parameters in dogs need to be verified in further studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Azabu University, Japan. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JH drafted the article, contributed to the neurological management of the patient, and acquired data. MS conceived the case report, performed VNS surgery, contributed to the neurological diagnosis and management of the patient, and critically revised the manuscript. DH diagnosed the dog with DRE and critically revised the manuscript. HI performed liver biopsy, contributed to the hepatic management of the patient, and critically revised the article. ST performed cholecystectomy and critically revised the article. All authors contributed to editing the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.708407/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Case Report: Corpus Callosotomy in a Cat With Drug-Resistant Epilepsy of Unknown Cause

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A 2-month-old, intact male domestic shorthair cat with dullness, bilateral central blindness, and recurrent epileptic seizures was presented to a local clinic. Seizures were the generalized myoclonic and tonic-clonic type. Phenobarbital was initiated and maintained; however, seizures were not controlled. Other anti-seizure drugs, including levetiracetam, zonisamide, and diazepam, also provided insufficient seizure control with seizures occurring hourly to daily. By 8 months of age, the cat displayed non-ambulatory tetraparesis and deep somnolence. Magnetic resonance imaging (MRI), cerebrospinal fluid analysis, and pre- and post-prandial total bile acid analyses were unremarkable. Scalp electroencephalography (EEG) revealed central dominant but generally synchronized spikes and multiple spikes. The cat was diagnosed with drug-resistant epilepsy of unknown cause and was included in a clinical trial of epilepsy surgery. Given the unremarkable MRI and bilateral synchronized EEG abnormalities, a corpus callosotomy was performed at 12 months of age, and partial desynchronization of spikes was confirmed on EEG. Incomplete transection was found in the genu of the corpus callosum on postoperative MRI. After surgery, the mental status and ambulation clearly improved, and seizure frequency and duration were remarkably reduced. Recheck with follow-up EEG and MRI were performed at 3, 6, and 12 months after surgery. Scores of activities of daily living and visual analog scales including cat's and owner's quality of life had also improved considerably. This case report is the first documentation of the one-year clinical outcome of corpus callosotomy in a clinical feline case with drug-resistant epilepsy.

Keywords: feline, corpus callosotomy, drug-resistant epilepsy, epilepsy surgery, quality of life

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INTRODUCTION

The corpus callosum (CC) comprises the largest commissural fibers connecting both cerebral hemispheres, lying in the center of the forebrain longitudinally. Corpus callosotomy (CCT), i.e., dividing hemispheres (split-brain) by disconnecting CC, is a type of palliative epilepsy surgery mainly applied to human patients with infantile epileptic encephalopathy and drug-resistant generalized epilepsy with atonic, absence, myoclonic, and tonic-clonic seizures (GTCS).

In particular, the efficacy of CCT has been established in atonic or astatic seizures, also known as drop attack, that shows sudden astasia causing head injury (1–3).

In veterinary medicine, the surgical procedure for CCT in normal dogs has been reported by Bagley et al. in 1995 (4), and the effect of CCT for epilepsy had been experimentally studied in feline seizure models such as kindling between the 1960s and 1990s (5–8). However, its clinical application and therapeutic outcome has not been reported so far. Here, we report the first client-owned feline case of drug-resistant epilepsy (DRE) of unknown cause undergoing CCT and the outcome after 1 year.

CASE PRESENTATION

History

A 2-month-old male domestic shorthair cat was presented to a local veterinary clinic with a complaint of recurrent generalized convulsions. The local veterinarian noticed the cat also had frequent facial twitching, ataxia, visual impairment, and slightly decreased mentation at the first consultation. Video clips recorded by the owner (**Supplementary Video 1**) showed generalized myoclonic seizures, which was momentary but clustered for 1 to a few minutes, and GTCS lasting about several tens of seconds to 1 minute. These seizures were initially observed several times per week; therefore, antiseizure drug (ASD) therapy with 1.0 mg/kg of phenobarbital (PB) every 12 h per os was initiated. The dose of PB was increased to 2.0 mg/kg q12h PO (achieved static serum level: 14.8 μg/ml) at 3 months of age due to insufficient seizure control. At this time, complete blood count, serum chemistry including fasted and postprandial ammonia and total bile acid, urinalysis, screening test of feline immunodeficiency virus and feline leukemia virus, cerebrospinal fluid (CSF) analysis including PCR for *Toxoplasma gondii* and feline corona virus, and low-field (0.4T) magnetic resonance imaging (MRI) of the brain were performed. All complementary tests were unremarkable. Due to severely increased seizure frequency to > 40 times daily (uncountable), the dose of PB was further increased (4.0 mg/kg q12 h PO), and zonisamide (8.0 mg/kg q12h PO), diazepam (DZP; 0.5 mg/kg q12 h PO) as well as levetiracetam (LEV; 20 mg/kg q8 h PO) were added. However, zonisamide was discontinued 3 days after the initiation due to the appearance of aphagia.

At 5 months of age, the cat was referred to a veterinary neurologist (SK) at the Veterinary Specialists Emergency Center (Saitama, Japan). Neurological examination revealed severe mental dullness, disorientation, difficulty in standing and walking, decreased postural reactions in all limbs, and bilateral menace response deficits with normal pupillary light reflexes. Appetite remained normal but the cat needed assisted feedings. Levetiracetam had been discontinued by the owner because the cat seemed to develop stupor after its administration. Doses of PB and DZP were maintained at 5.0 mg/kg q12 h PO (achieved static serum level: 27.1 μg/ml) and 0.8 mg/kg q12 h PO, respectively. Although the frequency and duration of GTCS decreased, myoclonic seizures and short tonic seizures still occurred 1–6 times per day (sz/d). Although other differential diagnoses at this point including inborn errors of metabolism, inherited neurodegenerative conditions, or immune-mediated limbic encephalitis could not be completely ruled out, further diagnostics such as the Tandem mass spectrometry and/or investigation of known antibodies such as anti-voltage-gated potassium channel and/or anti-leucine-rich glioma inactivated 1 receptor antibodies for limbic encephalitis were unavailable locally then. At 7 months of age, the cat was presumptively diagnosed as DRE of unknown cause with severely impaired quality of life (QOL), when the potential candidacy of the epilepsy surgery research project was discussed and the owner requested the candidacy to be evaluated.

The project team of veterinary epilepsy surgery (**Supplementary Data 1.1**) discussed this case based on the abovementioned history and data. All members considered this cat as a candidate for epilepsy surgery. Electroencephalography (EEG) and high-field MRI as presurgical evaluations were performed to determine the type of surgical procedure.

Presurgical Evaluations and Decision of Surgical Procedure

At 8 months of age, the cat was further referred to the Veterinary Medical Teaching Hospital of Nippon Veterinary and Life Science University (Tokyo, Japan) to undergo EEG and high-field MRI. At this point, seizure frequency increased reaching up to 7–10 sz/d, including GTCS. The cat was non-ambulatory and nearly stuporous but remained responsive to forced feeding, loud sounds, and nociceptive stimulation. Postural reaction deficits and bilateral menace deficits were noticed on neurological examination; no other abnormalities were recorded. Feeding, drinking, and urination had been fully assisted by the owner. Complete blood count and serum chemistry profile were re-evaluated and revealed mild dehydration, which was easily improved later by increasing the water intake. Scalp EEG under sedation (**Supplementary Data 1.2**) revealed frequent spikes and multiple spike complexes were observed on the central regions (C3, C4, Cz) dominantly but synchronized generally (**Figure 1A**). High-field MRI (3.0T) under general anesthesia (**Supplementary Data 1.3**) showed no appreciable structural abnormalities in the whole brain (**Figure 2A**). Cerebrospinal fluid analysis including cell count (0/μl; ref 0–5/μl), cytology (no cells observed), protein concentration (12.4 mg/dl; ref 0–25 mg/dl), glucose (126 mg/dl; ref 35–140 mg/dl), and electrolytes (Na^+ 158; K^+ 2.9; Cl^- 135 mEq/l) was also unremarkable. The serum level of PB at this point was 33 μg/ml. With these repeated MRI and CSF analysis, inherited neurodegenerative conditions were considered unlikely. Although other differentials could not be completely ruled out, DRE of unknown etiology was considered the most likely.

Based on these results, the epileptogenic zone of this case was not determined; therefore, the project team selected CCT as a suitable surgical procedure based on the following reasons: (1) epileptiform discharges distributed bilaterally; (2) unremarkable MRI; (3) severe seizure frequency that might result in decreased mental status; and (4) decreased QOL of the patient and the owner (**Supplementary Data 1.1**). As the final checkpoint to detect an identifiable epileptogenic focus,

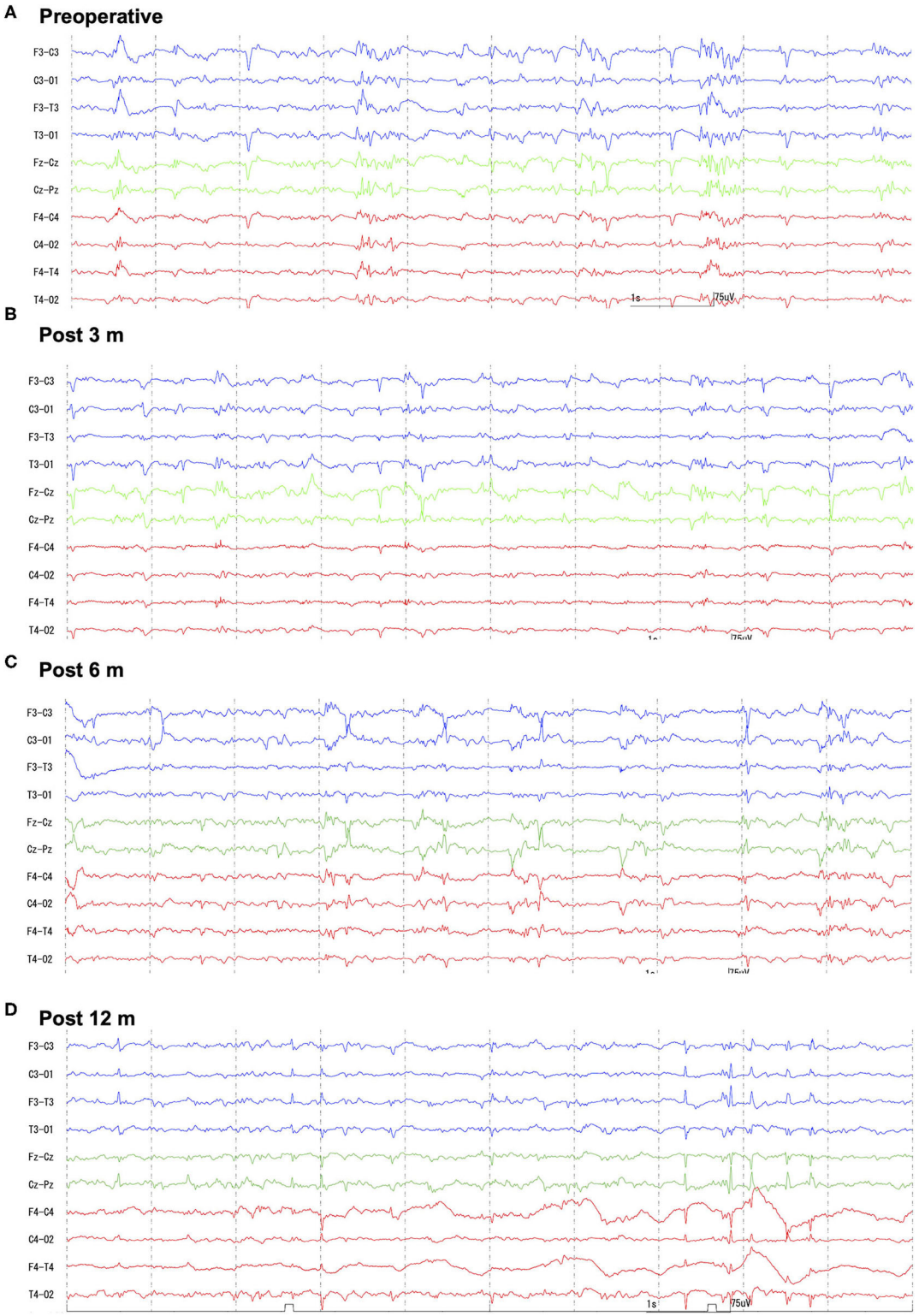

FIGURE 1 | Preoperative and follow-up scalp EEGs. Preoperatively **(A)** generalized spikes and multiple spike complex were frequently observed with phase reversal at the central region (C3, Cz, C4). Follow-up EEGs [**(B)** 3-months; **(C)** 6-months; **(D)** 12-months after surgery] showed decreased spike frequency, and some small spikes were found to be limited to one hemisphere. However, large spikes were always bilaterally synchronized. Bipolar montages: blue traces are left (F3-C3, C3-O1, F3-T3, T3-O1), greens are midline (Fz-Cz, Cz-Pz), and reds are right (F4-C4, C4-O2, F4-T4, T4-O2). Sensitivity = 15 μV, Time constant = 0.1, High-cut filter = 60 Hz, AC filter = ON.

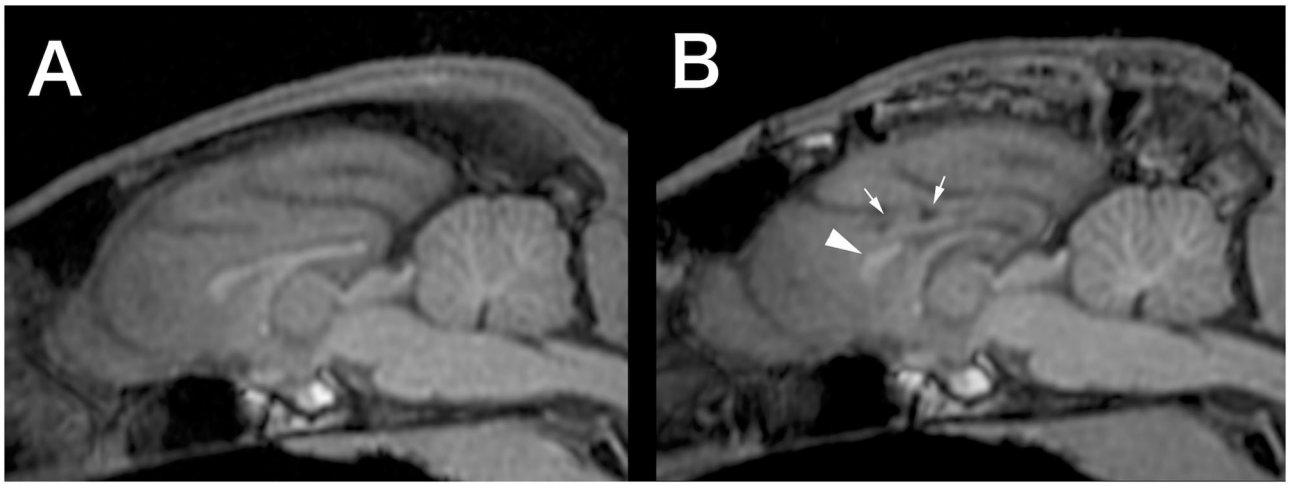

FIGURE 2 | Preoperative **(A)** and 12-months postoperative **(B)** midline sagittal T1-weighted MRI (generated by multiplanar reconstruction from 3D-T1 data). In the postoperative image, the genu of the corpus callosum (arrowhead) left intact and necrotic lesions in the cingulate gyrus (small arrows), which was damaged during the surgery, were observed.

an intraoperative electrocorticography (ECoG) recording was planned immediately following the craniotomy. If the intraoperative ECoG recording clearly identified the epileptogenic focus, a different procedure such as a focal resection and/or multiple subpial transections would have been considered more suitable (**Supplementary Data 1.1**).

The owner was requested to record seizures including the types, frequency, and duration, as well as the visual analog scales (VAS; **Supplementary Data 2**) before and 3, 6, and 12 months after surgery. An attending neurologist (DH) evaluated the activities of daily living (ADL) (**Supplementary Data 3**) and performed follow-up EEG and MRI during the same period.

Corpus Callosotomy

Corpus callosotomy (CCT) was performed at 12 months of age. General anesthesia was induced by propofol (6 mg/kg IV) and maintained with inhalation of isoflurane and oxygen. The cat was positioned in sternal recumbency in a Sphinx-like position. During surgery, electrocardiogram, oxygen saturation, end-tidal CO_2 concentration, indirect blood pressure, rectal temperature, and urine production volume were monitored. Lactated Ringer's fluid was infused at 3–5 ml/kg/h, and a constant rate infusion (CRI) of remifentanil as intraoperative analgesia was performed at 20 μg/kg/h. After shaving and sterilization, an H-shaped scalp incision was made with the skin opened like a double door. Bilateral temporal muscles were incised along to the external sagittal crest and detached from the parietal bones, and then retracted bilaterally. A 3 cm (left–right) × 4 cm (rostral–caudal) rectangular-shaped craniotomy straddling the left and right of the parietal bone was performed. The first four burr holes were made on each corner of the rectangle with the rostral pair at the level of the caudal end of the frontal bone and the caudal pair approximately 1.5 cm rostral to the external occipital protuberance. The craniotomy was completed by connecting these burr holes to expose the underlying cerebral surface of the parietal lobes. In the process of connecting the burr holes, the craniotomy lines connecting the two rostral and the two caudal burr holes, i.e., transverse craniotomy lines, had widened areas across the midline in order to visualize the underlying dorsal sagittal sinus well. The removed bone fragment was wrapped with saline-filled gauze and saved for the closure.

After the craniotomy, epidural ECoG was recorded. A 1.5 × 1.5 cm grid-type silicon-sheet electrode with nine exploration electrodes was placed on the dura covering each hemisphere's parietal lobe. On ECoG, bilaterally synchronized spike-containing burst-suppression was recorded (**Supplementary Data 1.4**).

As ECoG did not aid to identify the epileptogenic zone, a sagittal sinus-based U-shaped dural incision was made on the left hemisphere and inverted to the right side. The left hemisphere was gently retracted laterally with neurosurgical putties, spatula, and shafts of suction to widen the longitudinal fissure. By peeling off the cingulate gyrus on both sides under the falx gently, the bright white CC was exposed (**Supplementary Video 2**).

The CC was bisected using bipolar cautery and suction from the caudal, the splenium, to the rostral, the genu. During the bisecting of the CC, the great cerebral vein, where the confluence of the vein of the corpus callosum and internal cerebral vein, was observed at the caudal end of the splenium. The third ventricle was also observed under the body. During the approach of the genu, a cortical vessel on the cingulate gyral surface was damaged, and the hemostasis manipulation and the subsequent brain swelling made it difficult to identify the genu. Therefore, the rostral dissection was abandoned (**Supplementary Video 2**). Due to the brain swelling, 1 g/kg of mannitol was intravenously administered over 15 minutes.

The dura was sutured with 6-0 polydioxanone after confirming normalized brain volume and no bleeding from the intracranial surgical field. Then, a post-callosotomy intraoperative ECoG was recorded; Although large spikes were

still synchronized on both hemispheres, small independent spikes, not seen pre-CCT, were observed from each hemisphere (**Supplementary Data 1.3**). After recording ECoG, the stored bone fragment was returned and fixed with polymethylmethacrylate. Temporal muscles and scalp were sutured routinely. A percutaneous endoscopic gastrostomy (PEG) tube was placed to facilitate postoperative feeding.

The cat recovered from anesthesia uneventfully, and the patient's conscious level had moderately improved compared with the preoperative state. However, facial twitching was observed intermittently until the next day. As postoperative analgesia, CRI of fentanyl (2 μg/kg/h to tapered) was infused for 12 h after surgery, and a fentanyl patch (4.2 mg/head) was applied for 3 days. Although cefmetazole (25 mg/kg q2–12h IV) had been used from intra- to postoperative 12 h, the cat showed fever ($\sim$40°C) with mild neutrophilia (WBC 22,000 /μl) on the next day. Because bacterial meningitis was a potential concern, meropenem (25 mg/kg, CRI for 30 min q24h) for 3 days and enrofloxacin (5 mg/kg q24h PO) for 5 days were administered. The fever ceased within 4 h after initiation of meropenem, after that fever and leukocytosis had never been observed remaining the cause being unidentified.

The day after surgery, the cat was able to maintain a sitting position and eat wet food on his own, with the nutritional requirement maintained using a PEG tube. Furthermore, on the postoperative day (POD) 3, even though mild right-sided hemiparesis was observed, the cat became ambulatory and was able to voluntarily urinate and defecate. The PEG tube was removed at POD 10, and the cat was discharged from the hospital at POD 16. During the postoperative hospitalization, 5 myoclonic seizures were observed, while there were 12 seizure-free days. Neurological findings at the discharge time were slightly somnolence, mild disorientation, ambulate but generalized ataxia with postural reaction deficits in all limbs, and loss of biliteral menace response. Phenobarbital (3.5 mg/kg q12 h PO) and DZP (0.5 mg/kg q12 h PO) were continued throughout the hospitalization.

Outcome

After discharge, the cat was routinely consulted at a local clinic every 2–4 weeks, and presented to the teaching hospital at 3, 6, and, 12 months after surgery for rechecks and follow-up EEG and MRI. Antiseizure drug therapy with PB and DZP were maintained for the 1-year follow-up period. The neurological status, mild cognitive impairment, ataxia with decreased postural reactions, loss of bilateral menace response, remained unchanged for the 1-year follow-up period. Mental status fluctuated from alert to somnolent depending on the time of the visit or seizure frequency. Changes of seizure types were observed postoperatively: GTCS had completely disappeared, and generalized myoclonic seizures changed to momentary facial myoclonic seizures and shorter tonic seizures. On the other hand, a new seizure type manifesting as the initial left arm-extended fencing posture (fencing posture seizure; FPS) followed by contralateral (right) myoclonic or clonic seizures (**Supplementary Video 3**) emerged 7 months postoperatively. Sequential changes in seizure frequency, VAS, and ADL from the preoperative time to the 1-year follow-up are shown in **Figure 3**, **Table 1**, and **Supplementary Data 3**, respectively. Seizure frequencies of all seizure types were markedly decreased (7.2–1.7 sz/d; 76% reduction); however, 1–2 sz/d in average either myoclonic seizures or FPS, or both remained unchanged through the 1-year follow-up period.

Follow-up scalp EEGs showed clearly decreased spike frequency. Although a few spikes were observed in a hemisphere only, most were still synchronized bilaterally (**Figures 1B–D**). Follow-up MRI at 3, 6, and 12 months after surgery revealed the bisected CC with the remaining genu, loss of caudal part of the left cingulate gyrus, and old hemorrhagic and a necrotic lesion in the left cingulate gyrus likely related to intraoperative tissue handling as well as vascular damage (**Figure 2B**).

DISCUSSION

This is the first report of CCT in a clinical feline case with DRE of unknown cause. Although very short myoclonic seizures and occasionally FPS remained, a meaningful reduction in seizure frequency and duration was achieved for the 1-year follow-up period. Furthermore, it is also important that the mental status, activity, and the QOL of both patient and owner improved substantially after surgery.

Corpus callosotomy is categorized as a palliative surgery together with neuromodulations such as vagus nerve stimulation (VNS) and deep brain stimulation (DBS) in human epilepsy. Palliative epilepsy surgeries are generally applied to various types of generalized seizures and/or focal seizures evolving to GTCS with unknown, multiple, or bilateral epileptogenic zone(s). There are many comparative studies between CCT and VNS for human generalized DRE. In general, CCT is more invasive and requires an intracranial procedure with a higher risk of complications; however, CCT is superior in reducing seizures and fast acting compared with VNS. One study that compared CCT and VNS for refractory generalized seizures showed the percentages of patients who had a $\geq$50% seizure reduction was 79 and 40%, respectively (9). In the same study, however, the complication rate of CCT (21%) was higher than that of VNS (8%). In one systematic review focusing on atonic seizures and drop attacks only, $\geq$50% seizure reduction was 85.6% and 57.6% for CCT and VNS, respectively (10).

There is no information regarding the clinical outcome of CCT in veterinary patients. Corpus callosotomy in the present case achieved a 100% reduction in GTCS and a $\geq$75% reduction for all seizure types. The preoperative neurological status of this case was difficult to assess accurately because the severely disabled condition, i.e., abasia-astasia and stuporous mental status, which were considered to be attributed to multiple factors including the underlying condition of continuous seizure activities, the consequence of seizure activities, and the adverse effect of multiple ASDs. There was no major complication except a transient right-dominant tetraparesis after surgery. Postoperative improvement of neurological and QOL status was more conspicuous, which brought high satisfaction to the owner. The owner showed the greatest pleasure and excitement when

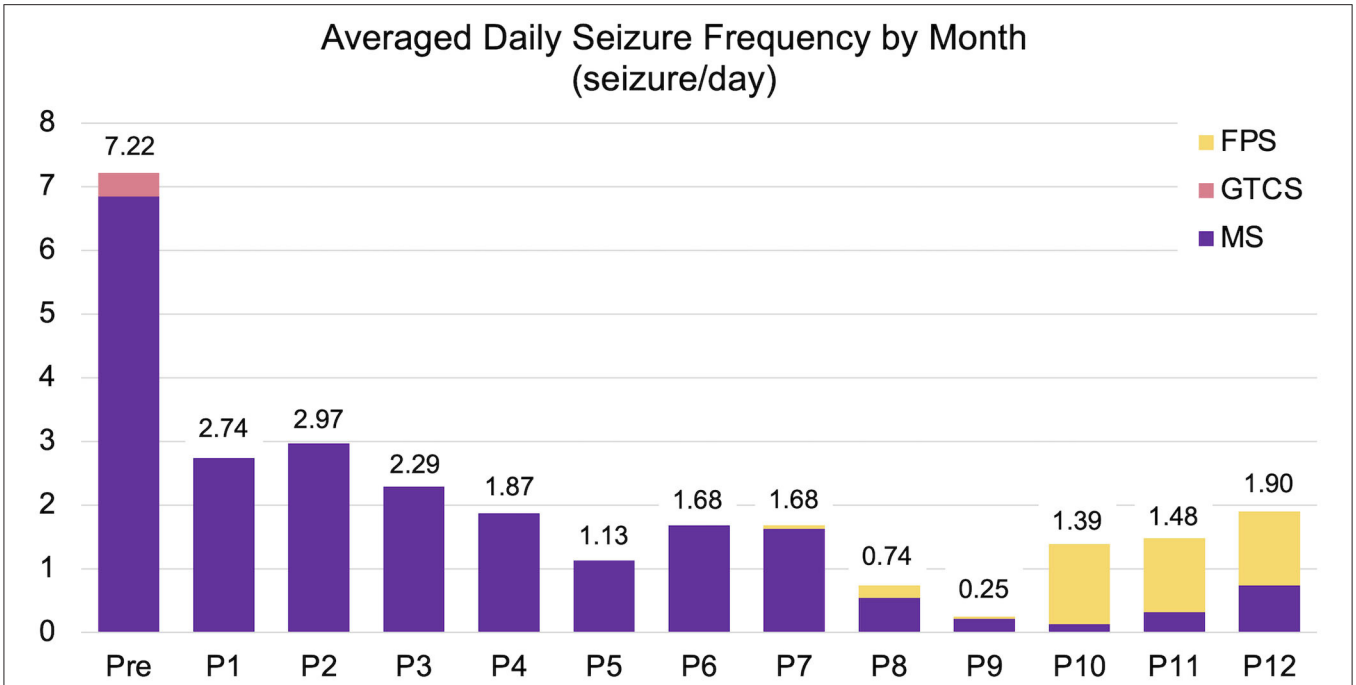

FIGURE 3 | Changes in averaged daily seizure frequency (seizure/day) for preoperative (Pre) to postoperative 12 months (P1–P12). The preoperative seizure frequency (Pre; 7.22 sz/d) was shown as the average for 3-months before surgery. The average one-year postoperative seizure frequency was 1.7 sz/d (76% reduction). FPS (yellow), fencing posture seizures; GTCS (pink), generalized tonic-clonic seizures; MS (purple), myoclonic seizures including generalized, facial myoclonic, and short tonic seizures.

TABLE 1 | Result of the visual analog scale (VAS).

	Definition of 0/100	Pre	Post 3m	Post 6m	Post 12m	Improved %*
Freq. Sz day	free/worst (everyday)	100	22	20	16	81
Freq. Myoclonic Sz§	free/worst	90	72	76	76	17
Duration of Myoclonic Sz§	0sec/2min†	100	13	11	17	86
Freq. GTCS	free/worst	69	0	0	0	100
Freq. Cluster Sz	free/worst	70	48	45	33	40
Freq. SE (≥5min)	free/worst	0	0	0	0	–
Severity of Sedation	none/comatose	78	37	48	48	43
Severity of Ataxia	none/non-ambulate	100	34	36	50	60
Degree of Activity	active/worst	84	18	37	27	67
Appetite¶	aphasia/polyphasia¶	25	80	32	79	128¶
Caregiver burden of Medication	not bothered/ extremely frustrated	84	80	100	87	−6
QOL of the animal	excellent/worst	49	16	18	24	60
QOL of the owner	excellent/worst	70	25	45	46	45
Satisfaction with surgery	satisfied/regret	NA	0	0	0	100

*The VAS sheet is shown in **Supplementary Data 2**. Values are actual measurements (mm) except the improved percentage. Pre, preoperative; Post 3, 6, and 12m; postoperative 3, 6, and 12 months; Freq., frequency; Sz, seizures; GTCS, generalized tonic-clonic seizures; SE, status epilepticus; QOL, quality of life; NA, not applicable. * Improved percentage (%) = 100−{(average of 3 postoperative values) × 100 / preoperative value}. § Myoclonic seizures included shorter tonic seizures. † Maximum value (100 mm) was defined as 2 min by the owner. ¶ Improved percentage of appetite was calculated as 50 mm indicated normal appetite (100%); thus, a 25 mm of preoperative value indicated 50% and a 64 mm of the average of postoperative values indicated 128%. Because the value of preoperative was not applicable, 0 mm of postoperative values were considered as 100% satisfied.*

the cat stood up, walked, ate, and excreted himself after surgery, as shown in VAS and ADL improvement. In fact, CCT efficacy in humans is observed not only in seizure reduction but also in overall daily function, behavior, cognition, and intelligence in over half of patients (2). The authors considered that the brain dysfunction associated with ongoing seizure activities restored its function relatively well along with decreased seizure episodes following CCT.

The CCT surgical procedure in cats has not been reported in the veterinary literature. Therefore, we performed it based on reports in dogs (4) and humans (2). Although the incomplete dissection may be the cause of the residual seizures, most

of the CC, including splenium, was clearly visualized and easily bisected compared with the authors' limited experience of CCT in dogs (unpublished information). On the other hand, a caudorostoral approach to the genu was challenging in this case due to the intraoperative injury to the cingulate gyrus and subsequent cerebral edema. More rostrally extended craniotomy allowing better visualization of the genu and rostral cerebral artery might be necessary to bisect the genu as in human anterior CCT. A stereotaxic bisection of the CC was used (7) in the previous experimental CCT studies in feline seizure models (5, 6, 8). This stereotaxic method with wire seemed easier to perform; however, the CC was not visually identified. With this stereotaxy-guided blind dissection, the surrounding structures, including cingulate gyrus and vessels were not protected and they might have been damaged. The authors elected not to utilize this technique given this potential lethal risk. Other feline experimental studies of binocularity had described CCT procedure with the aid of stereotaxic frame and surgical microscope (11, 12). Those studies, however, did not aim to inhibit seizures, and had bisected caudal CC mainly. In human CCT, neuro-endoscopic CCT and radiosurgical or laser interstitial thermal therapeutic CCT have been recently developed to decrease the invasiveness and the complication rates (2, 3, 9). If those types of equipment would be available in veterinary medicine, the surgical technique of CCT may change in the future.

Interestingly, in the present case, the seizure type showing the left arm-extended fencing posture at the onset, which is considered a specific seizure sign of the contralateral frontal lobe in humans, appeared after CCT. This seizure type suggested that (at least one of) the epileptogenic zone(s) of the present case localized in the right frontal lobe. Perhaps, the remaining genu may be the cause of the seizure activity transition from the right frontal lobe to the left hemisphere. FPS may also be an altered manifestation of preoperative GTCS. The recognition of laterality of epileptogenicity after CCT in human patients with secondarily generalized seizures without distinct laterality is considered an advantage of CCT, which will be an indicator for subsequential resection surgery (1, 2, 13). Although we do not plan the second resection surgery for the current case at present, it may be performed if seizures increase and/or the owner requests it in the future.

As mentioned above, CCT is often compared with VNS in humans for various factors. For veterinary patients in particular, the size, weight, and costs of the implantable pulse generator (IPG) of VNS (and also DBS) would be a considerable limitation for patients and owners, and there is no specific IPG for small veterinary patients. Currently available IPGs for human patients are quite large and heavy for cats or small breed dogs, and likely cost-prohibitive (10,000–15,000 USD). Furthermore, potential complications such as infection or malfunction of the device during long-term implantation must be taken into consideration. Therefore, we expect that CCT may be a reasonable epilepsy surgery for veterinary patients, although pros and cons of CCT and VNS will become clear in the future.

In conclusion, this is the first report of CCT in a client-owned feline patient with DRE describing relatively positive outcome. Although it will take a long time and the accumulation of both positive and negative cases to establish the utility of CCT in veterinary patients with DRE, the authors hereby present epilepsy surgery as one of the new options for the treatment of epilepsy in veterinary medicine and declare the dawn of a new era of epilepsy treatment in veterinary medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Veterinary Medical Teaching Hospital, Nippon Veterinary and Life Science University (accession no. H30-1). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DH and SK: conception, design, and writing the draft. All authors data acquisition and analysis, revising the draft, and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.745063/full#supplementary-material

Supplementary Data 1 | Supplementary explanations and results for (1) the Project team and clinical trial, (2) scalp EEG, (3) MRI, and (4) intraoperative ECoG.

Supplementary Data 2 | Visual analog scale sheet.

Supplementary Data 3 | Activities of daily living sheet and the results.

Supplementary Video 1 | Videos of the preoperative seizures.

Supplementary Video 2 | Video of the surgical procedure of corpus callosotomy.

Supplementary Video 3 | Videos of the postoperative seizures.

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CASE REPORT
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Abnormal Behavior Episodes Associated With Zonisamide in Three Dogs: A Case Report

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Psychiatric adverse effect associated with anti-seizure drugs has been well-recognized in human medicine. This case report describes three dogs with presumptive idiopathic epilepsy presented for abnormal behavior episodes. Abnormal behavior episodes included sudden rage and aggression to the family members, insomnia, restlessness, and/or constant attention-seeking behavior. MRI study and cerebrospinal fluid analysis in two dogs were unremarkable. The abnormal behavior episodes deteriorated along with gradual dose increment of zonisamide and these episodes almost completely disappeared within 5 days after discontinuation of zonisamide. The exact same episodes relapsed within days after re-administration of zonisamide and disappeared again shortly after discontinuation of zonisamide. Dose adjustments of other anti-seizure medications in case 2 did not result in significant changes in these behavior episodes. Although psychiatric adverse effects including aggressive behavior associated with zonisamide are widely recognized in humans, this is the first report in dogs in the clinical setting.

Keywords: episodic abnormal behavior, psychiatric adverse effect, anti-seizure drug, zonisamide, dog, epilepsy

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INTRODUCTION

Zonisamide (ZNS) is a benzisoxazole derivative with a non-arylamine sulfonamide group (1) and is chemically unrelated to other anti-seizure drugs (ASDs) used in veterinary medicine. With limited available evidence supporting its efficacy in the veterinary field, this medication has been widely used for anti-seizure purpose in dogs and in humans (2–7). In addition to its anti-seizure potency, ZNS may be efficacious in treating various human neurological and psychiatric diseases including migraine, neuropathic pain, essential tremor, and Parkinson's disease (8–12).

The recommended dose and monitoring strategy are as follows: oral starting dose at 3–7 mg/kg every 12 hours (q12h) or 7–10 mg/kg q12h in dogs with co-administered hepatic microsomal enzymes inducers such as phenobarbital, serum concentrations should be aimed between 10 and 40 mg/L according to the human target range, and the serum concentration measurements should be performed at least 1 week after treatment initiation or dosage adjustment given the approximate elimination half-life of 15 h (6, 7). Reported adverse effects of ZNS in dogs in the clinical setting include sedation, generalized ataxia, vomiting, inappetence, and a few idiosyncratic reactions such as cutaneous reactions (13, 14), acute hepatopathy (15, 16), and renal tubular acidosis (17). In addition, aggression has been reported in a research setting investigating chronic toxicity of ZNS in dogs (18). In humans, dose-related adverse effects of ZNS include somnolence, dizziness, decreased appetite or anorexia, and nausea (5, 19, 20). Other reported adverse effects include

fatigue, headache, psychiatric symptoms, cognitive disturbances, diplopia, weight loss, diarrhea, ataxia, oligohydrosis, urolithiasis, and rash (19–24). Here, we report three dogs with abnormal behavior episodes associated with ZNS.

CASE PRESENTATION

Case 1

An 11-year-old male castrated golden retriever that weighed 29.1 kg was evaluated at Saitama Animal Medical Center for recently increasing frequency of the generalized tonic–clonic seizures (GTCS). The owner described this dog had exhibited epileptic episodes once every 2–3 months since he was 8 years old and his episodes were becoming more frequent to once per month over the past 8 months.

Prior to the referral, this dog had been managed with ZNS starting at 2.5 mg/kg per os (PO) q12h then gradually increased in the dose up to 5 mg/kg q12h with the trough blood concentration at 21.2 μg/ml over the 15 months. No other ASDs such as phenobarbital or potassium bromide, listed as first-line ASDs in dogs, were prescribed. Zonisamide was further increased to 7.5 mg/kg q12h due to a few events of cluster of seizures. Two more months later, levetiracetam (LEV) at 17 mg/kg PO q12h was added on ZNS as the dog experienced another episode of cluster of seizures.

Upon the initial visit, the physical and neurological examinations were overall unremarkable other than severe degenerative joint condition in his bilateral elbow, coxofemoral, and stifle joints. Complete blood count (CBC), serum biochemical analysis, and urinalysis were within the reference intervals. Our initial plan was to gradually increase ZNS dosage as needed and to maintain LEV at the same dose. Firocoxib was also prescribed for the joint condition. Zonisamide was prescribed with the gradual dose increment plan potentially up to 13.7 mg/kg q12h if needed based on his seizure frequency. A 2-week telephone follow-up revealed this dog was exhibiting unusual behavior episodes of abrupt barking during sleep without obvious external stimuli. This dog had no pre-existing abnormal behavior problems before and no seizure episodes were witnessed in association with these behavior episodes. At this point, ZNS dose was 10 mg/kg q12h. Another week later, the owner reported the increasing frequency and the change in the episodic abnormal behavior. According to the owner, this dog suddenly stood up during the sleep to bite his tail or blanket for a minute. When the owner called him during the abnormal behavior episode, the dog was able to come out of it and went back to himself. Ten more days later, the owner described that abnormal behavior episodes were deteriorating in the following aspects: increasing in frequency to daily events, not limited to during the sleep, and deteriorating in its severity to bite the owner on her arm severe enough to visit an emergency hospital. The frequency of his GTCS decreased after increment of ZNS up to 13.7 mg/kg q12h while the abnormal behavior episodes increased in its frequency further to over 10 times a day, including abrupt growling at or attempts to bite the family members when they came into his sight while awake (**Supplementary Video 1**). An MRI and cerebrospinal fluid analysis were both unremarkable. Phenobarbital (PB) at 1.5 mg/kg q12h was added at this point concerning these abnormal behavior episodes as an atypical manifestation of sensory epileptic events. The owner reported 7 days later that no GTCS was witnessed while the episodic abnormal behavior remained at the same intensity and frequency. Zonisamide was discontinued abruptly and PB was increased to 2 mg/kg q12h. Seven days after the discontinuation of ZNS, the owner reported those frequent abnormal behavior episodes almost completely disappeared within 5 days after discontinuation of ZNS except for occasional gesture curling up the upper lip during the sleep for a few seconds. Within another week, the abnormal behavior episodes completely disappeared. Our recommendation of reintroducing ZNS for confirmation of direct association between the aggressive behavior and ZNS was rejected by the owner for safety concern of the family members. This dog was managed well with PB (2 mg/kg q12h) and LEV (20 mg/kg q8h) with no observable epileptic or aggressive behavior episodes for 13 months until this dog died of an unrelated condition.

Case 2

A 6-year-old female spayed miniature poodle weighing 5.05 kg was referred to Veterinary Specialists and Emergency Center for episodic aggression. The aggression was noted in the absence of precipitating causes or environmental triggers, occurring nightly predominantly toward females in the household. The aggressive behavior toward the female child was particularly noteworthy, biting her as she slept. Episodic aggression toward the adult female occurred while the dog is being picked up and held (**Supplementary Video 2**). The episodic aggression lasted up to 30 s in duration, returning back immediately following. Episodes showed no response to acepromazine (unknown dose or route).

Prior to referral, this dog had been on ZNS at the dose of 10 mg/kg PO q12h for presumptive idiopathic epilepsy over the past 3 years and epilepsy had been extremely well-controlled with no seizure episodes witnessed. Other than episodic aggression, the dog remained healthy and happy. No significant changes in the environment were reported.

Upon the initial visit, the physical and neurological examinations were overall unremarkable. CBC, serum biochemical analysis, and urinalysis were within the reference intervals. Serum level of ZNS was 47.8 μg/ml. MRI and cerebrospinal fluid analysis were rejected by the owner given the concerns associated with general anesthesia and long history of presumptive idiopathic epilepsy.

Due to serious detrimental impact on the family, ZNS was discontinued abruptly as a therapeutic trial with our concern of possible association between the episodic aggression and ZNS. Phenobarbital 2.5 mg/kg orally q12h was prescribed without loading. The nightly episodic aggression completely disappeared within 5 days after discontinuation of ZNS. This dog had an episode of cluster seizures. After failing to control daily epileptic episodes with PB, LEV, and potassium bromide over the next 10 days, ZNS at 50% reduced dose (5 mg/kg PO q12h) was resumed to confirm the direct association between ZNS and the aggressive episodes as well as to manage seizure episodes. Daily seizure episodes resolved after ZNS was added. While the dog

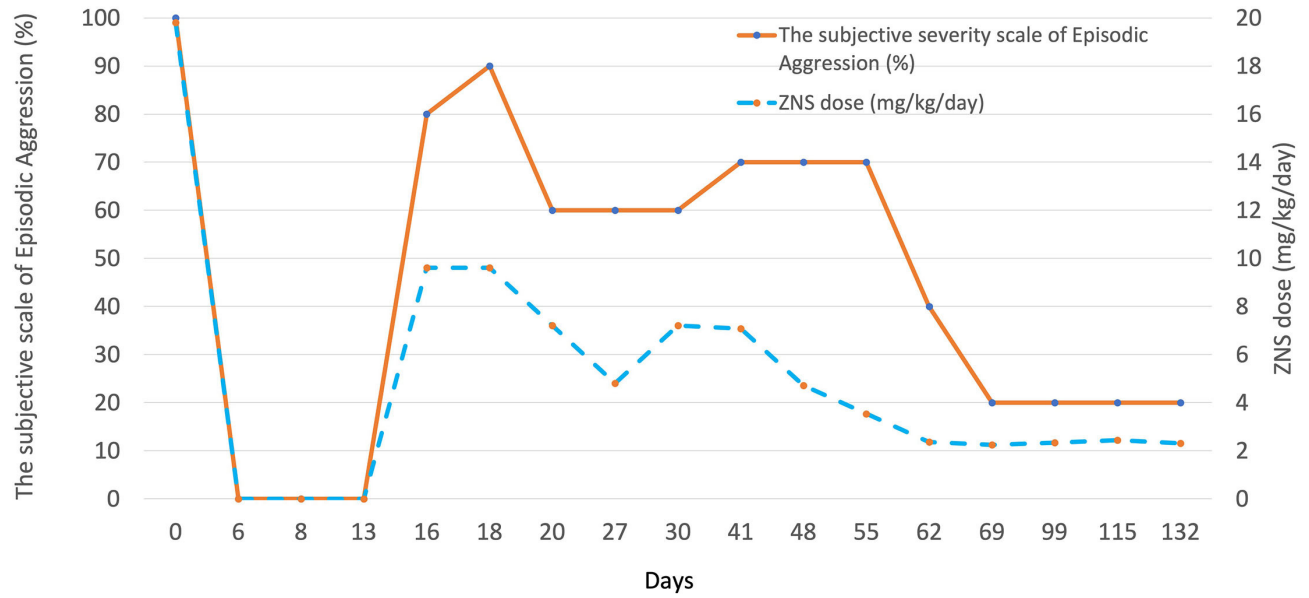

FIGURE 1 | A graph illustrating the association between the dose of ZNS and the severity of episodic aggression in case 2. Multiple dose adjustments resulted in significant changes in the severity of the abnormal behavior episodes in this case. The severity of episodic aggression was determined based on the client's oral description at each follow-up; 100% at the initial presentation.

remained herself over the next 3 days during hospitalization after reintroduction of ZNS, the exact same episodes relapsed on the night after discharge. The aggressive episodes deteriorated day by day over the next 5 days until ZNS dose was reduced to 5 mg/kg PO in the morning and 2.5 mg/kg PO in the evening. Seven days later, the owner reported episodic aggression was improved by 40% compared with that prior to dose reduction. No seizure episodes were recognized. Over the next 6 months, several dose adjustments of ASDs were made. Episodic aggression deteriorated with minor dose increment of ZNS and improved with minor dose reduction.

The severity of the episodic aggression was scaled as 100% at the initial appointment and the sequential change in the severity was evaluated by the client and provided at each follow-up appointment in the gross scale of 0–100%. Chronological changes in episodic aggression in association with dose adjustments of ZNS are summarized in **Figure 1**. The severity and frequency of the episodic aggression showed proportional relationship with the dose of ZNS. No similar episodic aggression was witnessed in association with dose adjustments or addition of other ASDs including PB, potassium bromide, and LEV.

Case 3

A 10-year-old male castrated miniature poodle was referred to Veterinary Specialists and Emergency Center for recent onset of generalized seizure episodes, decreased appetite, and abnormal behavior.

This dog had clinical onset of generalized seizure 12 days prior to referral. The seizure episode was described as sudden running fit with falling and defecation, followed by 1–2-min duration of opisthotonus, clonic motor activities, and copious amount of ptyalism. Within 24 h after ZNS was commenced at the dose of 5 mg/kg PO q12h, this dog started acting abnormal. His behavior was characterized by insomnia, agitation, constant attention-seeking behavior through the night, restlessness, and excessive reaction to the external stimuli. Routine bloodwork consisting of CBC and serum biochemical analysis were within the reference intervals. The dose of ZNS was increased to 6.6 mg/kg PO q12h by a referring veterinarian. MRI, cerebrospinal fluid analysis, and fasted and postprandial total bile acid analyses were within normal limits. Addition of LEV (27 mg/kg PO q8h) and glycerin (5 ml/head PO q8h) did not alter the abnormal behavior and decreased appetite.

Upon the initial visit, the physical and neurological examinations were overall unremarkable. Given the history and unremarkable clinicopathological analyses results, ZNS dose was reduced to 4.5 mg/kg PO q12h without any changes in LEV and glycerine to avoid multiple variables at one time for assessing the clinical response to the medication adjustment. The owner reported that significant improvements were recognized within 24 h after the dose reduction and no seizure episodes were witnessed. The dose of ZNS was further decreased to 2.3 mg/kg PO q12h without any changes in LEV and glycerine for another 3 days then completely discontinued. Seven days after complete discontinuation of ZNS, abnormal behavior episode completely disappeared, and the appetite was recovered by 80% according to the owner. However, sudden jerky movement of the upper body was increasingly noticed along with tapering off process of ZNS. Therefore, the dose of LEV was increased to 40 mg/kg PO q8h,

resulting in significant improvement of the jerky movements without relapse in the abnormal behavior.

Seven days after the dose increment of LEV, ZNS was re-introduced at 6.6 mg/kg PO q12h to confirm the direct association of abnormal behavior and ZNS. The owner reported the same abnormal behavior episodes relapsed within 12 h after re-introduction and gradually deteriorated over the next 3 days. Zonisamide was abruptly discontinued 4 days after re-introduction, which resulted in resolution of the abnormal behavior episodes within 24 h. No other modifications in other medications were made during this ZNS re-introduction trial.

DISCUSSION

This case series reports the occurrence of reversible behavior changes associated with zonisamide in three dogs with no pre-existing behavior problems prior to administration of zonisamide. Among various ASDs, ZNS has been frequently used in veterinary practice with several adverse effects being reported (2, 7, 13–17, 25–32). Reported type 1 adverse effects include sedation, vomiting, loss of appetite, and ataxia (2, 7, 25, 28). Reported type 2 adverse effects include two cases of acute toxic liver injury, three cases with dermatologic lesions, and one case of renal tubular acidosis (13–17). Other potential type II adverse effects include one case with keratoconjunctivitis sicca and one case with polyarthropathy, although the direct relationship was not confirmed in these cases (7). As for type 3 adverse effects, the possibility of affecting thyroid function (especially decrease in total T4) and the changes in blood chemistry profile within the reference range, including the elevation of ALP and Ca and the decrease of total protein and albumin compared with those prior to the administration of ZNS, were observed (7). Adverse effects classified as type 4 have not been reported (7). Recently, ZNS-related anticonvulsant hypersensitivity syndrome was reported in cats (27). However, aside from a previous report by Walker et al. in the research setting where three male dogs showed aggression while they received 75 mg/kg/day of ZNS for 52 weeks achieving the serum ZNS level around 80–120 μg/ml, (18) abnormal behavior episodes as we experienced have not been reported in the clinical veterinary setting to the best of our knowledge.

In human medicine, there were several reports of psychiatric and behavioral side effects associated with ZNS (1, 24, 33–36). A review article reported the most common adverse effects pertained to the CNS were ataxia, dizziness, somnolence, agitation, and anorexia (36). Another report described that psychiatric adverse events (PAEs) and cognitive adverse events (CAEs) were the most frequently identified reasons for terminating ZNS therapy in 433 epileptic patients who received ZNS (24). In this study, CAE was described as cognitive slowing, memory deficits, and language dysfunction, and PAE was described as depression, aggressive behavior, psychosis, irritability, or suicidal ideation (24). The incidence of PAE severe enough to result in discontinuation of ZNS was 6.9%; the incidence of CAE resulting in discontinuation of ZNS was 5.8%. These patients improved shortly after ZNS was discontinued. Changes in brain serotonin and dopamine levels are considered as a mechanism of ZNS-induced PAE (24, 35, 37) and human patients with a history of psychiatric disorder had significantly greater risk of developing PAE associated with ASDs including ZNS (24, 36, 38, 39). It was also interesting that the average maximum ZNS serum concentration in patients who discontinued ZNS attributable to PAE or CAE was significantly lower than maximum ZNS concentration of control group in the aforementioned study (24). This may suggest that PAE or CAE are not necessarily associated with higher serum concentration of ZNS than the reference range. While most of the human patients developed PAE or CAE in the first 3 months of exposure to ZNS and resulted in discontinuation of ZNS within 5 months, some human patients developed PAE or CAE after many months after ZNS treatment was started, often in association with late dose increment of ZNS (24).

Our cases showed clinical apparent direct association with ZNS and correlation with dose adjustment in case 1 and case 2. This suggests that ZNS-related PAE may be a dose-dependent adverse effect that could be overlapped with the therapeutic reference range in predisposed patients. Although therapeutic and toxic range of ZNS in dogs has not been well-established yet, serum concentration at 47.8 μg/ml in our case 2 could be considered in high end or above the therapeutic range (6, 7, 25, 28). The aggressive behavior resolved almost completely within 5 days after the abrupt cessation of ZNS in our cases. This may also support the possible dose-dependent nature of the aggressive episodes, given the reported elimination half-life of ZNS in dogs being approximately 15 h (6, 26).

In two recent reports on human medicine, LEV had the highest PAE risk (15.7–16.2%), which was significantly higher compared with those with other ASDs (38, 39). Similarly, a case series study described abnormal behavior episodes in dogs receiving LEV (40). Interestingly, 51.2% of LEV-attributed PAE symptoms in humans resolved with dose decrement, whereas only 15.4% patients of ZNS-attributed PAE symptoms resolved with dose decrement and the rest (85.6%) of ZNS-attributed PAE symptoms required complete cessation of ZNS (38). In our cases 1 and 2, ZNS was discontinued abruptly due to serious impact of the aggressive behavior episodes to the family and human data according to the aforementioned report (38).

Limitations of this report reside in the small number of cases, the retrospective nature, and difficulty in proving the direct association of abnormal behavior episodes and ZNS or in ruling out other potentially contributing conditions. Our case 2 did not have MRI or cerebrospinal fluid analysis to rule out other intracranial structural conditions, which was clinically considered relatively unlikely given stable condition through a long follow-up duration of this case. Nonetheless, the proportional association between ZNS dose and the severity of the clinical signs was shown with multiple dose adjustments in this case.

The clinical signs in our cases were similar to PAE in humans, which seriously and negatively affected the caregivers' quality of life. Another category of CNS-related adverse effect, CAE in humans, has also been demonstrated in the forms of decreased trainability or decreased activity level in dogs (41, 42). According to the human literature, PAE associated with ASDs appeared reversible in nature (38). Further studies are warranted to provide more insights in behavior change associated with ASDs in animals.

This is the first report describing abnormal behavior episodes, similar to PAE in humans, associated with ZNS in dogs in the clinical setting.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this case report describes a medical condition of client-owned dogs without serious iatrogenic consequence. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SK: conception, design, and writing the draft. SK, MU, and AH: data acquisition, figure and supplemental video preparation, and revision of the draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.763822/full#supplementary-material

Supplementary Video 1 | An abnormal behavior episode of case 1 showing aggressive growling at the family members while resting in the bed. This dog bit the client on the arm requiring the emergency room visit for deep bite wound treatment. The episode typically lasted for ∼1 min.

Supplementary Video 2 | An abnormal behavior episode of case 2 right after getting on the client's lap. This dog bit the client on the arm multiple times. The abrupt episode typically lasted for ∼20–30 s.

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Response to Levetiracetam Treatment and Long-Term Follow-Up in Dogs With Reactive Seizures Due to Probable Exogenous Toxicity

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Limited information is available on the long-term follow-up and seizure recurrence in dogs with reactive seizures due to suspected exogenous toxicity. The purpose of this study was to report the long-term follow-up of 13 dogs referred to a single referral hospital, diagnosed with reactive seizures and treated with a standardized levetiracetam protocol. All dogs received a loading levetiracetam dose of 60 mg/kg/IV once, followed by a maintenance dose of 20 mg/kg every 8 h as part of an open-label clinical study. Levetiracetam was withdrawn after a 6-months seizure-free period by reducing levetiracetam to 20 mg/kg every 12 h for a 4-week seizure-free period, followed by levetiracetam 20 mg/kg every 24 h for a 4-week seizure-free period, before levetiracetam treatment was stopped. No adverse effects of the treatment were reported. No dogs experienced any seizures after discharge or after levetiracetam withdrawal. Median follow-up time from time of discharge was of 78 months (=6 years 6 months). The result of this study supports the use of levetiracetam for treatment of reactive seizures due to exogenous substance intoxication. Moreover, our results do not support the need for long-term antiepileptic treatment in cases of reactive seizures due to exogenous intoxication.

Keywords: reactive seizures, levetiracetam, intoxication, long-term follow-up, canine epilepsy

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INTRODUCTION

Reactive seizures (RS) have been defined as seizures "occurring as a natural response from the normal brain to a transient disturbance in function (metabolic or toxic in nature) which is reversible when the cause or disturbance is rectified" (1). Reported prevalence of RS in dogs varies widely among the few studies available in the veterinary literature, ranging from 6.3 to 13.6%. Treatment of RS involves the use of antiseizure drugs (ASD) and treatment of the intrinsic underlying etiology when known (2–6) Levetiracetam (LEV) is a pyrrolidine derivative and is widely used in human medicine for various seizure types. In veterinary medicine, although it is not licensed, it is commonly used due to is safety profile (7), lack of hepatic metabolism (unlike most ASD), and minimal drug interactions. While there is fair evidence for recommending LEV as adjunctive treatment for seizures in dogs, data on efficacy of LEV as monotherapy is scarce (8). Levetiracetam was reported superior to placebo in a randomized, double-masked, placebo-controlled trial for the treatment of cluster seizure and status epilepticus when administered intravenously (IV). This

study included 19 dogs (9 receiving LEV and 10 receiving placebo) with epileptic seizure due to various etiologies (9). A prospective open-label clinical trial on intrarectal (IR) LEV administration reported good control of cluster seizures in 21 dogs with idiopathic epilepsy (10). Currently, there is limited information available on long-term follow-up and seizure recurrence in dogs with RS due to suspected exogenous toxicity. The purpose of this study was to report the long-term follow-up of 13 dogs diagnosed with RS treated with LEV, due to highly suspected exogenous toxicity.

MATERIALS AND METHODS

This was a prospective open-label clinical study including 13 dogs referred to a single referral hospital (Animal Health Trust, Center for Small Animal Studies) with seizures due to highly suspected exogenous intoxication between January 2009 and December 2015. The study was conducted as a prospective, observational cohort study, approved by the clinical research and ethical committees of the Animal Health Trust (Approval number: AHT42_2009; Approval date: 19th January 2009). Inclusion criteria were: evidence/high suspicion of exogenous toxin ingestion resulting in RS based on anamnestic information given by the owners who witnessed/suspected ingestion of foreign material with or without consecutive presence of gastroinstinal signs (vomiting/hypersalivation/diarrhea), a neurological examination performed by a board-certified neurologist and treatment with LEV (emergency treatment followed by maintenance treatment). Levetiracetam treatment was standardized as follow: at admission all dogs received a LEV loading dose of 60 mg/kg/IV once. The treatment was continued with LEV 20 mg/kg every 8 h orally (PO) or IV until the patient was able to receive medications PO. Levetiracetam treatment was continued in all dogs at a dosage of 20 mg/kg every 8 h (or the nearest dosage based on available formulation and body weight) for a 6-month seizure free period. Levetiracetam was withdrawn by reducing LEV to 20 mg/kg every 12 h PO for a 4-week seizure free period, followed by LEV 20 mg/kg every 24 h PO for a 4-week seizure free period, after which LEV treatment was interrupted. All owners were contacted, as last follow-up, in December 2019. Dogs with a previous history of seizures due to any etiologies prior to the known or suspected intoxication, were excluded from the study. Cluster seizures was defined as two or more seizures in 24 h with complete recovery of consciousness between seizures. Status epilepticus was defined as a single seizure lasting longer than 5 min, or two or more seizures without complete recovery of consciousness in between (1). The following information was collected for each case: signalment (breed, sex, age at presentation, and body weight), seizure type (e.g., single seizure, cluster seizures or status epilepticus) and occurrence, diagnostic investigation results (hematology, serum biochemistry, bile acids/ammonia serum levels, urinalysis, magnetic resonance imaging, and cerebrospinal fluid analysis), LEV adverse effects.

RESULTS

Population

Thirteen dogs met the inclusion criteria. The signalment of the 13 dogs is described in **Table 1**. All dogs presented for cluster seizures of generalized tonic-clonic seizures. Details of seizure activity are included in **Table 1**. Mean number of seizures from onset to first administration of LEV was 5.38 (range 2–9 RS). The owners of all 13 dogs reported onset of RS after highly suspected ingestion of an unknown toxic substance, although the named substance could not be traced. All dogs were referred after a mean period of 16 h (range 4–24 h) from the first RS. Nine of 13 dogs received subcutaneous apomorphine administration before referral (0.1 mg/kg; EMEDOG®1 mg/ml, solution for injection for dogs, TVM Animal Health Ltd) to induce emesis. Hematology and serum biochemistry results did not reveal any abnormalities that would result in seizure activity in any of the 13 dogs. In all but 2 dogs (case 5, 6) a bile acid stimulation test and ammonia serum level were performed, and results revealed no significant abnormalities. Urinalysis revealed no significant abnormalities in the 5 dogs in which it was performed (case 1, 2, 8, 9, and 12). Magnetic resonance imaging of the brain and cerebrospinal fluid analysis were performed in all dogs and no abnormalities were detected. Based on the medical history, clinical presentation, clinical course, diagnostic investigation results and follow-up, all dogs were diagnosed with RS triggered by highly suspected ingestion of an unknown toxic substance.

Antiseizure Treatment

Details of ASD treatment in each dog are presented in **Table 1**. Eleven of the 13 dogs received diazepam before referral at a median dose of 0.6 mg/kg/IR (range 0.5–1 mg/kg IR). Two dogs received no treatment prior to referral. According to protocol at admission all dogs received a LEV loading dose of 60 mg/kg/IV once. The treatment was continued with LEV 20 mg/kg every 8 h IV for 24 h in 4/13 dogs until able to receive medications PO (dog 5, 7, 9, and 10), and with LEV 20 mg/kg every 8 h PO in the remaining 9/13 dogs. After initial administration of LEV, the mean number of seizures was 0.38 (range 0–2 RS). Ten dogs experienced no further seizure after the LEV loading administration. Two dogs (dog 5, 10) experienced 1 seizure and 1 dog (dog 9) experienced 2 seizures after the loading LEV administration and no further seizure after the first maintenance (20 mg/kg/IV) LEV administration. In all dogs the LEV withdrawal protocol was followed by all clients without need to interrupt treatment earlier. No adverse effects due to LEV were reported in any of the 13 cases.

Follow-Up

No dogs experienced any seizures after discharge, nor after LEV withdrawal. Median follow-up time from time of discharge was of 78 months (=6 years 6 months; range: 48–117 months). Nine of the 13 dogs were alive at the time of last follow-up. Dog 4 was euthanised 77 months after discharge, due to recurrence of paraplegia secondary to intervertebral disc herniation. Dog 5 was euthanised after 48 months from discharge because of haemoabdomen due to a ruptured splenic neoplasia and

TABLE 1 | Signalment, body weight, treatment, and follow-up details of the 13 dogs with highly suspected exogenous toxicity.

	Patients' details	Age at RS onset	Pre-referral ApoM treatment	Pre-referral emergency ASD treatment	Seizure occurrence from onset to discharge	Duration of hospitalization	Follow-up duration
1	Labrador, MN, 20 kg	11 m	0.1 mg/kg/SC	Diazepam: 0.5 mg/kg/IR twice	4 seizures in 4 h before referral, 0 seizure after LEV loading	2 days	97 m
2	Cocker Spaniel, MN, 15 kg	24 m	None	None	3 seizures in 5 h before referral, 0 seizures after LEV loading	2 day	96 m
3	Border Collie, FE, 15 kg	6 m	0.1 mg/kg/SC	Diazepam: 1 mg/kg/IR twice	6 seizures in 8 h before referral, 0 seizures after LEV loading.	3 days	117 m
4	Cocker Spaniel, FS, 12 kg	38 m	0.1 mg/kg/SC	Diazepam: 0.5 mg/kg/IR twice	4 seizures in 12 h before referral, 0 seizures after LEV loading.	2 days	Euthanised after 77 m
5	Labrador, MN, 30 kg	60 m	0.1 mg/kg/SC	Diazepam: 1 mg/kg/IR twice	10 seizures in 24 h, of which 9 seizures before referral, 1 seizure after LEV loading.	3 days	Euthanised after 48 m
6	Beagle, MN, 15 kg	48 m	0.1 mg/kg/SC	None	4 seizures in 24 h before referral, 0 seizure after LEV loading.	2 days	60 m
7	French Bull Dog, FS, 11 kg	12 m	0.1 mg/kg/SC	Diazepam: 0.8 mg/kg/IR once	8 seizures in 23 h before referral, 0 seizure after LEV loading.	3 days	Euthanised after 84 m
8	French Bull Dog, FS, 10 kg	18 m	0.1 mg/kg/SC	Diazepam: 1 mg/kg/IR twice	5 seizures in 20 h before referral, 0 after LEV loading.	1 day	62 m
9	Irish Setter, MN, 28 kg	24 m	None	Diazepam: 0.5 mg/kg/IR three times	10 seizures in 24 h, of which 8 seizures before referral, 2 after LEV loading	3 days	Euthanised after 49 m
10	Maltese, FS, 5 kg	14 m	None	Diazepam: 0.5 mg/kg/IR three times	8 seizures in 22 h, of which 7 seizures before referral, 1 after LEV loading.	2 days	108 m
11	German Shorthair Pointer, FS, 30 kg	36 m	None	Diazepam: 0.5 mg/kg/IR once	4 seizures in 12 h before referral, 0 after LEV loading.	1 day	72 m
12	Hungarian Vizsla, FS, 20 kg	36 m	0.1 mg/kg/SC	Diazepam: 0.5 mg/kg/IR four times	3 seizures in 12 h before referral, 0 after LEV loading.	3 days	84 m
13	Weimaraner, FS, 28 kg	24 m	0.1 mg/kg/SC	Diazepam: 0.5 mg/kg/IR three times	6 seizures in 18 h before referral, 0 after LEV loading.	3 days	60 m

ME, male entire; MN, male neutered; FE, female entire; FS, female spayed; kg, kilograms; m, month; h, hours; CS, cluster seizures; ApoM, apomorphine; ASD, antiseizure drugs; LEV, levetiracetam; SC, subcutaneous; IR, intrarectally; IV, intravenously; PO, orally.

suspected secondary lung metastasis. Dog 7 was euthanised after 84 months from discharge after diagnosis of immune-mediated anemia and dog 9 was euthanised after 49 months from discharge due to gastric torsion.

DISCUSSION

Relatively little data is available on the efficacy of LEV monotherapy for the treatment of seizures or epilepsy in dogs, and to the best of the authors' knowledge there is no published data on LEV monotherapy for the treatment of RS due to suspected exogenous toxicity in dogs (7, 8). The role of LEV in the maintenance treatment of dogs with idiopathic and structural epilepsy has been described in a few studies (11–13). In this study, LEV was chosen to treat RS due to highly suspected exogenous intoxication due to its favorable metabolic profile compared to other ASD (7). All dogs were treated with a standardized LEV protocol following acute onset of cluster seizures. No seizures occurred after the LEV loading dose in 10 dogs and the first maintenance dose in 3 dogs (dogs 5, 9, and 10). It cannot be ruled out that in these three dogs the pre-referral emergency diazepam administration might have interfered with the plasma concentration of LEV (14). This suggests that LEV was efficacious as ASD in these dogs, however, this is challenging to demonstrate as there was no placebo control group. Similarly, a shorter maintenance treatment duration may have been associated with similar outcomes, but this was not assessed in this study. The decision to continue the LEV for 6 months following the initial presentations was based on current guidelines on initiation of ASD treatment in dogs with idiopathic epilepsy, which include the presence of 2 or more epileptic seizures within a 6-month period and the presence of

status epilepticus and cluster seizures, as well as other criteria (15). In fact in the 13 dogs reported in this study the choice of ASD could also be considered further complicated by the knowledge that the signalment of the dogs included in the study could be suggestive of idiopathic epilepsy. Considering that ASD treatment is frequently a life-long commitment, and that dogs affected with RS due to suspected exogenous intoxication might not be needing life-long ASD treatment, our study supports the possibility that LEV monotherapy offers a sensible alternative to the use of other ASD in these cases. In the 13 dogs included in this study, although the exogenous substances could not be traced, the presumptive diagnosis of exogenous intoxications was suspected based on owners' report of sudden onset of cluster seizures after ingestion of unwanted substances and response to antiepileptic treatment. Levetiracetam oral dosage was reduced by ∼20% monthly (15). Lack of seizure re-occurrence after LEV discontinuation and a mean follow-up of 78 months (=6 years 6 months; range: 48–117 months) further supports the diagnosis of RS secondary to exogenous toxicity. Interestingly, none of the 13 cases we reported has experienced RS long-term following treatment withdrawal. Similarly, also Zimmerman et al. (4) and Jull et al. (16) reported that no further RS occurred after discharge at a median follow-up time of 2 years 6 months and 2 years, respectively. Our case series therefore strengthen the evidence that cluster seizures triggered by exogenous intoxication does not cause/predispose to recurrent seizures in dogs based on a significantly longer follow-up period (6 years 6 months). Another possible conclusion the reader might take from this study is that the use of LEV might be useful after toxin exposure, equally efficacious or even superior to ASD in cases of reactive seizures due to probable exogenous toxicity. Limitations of the present study are the relatively small sample size which was related to the closure of the institution where the study was conducted, the lack of a control group, and the lack of laboratory evidence of intoxication and named substance, and as such the results of this study should be interpreted with caution. However, these preliminary results can be useful for the practicing veterinarians to inform treatment decision and prognosis discussions with dog owners. In addition, these results provide the foundation for further studies. To the authors' knowledge, this is the first study supporting the use of LEV for treatment of RS due to exogenous substance intoxication. Moreover, our results do not support the need for long-term antiepileptic treatment in cases of RS due to exogenous intoxication.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Health Trust Ethical Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

FS and LD have drafted, written, and corrected the paper together. All authors contributed to the article and approved the submitted version.

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Cyclooxygenase-2 Inhibition as an Add-On Strategy in Drug Resistant Epilepsy—A Canine Translational Study

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Drug-resistant epilepsy is a common complaint in dogs and affects up to 30% of dogs with idiopathic epilepsy. Experimental data suggest that targeting cyclooxygenase-2 (COX-2) mediated signaling might limit excessive excitability and prevent ictogenesis. Moreover, the role of COX-2 signaling in the seizure-associated induction of P-glycoprotein has been described. Thus, targeting this pathway may improve seizure control based on disease-modifying effects as well as enhancement of brain access and efficacy of the co-administered antiseizure medication. The present open-label non-controlled pilot study investigated the efficacy and tolerability of a COX-2 inhibitor (firocoxib) add-on therapy in a translational natural occurring chronic epilepsy animal model (client-owned dogs with phenobarbital-resistant idiopathic epilepsy). The study cohort was characterized by frequent tonic–clonic seizures and cluster seizures despite adequate phenobarbital treatment. Enrolled dogs ($n = 17$) received a firocoxib add-on therapy for 6 months. Tonic–clonic seizure and cluster seizure frequencies were analyzed at baseline (6 months) months during the study (6 months). The responders were defined by a substantial reduction of tonic–clonic seizure and cluster seizure frequency (≥50%). In total, eleven dogs completed the study and were considered for the statistical analysis. Two dogs (18%, 2/11) were classified as responders based on their change in seizure frequency. Interestingly, those two dogs had the highest baseline seizure frequency. The overall tolerability was good. However, given the low percentage of responders, the present data do not support an overall considerable efficacy of COX-2 inhibitor add-on therapy to overcome naturally occurring phenobarbital-resistant epilepsy in dogs. Further translational evaluation should only be considered in the canine patients with a very high baseline seizure density.

Keywords: idiopathic epilepsy, coxib, P-glycoprotein, seizures, dog, pharmacoresistance, treatment study, blood brain barrier

INTRODUCTION

Inflammation is considered as a major contributor to ictogenesis in the chronic epileptic brain (1). Controversial data have been obtained from the rodent experiments indicating beneficial, detrimental, or lack of effects of strategies targeting the inflammatory enzyme cyclooxygenase-2 (COX-2) (1). Studies supporting the targeting of COX-2 reported delays in seizure onset, reduced seizure duration, and increased seizure thresholds (2–4). Moreover, data from a chronic rat model of drug-resistant temporal lobe epilepsy suggested that COX-2 inhibition might even help to overcome drug resistance (5). According to the previous findings, this might be related to the prevention of a seizure-induced P-glycoprotein (Pgp) upregulation and associated increases in blood–brain barrier efflux (6–8). As emphasized in a recent review, no clinical studies have yet been conducted with COX-2 selective inhibitors (=Coxibs) in human patients with epilepsy due to the withdrawal of these drugs (1). In this context, the authors pointed out that there is a particular interest to further explore the potential therapeutic benefits although the evidence is still inconclusive. Dogs with idiopathic epilepsy may be particularly suited for further investigation of this treatment strategy, because several Coxibs are currently licensed for therapeutic management of inflammatory disorders in dogs, and difficult-to-treat epilepsy with tonic–clonic and cluster seizures is a common complaint in dogs (9).

Seizure freedom is only infrequently achieved despite lifelong treatment with antiseizure drugs marketed for dogs and humans (9–12). In this context it is important to note that indicators of neuroinflammation were present in the serum of dogs with the idiopathic epilepsy (13, 14). Analysis of post mortem tissue from canine patients demonstrated upregulation of endothelial Pgp in the brains of epileptic dogs following status epilepticus or cluster seizures (15). Moreover, a high-seizure density has been identified as a risk factor for drug-resistant epilepsy in client-owned dogs (9, 11, 12). Thus, canine patients with phenobarbital-resistant epilepsy might serve as a valuable translational model allowing the assessment of COX-2 selective inhibitors. Furthermore, evaluation in dogs with spontaneous epilepsy would allow further evaluation of this treatment strategy under the clinical conditions with a higher variance in the genetic background, etiology, and seizure history as compared with highly standardized experimental rodent studies (16).

We hypothesized that COX-2 inhibition can improve seizure control in dogs with the chronic epilepsy. Therefore, we obtained pilot data on the efficacy and tolerability of a COX-2 inhibitor add-on treatment in a canine cohort with phenobarbital-resistant idiopathic epilepsy and frequent tonic–clonic seizures.

MATERIALS AND METHODS

The present clinical trial was conducted as a non-controlled open-label prospective pilot study. The ethical approval of the project was granted by the ethics and welfare committee (URN 2012 1175). All the experiments were conducted in accordance with the German animal protection law (Tierschutzgesetz). All the clients signed permission for scientific use of the data following informed consent. Client-owned pet dogs diagnosed with phenobarbital-resistant idiopathic epilepsy and frequent tonic–clonic seizures were recruited. Idiopathic epilepsy was diagnosed if recurrent seizures occurred for at least 6 months, onset was between 6 months and 6 years and clinical examination, interictal neurological examination, laboratory investigation, and MRI or CT of the brain revealed no the relevant abnormalities. A consistent high seizure frequency ($\geq$2 tonic–clonic seizures/month in the 4 months before the study inclusion despite adequate phenobarbital therapy for at least 6 months (target steady state serum concentration 20–35 μg/ml) was required, regardless of type or dosage of other co-administered antiseizure medications. Exclusion criteria were age of onset $\geq$6 years, treatment with glucocorticoids in the previous 4 weeks, a history of gastrointestinal disease in the previous 6 month, a history of hepatic, renal, or cardiac disease or bleeding problems, poor documentation of the epileptic seizures and/or poor owner compliance. Owners were provided with seizure logs for documentation on first contact. Firocoxib[1], a selective COX-2 inhibitor licensed for the treatment of osteoarthritis and post-operative pain in dogs was used as study drug. Firocoxib was administered as add-on to antiseizure medications with an oral dose of 5 mg/kg SID. Dog owners were blinded as to the exact nature of the drug. The study consisted of a continuous and an alternating treatment phase (detailed study design is illustrated in **Figure 1**). Seizure data were analyzed at baseline (at least 4 months, when available 6 months and during firocoxib add-on treatment (at least 4 months, when available 6 months, prospectively) by screening of seizure logs on each control visit, at 1, 2, 4, and 6 months. Seizure frequency (mean number of tonic-clonic seizures/month; in the case of cluster seizures each seizure was counted as one event), seizure days (mean number of seizure days/month), and cluster seizures (mean number of cluster days/month; each day with $\geq$2 seizures was counted as one event) were assessed. Phenobarbital serum concentrations (Epilepsy centre Bethel, Bielefeld, Germany), blood tests (hematology, serum chemistry, and electrolytes), urinalysis (specific gravity, pH, protein, glucose, hemoglobin, microscopic examination, and urine protein creatinine ratio) and buccal mucosal bleeding time were measured at baseline and encouraged on each control visit. Bile acid stimulation test was only performed at baseline and study exit. Primary outcome was decrease in mean seizure frequency compared with the baseline. The responders were defined by a seizure frequency reduction of $\geq$50% compared with the baseline data (partial treatment success). Secondary outcome was a decrease in cluster seizures compared to baseline.

Dogs with a study participation of at least 4 months were considered for statistical analysis. Statistical analysis (GraphPad Prism version 5) was performed by the Wilcoxon matched-pairs signed rank test (two-tailed) for seizure data. Statistical significance was defined at $p \leq 0.05$.

[1]Previcox® (Boehringer Ingelheim) licensed for the control of pain and inflammation associated with osteoarthritis, soft-tissue, orthopedic, and dental surgery in dogs (EMA/CVMP/19896/2007&EMEA/V/C/000082).

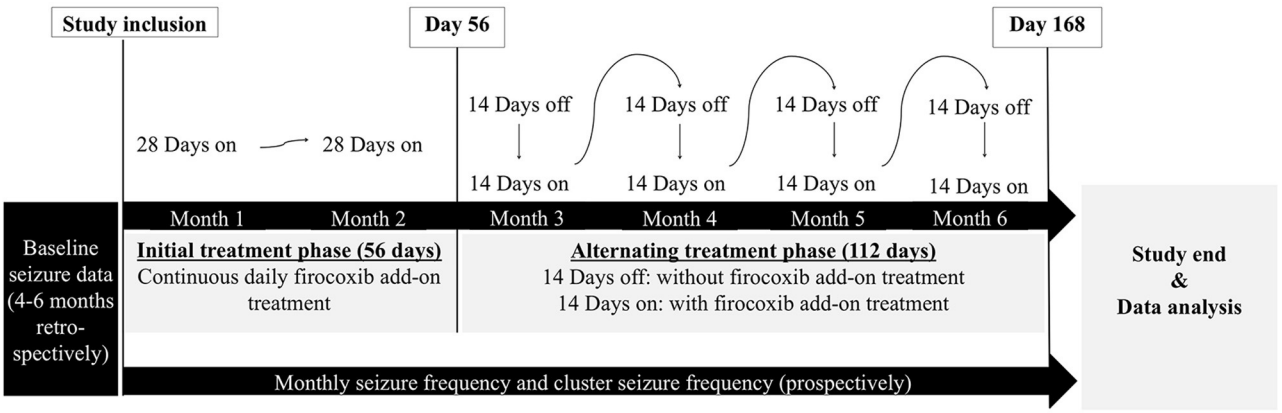

FIGURE 1 | Schematic illustration of the study design. The study course consisted of a continuous treatment period for the first 56 days, followed by a 14-day alternating treatment period for 112 days (total study period 168 days). The alternating treatment protocol was selected to lower the risk of potential firocoxib adverse events. Antiseizure medication was not changed during the firocoxib add-on phase, use of diazepam rectal tubes for emergency management of seizures was allowed. Clinical examinations and blood tests were performed on a regular base throughout the entire study period. Study participation was denied to any dog with pre-existing signs of gastrointestinal disease, renal disease or pre-treatment with any non-steroidal anti-inflammatory drug or glucocorticoids.

RESULTS

In total, seventeen dogs (7 females; 10 males; mean age 5.12 years, range 2.0 to 11.0 years) with phenobarbital-resistant idiopathic epilepsy were recruited. Ten dogs were treated with phenobarbital monotherapy, and seven dogs with combination therapy: phenobarbital/potassium bromide (4 dogs), phenobarbital/levetiracetam ($n = 2$) or phenobarbital/potassium bromide/levetiracetam (1 dog). In seven dogs, the study was terminated early (range 8 days−16 weeks). Reasons for early study exit were severe disease of the owner (1 dog), other diseases requiring additional medication (4 dogs; vomiting and diarrhea with suspicion of pancreatitis; diarrhea; atopic dermatitis; cranial cruciate ligament rupture) and worsening of seizure control (2 dogs, day 86, day 125). One of the drop-out patients completed the minimum required study period of 4 months and was therefore included in the statistical analysis.

In total, seizure data of eleven dogs with phenobarbital-resistant idiopathic epilepsy (7 males, 4 females; mean age 5.0 years; mean body weight 21.3 kg) were analyzed. The mean age of onset was 2.9 years (range 1.0–5.0 years), duration of the epilepsy 2.1 years (range 0.5–5.0 years), and the mean phenobarbital serum concentration 29.05 µg/ml (range 24.6–32.4 µg/ml; 10 dogs). One dog with a phenobarbital serum concentration of 18 µg/ml despite high daily dosages of phenobarbital (5.6 mg/kg q12h) was included (#9). Five of these dogs received additional antiseizure medications (#12, #15, #17 potassium bromide; #9 levetiracetam; #4 potassium bromide, and levetiracetam). Three other dogs were previously treated with potassium bromide but treatment was discontinued due to lack of efficacy (#3, #8) and side effects (#8).

The seizure frequency of the study population with pooled data ($n = 11$) was not relevantly altered by the firocoxib add-on treatment strategy [median seizure frequency (range): baseline 3.5 seizures/month (2.3–11.2); firocoxib add-on 4 seizures/month (2.3–8); $p = 0.59$] during the six-month treatment period (**Figure 2A**). However, considering individual data, six dogs (55%, 6/11) experienced a seizure frequency reduction in response to firocoxib add-on therapy ranging from 8 to 54% (**Figure 3**). Among these, two dogs (dogs #8, #17) showed a seizure frequency reduction of ≥ 50%. These were the dogs that suffered from the highest baseline seizure frequency (10.7 and 11.2 seizures/month, respectively), indicating that Pgp-mediated mechanisms may be only clinically relevant in a proportion of phenobarbital-resistant dogs with a very high baseline seizure frequency.

Considering that the frequent occurrence of cluster seizures and a high seizure density seems to be linked to a poor drug responsiveness in canine epilepsy patients and upregulation of p-glycoprotein and that cluster seizures have a major impact on quality-of-life in epilepsy patients, we separately assessed an effect on occurrence of cluster seizures defined as days with cluster seizures. There was no relevant alteration in cluster frequency with respect to the pooled data of the study population during firocoxib add-on treatment [$n = 11$; median cluster frequency (range): baseline 0.8 cluster seizure days/month (0–2); firocoxib add-on 1.0 cluster seizure days/month (0–2); $p = 0.5$] (**Figure 2B**). There was also no relevant change in seizures/cluster day (median 2.5 seizures/cluster day during baseline vs. 2.6 seizures/cluster day during firocoxib add-on treatment). Considering individual data, the number of days with cluster seizures increased in six dogs during the firocoxib add-on treatment and decreased in four dogs. One dog did not exhibit cluster seizures during the complete study phase. Yet again, one of the dogs with the highest baseline cluster day frequency (#17) showed a reduction of ≥50% in monthly cluster days.

The regular clinical and laboratory controls revealed no relevant or adverse effects of the firocoxib add-on

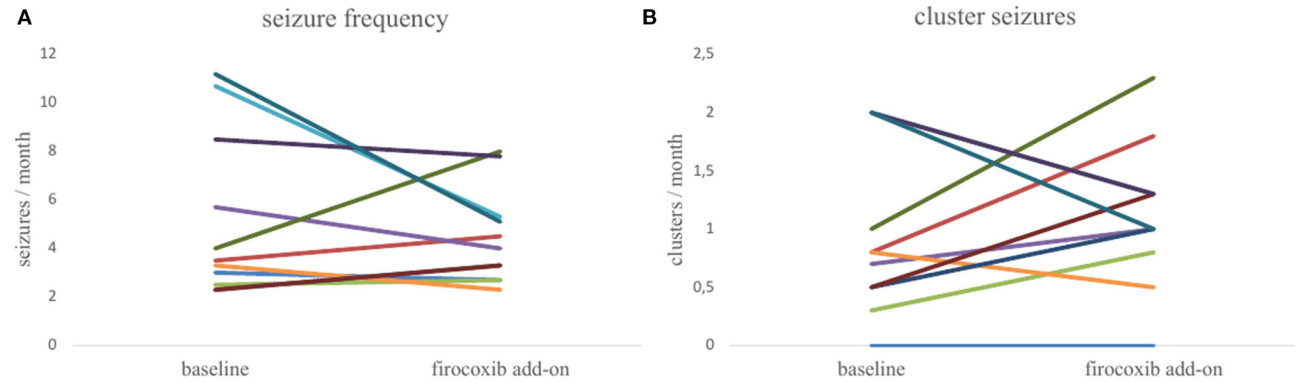

FIGURE 2 | Pairwise comparisons of the monthly seizure and cluster seizure frequency of the study population ($n = 11$) before and during firocoxib add-on treatment. **(A)** Seizure frequency: The median seizure frequency for the study population at baseline and during firocoxib add-on treatment was 3.5 (range 2–11) and 4.0 (range 2–8), respectively. The median seizure frequency did not significantly differ between the baseline period and the firocoxib add-on treatment phase ($p = 0.59$). Seizure frequency decreased considerably (≥50%) in two dogs with the highest seizure frequency. **(B)** Cluster seizure frequency: The median cluster seizure frequency at baseline and during firocoxib add-on treatment was 0.8 (range 0–2) and 1.0 (range 0–2), respectively. The cluster seizure frequency did not significantly differ between the baseline period and the firocoxib add-on treatment phase ($p = 0.57$).

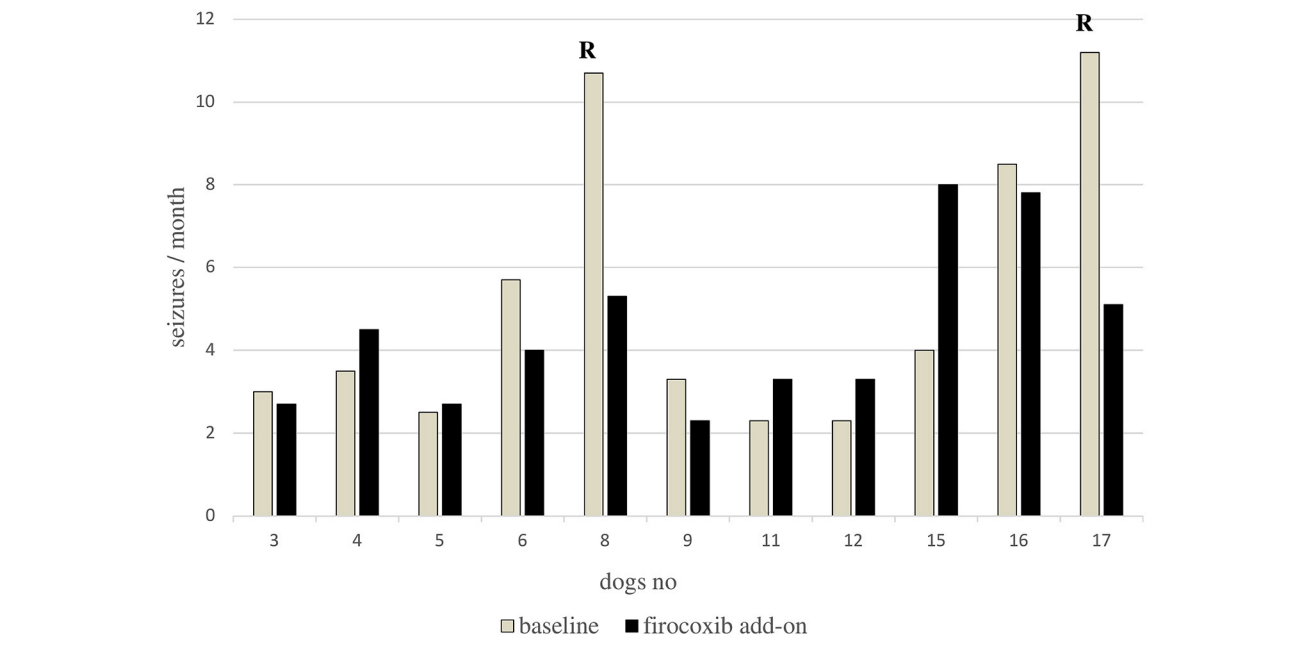

FIGURE 3 | Bar graph showing the individual monthly seizure frequency at baseline and during firocoxib add-on treatment. Six dogs showed a decrease in seizure frequency and five dogs showed an increase in seizure frequency. Only two dogs (cases 008,017) were classified as responders (R) defined by a seizure frequency reduction of ≥50%. None of the dogs became seizure-free.

treatment. Two of the dogs developed diarrhea, which was likely related to other reasons (1 endoparasites, 1 infectious gastroenteritis because other dogs in the household were also affected). Intermittent normoglycemic glucosuria was observed in one dog. Mean phenobarbital serum concentration was 29.05 µg/ml prior to study entry and 31.74 µg/ml after 4 months of treatment (10 dogs). However, fluctuations were observed, and trough samples were not consistently obtained.

DISCUSSION

This pilot study in dogs with phenobarbital-resistant idiopathic epilepsy does not confirm COX-2 targeting with firocoxib as a potent add-on therapeutic strategy for drug-resistant epilepsy. As mentioned above earlier findings from several rodent studies pointed to beneficial effects of COX-2 inhibition with an influence on seizure duration or seizure thresholds (2–4). Moreover, transient COX-2 inhibitor treatment significantly

improved the response to phenobarbital in a chronic rodent model of drug-resistant epilepsy suggesting beneficial effects of COX-2 inhibition (5). However, another experimental study with administration of the COX-inhibitor SC-58236 pointed to putative detrimental effects of COX-2 inhibition in the chronic phase of a poststatus epilepticus model with recurrent spontaneous seizures (17). In line with this finding, COX-2 inhibition aggravated seizure activity in a kainate-induced status epilepticus model (18–20).

The differences in the outcome between the experimental studies and the present findings in the dog model might be related to several factors including differences between species and strains as well as in the etiology, seizure history, and intrinsic disease severity. Especially, the disease duration (in our study mean disease duration was 2.1 years) might play an important role in the evaluation of new therapeutic strategies, as there might be a "point of no return" regarding certain epileptogenic pathways in the chronic epileptic brain.

Interestingly, the two dogs with partial treatment success that showed a relevant ≥50% reduction in seizure frequency during firocoxib treatment, exhibited the highest baseline seizure frequency. These data might indicate that only patients with a high intrinsic severity and high seizure density can be benefitted from COX-2 inhibitor as an additional add-on treatment strategy. However, considering the small number of dogs in this pilot study, the natural fluctuation of seizure frequency, and the potential for pseudo responders if some seizures are not documented, controlled studies in larger cohorts are required to confirm a link between seizure frequency and efficacy (21).

Provided that this finding receives further confirmation, it would be in line with the concept that the control of Pgp expression rates might be one factor contributing to the COX-2 inhibitor effects in the epileptic brain (22). Seizures are known to cause only a transient increase of Pgp, which in the rodent models might only last 1–2 weeks following a seizure (23). Thus, accumulation of Pgp induction resulting in a functionally relevant overexpression might only occur in the patients with a high seizure density. This is in line with observations from our pilot data where two dogs with a very high baseline seizure frequency responded with a >50% seizure frequency reduction. Interestingly, phenobarbital concentrations revealed no relevant change in the two dogs with partial treatment success, while we observed an overall increase in phenobarbital during the 6 months treatment period. However, completely different disease-associated factors linked with the intrinsic severity might also contribute to an effect of COX-2 inhibition or to the modulation of seizure activity in these two dogs (24).

In conclusion, our data argue against an overall relevant efficacy of COX-2 inhibitor add-on approaches in the canine phenobarbital-resistant. Overall data may only support a transitory beneficial effect in dogs with very high seizure frequencies which necessitates further confirmation in the controlled studies. Further evaluation and development of respective approaches may be considered in dogs with a very high seizure density.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the hospital board of the Clinics of Small Animal Medicine, Ludwig-Maximilians-University Munich, Germany and the Ethics Committee of the Royal Veterinary College, London, UK. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

HP, HV, and AF designed and supervised the study. VH and VM supervised case collections. AT, MK, FK, FG, and LA contributed cases. HP and VH wrote the first draft of the manuscript. AF finalized the manuscript. All authors provided input into the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Detection of Generalized Tonic–Clonic Seizures in Dogs With a Seizure Detection System Established Using Acceleration Data and the Mahalanobis Distance: A Preliminary Study

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Caregivers of dogs with epilepsy experience severe stress due to unpredictable seizures. Hence, they feel the need for a better management strategy. A seizure detection system (SDS), which can identify seizures and provide notifications to caregivers immediately, is required to address this issue. The current study aimed to establish a wearable automatic SDS using acceleration data and the Mahalanobis distance and to preliminarily investigate its feasibility among dogs. A generalized tonic–clonic seizure (GTCS) was targeted because it is the most common type of seizure and can have serious consequences (i.e., status epilepticus). This study comprised three phases. First, the reference datasets of epileptic and non-epileptic activities were established using acceleration data of GTCSs in 3 dogs and daily activities in 27 dogs. Second, the GTCS-detecting algorithm was created using the reference datasets and was validated using other acceleration data of GTCSs in 4 epileptic dogs and daily activities in 27 dogs. Third, a feasibility test of the SDS prototype was performed in three dogs with epilepsy. The algorithm was effective in identifying all acceleration data of GTCSs as seizures and all acceleration data of daily activities as non-seizure activities. Dogs with epilepsy were monitored with the prototype for 48–72 h, and three GTCSs were identified. The prototype detected all GTCSs accurately. A false positive finding was not obtained unless the accelerometer was displaced. Hence, a method that can detect epileptic seizures, particularly GTCSs, was established. Nevertheless, further large-scale studies must be conducted before the method can be commercialized.

Keywords: canine, dog, epilepsy, Mahalanobis distance, seizure detection, wearable device, accelerometer

INTRODUCTION

Epilepsy is a chronic brain disorder that causes epileptic seizures. It can impair the quality of life (QOL) of dogs and their owners due to various reasons (1–5). In particular, epileptic seizures are unpredictable in nature, and prolonged seizure (i.e., status epilepticus) can be life-threatening. Thus, anxiety correlated with sudden seizures causes severe stress among owners, and this may

reduce QOL (2, 4). However, most owners find it challenging to monitor dogs with epilepsy, and this notion is true for veterinarians and clinical staff who manage dogs with epilepsy in the hospital. A seizure detection system (SDS) that can be used for continuous monitoring and the detection and notification of seizures can reduce burden among owners and veterinarians. Based on a recent study, the owners of dogs with epilepsy want to use a seizure detection device (6). Such a system can accurately identify seizure frequency, which is important for the medical/surgical treatment of epilepsy.

An accelerometer is used to evaluate the behavior and activity of dogs (7–12). Moreover, it is applied for not only motion analysis but also for medical research, such as the evaluation of therapeutic effects of treatment among dogs with osteoarthritis and the assessment of pruritus degree due to dermatosis (13, 14). We hypothesized that epileptic seizures in dogs can be detected using acceleration data.

The Mahalanobis distance is a type of discriminant analysis, which determines the distance between the test dataset and several reference datasets. The test dataset belongs to the same category as the reference dataset with the shortest distance. Therefore, the Mahalanobis distance is used for outlier detection in the engineering field (15, 16). One study investigated whether the behaviors of cows, including resting, ruminating, and eating, can be identified using acceleration data and the Mahalanobis distance (17). In this study, the validity of behavior identification was 95%. We considered epileptic seizures as one motion and hypothesized that seizures can be differentiated from other motions and detected correctly.

The current research aimed to develop an automatic SDS using acceleration data and the Mahalanobis distance and to preliminarily investigate its feasibility among dogs. Moreover, a generalized tonic–clonic seizure (GTCS) was targeted because it is the most common type of seizures (18–20) and can cause serious complications (i.e., status epilepticus).

METHODS

Animals

In total, 24 laboratory dogs, including 7 dogs with epilepsy (Dogs A–G) and 17 healthy dogs, 2 patient dogs (Dogs 1 and 2) with epilepsy that were hospitalized in Azabu University Veterinary Teaching Hospital, and 11 pet dogs were included in this study. **Supplementary Tables 1–3** depict the information of these dogs. Seven laboratory dogs with epilepsy (Dogs A–G) were diagnosed with idiopathic epilepsy (IE), which was made using the International Veterinary Epilepsy Task Force (IVETF) criteria (21). The seizure types included GTCSs and/or focal motor seizures that evolved to GTCSs. Dog 1 was diagnosed with IE based on tier III level of the IVETF consensus proposal (21). Dog 2 had structural epilepsy caused by brain tumor, which was diagnosed via magnetic resonance imaging. Both the patient dogs had GTCSs. In total, 17 laboratory dogs and 11 pet dogs had good general condition and did not experience seizures. The owners of 11 pet dogs were acquaintances of the investigators, and they volunteered to join in this study. This study was approved by the Institutional Research Committee at Azabu University, and all procedures were conducted following the guidelines approved by this committee (approval number: 110606, 200330-1, 201007-4).

Abbreviations: RDE, Reference dataset of epileptic seizures; RDNE Reference dataset of non-epileptic activities; GTCS, Generalized tonic–clonic seizure; SDS, Seizure detection system.

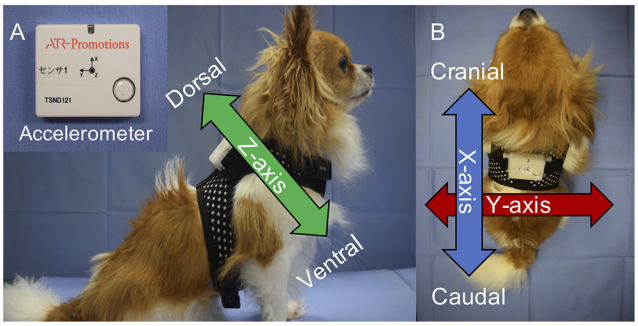

FIGURE 1 | The accelerometer (TSND121, ATR-Promotions) and its position on the dogs. The width of the device was 37 mm; height, 46 mm; depth, 12 mm; and weight, 22 g **(A)**. The accelerometer was placed on the interscapular region with the harness **(A,B)**. The X-axis was craniocaudal; the Y-axis, lateral; and the Z-axis, dorsoventral **(A,B)**.

Accelerometer

A wireless three-axis accelerometer (TSND121, ATR-Promotions, Japan) was used to obtain acceleration data (**Figure 1A**). The sensor was programmed at a sampling frequency of 50 Hz and at an acceleration range of ± 8 g. The accelerometer was tied to a harness with plastic clamping bands. This harness was worn by the dogs during all experiments. The accelerometer was positioned at the interscapular region. Hence, the X-axis was in the craniocaudal direction, the Y-axis in the lateral direction, and the Z-axis in the dorsoventral direction (**Figures 1A,B**). To prevent rotation of the harness and accelerometer, the former was selected according to body size, and it fitted well. Acceleration data were recorded and saved on a computer using the accelerometer software (Sensor Controller, ATR-promotions, Japan). The recorded acceleration data were exported into Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and were processed.

Experimental Protocol

Figure 2 shows the flowchart of this study and information about the dogs that participated in each phase of the experiment. This study comprised three phases. In the first phase, the reference dataset of epileptic seizures (RDE) and the reference dataset of non-epileptic activities (RDNE) were established using the acceleration data of GTCSs and daily activities, respectively. In the second phase, the GTCS-detecting algorithm was established and validated using the RDE and RDNE. Finally, a feasibility test of the prototype of SDS that implemented the algorithm was performed on dogs with epilepsy.

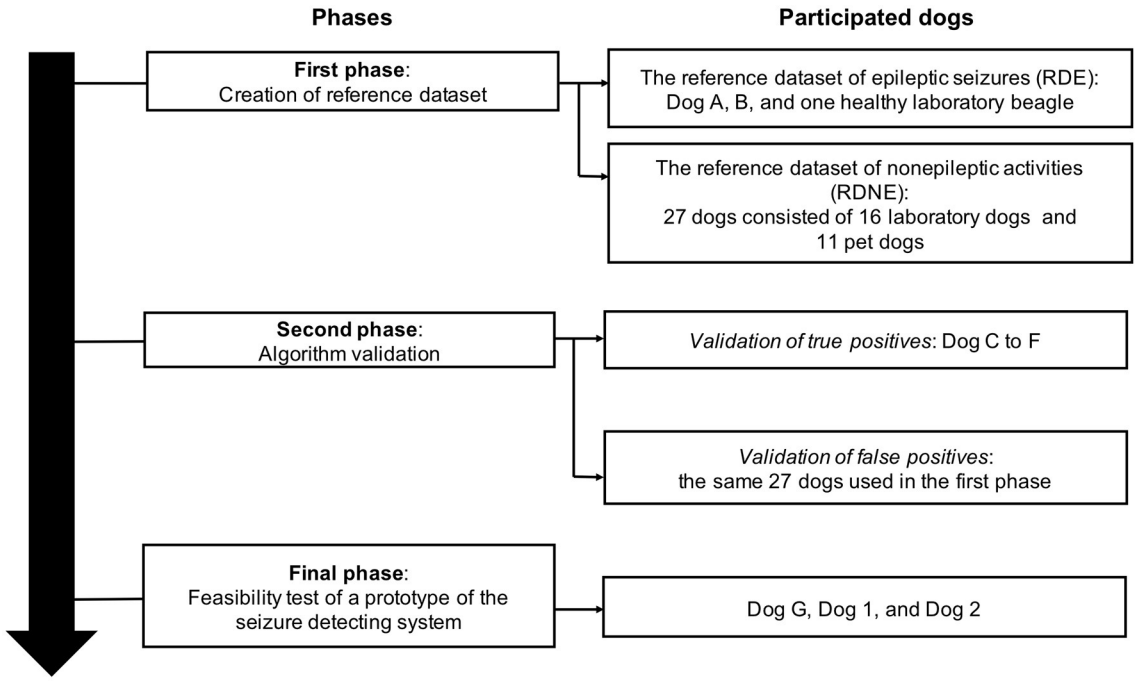

FIGURE 2 | The flowchart of this study, which comprised three phases: creation of the reference datasets in the first phase, validation of the algorithm in the second phase, and feasibility testing of the prototype of the seizure detection system in the final phase.

First Phase: Creation of Reference Dataset

To establish the RDE, GTCSs acceleration data were obtained from three beagles (Dogs A and B and one healthy laboratory dog). However, the frequency of seizure in Dogs A and B was extremely low (once to twice a year). Thus, to collect acceleration data of GTCSs, bemegride (Medibal; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), a seizure inducer, was used in the three dogs (22). Bemegride 20 mg was administered intravenously for over 30 s to induce seizure based on the methodology in a previous study conducted by the authors (23). Although bemegride induces seizures, it had no other undesirable adverse effects in dogs (23). If a seizure occurred, bemegride was discontinued immediately, even before the dosage reached 20 mg. Immediately after the seizure ceased or 5 min had elapsed after seizure onset, we administered intravenous diazepam (0.5 mg/kg; Horizon Injection 10 mg; Maruishi Pharmaceutical, Osaka, Japan), followed by phenobarbital (3 mg/kg; Phenobal Injection 100 mg; Fujinaga Pharma Corporation, Tokyo, Japan), which are bemegride antagonists (24, 25). If the seizure did not cease, two doses of diazepam were administered additionally. In case the seizure continued, standard treatment for status epilepticus was started.

After seizure cessation, the dogs were monitored constantly for 24 h. Moreover, oral levetiracetam was administered if seizures occurred during observation. The dogs were video-recorded from the start of bemegride administration to seizure cessation. A radio clock was used to record the videos to validate the time of events. The times of the radio clock and the accelerometer were synchronized before the start of each experiment. Only acceleration data recorded during the tonic–clonic phase were used for the RDE creation. The average of the GTCS acceleration data was obtained, and their variance-covariance matrix was calculated. The average and the variance-covariance matrix comprised the RDE.

To create the RDNE, daily activity acceleration data were collected from 27 dogs (16 healthy laboratory dogs and 11 pet dogs). The acceleration and video data of different daily activities (non-epileptic activities) were collected using the following method. The laboratory dogs stayed in a test room, which was a 2.8 × 4.1-meter space, and they moved freely during data recording. The radio clock, which synchronized its time with the accelerometer, was video-recorded. The data of pet dogs were recorded in their house or outside, and they moved freely and performed their daily activities. The investigator followed the dogs and recorded the video. The times of the accelerometer and the clock in the video camera were synchronized. In both laboratory and pet dogs, data recording was ended if a dog stopped moving spontaneously or 60 min had passed. This experiment was performed once on each dog. The daily activities of the dogs were classified into several movements such as walking and sitting. The dataset of each movement was created by extracting consecutive 9-s acceleration data from dogs that performed the movement. For example, if 10 dogs walked, the total 90-s acceleration data comprised the dataset of walking. For movements that did not last for 9 s, the 9-s acceleration data were created by combining data obtained at different timings. Nine seconds was used because at least nine data were required to calculate the variance-covariance matrix. The average of the

dataset of all movements was obtained, and their variance-covariance matrix was calculated. The average and the variance-covariance matrix comprised the RDNE.

Second Phase: Algorithm Validation

Algorithm Procedure

In the second phase, we created the GTCSs detection algorithm* using data generated in the first phase. The algorithm procedure was as follows: First, the resultant force was calculated using three-axis acceleration data. The average and coefficient variation of the three-axis and the resultant force for each second were calculated. Thus, eight parameters (i.e., the average and coefficient variation of the X-, Y-, and Z-axis and the resultant force) existed in each second (**Supplementary Figure 1**). A test dataset comprised the 9-s epoch of the eight parameters (**Supplementary Figure 1**). Subsequently, the Mahalanobis distance between the test dataset and the two pre-generated reference datasets; the RDE and RDNE, was calculated. If the Mahalanobis distance of the test dataset obtained from a dog was shorter in the RDE than in the RDNE, the test dataset belonged to the RDE. Hence, the dog had epileptic seizures. However, if the Mahalanobis distance of the test dataset recorded from a dog was shorter in the RDNE than in the RDE, the test dataset belonged to the RDNE. Therefore, the dog did not present with seizures. The Mahalanobis distance calculation was performed in Microsoft Excel by programming its formula into Excel VBA (Microsoft Corp., Redmond, WA, the USA).

Algorithm Validation

Four laboratory dogs (Dogs C–F) were used to validate true positive findings. In the second phase, the presence of true positive results indicated that the algorithm was accurate in identifying that the GTCSs test dataset belonged to the RDE. The acceleration data of GTCSs in these dogs were collected using the following method. Dogs C and D were treated with anti-seizure drugs (ASDs) for epilepsy. Treatment with ASDs was discontinued temporarily, and the dogs were monitored continuously until a seizure occurred. Immediately after the seizure ceased or 5 min had elapsed after the seizure onset, diazepam (0.5 mg/kg; Horizon Injection 10 mg; Maruishi Pharmaceutical, Osaka, Japan), followed by phenobarbital (3 mg/kg; Phenobal Injection 100 mg; Fujinaga Pharma Corporation, Tokyo, Japan), was administered intravenously. If the seizures did not cease, the same treatment as in phase 1 was provided. Acceleration data were collected throughout this experimental period. After the seizure cessation, the dogs were assessed continuously for 24 h. Treatment with maintenance ASDs was restarted. Additionally, oral levetiracetam (Ekeppra 500 mg; Otsuka Pharmaceutical, Tokyo, Japan) was administered at a dose of 20 mg/kg every 8 h for 3 days to prevent the development of another seizure. Dogs C and D were video-recorded from the time when ASDs were withdrawn to the time when the seizure occurred and then ceased. In case Dog C or D did not develop a seizure 36 h after ASD withdrawal, bemegride induction was started. Since the frequency of seizures was extremely low in Dogs E and F, bemegride was used to induce a GTCS in the same manner as Dogs A and B. The 9-s epoch of the eight parameters immediately after the GTCS onset in each dog was extracted as the GTCSs test dataset. The GTCSs test datasets were processed with the algorithm.

The acceleration data of daily activities obtained from 27 dogs (16 healthy laboratory dogs and 11 pet dogs) in the first phase were used to validate false positive findings. In the second phase, the presence of false positive results indicated that the algorithm was not accurate in identifying that the non-epileptic activity test dataset belonged to the RDNE. All recorded acceleration data from each dog were combined as continuous data. This combined data was divided into 9-s epochs in the non-epileptic activity test dataset. The test datasets were created repeatedly by shifting the beginning of the 9-s epoch every 1 s (**Supplementary Figure 2**). The non-epileptic activity test datasets were processed with the algorithm.

Final Phase: Feasibility Test of a Prototype of the SDS

A prototype of SDS, which implemented our algorithm, was established, and the feasibility test of the SDS was performed on three dogs with epilepsy. This system comprised a wireless three-axis accelerometer, a monitoring device that used the seizure algorithm created in the second phase, and a smartphone (**Supplementary Figure 3**). The sampling frequency and the acceleration range of the accelerometer were 50 Hz and ±8 g, respectively. The accelerometer linked to the monitoring device via Bluetooth was placed on the midline of the dorsum of the dog using a harness or custom-made jacket. The monitoring device provided seizure notifications to the smartphone, and it detects GTCSs real-time. Dog G and Dogs 1 and 2 with epilepsy were monitored with the system for 72 and 48 h, respectively. These dogs were continuously monitored by the investigators during the feasibility test to determine whether the prototype detected seizures accurately (true positive). Moreover, the occurrence of false positive findings (detecting a seizure even though a dog did not actually have one) and false negative findings (failure to detect a seizure in a dog that actually experienced one) was assessed by the investigators.

RESULTS

First Phase: Creation of Reference Dataset

Regarding the RDE, three beagles presented with GTCSs immediately after starting treatment with bemegride. However, it ceased spontaneously within 5 min. The dogs were managed with diazepam and phenobarbital in accordance with the abovementioned protocol. No additional seizures were observed. Hence, the three GTCSs acceleration data comprised the RDE.

Regarding the RDNE, the median acceleration recording time in 27 dogs was 21 (range: 8–60) mins. The total recording time was 696 min (41,760 s). Fifteen different types of movements in dogs confirmed in the video, such as walking and lying, were selected as daily activities (**Table 1**). **Table 1** presents the number of dogs that performed each movement during data recording.

TABLE 1 | Fifteen movements comprising the reference dataset of non-epileptic activities and the number of dogs comprising the dataset of each activity.

Movements	Number of dogs
Walking	23
Standing	15
Shaking	24
Drinking	7
Running	4
Jumping on a sofa	3
Jumping off a sofa	3
Lying on the stomach	12
Lying on the side	5
Scratching	4
Playing with a toy	3
Being stroked	8
Sitting	14
Changing position from lying on their stomach to lying on their side	5
Changing position from lying on their side to lying on their stomach	5

For instance, acceleration data during walking in 23 dogs comprised the dataset of walking. Thus, the walking dataset comprises 207-s acceleration data (9 s for each dog). The cumulative total was 135 dogs; therefore, the 1,215-s acceleration data comprised the reference dataset of non-epileptic activities.

For example, 23 dogs performed walking. Hence, the total 1,215-s acceleration dataset obtained from 15 movements comprised the RDNE.

Second Phase: Algorithm Validation

In total, there were four GTCSs recorded (**Supplementary Video 1**). Dog C experienced a spontaneous GTCS after withdrawal of ASDs. In Dogs D, E, and F, it was induced by bemegride. Hence, their acceleration data were used for validation. Three drug-induced GTCSs ceased spontaneously within 5 min. Four GTCSs test datasets were created. All datasets had shorter Mahalanobis distances in the RDE than in the RDNE (**Table 2**). Therefore, they were classified accurately. That is, there were only true positive and no false negative results.

As 1,215 of 41,760-s acceleration data were used for creating the RDNE, the remaining 40,545-s daily activity acceleration data were used for validation. Thus, 40,537 non-epileptic activity 9-s epoch test datasets were established. All non-epileptic activity test datasets had shorter Mahalanobis distances in the RDNE than in the RDE. Therefore, all non-epileptic activity test datasets were classified accurately; that is, there were no false positive findings.

Final Phase: Feasibility Test of a Prototype of the SDS

The three epileptic dogs were under the SDS monitoring for 168 h. Dog G had three seizures during monitoring. The seizures were focal motor epileptic seizures that evolved into GTCSs. The prototype detected all three seizures accurately, and the seizure notification was sent to the smartphone. Thus, there were three true positive findings. Two patient dogs did not have seizures during monitoring, and a false negative finding was not obtained. In one patient dog, a false positive finding was observed 6 times when the dog was walking around in the cage. However, the accelerometer was displaced on the harness laterally during these times. Other than that, there were no false positive findings in all three dogs.

DISCUSSION

This preliminary study investigated whether GTCSs can be detected using acceleration data and the Mahalanobis distance, which is the initial step toward the development of the SDS. Via algorithm validation and feasibility testing of the SDS prototype, our algorithm was accurate in identifying all four acceleration data acquired from each GTCS as seizures. Further, the prototype of the SDS was effective in detecting all three seizures in one dog. False positive findings were not obtained except in a case in which the accelerometer was displaced in 40,545-s daily activity acceleration data in 27 dogs and during the 168-h monitoring period in three dogs with epilepsy. Therefore, the development of the SDS with our algorithm should be further advanced.

Several studies aimed to detect or predict epileptic seizures in humans (26–32). If the target is limited to GTCSs, the sensitivity of seizure detection using an accelerometer is 79–91% (26–28). Interestingly, our canine study revealed accurate seizure detection. In humans, GTCSs, particularly clonic seizures, is characterized by a burst-like acceleration pattern caused by intense movement (31). Dogs with clonic seizures had an intense movement. Therefore, GTCSs were likely detected using an accelerometer in dogs. The current study supports the hypotheses that seizures can be differentiated from other motions and GTCSs can be detected accurately.

A recent study using an accelerometer on a collar for detecting seizures in dogs revealed that generalized seizures could be detected with an accelerometer (33), which is consistent with our results. However, the overall sensitivity of identifying generalized seizures in this previous research was ∼20%. Although the sample size was small and ∼50% of dogs experienced drug-induced seizures (3 bemegride-induced and 1 spontaneous in the second phase and 3 spontaneous in the final phase) in this study, our algorithm detected all seven GTCSs without any false positive findings if the system was used properly. The prior study used machine learning (random forest classifier) to detect seizures. In this report, the Mahalanobis distance was utilized. Machine learning has been applied in human seizure detection studies (26–32). Humans take different postures and perform complex movements. Thus, machine learning that detects patterns or rules from large datasets could be used as seizure-detecting methods. Alternatively, the postures and movements of dogs are relatively uncomplicated compared with those of humans. Thus, a simpler algorithm can detect seizures in dogs. Using the Mahalanobis distance, we created a simple two-choice algorithm (seizures or not seizures). The conciseness of this algorithm is likely one of the reasons why our method could detect convulsive seizures more accurately.

Our method requires a test dataset with 9-s epoch. We preliminarily assessed the algorithm using datasets with different

TABLE 2 | The Mahalanobis distance between the GTCS test dataset and the reference dataset.

Dogs	Type of seizures	Number of recorded seizures	Distance to the RDE	Distance to the RDNE	Reference data to which the test dataset belongs to
C	GTCS	1 (spontaneous)	5.3	5.38	RDE
D	GTCS	1 (drug-induced)	3.43	6.2	RDE
E	GTCS	1 (drug-induced)	4.75	5.32	RDE
F	GTCS	1 (drug-induced)	4.77	5.43	RDE

All GTCS test datasets recorded from each dog had a shorter distance to the reference dataset of epileptic seizures (RDE) than the reference dataset of non-epileptic activities (RDNE). Therefore, all GTCS test datasets belonging to the RDE were identified accurately.

time durations. If a 3-s or 6-s epoch was used, GTCSs were detected accurately. However, false positive findings were obtained several times. That is, the algorithm determined that non-epileptic activity test dataset belongs to the RDE. Meanwhile, in a 9-s epoch, there were few false positive findings, as shown in the current study. At least 9-s data were required to differentiate seizures from non-seizure activities. Generalized tonic–clonic seizures lasts for more than 9 s in most cases, and rapid treatment is important to prevent status epilepticus. Hence, the algorithm using 9-s data is feasible for practical use.

Our seizure detection algorithm targets GTCSs. Several studies have shown that 68–94% of dogs with epilepsy present with GTCSs (18–20). Therefore, most dogs with epilepsy experience GTCSs. In addition, status epilepticus, which can be life-threatening, often presents with GTCSs. Thus, a device targeting GTCSs is clinically important in veterinary medicine.

The SDS appears to work regardless of dog breed. Whether breeds affected the detection of GTCSs needs to be investigated. The RDE using a single breed of the beagle was first established, and the algorithm was verified with other different breeds. One healthy beagle was included because only two epileptic laboratory beagles were available during the conduct of the first phase of the study. Although the RDE comprised data from three dogs of a single breed, seizures were detected accurately in five different breeds. Further, no false positive finding was confirmed in 10 different breeds. Therefore, our algorithm is likely to be applicable to any dog breeds.

A harness or jacket was used to place the accelerometer on dogs in this study. We believe that a change in the three-axis direction of the accelerometer could affect the detection of epileptic seizures. Moreover, the position of the accelerometer could change due to collar rotation. Thus, a harness or jacket that does not easily rotate was used. Indeed, a false positive result was only obtained in cases in which the accelerometer was displaced in one patient dog using the prototype. Therefore, the accelerometer should be maintained in the correct position to ensure that the seizure detection algorithm is working accurately. However, the three dogs enrolled in the final phase were cage-confined during the monitoring. Thus, false-positive findings may increase in dogs living at home.

The SDS can have several benefits in veterinary medicine. Numerous studies showed that the caregivers of epileptic dogs, particularly those who have less time to supervise the dog directly, experience stress and anxiety due to unpredictable seizures (2, 4, 6). Medical staff may have a similar experience because continuous monitoring is not always easy in some busy clinics. Our algorithms can allow the SDS to send automatic notification to the smart phone of caregivers if a seizure is detected. The person who receives the alert can check the dog's safety remotely by watching the live streaming monitor. Moreover, emergency medications may be administered more readily. Seizure frequency is an indispensable information for the seizure management of individual dogs and epilepsy research (34). However, currently, the assessment of seizure frequency is based on the subjective report of caregivers alone. Previous reports in human medicine showed that patients occasionally underestimate seizure frequency (35, 36). Seizure management and epilepsy research can improve with the SDS use as it can accurately evaluate seizure frequency. For epilepsy surgery, the epileptogenic area should be identified for resection or disconnection. The symptomatogenic zone is an area in the cortex that produces the initial clinical sign if a seizure occurs, and it often becomes important in identifying the epileptogenic area (37, 38). Therefore, the initial clinical sign of a seizure onset must be identified accurately. However, video recording of seizures upon onset is often challenging and missed. Hence, the SDS can resolve this issue as it can record the video tracing back several minutes before a seizure onset.

The current study had some limitations. Firstly, the RDE for our seizure-detecting algorithm was established from drug-induced, not spontaneous, seizures. In some cases, the seizures used in algorithm validation were drug-induced. However, we accurately detected spontaneous seizures with the prototype implementing our algorithm. Thus, the reference dataset generated from the drug-induced seizures acceleration data could suffice if it is used as the reference dataset for spontaneous seizures. Secondly, the validation of false-positive findings in the second phase was conducted using acceleration data obtained in the first phase. Using acceleration data recorded from other dogs for the validation would be ideal although no data overlaps were noted between the RDNE and non-epileptic activity test datasets. Lastly, the specificity and sensitivity of our system have not been evaluated because of the small sample size. Obtaining an adequate number of GTCSs acceleration data without inducing was difficult because seizures are unpredictable and the seizure frequency of the most epileptic laboratory dogs in this study was low (less than once per 3 months). Therefore, bemegride, one of the inducers of EEG abnormalities in humans (39), was used.

However, the induction of GTCSs to obtain acceleration data in a sufficient number of dogs for specificity and sensitivity evaluation may have ethical concerns. In some countries, medical induction of seizures would not be approved in dogs. Therefore, although the number of dogs included was limited, it was still sufficient for the pilot study. Nevertheless, further large-scale studies must be conducted to obtain clinical data from dogs with epilepsy using a plurality of the SDS prototypes.

We created a method for detecting epileptic seizures (particularly GTCSs) from other movements using acceleration data and the Mahalanobis distance. The algorithm accurately identified all seven GTCSs (four in the validation of our algorithm and three in the feasibility test of the prototype) without any false positive or negative results. Further, the prototype of the SDS implementing this algorithm was effective in detecting all three GTCSs. Thus, the development of the SDS with our algorithm should be further advanced and validated before it can be commercialized.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Azabu University, Japan. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JH: acquired the data, drafted, and wrote the manuscript. MS: conceived and designed the study, wrote the manuscript, and critically revised the article. TK and TA: acquired the data and provided critical feedback. MY: acquired the data, contributed to the initial design of the study, and provided critical feedback. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.848604/full#supplementary-material

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Conflict of Interest: The patent of the seizure detection method and monitoring system in this manuscript has been granted and registered. Japanese Patent No. 6193613.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phenotypic Characterization of Idiopathic Epilepsy in Border Collies

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The prevalence of idiopathic epilepsy (IE) within the Border Collie (BC) dog breed is high. The aim of this retrospective study was to describe the phenotype of BCs with IE and assess correlations between phenotypic variables and owner-provided quality-of-life (QoL) scores. Data of BCs diagnosed with IE during the period of five consecutive years were retrospectively analyzed. All the dogs were presented at least once to a veterinary neurology specialist at one of three veterinary referral hospitals and most were under the continued medical care of that specialist. Owners were requested to complete a standardized online questionnaire including quality-of-life (QoL) scoring questions. Data of a total of 116 BC dogs were included for analysis. The median age at onset of the first epileptic seizure (ES) was 33.5 months (6–188). A total of 34/86 (40%) of medically treated dogs received 1 antiseizure medication (ASM) and 52/86 (60%) received ≥2 ASMs. Phenobarbital was the most commonly employed ASM, used in 70/86 of treated dogs (81%). Four or more side effects were observed in 20/86 (23%) of treated dogs. Age at onset of first ES was significantly lower for dogs having experienced cluster seizures (CSs), status epilepticus (SE), or both (median 27 months) vs. dogs that had not experienced CS or SE (median 43 months). The QoL of BC with IE was scored with a median score of 7 out of 10. Owners scored their dog's QoL to have declined by a median of 30% during the course of life with IE with 39% (37/95) of owners scoring their dog's QoL to have declined by ≥50%. This study confirms the association of age at onset of first ES with the severity of epilepsy (e.g., presence of CS and/or SE) and further characterizes the phenotype of IE in BC dogs. QoL of BC can be heavily impacted by IE.

Keywords: canine epilepsy, dog, idiopathic epilepsy, seizure, hereditary

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INTRODUCTION

The prevalence of epilepsy in the general dog population is estimated to be 0.6–0.75% (1–6). Breed-specific characteristics (phenotype or clinical manifestation) of idiopathic epilepsy (IE) have been reported for a variety of breeds, but most are based on single studies from specific countries with notable exceptions such as the Belgian Shepherd, Golden Retriever, and Finnish Spitz (6). Although it is well-established within the veterinary neurology community that Border Collie (BC) dogs show a high prevalence of IE (1–6), there is only one report that focuses on IE in this breed, based on a cohort of 49 BC dogs from Germany (7). Another study investigated IE in Labradors and Border Collies from the UK and the impact of neutering (8). Breed-specific differences regarding IE in dogs

may hold important implications for owners, prognostication and successful clinical management (6). Observations of particular breeds with severe [exhibiting high-frequency epileptic seizures (ES), cluster seizures (CS), or status epilepticus (SE)] or antiseizure medication (ASM)-resistant IE in particular, may provide incentives for epilepsy research (e.g., genetic studies). Although a genetic basis was strongly indicated by observations from the earlier study on BC dogs (7) and clinical experience of many veterinary neurologists has suggested this for a long while, no causative genetic mutations or variations have been identified yet in the BC breed. The phenotype reported by Hülsmeyer et al. on a cohort of German BC dogs with IE was characterized by a high prevalence of CS occurring in almost all dogs (94%) and was further complicated by SE in about half of BCs (7). The presence of CS and/or SE is interpreted as IE being "severe" (4, 7). The main aim of this retrospective study was to evaluate and describe the phenotype of BCs with IE and assess correlations between phenotypic variables and owner-provided quality-of-life (QoL) scores.

MATERIALS AND METHODS

Data Collection

Data were collected manually and retrospectively by reviewing digital patient files in the database of the Department of Small Animal Medicine, Faculty of Veterinary Medicine, Utrecht University, The Netherlands; Small Animal Department, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium and Evidensia Small Animal Hospital Arnhem, The Netherlands and review of data collected *via* an online questionnaire (see later). All the dogs were privately owned and participating owners gave informed consent for participation in the study. The dogs entered in this study were examined and handled by licensed veterinarians in compliance with local legislation and approval of an ethics committee for the study was not necessary. BC dogs were included if dogs had been presented for clinical consultation (including a general and neurological examination) with a specialist in veterinary neurology at one of three veterinary referral hospitals during a period of five consecutive years (2016–2021) and diagnostic test results were consistent with a diagnosis of IE [minimum tier I, International Veterinary Epilepsy Task Force (9)]. Most dogs were under continued care by that specialist. All the BCs had to have a pedigree or descended directly from BCs with a pedigree. Cases were excluded when they did not fit the inclusion criteria noted earlier or when data were insufficient. Insufficient data were defined as data for a particular dog that did not include parameters noted below marked with "*" or ≥3 of the remaining parameters under investigation were not recorded in the files. Data recorded consisted of: breed (Border Collie)*; sex*; neuter status*; alive (yes/no)*; age at death (when dead)*; age at the time of data collection*; age at onset of first ES (first ES noticed by owner)*; ASM treatment (yes/no)*; ASM or ASMs used*; occurrence of side effects (yes/no); type of side effects; type of ES (generalized, focal or combination); type of focal ES (motor, autonomic, behavioral); ES frequency in the last 3 months; occurrence of CS (yes/no); occurrence of SE (yes/no); owner-identified triggers or temporal pattern (yes/no); type of trigger or temporal pattern; occurrence of pre-ictal signs (yes/no); type of pre-ictal signs (yes/no); occurrence of post-ictal signs (yes/no); type of post-ictal signs (yes/no); vaccination status up-to-date (yes/no); anthelmintic drug use (yes/no); and anti-ectoparasitic drug use (yes/no; flea/tick prevention). Data were transferred to Microsoft® Excel (2021) for analysis. Cluster seizure and SE were defined as: CS = two or more ES within a 24-h period, SE = >5 min of continuous ES or two or more discrete ES between which there is incomplete recovery of consciousness (for generalized convulsive ES) (10). ES pertaining to 1 CS event were counted as 1 ES for ES frequency.

Quality-of-Life

Owners of included BCs were requested to complete a standardized online questionnaire including quality-of-life (QoL) scoring questions adapted from other studies (11) at variable times during their dog's clinical course. Owners were asked to provide further detailed information on the dog (used to confirm data stored in patient files, such as date of birth and yes/no answer to questions already noted during consultations) and their personal information (e.g., name, e-mail address and phone number; voluntarily provided with owners' permission, used in case answers to the questions needed further explanation). Owners were asked to score the QoL of their dog with IE on a scale of 1 (very bad)-10 (very good), and also to score the QoL of their dog with IE as a percentage compared to the QoL before the onset of IE (baseline score of 100%). The original questionnaire was in Dutch. Questions were translated into German and English for owners not able to complete the Dutch questionnaire.

Statistical Analysis

Descriptive statistics are reported, a chi-square or the Mann–Whitney *U*-test (2-tailed) was performed to test for significant differences ($P < 0.01$ was considered significant) between subgroups of the study population for categorical and continuous data, respectively, and a McNemar test was used to evaluate the association between clinical variables within a group (paired, nominal data). Pearson correlation tests [reported as r(df) = r, P] were performed to evaluate and describe if there were significant relationships ($P < 0.01$ were considered significant) between clinical variables using Microsoft® Excel (2021). Correlations (r) were interpreted as: 0.90–1.00 (−0.90– −1.00) = very strong to perfect positive (negative) correlation; 0.70–0.90 (−0.70– −0.90) = strong positive (negative) correlation; 0.50–0.70 (−0.50– −0.70) = moderate positive (negative) correlation; 0.30–0.50 (−0.30– −0.50) = weak positive (negative) correlation; 0.00–0.30 (−0.00– −0.30) = no to very weak positive (negative) correlation. Odds ratios were calculated and reported with 95% CIs. All the other results are presented as medians and ranges: median (range lowest–highest) unless stated otherwise.

RESULTS

A total of 185 BC dogs with IE were identified in the databases of included institutions. A total of 69 cases were excluded because did not meet inclusion criteria. A total of 116 BC dogs met the inclusion criteria, had sufficient data recorded, and were included for analysis. In total, 78 of the included dogs originated from The Netherlands, and 33 from neighboring countries (Germany, Belgium), and 5 dogs were of undefined origin. Study population characteristics are summarized in **Table 1**.

Age at the Time of Data Collection, Age at Onset, Age at Death

The median age of all included dogs, either age at the time of data collection or age at the moment of death, was 45 months (6–188). Of those BCs still alive at data collection ($n = 88$), the median age at the time of data collection was 48 months (9–166). Of those BCs that had died ($n = 28$), the median age at death was 33.5 months (6–188). Causes of death were not recorded for any of the dead patients. The median age at onset of first ES was 33.5 months (6–174); there were no significant differences in age at onset of first ES between dogs that were dead or alive. No sex predisposition or association with neuter status was found regarding ES frequency, age at onset of first ES or mortality.

ASM Treatment, ASMs Used, and Side Effects of Treatment

A total of 86/116 dogs (74%) were treated with ASM, 30/116 dogs (26%) were not treated with ASM. There were no differences in age at the time of data collection or age at onset of first ES between dogs treated or untreated. There were no statistically significant differences between male and female dogs, neutered and intact dogs, and alive or dead dogs with respect to being treated or not. A total of 34/86 (40%) of treated dogs were treated with 1 ASM and 52/86 (60%) were treated with ≥2 ASMs. Phenobarbital was the most commonly employed ASM (70/86, 81%), followed by potassium bromide (39/86, 45%), imepitoin (26/86, 30%), diazepam (18/86, 21%), levetiracetam (12/86, 14%), and gabapentin (2/86, 2%). The combination of phenobarbital/potassium bromide was the most common polytherapy employed (31/86, 36%).

The occurrence of side effects was reported in 70/86 (81%) of dogs treated with ASMs based on owner reports and clinical assessments. No significant associations were found between specific ASMs and the occurrence of side effects (yes/no) or the number of ASMs used in the treatment and the number of side effects. The most common side effect reported was lethargy (53%). Side effects are summarized in **Table 2**.

Frequency of ES, Type of ES, and Occurrence of CS and SE

The ES frequency during the last 3 months was reported in 76 dogs. The median ES frequency per month was 1 (0–17). There was no significant difference in ES frequency between dogs <24 months of age at onset of first ES vs. dogs ≥24 months of age at onset of first ES. No significant correlation was found between age at onset of first ES or age at the time of data collection and ES frequency. Dogs that were treated with ASMs did not have

TABLE 1 | Study population characteristics.

			Number	Percentage	
Total no. of dogs			116	100%	
Sex					
	Male	Total	66	57%	100%
		Intact	38		58%
		Neutered	28		42%
		Alive	52		79%
		Dead	14		21%
	Female	Total	50	43%	100%
		Intact	26		52%
		Neutered	24		48%
		Alive	36		72%
		Dead	14		28%
Neuter status		Intact	64	55%	
		Neutered	52	45%	
Alive			88	76%	
Dead			28	24%	

TABLE 2 | Side effects of antiseizure medication (ASM) treatment.

	Number of dogs (*n*)	%
Dogs treated with ASM	86	100%
Yes (side effects)	70	81%
No (no side effects)	16	19%
Type of side effect (n = 70 dogs experiencing side effects)		
Lethargy	43	61%
Polyphagia	31	44%
Ataxia	30	43%
Polydipsia	24	34%
Weakness	23	33%
Weight gain	20	29%
Polyuria	19	27%
Restlessness	13	19%
Defecation in house	3	4%
Diarrhea	2	3%
Skin rash	1	1%
Coughing	1	1%
Number of side effects (n = 86 dogs treated with ASM)		
No side effects	16	19%
1 side effect	14	16%
2 side effects	15	17%
3 side effects	20	23%
≥4 side effects	21	24%

There were no statistically significant associations between types of side effects and ASM used.

a significantly higher ES frequency than untreated dogs in the previous 3 months.

The occurrence of CS and SE was reported in 114 dogs (**Table 3**). Dogs reported having experienced SE were significantly more likely to have experienced CS than not (and *vice versa*). Age at onset of first ES was significantly lower for dogs having experienced CS, SE or both [27 months (4–165)] vs. dogs that had not experienced CS or SE [43 months (5–174)]. Dogs having experienced CS, SE, or both were more likely to be dead than dogs not having experienced CS or SE with an odds ratio of 3.33 (1.54–8.00, 95% CI). There was no significant association between dogs being treated or not and being treated with 1 or >1 ASMs vs. having experienced CS, SE, or both.

The most common type of ES reported was generalized tonic–clonic (102/116, 88%) with only 8/116 (7%) of dogs experiencing focal ES and 5/116 (4%) experiencing a combination of both types. Out of 8 dogs experiencing focal ES, 4 were reported to present as motor dysfunction, 3 were reported to present as autonomic dysfunction and 1 was reported to present as behavioral dysfunction.

Triggers, Temporal Pattern, Pre-Ictal Signs, and Post-Ictal Signs

The occurrence of triggers or temporal patterns for ES was reported in 114 dogs (**Table 4**). In 68/114 (60%) dogs, no triggers or temporal patterns were identified. In the 46/114 (40%) dogs where triggers or temporal patterns were identified, stress was reported as a trigger in 22/46 (48%).

The occurrence of pre- and post-ictal signs was reported in 115 dogs (**Table 5**). In 64/115 (56%) dogs, pre-ictal signs were reported. Seeking contact with the owner (29/64, 45%) and restlessness (26/64, 41%) were the most frequently reported pre-ictal signs. In 95/115 (83%) dogs, post-ictal signs were reported. Walking aimlessly (79/95, 83%) and lethargy (60/95, 63%) were the most frequently reported post-ictal signs.

Vaccination Status, Anthelmintic Drug Use, and Anti-Ectoparasitic Drug Use

The vaccination status was reported for 104 dogs and was up-to-date in 82 (79%) of dogs. For 103 dogs, the application of anthelmintic and anti-ectoparasitic was reported. Anthelmintic drugs were used in 72/103 (70%) dogs and anti-ectoparasitic were used in 66/103 (64%) dogs. No associations were found between these variables and age at onset of first ES, age at the time of data collection, the occurrence of CS, SE, or both, ES type, or ES frequency.

Quality-of-Life

The QoL of BC with IE was scored by 95 out of 116 (82%) owners with a median score of 7 (1–10). Dogs having experienced CS, SE, or both were scored significantly lower QoL scores [7 (1–10)] by their owners than dogs that had not experienced CS, SE, or both 9 (1–10) ($P = < 0.01$). Dead dogs (scored QoL pertaining to when they were alive) were scored a median of 2 (1–10), which was statistically significantly different from dogs that were

TABLE 3 | Number and percentages of dogs experiencing cluster seizures (CS) and status epilepticus (SE) ($n = 114$).

	CS		
	Yes	**No**	**Total**
SE			
Yes	25 (22%)	8 (7%)	33 (29%)
No	42 (37%)	39 (34%)	81 (71%)
Total	67 (59%)	47 (41%)	114 (100%)

TABLE 4 | Triggers or temporal patterns identified by owners.

	Number of dogs (*n*)	**%**
Trigger or temporal pattern (*n* = 114 dogs for which these were or were not reported)		
Yes (identified)	46	40%
No (not identified)	68	60%
Type of trigger or temporal pattern (*n* = 46 dogs for which these were reported)		
Stress	22	48%
Time of day (morning, evening or night)	17	37%
Influence of weather	7	15%
Physical exercise	6	13%
Sexual excitement	4	9%
Visit veterinarian	4	9%
Seasonal influence	1	2%
Other	2	4%

TABLE 5 | Pre- and post-ictal signs.

	Number of dogs (*n*)	**%**
Pre-ictal signs	115	100%
Yes (identified)	64	56%
No (not identified)	51	44%
Post-ictal signs	115	100%
Yes (identified)	95	83%
No (not identified)	20	17%
Type of pre-ictal signs	64	
Seeking contact with owner	29	45%
Restlessness	26	41%
Salivation	4	6%
Vomiting	2	3%
Aggression	2	3%
Other	5	8%
Type of post-ictal signs	95	
Walking aimlessly	79	83%
Lethargy	60	63%
Drinking	28	29%
Blindness	25	26%
Eating	24	25%
Aggression	7	7%
Other	13	14%

alive (median score of 8, 3–10) ($P = < 0.01$). QoL scores were not significantly impacted by treatment with ASM(s) (yes/no) or the occurrence of side effects (yes/no) and were not significantly associated with age at the time of data collection, age at onset of first ES or ES frequency.

Owners scored their dog's QoL to have declined by a median of 30% during the course of life with IE with 39% (37/95) of owners scoring their dog's QoL to have declined by ≥50%. The owners' perception of change in QoL showed a very weak positive correlation with age at the time of data collection [i.e., the older the dog was, the lower the change in QoL (less negative impact)] [r (93)= 0.30, $P = <0.01$]. Dogs that were dead (QoL pertaining to when the dog was still alive) were scored to have 90% (0–100) reduction in QoL, which was a statistically significantly higher change compared with a reduction of 30% (0–80%) for those dogs that were still alive ($P = < 0.01$). The amount of change in QoL was not significantly impacted by treatment with ASM(s) (yes/no) or the occurrence of side effects (yes/no) and was not significantly associated with age at onset of first ES or the occurrence of CS, SE, or both.

DISCUSSION

This study provides additional information characterizing the phenotype of IE in BC dogs. Several findings reported previously were mirrored in our study that included a larger number of BC dogs (7). The median age at onset of first ES was 33.5 months (2.8 years) compared with 2.4 years in the study by Hülsmeyer et al. (7). There was no sex predisposition or association with neuter status. The most frequently recorded owner-reported trigger was unspecified stress. Restlessness and seeking owners' attention were recorded as the most prevalent pre-ictal signs. The predominant ES type was a generalized tonic-clonic ES. Only a few dogs had (additional) focal ES that were characterized as either autonomic, behavioral or motor. Walking aimlessly and lethargy were the most common identified post-ictal signs. Although restlessness was reported as the most common post-ictal sign in the earlier study, "walking aimlessly" may be described as "restlessness". This elucidates the difficulty in the interpretation of signs reported by owners and their subjective nature. In total, 74% of dogs were treated with ASMs, compared with 78% of dogs in the study by Hülsmeyer et al. (7). However, as only 33 treated dogs were included for further analysis in that study, our subset of 86 dogs provided further information on which ASMs were mostly employed (phenobarbital in 81%) and how many were used to treat these dogs. In total 60% of BC treated with ASMs were treated with 2 or more ASMs. While we did not evaluate response to treatment or ASM resistance, this finding is speculatively suggestive of a difficult to treat form of IE, in accordance with the study by Hülsmeyer et al. (7). In the same study, ASM side effects were reported by 67% of owners of BCs with IE, vs. 81% of owners of BCs with IE in our study. In almost a quarter of these dogs, 4 or more side effects were reported. These findings highlight the importance of discussing the possibility of the occurrence of side effects with owners of BCs with IE when treatment is contemplated or started. Most reported side effects are well-known to be associated with the use of ASMs in the treatment of dogs with IE (12). An important difference in findings between our study and that of Hülsmeyer et al. is the prevalence of SE and CS in BC with IE (7). CS occurred in 94% of dogs in that study vs. 59% in our study and SE occurred in 53 vs. 29%, respectively. These differences may be explained, for instance, by differences in treatment, collection bias (differences in methods and the possibility that owners of severely affected dogs are more likely to respond to questionnaire calls), dependence on owner reports, and differences in the severity of IE in the cohorts studied. Still, a significant number of dogs in our study experienced CS and SE. The occurrence of CS and/or SE in various dog breeds with IE has been reported and is highly variable, but consistently high in BCs and notably higher than in some other breeds, such as the poodle or Finnish Spitz (13–18).

We chose to include an owner-reported ES frequency over the last 3 months as owner-recall bias would be expected to have a larger influence on ES frequency calculated over longer periods of time than shorter periods of time. The time frame of ES frequency calculated over the last 3 months has been applied in the recent studies and we chose this time frame as most owners had ES diaries pertaining to that period (19). Still, as the moment of data collection was not standardized (e.g., before starting ASM or after, the time elapsed since age at onset of first ES was variable et cetera), the interpretation and analysis of these data are confounded. This should be taken into account when interpreting findings relating to ES frequency and associations with other variables. No significant associations between ES frequency and other clinical variables were found in this study.

The finding that age at onset of first ES was significantly lower for dogs having experienced CS, SE, or both and that these dogs were more likely to be dead than dogs not having experienced CS or SE is noteworthy and implies a worse prognosis. QoL scores for dead dogs were significantly low and QoL score changes were significantly higher than that for dogs that were alive. Although the authors would speculate that it is likely that euthanasia or epilepsy-related death was the reason for death in most of these cases, the reason for death was not recorded in this study and so this remains unclear. The occurrence of probable sudden unexpected death in dogs with epilepsy (pSUDED) has been studied recently and researchers found it likely that dogs with CS have an increased risk of pSUDED (20). We did not find any associations between the implementation of vaccinations or the use of anthelmintic or anti-ectoparasitic drugs and clinical variables and ES frequency or the occurrence of CS/SE or both. However, our data did not include information on the types of drugs used. This finding may provide dog owners in general and also those of dogs with IE with more evidence-based information to make their decisions on whether or not to vaccinate their dog with IE.

To evaluate the QoL of BC with IE, we asked owners to score the QoL of their dog with IE and separately also score the QoL of their dog as a percentage compared with the QoL before the onset of ES. The median QoL score was 7 out of 10. This may be subjectively interpreted as good despite the seriousness of IE in BCs. QoL scores were not found to be associated with treatment

with ASMs. This may be explained by either a type II error (i.e., too small sample) or a multitude of conflicting effects of treatment (i.e., improvement of ES frequency or severity vs. the occurrence and severity of side effects). We did find a significant association between QoL scores of dogs with or without CS and/or SE: dogs with CS and/or SE had a significantly lower score than dogs without CS and/or SE. This finding implies that increased severity of IE leads to a decreased owner-scored QoL. This seems very logical and the presence of CS and/or SE has indeed been interpreted in the literature as an indicator of the severity of epilepsy (4, 7). More detailed studies on the QoL of BC with IE based on more exhaustive questionnaires may provide more insights on this matter (21). In total, thirty-nine percent (39%) of owners scored their dogs' QoL to have declined by ≥50%, which exemplifies the impact IE can have on the QoL of dogs. The very weak positive correlation of QOL with age at the time of data collection should not be overinterpreted. It is very weak to start with and may simply reflect, for instance, that dogs of older age at the time of data collection are less heavily impacted by side effects of treatment (e.g., lethargy may be less noticeable in a 10-year-old BC than in a 1-year-old BC) or less severe course of IE in elderly BCs.

The prevalence of IE in the BC breed has been shown to be high in many studies (1–6). Although a strong indication for a genetic background to the occurrence of IE in BC dogs is reported based on pedigree analysis (7), the causal genetic mutation(s) have not been identified yet. This holds true for many other dog breeds with a reported high prevalence of IE (6). It should be noted that a higher than average prevalence (or incidence) has been reported in some Dutch dog breeds specifically (e.g., Friesian Stabijhoun and Drentse Patrijshond) (6). A geographical predisposition for IE in BC (i.e., the Dutch BC) may be investigated by comparing incidence or prevalence between different geographical regions or countries. This would be best implemented by comparing the diagnostic percentage of national databases for IE in the BC (e.g., UK "VetCompass^{TM}" and the Dutch "Petscan") (5). If national databases become widely implemented, a geographical "heat map" of high incidences of IE could be constructed that may help select subpopulations of BC to investigate further. As the genetic basis for IE is likely complex and cooperation of kennel clubs, owners, and veterinarians is necessary to acquire large volumes and adequate samples for genetic analysis, this may provide helpful insights. Also, up-to-date information on the incidence of IE in the BC is very helpful in evaluating measures taken by breeders to reduce the prevalence of IE within the breed.

There are some notable limitations to our study. Due to its retrospective nature, the diagnostic work-up varied within the study population. By including dogs that fit the criteria for a tier I International Veterinary Epilepsy Task Force diagnosis of IE, we aimed to exclude to a degree dogs with structural causes of epilepsy or reactive seizures (9). However, we cannot exclude the possibility that some of the included dogs did not have IE. In addition, some of the recorded data were unspecific. For instance, by only noting the type of ES as "generalized tonic–clonic", "focal" or "combination", we were unable to discriminate between dogs having focal ES evolving to become generalized and those that had immediately generalized ES with certainty (6, 7, 10). Indeed, ES with the focal onset and secondary generalization were indicated in BC in the study by Hülsmeyer et al. but the definition thereof remains open to discussion (4). Other limitations are, among others, that the data did not allow for evaluation of the response to treatment or drug resistance and the subjective interpretation of some of the parameters by owners. Despite these limitations, this study provides valuable insights into the phenotype of BC with IE.

In conclusion, this study confirms the association of age at onset of first ES with the severity of IE (e.g., presence of CS and/or SE) and provides additional information characterizing the phenotype of IE in BC dogs. Age at onset of first ES was significantly lower for dogs having experienced CS, SE, or both. QoL of BC can be heavily impacted by IE and especially in BCs with CS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PM was responsible for the study conception and data collection in cooperation with co-authors and supervised data analysis and manuscript editing, in cooperation with EB, SB, PL, and AF. The majority of Dutch cases were seen by PM and the majority of Belgium cases by SB. Statistical analysis, data analysis, and manuscript writing were performed by KS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.880318/full#supplementary-material

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Bromide Dose in Dogs With Epilepsy Living Close to Coastal Areas and Living More Inland: A Retrospective Observational Study

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Potassium bromide is a frequently used antiseizure medication with a half-life time of over 25 days. Oral intake of sodium chloride as well as renal function influence this half-life time and may have an influence on the needed dose to *get proper serum* levels. The hypothesis is that dogs living close to coastal areas require a greater potassium bromide dose than dogs living more inland. The main study objective was to determine the relationship between bromide dose, serum bromide concentration, treatment duration, type of food, concurrent therapies and the proximity of the dog's residency to a coastal area. A retrospective cross-sectional study was performed. A total of 658 bromide serum measurements were retrieved from the veterinary faculty's laboratory archive, Utrecht University, The Netherlands. Information on the bromide dose, renal function, treatment period, type of food, concurrent therapies and residence was obtained retrospectively from owners of all dogs using a postal survey. A dataset of 220 unique study units was created. The dogs were grouped based on their residence (proximity to the coast > or < 50 km). Differences between the groups of dogs regarding bromide dose, serum bromide concentration, treatment duration, type of food and concurrent therapies were analyzed to evaluate the effect of residence on bromide dose and serum concentration.

Results: Although not statistically significant there is a trend that dogs living in close proximity to the sea may require a higher dose of potassium bromide to maintain therapeutic concentrations compared to dogs living more inlands. Additional studies are needed to further explore this observation.

Keywords: epilepsy, potassium bromide, anticonvulsant, salt, aerosols, sea

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INTRODUCTION

Bromide (most often administered as potassium bromide) was the first drug used for the treatment of epilepsy in humans (1–3), but it is nowadays used less commonly as there are many newer drugs with a superior safety and efficacy profile available (4, 5). Phenobarbital, introduced around 1,910, was the first anti-convulsant drug replacing the use of bromide and is, in veterinary medicine, still first-choice treatment for canine epilepsy (6). Unfortunately, few alternative options remain in veterinary medicine, as the canine metabolism degrades most newer human medications

preventing optimal seizures control in dogs (1). Hence the need of bromide in veterinary neurology. The use of bromide, as a possible treatment of canine epilepsy, was first described by Schwartz-Porsche et al. (7) and Podell and Fenner (8), where it proved to be an efficacious add-on therapy for refractory, phenobarbital-treated, dogs. Bromide can also be used as monotherapy (9), with an efficacy comparable to fenobarbital (10). However the authors concluded that there were, compared with fenobarbital, several disadvantages to its use (10). Side effects are more frequent, and can include increased appetite, drinking, urinating, drowsiness and loss of activity. Further, it's half-life is long, and it can be highly variable amongst dogs, ranging from 25 days (in patients) to 46 days (in laboratory Beagles) (11, 12). Given that steady state serum concentrations are only achieved after five half-lives (13, 14), it might feasibly take approximately 125 to 230 days before stable and efficacious serum levels can be achieved. Its uptake and secretion is influenced by both dietary chloride intake and renal function (9, 11). Therefore, a clinician using bromide therapy must take into account dietary chloride intake (since a high chloride intake will tend to shorten half-life and lead to lower serum concentrations) and renal function (which decreases bromide excretion, thereby increasing circulating concentrations) (15). Although chloride intake is predominantly through ingestion of food, it is feasible that other environmental sources contribute. The ingestion of sea water will most likely influence serum levels and the same could be applicable to dogs living close to sea as air in a coastal environment has a high concentration of salt in aerosol form (16, 17). It has been reported that salt content diminishes steadily to negligible concentrations once beyond 50 km inland (16, 17). Hence, it is possible that a greater salt exposure in a coastal region could decrease bromide half-life in dogs receiving bromide, meaning that a greater dose is required to reach effective serum concentrations. The aim of the current study was to determine whether there were differences in the use of bromide as an antiseizure therapy for dogs living in coastal regions, compared with dogs living inland.

MATERIALS AND METHODS

Study Design and Eligibility Criteria

The study is a retrospective cross-sectional study of epileptic dogs on bromide. Dogs were eligible for inclusion if they were suffering from idiopathic epilepsy as defined by Berendt et al. (18) and were being treated with potassium bromide either as monotherapy or in combination with other antiseizure medications. Further, a serum sample had to have been submitted to the University Veterinary Diagnostic Laboratory (UVDL), Utrecht, the Netherlands, for measurement of therapeutic bromide concentration. A time frame of 2 years, for the selection of cases, was chosen. Samples were either submitted by primary care veterinarians or referral specialists. Study units were defined as the individual dogs from which the samples were obtained, rather than the serum bromide concentrations (19). Therefore, when multiple samples had been submitted from the same dog, only the result of the most recent sample was used during a period when the bromide dose and food given had not been altered. Dogs were excluded if the veterinarian had used a loading dose protocol when bromide therapy was commenced, if dogs living inland had visited the coastal areas during this study period, or if their serum creatinine concentration was > the reference value used by the laboratory (i.e. reference value [in umol/L] = 60 ± the bodyweight of the dog).

Abbreviations: UVDL, University Veterinary Diagnostic Laboratory.

Serum Bromide Measurement

All serum bromide measurements were performed at the University Medical Center, Division Laboratory and Pharmacy, Utrecht University, Netherlands, using a slightly modified version of the gold-trichloride assay as previously described (11, 20). To a total of 500 µl of test serum 3,0 ml of 0,5% sodium chloride was added. Precipitation of proteins in the test serum was accomplished by adding 500 µl of 25% trichloroacetic acid. Samples were then centrifuged for 5 min. Two ml of supernatant was mixed with 250 µl 0 5% gold chloride and centrifuged for another 5 min. The clear supernatant was used to spectrophotometrically measure the resulting gold color at 440 nm. Quantification of bromide concentrations in this assay is based on the principle of bromide and gold chloride reacting and forming gold bromide, resulting in a specific absorption at 440 nm. The limit of quantification was 100 mg/ml and linearity was validated between 500 and 3,000 mg/l of bromide.

Collection of Patient Information

A time frame of 2 years, during which there was only one formulation of potassium bromide available (Epikal, AST Pharma), for the selection of cases, was chosen to identify dogs eligible for inclusion. A postal questionnaire survey was sent to the veterinarians who submitted the sample, in order to gather information on the dog, and to confirm eligibility. Data obtained included: residence, type of food, diagnostic tests performed (including renal function), bromide formulation, starting date of the therapy, sampling date, bromide dose during this period, and any other treatments administered.

Statistical Analysis

Data were analyzed with computer software IBM® SPSS® Statistics. Statistical significance was set at $P < 0.05$ with P values between 0.05 and 0.1 were considered to suggest a statistical tendency.

The main objective was to determine if there was a difference in bromide dose and bromide serum concentration in dogs living in different residences. Other factors taken into association included treatment duration, type of food, additional antiepileptic drugs (AED). The serum bromide dose was measured in mg/kg bodyweight and the bromide serum concentration was measured in mg/l serum. The treatment duration for each dog was defined as the period between the start of bromide therapy at a certain dose and sampling date. As mentioned above, no alterations in bromide dose were allowed during the treatment period. Treatment duration was converted to a binary variable with treatment duration <120 days and >120 days. The different types of food the dogs

were receiving were grouped in "commercial diets", "gluten-free antigen-limited diets", "fresh meat diets" and "unknown/mixed diets". Also, groups were made for concurrent therapies. The first group "None" received no concurrent therapy, another group was treated concurrently with phenobarbital, group 2 with phenytoin and group 3 received other types of medication. The different residences were determined by calculating the distance of the dog's geographic location to the closest coastal region with the "Measure distance" function in Google Maps. Dogs were grouped as follows: those living ≤50 km from a coastal region were assigned to group "sea side," dogs >50 km from a coastal region were assigned to group 'inlands. The relation between treatment period, type of food and concurrent therapies in the dogs living in the different residences were assessed using a Chi-square test. An ANCOVA was used to determine the influence of residence, concurrent therapy, treatment period and diet on serum bromide concentration and bromide dose, whilst the influence of residence on serum bromide concentration and bromide dose was assessed by an ANCOVA and a regression analysis.

RESULTS

Details of Study Dogs

In the initial computer records search, a total of 658 dogs were identified that had samples submitted within the study period. Of these, postal survey results were returned for 345, of which 220 were filled in completely, enabling a dataset of 220 unique single study units to be created (**Table 1**). Veterinarians did not report using a loading dose protocol in any of these 220 dogs, and serum creatinine concentrations were never > the laboratory reference value.

Bromide Dose, Serum Bromide Concentrations

The mean bromide dose daily given was 31 mg/kg (2 mg/kg to 102 mg/kg), whilst the mean serum bromide concentration was 1,288 mg/L (351 mg/L to 3,257 mg/L) (**Table 1**).

Residences

All study dogs lived in the Netherlands, but came from various regions. A total of 117 dogs (53%) were assigned to group seaside (< 50 km from a coastal region), 103 dogs (46%) were assigned to group inlands (> 50 km from a coastal region) (**Table 1**).

TABLE 1 | Baseline data of the study population.

Number of study units (dogs)	**220**
	Residence seaside: 117 (53%)
	Residence inlands: 103 (46%)
Mean bromide dose	31 mg/kg (2 mg/kg to 102 mg/kg)
Mean bromide serum concentration	1288 mg/L (352 mg/L to 3257 mg/L)

Treatment Duration

There was no minimum duration of treatment required. Although with a treatment period <120 days there is a risk that a steady-state bromide serum concentration has not been reached. There were no significant differences in treatment period of the dogs (>120 days or <120 days) between the two residences ($P = 0.87$). There is no significant effect of treatment duration on the bromide dose and serum bromide concentration ($P = 0.33$).

Types of Food

The dogs were fed a wide variety of types of food, with the majority ($n = 159$, 72%) receiving a variety of commercial diets from a range of manufacturers (e.g. >25). A further four dogs (2%) received a commercial gluten-free antigen-limited diet, 21 dogs (10%) received fresh meat only, whilst the type of diet was not known for the remaining 36 dogs (16%) (**Figure 1**). Diets were not changed between the start of treatment and sampling date. There was no difference in type of food given to the dogs between the two residences ($P = 0.97$) or influence of the types of diet on the serum bromide concentration and the bromide dose ($P = 0.39$).

Concurrent Therapies

Phenobarbital, slow-release phenytoin and levetiracetam were administered as concurrent AED. Twenty-four dogs (11%) received no concurrent therapy, where 168 dogs (76%) were treated with phenobarbital and 20 dogs (10%) with slow-release phenytoin. Eight dogs (4%) were treated with other medication such as levetiracetam, carprofen, benazepril, phenylephrine, prednisone or levothyroxine (**Figure 2**). Interestingly there was a significant difference in concurrent therapies between residence seaside and residence inland ($P = 0.02$). There is no significant influence of concurrent therapies on bromide dose and serum bromide concentration ($P = 0.12$).

Effect of Residence on the Bromide Dose and Serum Bromide Concentration

Figure 3 shows the linear regression for potassium bromide dose and serum concentration for the two residences. The regression coefficients were 0.15 for residence seaside and 0.31 for residence

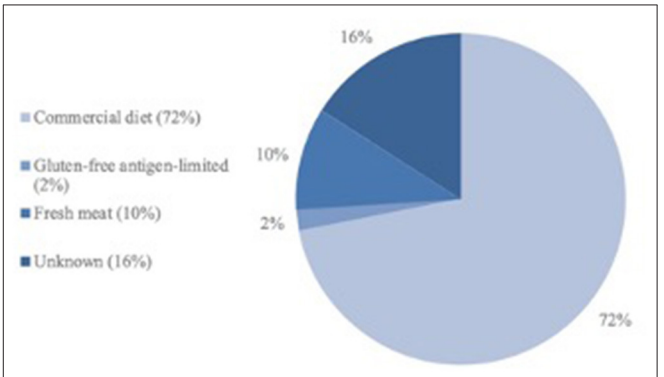

FIGURE 1 | Types of food of the study population.

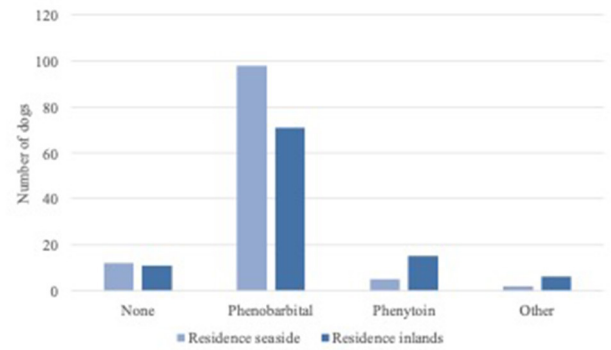

FIGURE 2 | Concurrent therapies of the dogs grouped by residence.

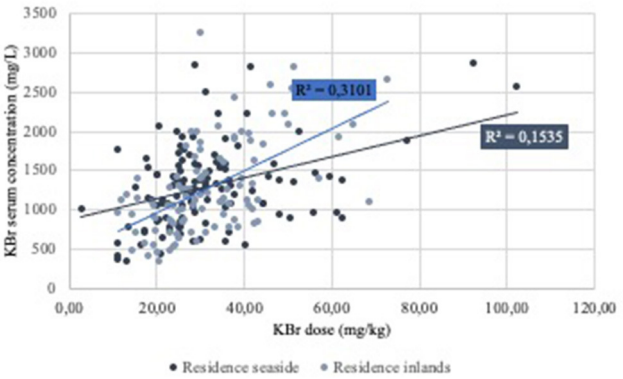

FIGURE 3 | Regression analysis of the potassium bromide (KBr) dose and serum concentration of the dogs grouped by residence.

inlands. Both were statistically significant by itself ($P < 0.01$). This shows the need of higher doses of potassium bromide to reach a specific serum bromide concentration when living in residence seaside ompared to the other residencies. However, residence had no statistically significant influence on serum bromide concentration and bromide dose ($P = 0.82$).

DISCUSSION

The purpose of this study was to investigate the effect of residence on the bromide dose and serum concentration and to describe the correlation between residence, bromide dose, treatment duration, type of food, concurrent therapies and serum concentration in dogs treated with bromide. This study shows that there is a tendency for the need of higher doses of potassium bromide for dogs living close to the sea to reach an effective serum concentration, than for dogs living more inland. However, this effect was not statistically significant.

As bromide is not subject to hepatic biotransformation the only factor that can influence the bromide excretion is the amount of dietary chloride, renal function and external salt intake (9, 21). Chloride can decrease the half-life time of bromide. After the glomerular filtration, bromide will be reabsorbed in the tubules (22). The reabsorption of bromide is, compared to chloride, stronger unless high quantities of chloride are present in the tubules (11, 23). If higher quantities of chloride are present in the diet the half-life time will decrease (11). This was most likely the reason that Trepanier and Babish (11) found a significantly longer half-life time of 46 ± 9 days compared to the half-life time of 25 days found by Schwartz-Porsche and Jurgens (12). The dogs used to calculate the half-life time from Schwartz-Porsche and Jurgens (12) were fed a normal diet and the dogs of Trepanier and Babish (11) a low salt diet. For this reason, we tried to exclude the food factor as well as the renal function in this study. The exact amount of chloride was not known for most types of food, nor could be retrieved how much food they actually gave to their dogs which made it impossible to get a clear idea of the actual amount of dietary chloride given to the dog. Although all dogs were fed a wide variation of food types there was no statistically significant difference when looked at food and residence ($P = 0.66$) and a difference in dietary salt intake was therefore unlikely. And although a normal creatinine level does not exclude a decreased renal function all dogs included had creatinine levels below the reference value of the lab. So most likely the only factor that could influence the bromide serum concentration would be the residence of the dog.

There are some limitations for this study. The data we used was obtained retrospectively, which leads automatically to less controlled data collection and thus a great variation in external influences. However, the external influences considered to be important in potassium bromide therapy have been accounted for in this study by showing that there were no differences in treatment duration or type of food between the residences. Treatment duration can influence the serum concentration of bromide, when a steady state has not been reached. Steady state will be reached after five half-live times (13, 14). The half-life time of bromide is shown to be between 25 and 46 days and therefore steady state can be reached after 125–230 days (11, 12). In this study no minimum treatment duration was required, and therefore the serum concentrations of bromide might not have been stabilized yet for the patients treated <120 days. However, no significant difference in treatment duration has been found between the residences ($P = 0.87$) and therefore influence of treatment duration on the shown tendency for the need of higher potassium bromide dosages in dogs living closer to the sea was deemed low. A significant difference is found between the two residences for concurrent therapies. As can be seen in graph 2, other drugs are used in dogs living inland compared to those living closer to the sea. It is however unlikely that this finding influences the regression coefficient for potassium bromide dose and serum concentration, as no other influences on bromide absorption and excretion other than renal function and salt intake are known (9, 21). Future prospective studies with controlled study groups would be beneficial to rule out this uncertainty.

It would have been of interest to have accurate measurements of chloride levels as well but in cases treated with bromide, chloride measurement becomes with routine laboratory equipment as used in veterinary medicine impossible as it cannot

differentiate properly between these two molecules. For this reason chloride is first removed from the serum sample before measuring bromide (11, 20). To measure both molecules in the same sample mass spectrometry is needed but this equipment is not routinely used in veterinary laboratories.

Based on our results dogs living close to sea likely need a higher bromide dose compared to dogs living inland. The only reason that could account for this is most likely that dogs living close to sea inhale more salty aerosols or ingest sea water through drinking (16, 17). which causes a decrease in half-life time of bromide.

CONCLUSION

In this study residence, living close to sea or not, may have a potential influence on the bromide dose needed to reach an effective bromide serum concentration. Dogs that live close to see seem to need a higher amount of bromide compared to dogs living inland. This study demonstrates that if a dog is put on bromide, it is important to take into account all possible sources of sodium chloride the dog can ingest as it will decrease half-life time and hence increase the dose needed to achieve optimal serum concentrations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PM was responsible for the study conception and PM and EL for data collection. Statistical analysis, data analysis, were performed by EL and JB. EL was responsible for writing. PM and BE supervised data analysis and manuscript editing. All authors contributed to the article and approved the submitted version.

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Urinary Neurotransmitter Patterns Are Altered in Canine Epilepsy

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Epilepsy is the most common chronic neurological disease in humans and dogs. Epilepsy is thought to be caused by an imbalance of excitatory and inhibitory neurotransmission. Intact neurotransmitters are transported from the central nervous system to the periphery, from where they are subsequently excreted through the urine. In human medicine, non-invasive urinary neurotransmitter analysis is used to manage psychological diseases, but not as yet for epilepsy. The current study aimed to investigate if urinary neurotransmitter profiles differ between dogs with epilepsy and healthy controls. A total of 223 urine samples were analysed from 63 dogs diagnosed with idiopathic epilepsy and 127 control dogs without epilepsy. The quantification of nine urinary neurotransmitters was performed utilising mass spectrometry technology. A significant difference between urinary neurotransmitter levels (glycine, serotonin, norepinephrine/epinephrine ratio, ɣ-aminobutyric acid/glutamate ratio) of dogs diagnosed with idiopathic epilepsy and the control group was found, when sex and neutering status were accounted for. Furthermore, an influence of antiseizure drug treatment upon the urinary neurotransmitter profile of serotonin and ɣ-aminobutyric acid concentration was revealed. This study demonstrated that the imbalances in the neurotransmitter system that causes epileptic seizures also leads to altered neurotransmitter elimination in the urine of affected dogs. Urinary neurotransmitters have the potential to serve as valuable biomarkers for diagnostics and treatment monitoring in canine epilepsy. However, more research on this topic needs to be undertaken to understand better the association between neurotransmitter deviations in the brain and urine neurotransmitter concentrations in dogs with idiopathic epilepsy.

Keywords: neurotransmitter, epilepsy, biomarker, urinary, canine

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INTRODUCTION

Dogs and humans are affected by naturally occurring epilepsy, a complex brain disorder characterised by a predisposition to experience recurring seizure events (1–3). It is one of the most common chronic neurological diseases in both species, with many shared clinical and epidemiological characteristics (4–6). Around two-thirds of the affected dogs and half of the human patients do not become seizure free, despite pharmacological treatment (7, 8). Persistent uncontrollable seizures are a health concern increasing mortality, causing psychological and physical stress, and culminating in a negative impact on the overall quality of life (9, 10).

Epileptic seizures are initiated by abnormally excessive or synchronous neuronal activity in the cerebral cortex or hippocampus of the brain (11). The exact pathogenesis of this process has not yet been solved. However, a contributing factor to the underlying pathophysiology of seizures may arise from the imbalance of excitatory and inhibitory neurotransmission, caused by neurotransmitter and receptor alterations (12, 13).

In the past decades, evidence in humans and primates has suggested that seizures were correlated to altered neurotransmitter concentrations of glutamate, ɤ-aminobutyric acid (GABA) and serotonin, which were measured in the extracellular fluid, cerebrospinal fluid (CSF) and serum (14–17). A deviating neurotransmitter composition, caused by a dysfunctional neurotransmitter metabolism in humans, can also result in seizures and other neurological signs (18).

Emerging seizures can also be linked to changes in neurotransmitter receptors. In earlier studies, the altered GABA or dopamine (DA) receptor density was accompanied by seizures or seizure susceptibility in human patients and rodents (19–22). Changes in receptor function, such as binding potential or endogenous activity of glutamate, GABA or serotonin receptors were found in humans suffering from temporal lobe epilepsy (23–27). Additionally, a divergent composition of glutamate or GABA receptor subunits has also been associated with recurring seizures in animal models and humans (28–31). Those detected subunit compositions were similar to those of the more excitatory immature brain and facilitated further seizures and epileptogenesis (32).

In the central nervous system (CNS), glutamate is the major excitatory neurotransmitter, whereas GABA is the primary inhibitory counterpart (33, 34). The equilibrium of these two neurotransmitters maintains the balance of cell excitability. The aforementioned alterations that affect one or both of these neurotransmitters are likely to elicit a shift to arousal in the brain, followed by seizures (13). The neurotransmitter serotonin is known for its anticonvulsant properties and regulation of mood and cognition (35, 36). Therefore, disturbances in the serotonergic system are assumed to evoke seizures and promote frequently developed neurobehavioural/-psychiatric comorbidities associated with epilepsy in dogs and humans (37–43).

In the body, intact neurotransmitters of the CNS are transported through the blood-brain barrier (BBB) to the peripheral systemic circulation, from where they are primarily excreted through the kidney into urine (44–47). The neurobiological basis of this process is poorly understood. It is substrate-specific and can vary for each neurotransmitter (48). However, several animal studies demonstrated an association between central and peripheral neurotransmitter output into the urine (49–52). Moreover, positive correlating neurotransmitter concentrations of serine, glycine and norepinephrine (NE) between the CSF, blood and urine in dogs were recently revealed, emphasising a connection (53).

In human medicine, non-invasive urinary neurotransmitter analysis is used to manage medical conditions such as depression and attention-deficit hyperactivity disorder (ADHD) (54–57). Patients affected by depressive and anxiety symptoms showed increased urinary catecholamines, like NE and epinephrine (E) (58–60). Suicide attempts in depression were strongly associated with urinary excreted DA, even greater than the CSF concentration (61). ADHD symptoms correlated with alterations of the urinary catecholamines NE and E, and a decrease in urinary phenylethylamine (PEA), which is linked to inattentiveness (62–66).

Urinary neurotransmitter analysis is not as yet used for epilepsy management in either humans or dogs, to the authors' knowledge. However, promising evidence was provided in a recent study, indicating altered urinary neurotransmitter patterns associated with the treatment efficacy of medium-chained triglyceride (MCT) oil in drug-resistant canine epilepsy (67). Intake of MCT oil increased urinary GABA concentration in dogs with IE. Also, the GABA/glutamate ratio changed significantly by decreased glutamate levels compared to GABA levels in dogs affected by epilepsy. Furthermore, non-responders without a reduction in seizure frequency below 50% excreted higher glutamate, histamine and serotonin levels in their urine (67).

This study investigated whether urinary neurotransmitter profiles differ between dogs with epilepsy and non-epileptic controls. We hypothesised that urinary neurotransmitter analysis could provide a non-invasive diagnostic tool, where characteristic neurotransmitter deviations can serve as valuable biomarkers in epilepsy research and clinical management.

MATERIALS AND METHODS

Sample Acquisition

In this multicentre study a total of 223 urine samples were collected from 190 privately owned dogs (both sexes; mixed or pure breed) and divided into two cohorts. From the first cohort, 96 urine samples from 63 subjects with idiopathic epilepsy (IE) were obtained. Dogs in the IE cohort had no acute or chronic diseases of the gastrointestinal tract, kidney, liver or heart failure. They met at least the requirements of Tier I ($n = 15$) confidence level of the International Veterinary Epilepsy Task Force (IVETF) for the diagnosis of IE, however, most dogs met Tier II ($n = 48$). Two adjustments to IVETF criterion were applied, as long as magnetic resonance imaging was unremarkable: firstly, abnormalities in the interictal neurological examination caused by antiseizure drug (ASD) treatment were tolerated and secondly, the maximum age at seizure onset was increased to 12 years (68). Samples were collected and analysed as part of three former epilepsy studies, between October 2012 and September

Abbreviations: GABA, ɤ-aminobutyric acid; CSF, cerebrospinal fluid; DA, dopamine; CNS, central nervous system; BBB, blood-brain barrier; NE, norepinephrine; ADHD, attention-deficit hyperactivity disorder; E, epinephrine; PEA, phenylethylamine; MCT, medium-chained triglyceride; IE, idiopathic epilepsy; IVETF, International Veterinary Epilepsy Task Force; ASD, antiseizure drug; RVC, Royal Veterinary College; TiHo, University of Veterinary Medicine Hannover; HPLC-QqQ MS/MS, High-performance liquid chromatography triple-quadrupole mass spectrometry/mass spectrometry; ANOVA, analysis of variance; SSRIs: selective serotonin reuptake inhibitors; peripheral nervous system; SSADH, succinic semialdehyde dehydrogenase, AADC, aromatic L-amino acid decarboxylase.

2017, at international study sites: Queen Mother Hospital for Animals, Royal Veterinary College, London, UK (RVC) (n = 59: 29 paired samples collected from the same individual at certain study stages; 30 unpaired); University of Veterinary Medicine Hannover, Hannover, Germany (TiHo) (n = 3, paired); University of Helsinki, Helsinki, Finland (n = 1, paired) (69–72).

The second cohort was a control group of 127 healthy dogs. All control cohort subjects were at least 1 year of age, did not receive any medication and had no chronic diseases. One hundred (n = 100) of the second cohort samples were collected from dogs owned by TiHo staff and students, between January and June 2020. The remaining 27 samples were obtained from healthy control dogs at the RVC study site.

To avoid bias of the study results, the dogs were not fed milk products, fruits and vegetables 48 h before sample acquisition. Exposition to strenuous exercise was also avoided for 24 h before sampling. Bitches were not in heat during the collection process. The urine samples were collected via the free catch method. The first or second void of morning urine from the fasting dog (preferable midstream) was used for urinary neurotransmitter analysis. Samples were transferred into a tube containing a preservative to ensure sample stability (50 mg oxalic acid/10 ml urine), followed by an immediate transport to the TiHo laboratory. Samples from the other study sites (London, Helsinki) were collected as part of an enrolment or study visit for epilepsy trials with MCT, epilepsy behaviour studies or from healthy controls of the previously mentioned studies and were directly cooled on ice (69–72).

Sample Preparation and Analysis

Samples were aliquoted and quickly frozen at the different study site laboratories. They were stored at −80°C for at least 4–6 h prior to shipment. The preserved urine samples were continuously frozen and shipped on dry ice for external analysis of neurotransmitter concentrations to "Doctor's Data," St. Charles, IL, USA. Nine urinary neurotransmitter levels (serotonin, histamine, glycine, phenylethylamine, DA, E, NE, glutamate, GABA) were quantified utilising High-performance liquid chromatography triple-quadrupole mass spectrometry/mass spectrometry (HPLC-QqQ MS/MS) technology. In addition, creatinine levels were measured by Enzymatic Colorimetric—Kinetic Jaffé method. Those were used as a reference value to determine urine concentrations and to evaluate neurotransmitter levels relative to creatinine levels. The applied neurotransmitter screening method is usually utilised in human patients. In previous canine studies, the method was used multiple times and the archived data revealed biologically reasonable results for this species as well (53, 67).

Statistical Analysis

Statistical analyses were performed with the R software (v4.0.3) to test the hypothesis, that there is a difference between the urinary neurotransmitter excretion of dogs affected by epilepsy and a healthy control group (H1) (73). Additionally, whether the urinary neurotransmitter excretion of dogs with epilepsy is affected by ASD administration was explored by comparing neurotransmitter levels of ASD- treated and untreated dogs with epilepsy (H2). First, data were log10-transformed to compensate for wide ranges. Then, the transformed data were tested against the hypothesis of normal distribution using Shapiro–Wilk's test. Finally, in the case of normally distributed data, group comparisons were analysed with an unpaired two-sided two-sample t-test. When data did not follow a normal distribution, the Wilcoxon-Mann-Whitney test was used in the analysis. Next, multiple group comparisons were analysed with a one-way analysis of variance (ANOVA) to find between-factor differences. Finally, a Games-Howell *post-hoc* test with the Holm correction for multiple comparisons was used to analyse multiple group contrasts and compensate for potential heteroscedasticity. If multiple group data did not follow a normal distribution, the Kruskal-Wallis test was used. Results were considered significant at the following p-value thresholds: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$(***), and $p \leq 0.0001$ (****).

RESULTS

Study Population

For the current study, 223 urine samples from 190 dogs of more than 21 breeds were collected, including the following: Australian Shepherd (n = 5), Beagle (n = 6), Belgian Shepherd (n =2), Bernese Mountain Dog (n = 1), Border Collie (n = 4), Chihuahua (n = 2), Dachshund (n = 3), Boxer (n = 1), German Shepherd (n = 4), French Bulldog (n = 1), Golden Retriever (n = 2), Havanese (n = 1), Jack Russell Terrier (n = 1), Labrador (n = 2), Vizsla (n = 3), Maltese (n = 1), Poodle (n = 2), Rhodesian Ridgeback (n = 1), Siberian Husky (n = 2), cross breeds (n = 46) and other breeds (n = 43). For n = 57 dogs, no information of their breed was available. The study population consisted of n = 89 males, of which n = 32 were intact, n = 57 were neutered, and n = 98 females, of which n = 37 were intact and n = 61 were neutered. For three dogs, the gender status is not available. The dogs had a mean age of 5.31 (±SD 3.41) years and weighed a mean of 20.08 (±SD 12.25) kg. Of the n = 63 dogs with IE, n = 42 were treated with phenobarbital (66.67%) and n = 27 were treated with potassium bromide (42.86%), of which 26 received potassium bromide additional to the administered phenobarbital, and one dog was solely treated with potassium bromide. Forty-four (n = 44) dogs in the IE cohort received additional ASD treatment in addition to or instead of the aforementioned ASDs (69.84%): levetiracetam (n = 16 chronically; n = 3 pulse therapy) (74), imepitoin (n = 6), Gabapentin (n = 2), rectal diazepam rescue therapy (n = 7), MCT oil (n = 36), cannabidiol oil (n = 2), coconut oil (n = 1). Seven (n = 7) dogs with IE did not receive ASD treatment at the time of sample acquisition and for n = 6 dogs no treatment data were available. Thirty-three (n = 33) (52.38%) of the affected dogs had at least three generalised seizures in the past 3 months before study enrolment. They were chronically treated with at least one ASD without improving seizure frequency. Seven (n = 7) dogs (11.11%) of the IE cohort were seizure free during the past 3 months before sample collection. For n = 23 dogs, the seizure frequency was not accessible.

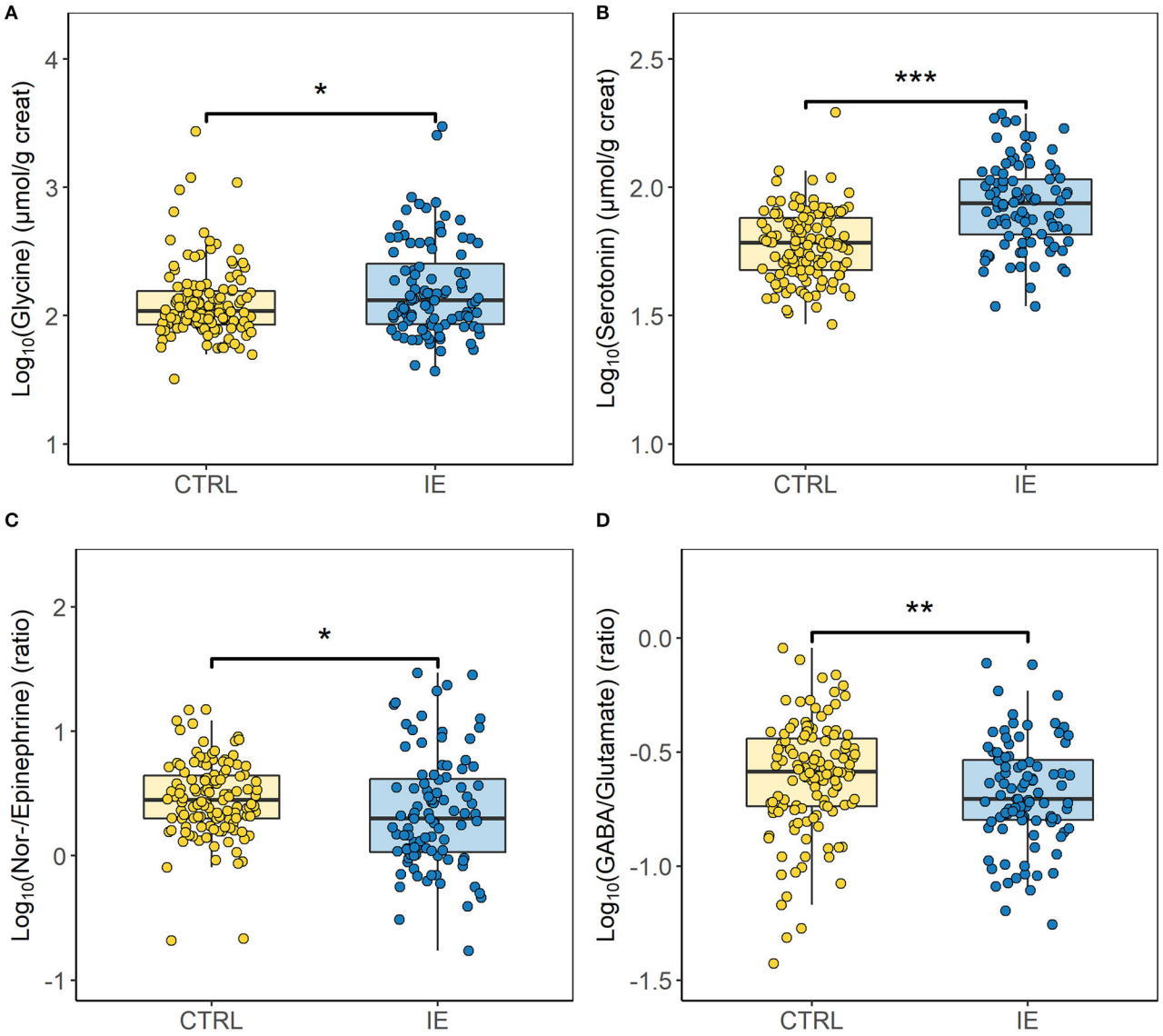

FIGURE 1 | Differences in the urinary neurotransmitter profile between dogs with idiopathic epilepsy (IE) ($n = 63$ dogs/96 samples) and the healthy control group (CTRL) ($n = 127$ dogs/127 samples). Figure shows an increase of urinary **(A)** glycine ($p \leq 0.05$) and **(B)** serotonin ($p \leq 0.001$) concentrations and a decrease of the **(C)** norepinephrine/epinephrine ratio ($p \leq 0.05$) and the **(D)** ϫ-aminobutyric acid (GABA)/glutamate ratio ($p \leq 0.01$) in dogs affected by idiopathic epilepsy. Data are presented as box-and-whisker plots (the median is represented by the central line, the 25th and the 75th percentile represent the lower and upper limit, and the length of the whiskers represent the 1.5 multiple of the interquartile range). Unpaired two-sided two-sample *t*-test and Wilcoxon–Mann–Whitney test were used to compare the urinary neurotransmitter excretion between dogs with epilepsy and the healthy control group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Neurotransmitter Analysis

A significant difference between urinary neurotransmitter levels of dogs diagnosed with IE and the control group was revealed, when sex and neutering status were accounted for (**Figures 1**, **2**). Urinary glycine ($p \leq 0.05$, **Figure 1A**) and serotonin concentration ($p \leq 0.001$, **Figure 1B**) were significantly increased in dogs with IE. Whereas, the NE/E ratio ($p \leq 0.05$, **Figure 1C**) and the GABA/glutamate ratio ($p \leq 0.01$, **Figure 1D**) was significantly decreased in dogs with epilepsy. The sex and neutering status of the dogs substantially affected the urinary neurotransmitter excretion (**Figure 2**). Glycine concentration was significantly increased in neutered females with epilepsy ($p \leq 0.05$, **Figure 2A**). The serotonin concentration was significantly increased in intact males ($p \leq 0.01$), neutered males ($p \leq 0.001$) and neutered females ($p \leq 0.001$) with epilepsy (**Figure 2B**). The NE/E ratio was significantly decreased in neutered females ($p \leq 0.05$, **Figure 2C**) with epilepsy. The GABA/glutamate ratio was significantly reduced in intact males ($p \leq 0.05$) and neutered females ($p \leq 0.05$) with epilepsy (**Figure 2D**). Finally, an influence of ASD treatment on

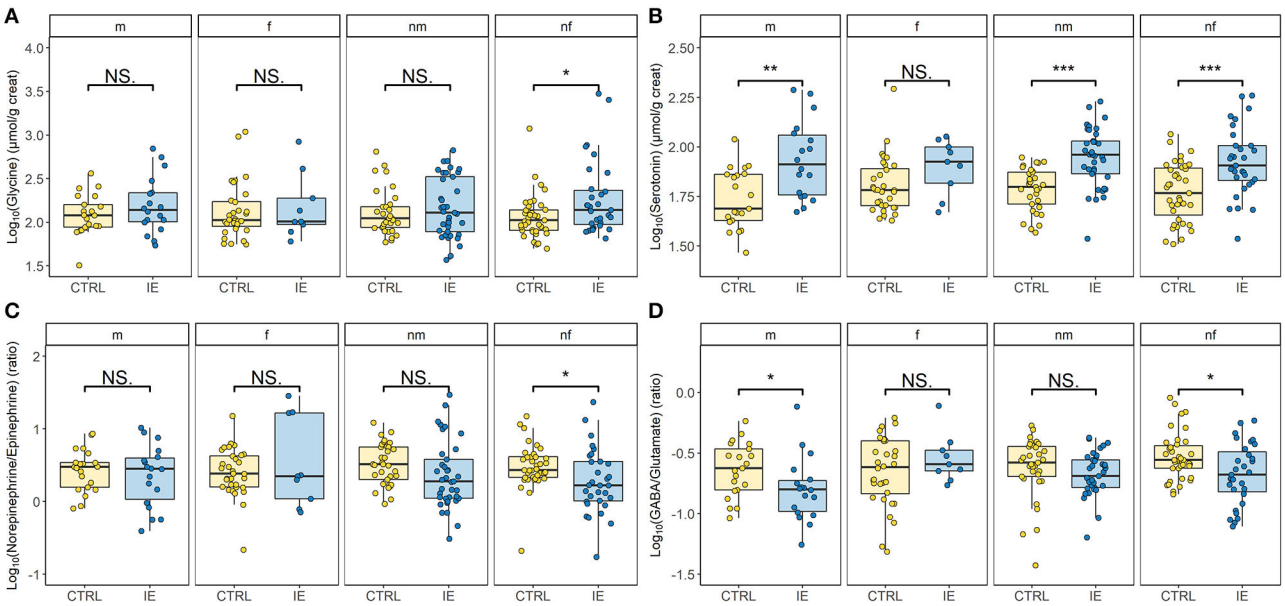

FIGURE 2 | Effects of the sex and neutering status on the urinary neurotransmitter profile of dogs with idiopathic epilepsy (IE) compared to the healthy control group (CTRL). **(A)** Glycine levels differ significantly in neutered females (nf) ($p \leq 0.05$) (IE: $n = 22$ dogs/31 samples vs. CTRL: $n = 39$ dogs/39 samples). **(B)** Serotonin levels differ significantly in intact males (m) ($p \leq 0.01$) (IE: $n = 11$ dogs/18 samples vs. CTRL $n = 21$ dogs/21 samples), neutered males (nm) ($p \leq 0.001$) (IE: $n = 24$ dogs/37 samples vs. CTRL $n = 33$ dogs/33 samples) and neutered females ($p \leq 0.001$) (IE: $n = 22$ dogs/31 samples vs. CTRL $n = 39$ dogs/39 samples). **(C)** The norepinephrine/epinephrine ratio differs significantly in neutered females ($p \leq 0.05$) (IE: $n = 22$ dogs/31 samples vs. CTRL $n = 39$ dogs/39 samples). **(D)** The ɤ-aminobutyric acid (GABA)/glutamate ratio differs significantly in intact males ($p \leq 0.05$) (IE: $n = 11$ dogs/18 samples vs. CTRL $n = 21$ dogs/21 samples) and neutered females ($p \leq 0.05$) (IE: $n = 22$ dogs/31 samples vs. CTRL $n = 39$ dogs/39 samples). In intact females (f) no significant difference (NS.) of all analysed neurotransmitter between the groups was found (IE: $n = 5$ dogs/9 samples vs. CTRL $n = 21$ dogs/21 samples). Data are presented as box-and-whisker plots (the median is represented by the central line, the 25th and the 75th percentile represent the lower and upper limit, and the length of the whiskers represent the 1.5 multiple of the interquartile range). Unpaired two-sided two-sample *t*-test and Wilcoxon–Mann–Whitney test were used to compare the urinary neurotransmitter excretion between dogs with epilepsy and the healthy control group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

urinary neurotransmitter excretion was observed in dogs with epilepsy (**Figure 3**). Treatment significantly increased serotonin concentration in dogs with epilepsy compared to untreated dogs ($p \leq 0.05$, **Figure 3A**), to an even higher level as in healthy controls (p Holm-corrected = 1.02e-12, **Figure 3B**). GABA concentration was significantly decreased in untreated dogs with epilepsy compared to those treated dogs with IE ($p \leq 0.05$, **Figure 3C**). The ASD treatment increased GABA concentration and increased it to a similar level to healthy control dogs (p Holm-corrected = 0.607, **Figure 3D**). For the remaining urinary neurotransmitters (histamine, PEA, DA, E, NE, glutamate) no statistically significant differences between the two cohorts or an ASD treatment effect were identified (**Supplementary Table 1**).

DISCUSSION

The objective of this study was to evaluate the suitability of urinary neurotransmitter analysis as a non-invasive diagnostic tool, where characteristic neurotransmitter deviations serve as valuable biomarkers for canine IE. It was hypothesised that urinary neurotransmitter profiles differ between dogs with epilepsy and healthy controls (H1). Sex and neutering status substantially affected urinary neurotransmitter excretion. In the present study, urinary neurotransmitter patterns were significantly altered in dogs with IE, when sex and neutering status were accounted for, which confirmed the first hypothesis of our study. Urinary glycine and serotonin concentration were significantly increased in dogs with IE, whereas the GABA/glutamate ratio and the NE/E ratio was significantly decreased. Additionally, it was hypothesised that the urinary neurotransmitter excretion of dogs with epilepsy was affected by administered ASD, with hypothesised differences between ASD-treated dogs compared to untreated dogs with epilepsy (H2). Results demonstrated that ASD treatment increased GABA concentration in dogs with epilepsy to the level seen in the healthy control population.

Glycine serves primarily as an inhibitory neurotransmitter in the CNS (75). It generally improves mood, mental performance and memory skills (76, 77). However, elevated levels can compromise cognitive processing and provoke seizures (78, 79). In humans, a rare inherited error of glycine metabolism, called non-ketotic hyperglycinemia, causes an excessive accumulation of this neurotransmitter in the body, particularly in the nervous system (80). Clinical signs of this disease include refractory seizures, hyperactivity and in adults cognitive impairment (79, 80). Affected patients also excrete high levels of glycine in their urine (80). Non-ketotic hyperglycinemia and epilepsy are two different diseases, however, parallels in clinical signs exist. The

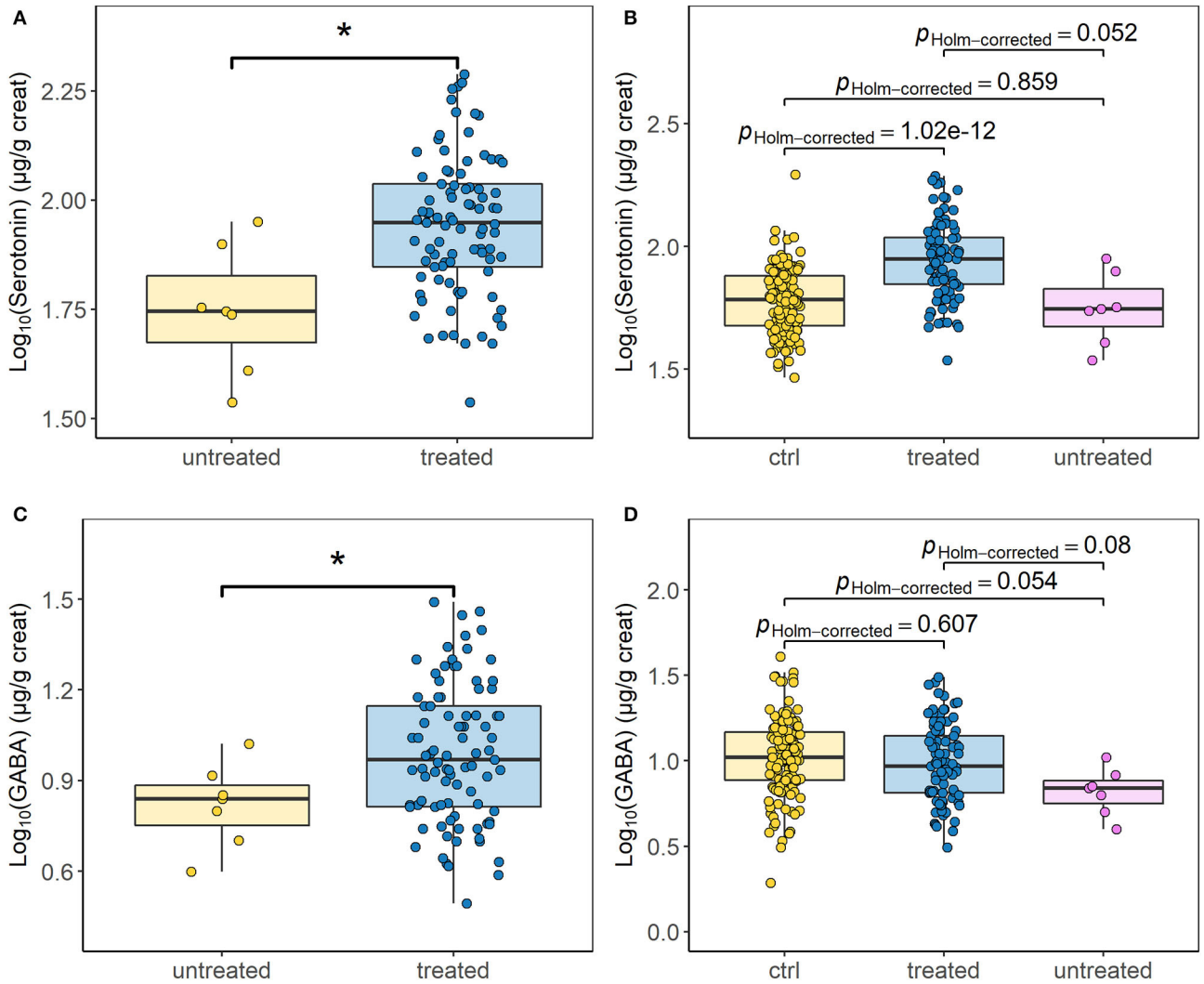

FIGURE 3 | Influence of the antiseizure drug treatment on the urinary neurotransmitter profile. **(A)** Urinary serotonin concentration compared between treated ($n = 56$ dogs/89 samples) and untreated dogs ($n = 7$ dogs/7 samples) affected by idiopathic epilepsy ($p \leq 0.05$). **(B)** Multiple group comparison of the urinary serotonin excretion between treated ($n = 56$ dogs/89 samples) and untreated dogs ($n = 7$ dogs/7 samples) with idiopathic epilepsy and the healthy control group (ctrl) ($n = 127$ dogs/127 samples). **(C)** Urinary ɤ-aminobutyric acid (GABA) concentration compared between treated ($n = 56$ dogs/89 samples) and untreated dogs ($n = 7$ dogs/7 samples) with idiopathic epilepsy ($p \leq 0.05$). **(D)** Multiple comparisons of the urinary ɤ-aminobutyric acid excretion between treated ($n = 56$ dogs/89 samples) and untreated dogs ($n = 7$ dogs/7 samples) with idiopathic epilepsy and the healthy control group ($n = 127$ dogs/127 samples). Data are presented as box-and-whisker plots (the median is represented by the central line, the 25th and the 75th percentile represent the lower and upper limit, and the length of the whiskers represent the 1.5 multiple of the interquartile range). Unpaired two-sided two-sample *t*-test and Wilcoxon–Mann–Whitney test were used for group comparison. For multiple group comparisons a one-way analysis of variance (ANOVA) and a Games-Howell *post-hoc* test with the Holm correction was utilised. *$p \leq 0.05$.

most prominent clinical sign of human and canine epilepsy are recurrent seizures. Cognitive impairments and hyperactivity are often associated as well (81–87). The elevated urinary glycine levels in dogs with IE found in the current study are another similarity. The results indicating, increased glycine concentration might be a contributing factor inducing seizures and associated cognitive impairment, as well as hyperactivity in affected patients. However, elevated urinary glycine in dogs with epilepsy found in this study, should be differentiated from the massively increased concentrations in human patients with non-ketotic hyperglycinemia. Further studies are needed to evaluate whether urinary glycine can serve as a potential biomarker in canine epilepsy, too. It must be considered, that for laboratory diagnosis of non-ketotic hyperglycinemia CSF and serum glycine concentrations are determined. In a previous study canine glycine levels correlated between CSF, serum and urine, suggesting non-invasive urinary neurotransmitter analysis as a good option for glycine screening in dogs (53). A treatment effect of the ASDs (phenobarbital and potassium bromide), which might have caused the detected glycine increase, was not revealed in this study. For valproate, an anticonvulsive drug administered in human medicine, an elevating effect on urine and plasma glycine levels exists (88). To the authors' knowledge, such an effect is not known from first and second-line drugs

(phenobarbital and potassium bromide) authorised for canine epilepsy treatment.

Urinary serotonin levels were increased in dogs with IE, compared to dogs without epilepsy and were substantially affected by their sex and neutering status. These findings match those of a recent study in which urinary serotonin excretion was altered after ovariohysterectomy in bitches (89). Serotonin plays a role in regulating sleep, appetite and mood (36). Grouping the data into treated and untreated epileptic dogs revealed that untreated dogs with epilepsy excreted significantly lower urinary serotonin levels than ASD-treated dogs with epilepsy or healthy control dogs. Decreased serotonin concentrations are related to the pathogenesis of various psychiatric and neurological disorders (41). Alterations in the serotonergic system can lower the seizure threshold and are associated with frequently co-occurring neurobehavioural comorbidities (35, 37). Psychological conditions/behavioural abnormalities are commonly treated with selective serotonin reuptake inhibitors (SSRIs) in humans and dogs (90, 91). However, drug manuals suggest that SSRIs are contraindicated in dogs with epilepsy or a history of seizures (92). In contrast, the International League Against Epilepsy and experimental data suggest SSRIs to be of low risk to patients with a history of seizures or epilepsy, indicating that they can be cautiously used for the treatment of anxiety in some epilepsy patients (93). Some experimental data even exists that SSRIs might be anticonvulsive. For example, the SSRI fluoxetine is effective in dogs with fly catching syndrome, a condition which has been considered by some as limbic epilepsy, but others as a compulsive behavioural disorder (94). In the current study, ASD treatment significantly increased the serotonin concentration in dogs with epilepsy compared to untreated dogs, to an even higher level than in healthy controls dogs. Elevated serotonin levels can be an amplifying and beneficial effect of ASD treatment, due to the protective properties of serotonin against seizures. Increased serotonin concentration may also improve associated neurobehavioural disorders in affected dogs, without prescribing contraindicated SSRIs, however, further studies are required to explore this potential positive effect.

The current study also revealed a diminished GABA/glutamate ratio in dogs with IE, which reflects low GABA levels or high glutamate levels in the examined urine samples, respectively. Both neurotransmitters are amino acids with contradictory effects on the body. GABA acts as the primary inhibitory neurotransmitter, while glutamate is the major excitatory counterpart in the CNS (33, 34). These findings in the urine potentially mirror neurotransmitter alterations in the epileptic brain. Furthermore, low urinary GABA concentrations in drug naïve dogs, compared to treated dogs with IE and healthy controls were shown. Dogs with IE who received ASD treatment excreted a higher urinary GABA concentration, which was almost at the same level as healthy controls. These findings reflect the expected lower GABA concentration in untreated epilepsy and corroborate a treatment effect, which may have corrected the GABA values up to the healthy controls state. However, the ASD administered in this study (phenobarbital and potassium bromide) are not known to directly influence GABA concentrations. Their anticonvulsant effect is mediated by other action mechanisms, including GABA receptor interactions (95, 96). Ultimately, however, acute and chronic phenobarbital treatment reduce brain GABA levels (97). Why urinary GABA levels behave differently requires further research.

The NE/E ratio was found to be decreased in dogs affected by IE, representing low NE levels or high E levels in the examined urine samples, respectively. These monoamine neurotransmitters are catecholamines and act receptor-binding-dependent either as excitatory or inhibitory stimulants in the CNS (98, 99). NE is known for its anticonvulsant properties in epilepsy, even though it can also be proconvulsive under certain circumstances (13, 100–102). Reduced NE levels of dogs with IE compared to healthy controls in the presented study corroborate the generally anticonvulsive effect of this neurotransmitter. The lack of NE might contribute to epileptogenesis and induction of seizures in the examined dogs. Furthermore, NE affects cognition, attention and memory ability (103). The noradrenergic system changes cause various neuropsychiatric and -degenerative disorders, such as Alzheimer's disease and ADHD in humans (103–105). As aforementioned, canine epilepsy can be associated with cognitive impairments (72, 82–84). The low NE concentrations detected in this study might contribute to the development of those impairments. These findings consistent with those of decreased NE levels assessed in the brain of human patients with Alzheimer's disease, which were correlated with the degree of cognitive impairment (106). Another comorbidity of human epilepsy is ADHD, with behavioural similarities also documented in canine epilepsy patients (86, 87). Previous research has indicated that ADHD can be associated with imbalances in the noradrenergic and dopaminergic systems (105, 107). Several studies reported a correlation between ADHD in children and altered urinary catecholamine excretion (56, 63, 108). Pliszka et al. detected elevated urinary excretion of NE metabolites in children with ADHD compared to healthy controls and increased urinary E excretion when ADHD was accompanied by anxiety (62). Anxiety disorders are also common in human and canine epilepsy (39, 109). A relationship between increased anxiety and exaggerated stress response of the neuroendocrine system haven been previously described (60, 110). E regulates many important body functions and is substantially involved in stress response (99). Elevated E concentrations in dogs with IE may have caused the identified deviation in the urinary NE/E ratio and may also be responsible for the co-occurring anxiety in canine epilepsy. Finally, sleep disturbances are often associated with epilepsy in humans (111). They are assumed to occur in dogs as well, although evaluation remains difficult (112). In former studies, poor sleep quality and disordered sleep were linked to a profuse activation of the sympathetic nervous system, resulting in increased nocturnal serum catecholamine levels (113, 114). As a comorbidity of epilepsy, altered sleeping patterns might also have existed in the canine participants of this study. This may have caused elevated nocturnal E levels, which were excreted and detected in the analysed morning urine. Overall, the evidence presented indicates that alterations in the NE/E ratio of dogs with IE in the current study may

be associated with the development of seizures and common comorbidities, such as sleep disturbances, ADHD- and anxiety-like behaviour. The results of this study suggest a potential role of the nor-/adrenergic pathway alterations in canine epilepsy and neurobehavioural comorbidities.

A few limitations of the present study should be noted. First, the multicentred sample acquisition enabled a large sample size of urine from participants with IE and healthy controls, however, variability in sample collection and storage may have impacted results. The number of untreated dogs with IE concerning the total study population of dogs with epilepsy was small, therefore, caution must be applied, as findings referring to this population might not be representative. Another limitation of this study is that a direct correlation between CNS and urinary neurotransmitters levels has only been shown to a limited extent in previous research. Finally, despite us finding differences, these might not be clinically discriminatory and only be considered as a monitoring tool. Future studies are needed to identify for which patient these changes are clinically relevant.

Numerous factors can influence the eliminated urinary neurotransmitter concentrations. Neurotransmitter passage from the CNS to the periphery is regulated by the BBB, being formed by specific endothelial cells, through which the transport differs for each substrate (48). For glycine a non-carrier-mediated process for BBB crossing is assumed in rats, whereas for dogs no significant transfer through the BBB could be shown so far (115, 116). Serotonin is shuttled via a serotonin transporter, which enables a bidirectional permeation through the luminal membrane of the endothelial cells, but only unidirectional transport to the brain on the abluminal side (117, 118). GABA can cross the abluminal endothelial membrane through a transport system and a luminal membrane passage is presumed as well, even though the transporter has not yet been identified (45, 119). Glutamate can also pass the BBB across the abluminal side via several transporters into the endothelial cells, from where a bidirectional luminal transport is possible (44, 120). NE is shuttled via an abluminal transporter out of the brain into the endothelial cells (117, 121). E is proven to be assimilated into endothelial cells, however the exact process remains elusive (122). BBB function might be altered during seizure and the neurotransmitter could pass more readily. The presented evidence emphasises that the neurotransmitter transfer through the BBB is still not completely revealed. In the interpretation of the current study results, substrate-specific permeability and transport directions of the respective neurotransmitters through the BBB endothelial cells, should be considered.

After crossing the BBB, the neurotransmitters circulate in the bloodstream, from which they are subsequently eliminated by the kidneys into the urine (48). Renal excretion of monoamine neurotransmitters is affected by two mechanisms: glomerular ultrafiltration from the arterial blood and active reabsorption and secretion through specific transporters (48, 52, 123, 124). All participating dogs had normal renal function and were not affected by renal diseases. Nevertheless, the above-mentioned processes can modulate the detected amount of urinary excreted neurotransmitters in healthy dogs as well and this might have affected the acquired results of this study.

Another impact on urinary neurotransmitter levels could arise from additional synthesis outside the CNS. Neurotransmitters are also produced in the peripheral nervous system (PNS), as in serotonin secreting enterochromaffin cells of the enteric nervous system or in norepinephrine producing renal nerves (125–127). Even bacteria, hosted in the body as microbiota, are capable of synthesising neuroactive molecules by themselves (e.g., GABA), or regulating their host's neurotransmitter metabolism (e.g., serotonin), resulting in a modified overall neurotransmitter pool (128–131). Moreover, neurotransmitters are additively produced in many other body organs beside the nervous system, such as the pancreas (e.g., GABA), the adrenal glands (e.g., NE, E, DA) and the kidneys (e.g., GABA, E, NE) (132–137). Ingesting nutritional sources of neurotransmitters or their precursors, can have an ancillary influence on the neurotransmitter pool of the body (128, 138, 139). To minimise this external impact, dairy products, fruits and vegetables were not fed before sample acquisition. However, the dog's personal standard diet, containing meat and seafood as neurotransmitter sources, might also have influenced their urinary neurotransmitter concentration on an individual level (128). It is assumed, that animals and processed foods contain more stable levels of neurotransmitters, than the avoided plants, which might have reduced the individual variety (128). Moreover, endogenous mechanisms of the body, as inactivating enzymes, intestinal metabolism and certain barriers, limiting the effect of nutritional neurotransmitter and as well their urinary excretion (128). Anyhow, nutrition is an important factor for the neurotransmitter metabolism of the body. In further studies it needs to be addresses to which extent dietary factors influence the canine urinary neurotransmitter excretion.

Although the mentioned factors influence on the urinary neurotransmitter concentration, former studies revealed an association between central and peripheral neurotransmitter excretion into the urine. In early studies labelled NE was injected into the cisterna magna of dogs, followed by detection in their blood and fast metabolite excretion via the urine (140). Following these findings, a more recent study showed positively correlating neurotransmitter concentrations of serine, glycine and NE mirrored in three canine body fluids: CSF, blood and urine (53). Orally administered serotonin substrates in rats enhanced the serotonergic activity in the CNS and urinary serotonin levels, indicating a shared regulation mechanism (49). In another study injecting a neurotoxic compound into rat brains induced diminished DA levels in their brain and urine (50). Furthermore, a relationship between urinary excreted neurotransmitters and psychological disorders in humans has been identified. Elevated concentrations of urinary catecholamines, such as NE and E were associated with depression and anxiety (58–60). Urinary excreted DA correlated with suicide attempts in depressed patients even stronger than the CSF levels (61). In addition to the presented evidence, a crosstalk between the CNS and PNS was demonstrated in different studies, further strengthening the central and peripheral neurotransmitter association (141–143).

In human medicine rare inherited disorders exist, which are causing seizures and are also associated with neurotransmitter alterations in the body (144). Disorders of the pyridoxine metabolism evoke ASD resistant seizures in neonates, responding

to administered pyridoxine (pyridoxine dependency) or in rare cases solely to its active form, pyridoxal phosphate (pyridoxal phosphate dependency) (144–146). Pyridoxal phosphate is involved in neurotransmitters metabolism of glutamate, GABA and glycine, but the contribution to the epilepsy remains controversial (146–148). Cerebral folate deficiency manifests with late infantile onset seizures and is treatable with folate acid supplementation (149). It is caused by folate transport or metabolism disorders, resulting in low CNS folate concentrations, which can be accompanied with a peripheral folate deficiency (149, 150). Folate is required in the neurotransmitter metabolism of glycine and its influence on serotonin and catecholamine homeostasis is discussed (144, 151, 152). Further inherited neurotransmitter disorders associated with seizures are succinic semialdehyde dehydrogenase (SSADH) deficiency (GABA metabolism disorder), aromatic L-amino acid decarboxylase (AADC) deficiency (dopamine/serotonin synthesising enzyme disorder, seizures are described but uncommon) and the aforementioned non-ketotic hyperglycinemia (glycine metabolism disorder) (80, 153–156). These disorders are not known in dogs so far. However, in canine IE the underlying cause of seizures remains unknown, which might generate a heterogenous group with different yet undiscovered diseases (1). The dogs of the current study met all requirements of the IVETF for the diagnosis of IE (Tier I/Tier II), but the mentioned human disorders require specific diagnostic screening and are not included in these clinical work-up guidelines (68). It is possible that similar inherited metabolic disorders exist in canine patients undetected, evoking seizures and altered neurotransmitter concentration. Nevertheless, in the mentioned human diseases additional abnormalities in the neurological examination/brain imaging are present, which are exclusion criteria of canine IE (Tier I/Tier II) and are therefore considered unlikely (144, 146, 150, 155). The exact underlying pathophysiology of seizures, and the associated urinary neurotransmitter alterations revealed in this study, remains to be elucidated further in the future. The current study can only be seen as a starting point.

Imbalances in the neurotransmitter system that cause epileptic seizures also lead to altered neurotransmitter elimination in the urine of affected dogs and, therefore, can serve as valuable biomarkers in epilepsy. Urinary neurotransmitter analysis with its non-invasive collection technique offers a major advantage over determining neurotransmitters from other body fluids (e.g., CSF, serum). Recent evidence revealed an association between urinary neurotransmitter patterns and treatment efficacy in drug-resistant dogs with IE, suggesting a benefit of utilising this diagnostic tool, particularly in epilepsy patients (67). In the future, neurotransmitter analysis could allow for a better understanding of the underlying pathomechanisms of epilepsy. These biomarkers may indicate specific subtypes of epilepsy in this heterogeneous disease, associated with pharmacoresistance. Applied in a clinical setting, the non-invasive urinary neurotransmitter analysis could be used for individual treatment monitoring and customised adjustments of therapeutic interventions in canine or even human epilepsy.

CONFERENCES

Preliminary results of the current study were presented at following conferences: 33rd ESVN -ECVN Symposium 2021; 30. Jahrestagung der Fachgruppe "Innere Medizin und klinische Labordiagnostik" der DVG (InnLab) 2022 (awarded with the 1st poster prize).

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed within the current study are available as **Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from all owners. The study was conducted following the guidelines of the University of Veterinary Medicine Hannover and approved by the thesis committee of the University. In addition, data and urine samples from multiple epilepsy studies were used. These studies were approved by the local Ethics and Welfare Group (EWG) (URN 2011 1132, URN 2016 1558 and URN 2017 1743-2).

AUTHOR CONTRIBUTIONS

TS participated in the planning of the study, carried out the main practical work, the recruitment and the sample acquisition of the control group samples in Hannover, interpreted the results, and drafted the manuscript. BB, TL, SH, RP, and HV provided clinical and laboratory data of the dogs with idiopathic epilepsy of former epilepsy trials and studies. HV designed and coordinated the study. SM supported sample acquisition. SM and HV made essential contributions to the conception and acquisition of data. ST performed the statistical analysis and wrote sections of the manuscript. ST, BB, SH, NM, RP, and HV critically reviewed and edited the manuscript for important intellectual content. All authors contributed to the manuscript revision, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.893013/full#supplementary-material

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Conflict of Interest: BB was employed by company BrainCheck.Pet®.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pharmacokinetics of Cannabidiol Following Intranasal, Intrarectal, and Oral Administration in Healthy Dogs

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The therapeutic potential of cannabidiol (CBD), a non-psychtropic component of the *Cannabis sativa* plant, is substantiated more and more. We aimed to determine the pharmacokinetic behavior of CBD after a single dose *via* intranasal (IN) and intrarectal (IR) administration in six healthy Beagle dogs age 3–8 years old, and compare to the oral administration route (PO). Standardized dosages applied for IN, IR and PO were 20, 100, and 100 mg, respectively. Each dog underwent the same protocol but received CBD through a different administration route. CBD plasma concentrations were determined by ultra-high performance liquid chromatography-tandem mass spectrometry before and at fixed time points after administration. Non-compartmental analysis was performed on the plasma concentration-time profiles. Plasma CBD concentrations after IR administration were below the limit of quantification. The mean area under the curve (AUC) after IN and PO CBD administration was 61 and 1,376 ng/mL*h, respectively. The maximal plasma CBD concentration (C_{max}) after IN and PO CBD administration was 28 and 217 ng/mL reached after 0.5 and 3.5 h (T_{max}), respectively. Significant differences between IN and PO administration were found in the T_{max} ($p = 0.04$). Higher AUC and C_{max} were achieved with 100 mg PO compared to 20 mg IN, but no significant differences were found when AUC ($p = 0.09$) and C_{max} ($p = 0.44$) were normalized to 1 mg dosages. IN administration of CBD resulted in faster absorption when compared to PO administration. However, PO remains the most favorable route for CBD delivery due to its more feasible administration. The IR administration route is not advised for clinical application.

Keywords: Canine, cannabinoid, *Cannabis sativa*, phytocannabinoid, tetrahydrocannabinol

INTRODUCTION

Several lines of evidence have supported a therapeutic potential of cannabis derivatives, in particular phytocannabinoids, in human and veterinary medicine (1–7). Cannabidiol (CBD) and Δ9-tetrahydrocannabinol are the most abundant phytocannabinoids extracted from the *Cannabis sativa* plant (8–10), with CBD being the most promising since this molecule is devoid of the psychoactive side effects exhibited by Δ9-tetrahydrocannabinol (9, 11, 12).

Phytocannabinoids have a complex and variable pharmacokinetic and pharmacodynamic profile. They show a prominent hepatic first-pass effect and therefore have a low oral bioavailability (13). Their target, the endocannabinoid system, is composed primarily of CB1 receptors, expressed mainly by central and peripheral neurons, and CB2 receptors, expressed mainly by immune cells (14–16), suggesting a therapeutic value of CBD for numerous medical conditions in humans because of its potential neural (2, 17) and immunomodulatory properties (18). Therapeutic applications of CBD in humans include epilepsy (1, 19–21), Alzheimer's disease (22) and multiple sclerosis (23, 24). In the veterinary medicine, therapeutic applications of CBD in dogs include osteoarthritis-associated pain (6, 25, 26), aggressive behavior (27), and epilepsy (7, 28). CBD is generally administered orally, but its low bioavailability, which is estimated to be <10% in humans (19, 29), continues to be a main issue in clinical trials (30). In healthy dogs, it has been shown that administration of oral CBD is associated with a low bioavailability as well, ranging from 13 to 19%, most likely due to its first-pass phenomenon in the liver (31). The aforementioned limitations indicate the necessity to explore alternative delivery routes.

The purpose of this study was to determine the pharmacokinetic behavior of CBD after a single dose *via* intranasal (IN) and intrarectal (IR) administration in healthy Beagle dogs and compare this to the more widely used oral administration route (PO). The plasma CBD concentrations were evaluated over a period of 60 h post-administration. We hypothesized that CBD delivered *via* IN administration would avoid first-pass liver effect and CBD delivered *via* IR administration would partially avoid liver metabolization and therefore higher plasma concentrations and subsequent exposure would be achieved compared to the PO administration route.

MATERIALS AND METHODS

Animals

Six neutered adult laboratory Beagle dogs (four females, two males), 3–8 years of age, weighing an average of 12 kg (range, 7.3–14.4 kg) were included in a randomized crossover study. A sample size of 6 was found to be the minimum sample size based on a power analysis with the following settings: the smallest relevant difference of 5 [with $\sigma = 4$, values based on (32)], based on a one-sample *t*-test exact solution with a non-central t-distribution, taking none-detection in one dog into account (31), with a power of ≥0.8 and at the α-level of 0.05. The study protocol was reviewed and approved by the Ethical Committee of the Faculties of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2018-42) and all manipulations were performed according to good animal practice. Care was taken to avoid stress and anxiety. No animals were sacrificed. The dogs were provided by the Small Animal Department of the Faculty of Veterinary Medicine and were purchased from Marshall BioResources (North Rose, New York, United States of America). The dogs were socially-housed in small groups (2 to 8 dogs), according to the European and Belgian legislation and received environmental enrichment (Directive 2010/63/EU, KB 29/05/2013). The bedding material in the inner part of the housing facility consisted of wood shavings. The dogs had permanent access to an outside area of 15 m² and twice a day they were allowed to run and play outside in an enclosed play area, enriched with climbing platforms, hiding places and small bushes. In addition, the dogs were regularly walked by students of the Faculty of Veterinary Medicine. Food was withheld for at least 12 h before the start of the experiments, but water was provided *ad libitum*.

Study Design

The dogs were randomly allocated to a 3-way crossover design by the principal investigator (DP), using an online randomization program (www.randomizer.org). Following a two-week wash-out period, each dog underwent the same protocol but received CBD through a different administration (IN, IR, PO) route. The first blood sample (T0) was taken 10 min before the CBD administration.

CBD Administration and Sample Collection

For the IN administration, a polyethylene glycol (PEG):sodium chloride (NaCl) 0.9% (50:50) solution containing 20 mg of synthetic CBD (2-[(1R,6R)-3-methyl-6-prop-1-en-2-yl-1-cyclohex-2-enyl]-5-pentylbenzene-1,3-diol) per dog was given *via* a mucosal atomization device (MAD NasalTM, Wolfe Tory Medical, South Salt Lake City, Utah, United States). The total volume was fixed at 1 mL and was divided equally over the two nostrils. During the intranasal delivery, dogs were held in sternal recumbency with the head and neck gently dorsoflexed and were kept in this position for ∼0.5 min after intranasal administration.

For the IR administration, dogs were first taken outside for a walk to avoid defecation during and after administration. Thereafter, a suppository containing 100 mg of CBD (Cannef® synthesized CBD suppositories 100 mg, CB21 Pharma s.r.o., Prague, Czech Republic) was gently administered in each dog manually in the rectum.

For the oral administration, a tablet containing 100 mg of CBD (Cannamed® synthesized CBD tablets 100 mg, xMed 21 s.r.o., Prague, Czech Republic) was administered together with a small amount (≅10 g) of highly digestible commercial canned food (Hill's® Prescription Diet® i/d® Canine, Hill's Pet Nutrition Inc., Topeka, USA).

Abbreviations: AUC, area under the curve; CBD, cannabidiol; C_{max}, maximal plasma concentration; IN, intranasal; IR, intrarectal; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LOQ, limit of quantification; NaCl, natrium chloride; PEG, polyethylene glycol; PO, oral; T_{max}, time to maximal plasma concentration.

The tablets for oral administration and suppositories for rectal use (100 mg), according to the manufacturers' specification, were commercially available (www.CBDepot.eu), and the IN dose was a self-developed formulation using analytical standard dissoluted in PEG.

Blood samples (2 mL) were collected from the *vena jugularis* 10 min before CBD administration (T0) and at 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, and 60 h after all routes of CBD administration. Blood samples were immediately transferred into tubes containing ethylenediamine tetraacetic acid and the plasma was immediately separated by centrifugation at 3,500 rpm for 5 min at 2°C. The plasma was then stored at −80°C until analysis.

Adverse reactions during and after CBD administration were recorded. Attention was given to sneezing and reverse sneezing, coughing, head shaking, snorting and licking during IN administration, nausea, vomiting and salivation during PO administration and defecation after IR administration.

Quantification of CBD in Plasma

Chemicals and Reagents

Ultrapure H_2O was obtained *via* a Milli-Q water purification system (Merck Millipore©, Overijse, Belgium). Standards of CBD (10 mg/mL in EtOH) and internal standard CBD-d3 (100 μg/mL) were purchased from Sigma-Aldrich (Overijse, Belgium). All solvents were of analytical grade. Acetonitrile was purchased from Thermo Fisher Scientific Inc.© (Erembodegem, Belgium). Formic acid was obtained from VWR™ (Leuven, Belgium).

Sample Preparation

One hundred microliter of the plasma sample was spiked with 25 μL of internal standard (CBD-d3) solution (400 ng/mL) and vortex mixed (±15 s). Next, 275 μL of acetonitrile was added to the samples and again vortex mixed (±15 s). After mixing, the samples were centrifuged (13,000 rpm, 10 min). Thereafter, the liquid layer was transferred to an autosampler vial. Finally, an aliquot (5 μL for concentrations below 250 ng/mL, 1 μL for concentrations between 250 and 1,500 ng/mL) was injected into the ultra-high-performance liquid chromatography with tandem mass spectrometry (LC—MS/MS) system.

LC-MS/MS

Chromatographic separation was performed using an Acquity UPLC HSS-T3 column (100 × 2.1 mm, dp: 1.8 μm) in combination with a guard column of the same type (Waters NV/SA, Asse, Belgium). The gradient elution programme consisted of two mobile phases (A and B). Mobile phase A and B were 0.1% formic acid in ultrapure H_2O and 0.1% formic acid in methanol, respectively. The following program was applied: 0 min (60% A, 40% B), 0–2 min (linear gradient to 100% B), 2–4 min (100% B), 4–4.1 min (linear gradient to 60% A, 40% B) and 4.1–8 min (60% A, 40% B). Flow rate was set at 0.4 mL/min. The LC eluent was interfaced to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters NV/SA, Asse, Belgium) with ion source heated electrospray ionization operating in positive ionization mode. Acquisition was performed in selected reaction monitoring mode. For CBD and internal standard, the following two most intense product ions were followed: CBD: mass-to-charge ratio 315.08 > 193.00/122.96 and CBD-d3: mass-to-charge ratio 318.12 > 196.03/122.96. The LC-MS/MS analytical methods were validated using matrix-matched calibrator and quality control samples, based on blank plasma of untreated dogs. The limit of quantification (LOQ) was 1 ng/mL. The LC-MS/MS analyses were conducted in accordance with the international guidelines (33–35).

Pharmacokinetic Analysis

Non-compartmental analysis was performed on the plasma concentration-time profiles using Phoenix 8.4 (Certara LP, NJ, USA). All dosing groups were included in the analysis, except for the IR administration data because of the low plasma concentrations (around LOQ). The following pharmacokinetic parameters were calculated: area under the plasma concentration-time curve, from 0 to infinity (AUC_{0-inf}); maximal plasma concentration (C_{max}) and time to maximal plasma concentration (T_{max}); terminal elimination half-life; elimination rate constant and mean residence time. Total body clearance and volume of distribution after IN and PO administration were not corrected for their respective bioavailabilities. The relative bioavailability of the IN administration when compared with the commercial oral product was calculated according to the following formula:

$$relative\ F = 100 \times \frac{mean\ AUC\ IN_{(0-inf)} \times Dose\ PO}{mean\ AUC\ PO_{(0-inf)} \times Dose\ IN}$$

Due to the dose discrepancy between the PO and IR administration (100 mg) and the IN administration (20 mg) and to facilitate comparison of the systemic exposure between the administration routes, the AUC and C_{max} of PO/IR and IN were normalized for dose by dividing by 100 and 20, respectively.

Statistical Analysis

The statistical analysis was conducted in R version 4.0.2 ("Taking off again"). Significance was set at $\alpha \leq 0.05$. A Wilcoxon signed rank test was used to compare the IN and PO administration routes.

RESULTS

Mean ± SD plasma CBD concentrations after PO, IN and IR administration were determined at 17 time points over 60 h post administration and are displayed in **Figure 1**. The mean AUC_{0-inf} after IN and PO CBD administration before dose normalization was 61.31 and 1376.03 ng/mL*h and after normalization to 1 mg dosages was 3.06 and 13.76 ng/mL*h, respectively. The maximal plasma CBD concentration (C_{max}) after IN and PO CBD administration before dose normalization was 27.96 and 216.76 ng/ml and 1.39 and 2.16 ng/mL after dose normalization, reached at 0.5 and 3.5 h (T_{max}), respectively. Significant differences between IN and PO administration routes were found in the T_{max} ($p = 0.04$) but no significant differences were found in the AUC normalized for dose ($p = 0.09$) and C_{max}

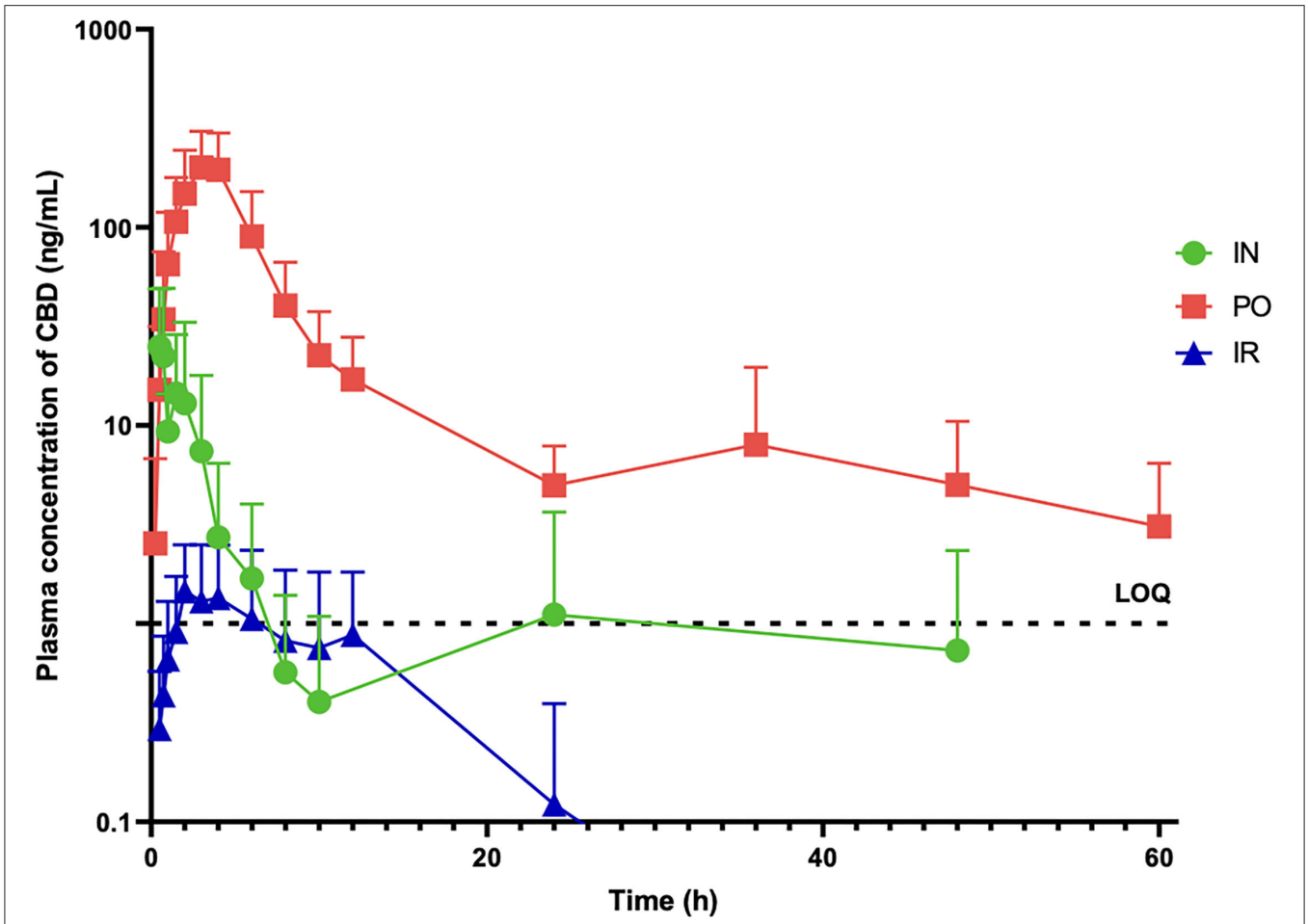

FIGURE 1 | Mean ± SD plasma concentrations of CBD (ng/mL) in six dose administered a single dose of CBD intranasally (20 mg) (IN), orally (100 mg) (PO) and intrarectally (100 mg) (IR).

normalized for dose ($p = 0.44$). The different pharmacokinetic parameters after PO and IN administration are shown in **Table 1**.

Due to the very low (below LOQ) plasma CBD concentrations (**Figure 1**) obtained after IR administration, no pharmacokinetic analysis was conducted for this administration route.

Two dogs showed sneezing and 1 dog showed head shaking after IN administration. These symptoms were seen immediately after IN administration and disappeared over a period of 30 s. All 6 dogs showed good compliance with PO administration, where the tablets were spontaneously ingested together with the small amount of highly digestible food. The IR administration of the CBD suppository went uneventful. No signs of nausea, vomiting or salivation were seen during and after PO administration and no defecation was observed during and after the IR administration.

DISCUSSION

To the best of our knowledge, this is the first study examining the pharmacokinetic profile of CBD in healthy dogs after IN and IR administration. The pharmacokinetic parameters were compared with the more widely used oral route of administration. The plasma CBD concentrations after IR administration were below the LOQ and were therefore not used in our analysis. We hypothesized that CBD delivered *via* IN administration would bypass liver metabolization largely and *via* IR administration partially and therefore higher plasma concentrations would be achieved, in comparison with the PO administration route. However, this hypothesis was not confirmed, since the oral administration route still showed a numerically higher mean exposure and maximal concentration normalized for dose when compared to the IN and IR administration routes albeit not statistically significant.

IN drug delivery is non-invasive, pain-free and easy. The mucosal atomization device converts the liquid drug into a fine mist and is used to deliver it into the nasal cavity consequently reaching the nasal mucosa. The nasal mucosa provides a large, particularly vascular absorptive surface adjacent to the brain and offers a direct pathway for drug absorption into the bloodstream, avoiding the first-pass hepatic phenomenon (36–39). As the IN route, the oromucosal route is also an easy and pain-free drug delivery method able to circumvents some of the

TABLE 1 | Pharmacokinetic parameters (mean ± SD) of CBD in 6 dogs administered a single dose of CBD intranasally (20 mg) (IN) and orally (100 mg) (PO).

Pharmacokinetic parameter	IN	PO
$AUC_{(0-\infty)}$ (ng/mL*h)*	61.31 ± 88.22	1376.03 ± 828.95
C_{max} (ng/mL)*	27.96 ± 25.29	216.76 ± 108.51
$AUC_{(0-\infty)}$ (ng/mL*h)**	3.06 ± 4.41	13.76 ± 8.28
C_{max} (ng/mL)**	1.39 ± 1.26	2.16 ± 1.08
T_{max} (h)†	0.49 ± 0.29	3.50 ± 0.55
$T_{1/2el}$ (h)	7.02 ± 7.97	15.65 ± 2.82
k_{el} (1/h)	0.52 ± 0.54	0.045 ± 0.007
MRT (h)	10.30 ± 14.04	13.07 ± 3.61
Relative F (%)	22.28%	/

$AUC_{(0-\infty)}$, area under the plasma concentration-time curve from 0 to infinity post-administration; C_{max}, maximal plasma concentration; T_{max}, time to maximal plasma concentration; $T_{1/2el}$, terminal elimination half-life; k_{el}, elimination rate constant; MRT, mean residence time; F, absolute bioavailability.

**Doses for $AUC_{(0-\infty)}$ and C_{max} before dose normalization.*

***Doses for $AUC_{(0-\infty)}$ and C_{max} after dose normalization to 1 mg CBD.*

† Significant differences between administration routes.

problems associated with the PO route, such as avoidance of first-pass hepatic metabolism (40). The oromucosal route can also provide a rapid onset of action (41), as long as the exposure times to the oral mucosal lining are adequate and a method of preventing washout of the drug by saliva is present (42), which is practically not possible in awake dogs due to lack of compliance (40). In our study, the mean T_{max} after IN administration was reached significantly faster (0.49 h) compared with the PO administration (3.5 h), but on the other hand, there was no significant difference between the mean AUC normalized for dose and mean C_{max} normalized for dose when comparing IN and PO administrations. It is worth to mention that a substantial proportion of the oromucosal delivered dose of CBD may actually be absorbed through the gastrointestinal tract (43) and this phenomenon may also be seen with IN drug administration (44), which could have influenced the velocity of the IN T_{max} concentrations in this study. Although drug delivery *via* IN administration is quickly absorbed and bypasses first-pass effect, other factors may have influenced IN CBD plasma concentrations in our study, such as the solvent formulation (PEG) used and the high lipophilicity of CBD. It is believed that PEG-only formulations are associated with a higher CBD permeation (45). In our study, CBD was intranasally delivered in a PEG:NaCl 0.9% (50:50) solution. The viscosity of a PEG-only formulation would be too high and would not turn into a fine mist of particles when administered *via* the mucosal atomization device. Paudel et al. (45) evaluated the pharmacokinetic parameters after IN CBD administration *via* a surgical procedure in anesthetized rats, using different solvent formulations containing PEG. Rats that received IN CBD with PEG alone, showed a 3.5-fold increase in mean AUC when compared to the group of rats that received IN CBD containing 50% of PEG, 35% saline and 15% ethanol in the solvent solution. Another explanation for the lower IN absorption could be the extreme lipophilicity of CBD (Log *P* 6.3) (46), which may make crossing the aqueous media of the nasal mucosa and other polar secretions difficult (45). Furthermore, the dogs in our study were not sedated nor anesthetized, which might have facilitated nasal drug delivery and possibly increased the AUC as well. Vlerick et al. (39) achieved a complete bioavailability when ketamine was administered intranasally in sedated dogs and this was associated with a lower risk of spilling and swallowing of the drug. Two dogs in our study sneezed and one dog was head shaking after the IN administration, which could have led to partial spilling of the drug out of the nasal cavity. On the other hand, we believe that not anesthetizing or sedating the dogs would avoid any possible drug-drug interaction that could consequently affect CBD pharmacokinetics and would not reflect the in-practice situation. The interaction between cannabinoids and volatile and intravenous anesthetic agents is equivocal, with evidence limited to animal studies, case reports and limited human studies (47). Also, it might have been possible that CBD concentrations after IN delivery were higher in the cerebrospinal fluid compared to plasma, but this was not analyzed in our study.

Main advantages of the oral administration of CBD include standardized concentrations and doses, and an easy administration route, where oils and capsules currently allow for more convenient and accurate dosing in comparison with other oral formulations (48). Besides the CBD oil, other oral formulations for dogs can be acquired, including soft chews, soft gel capsules and tablets (49). In general, CBD in oil suspensions designed for oral and oromucosal administration are currently favored and appears to be the preferred method of delivery for absorption (7, 40, 50). Pharmacokinetic analysis demonstrated that the CBD-infused oil formulation resulted in higher C_{max} and AUC than oral microencapsulated oil beads and CBD-infused transdermal cream (50). Small volumes of CBD oil might slowly transcend the esophagus into the stomach, which could possibly prolong its absorption, but on the other hand, CBD in a soft chew presentation is more likely to create a food bolus that it is delivered quickly to the stomach, resulting in a quicker digestion and absorption (51). Soft chews are currently the most popular dosage-form treats available in the marketplace for dogs (52). CBD has a high lipophilicity and its administration in a lipid solvent, such as medium-chain triglycerides oil for example, may increase the bioavailability of CBD (53). In a study in rats, the administration of oral CBD together with lipid compounds increased the bioavailability of CBD by almost 3 times when compared to non-lipid formulations (54). Oral dosing with CBD in an oil base may enhance absorption, but may enhance further by inclusion in a food matrix (51). Drugs with a high lipophilicity and that are administered orally in a lipid solution can precipitate in the gastrointestinal tract, resulting in an absorption rate slower than elimination (55). In a human study, the administration of CBD together with a high-fat caloric meal is used as a potential method to increase the oral bioavailability of CBD (43). This method has also been used in dogs receiving oral cannabinoid, where they were fed at the time of administration to promote cannabinoid absorption (56).

Higher plasma concentrations and exposure were achieved for practically applicable oral dosages than for applicable IN dosages for the examined products. The PO CBD dose in our

study ranged from 7 to 13 mg/kg. Deabold et al. (51) was able to obtain similar concentrations when determining single-dose oral pharmacokinetics of CBD in healthy dogs using a lower dose (2 mg/kg), achieving a mean C_{max} of 301 ng/mL at 1.4 h (T_{max}) and a mean AUC of 1,297 ng-h/mL. This is possibly due to the use of an infused soft chew treat made with a glycerol/starch/fiber base which should be easily digestible and appears to deliver approximately two and a half times the concentration. As in our study, other research groups also used a higher oral dose in dogs (10 mg/kg) and a mean C_{max} was reached, between 578 and 1,868 ng/mL, respectively (50, 57). The mean AUC found in our study after PO administration was 1,376 ng/mL*h. Bartner et al. (50) showed a mean AUC between 8,820 ng/mL*h (10 mg/kg of CBD-infused oil) and 6,180 ng/mL*h (10 mg/kg of microencapsulated oil) in healthy dogs. CBD has a high affinity for lipids and low water solubility (58), and consistent with its lipophilicity CBD administered orally was not detected in 50% of the dogs, in which CBD was administered as a powder within a gelatin capsule (31). Therefore, if given orally, it is best absorbed in the presence of fat, oils or polar solvents (59). In our study, oral CBD was administered within a tablet with a small amount of highly digestible wet food containing 15% of fat (as previously mentioned) and this could explain why we observed a lower CBD exposure compared to studies using oil-based CBD (50) but still a better absorption when comparing our results to other studies (31).

The rectum offers a practical delivery route for several drugs and is a relatively easy and quick method when oral administration is not feasible or when intravenous access is not available. In one study with healthy Beagle dogs, THC administered rectally with suppositories in a lipophilic base (Witepsol H15), had a bioavailability of ∼67% (60). Intestinal absorption and bioavailability depend on several factors such as drug solubility in the gastro-intestinal environment, permeability of the drug through the enterocyte membrane, activity of efflux transporters and metabolizing enzymes (61). In our study, plasma CBD concentrations after IR administration were extremely low and therefore the pharmacokinetic parameters were not analyzed. The suppositories used in our study contained a formulation of glycerol monostearate, which is a more hydrophilic substance (Log *P* 7.4) (62). The use of suppository formulations in lipophilic bases was previously associated with a higher absorption and bioavailability of cannabinoids (60), thus use of more lipophilic bases could have increased plasma CBD concentrations after IR administration in our study. Overall, IR administration of CBD under the currently used formulation is not advised due to its inconsistent and low plasma concentrations.

The most frequently observed adverse effect associated with the IN route was short sneezing in two dogs and head shaking in one dog. Sneezing or snorting reaction and head shaking during or after intranasal administration have also been reported in three other studies, where dogs received IN diazepam (44), IN midazolam (38, 63) and IN ketamine (39). In our study, a PEG:NaCl 0.9% (50:50) solution was used as a solvent for the IN CBD administration. In humans, PEG has been reported to induce mild local toxicity to the nasal mucosa (64, 65), which could induce local irritation and a displeasing sensitivity. Nevertheless, all three administration routes were easy to perform and generally well-tolerated by all dogs.

The major limitation of this study was the lack of an IV route group, which could have provided some more consistent pharmacokinetic data. There are also potential limitations regarding the IN route, including spilling of the drug due to sneezing and swallowing of a part of the dose administered.

CONCLUSION

The IN, IR and PO single administration of 20, 100, and 100 mg CBD, respectively, was well-tolerated by all of the dogs. PO remains the most favorable route for CBD delivery due to its more feasible administration. Nevertheless, IN administration of CBD provided a faster blood absorption when compared to the PO and IR CBD administration. These findings encourage the use of IN CBD in veterinary medicine as a possible alternative when PO route is not possible. The IR administration route is not advised for clinical application.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of the Faculties of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2018-42).

AUTHOR CONTRIBUTIONS

DP and SB were responsible for conception of the study, writing–original draft, and approval of the submitted manuscript. RT and MD were responsible for conception of the study, acquisition of the data, data analysis and interpretation, pharmacokinetic evaluation, and writing–review and editing of the manuscript. BB was responsible for statistical analysis and writing–review and editing of the manuscript. MC, LH, IC, PM, AF, and JS were responsible for writing–review and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JS was employed by company CBDepot.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcutaneous Cervical Vagus Nerve Stimulation Induces Changes in the Electroencephalogram and Heart Rate Variability of Healthy Dogs, a Pilot Study

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Transcutaneous cervical vagus nerve stimulation (tcVNS) has been used to treat epilepsy in people and dogs. Objective electroencephalographic (EEG) and heart rate variability (HRV) data associated with tcVNS have been reported in people. The question remained whether EEG and electrocardiography (ECG) would detect changes in brain activity and HRV, respectively, after tcVNS in dogs. Simultaneous EEG and Holter recordings, from 6 client-owned healthy dogs were compared for differences pre- and post- tcVNS in frequency band power analysis (EEG) and HRV. The feasibility and tolerance of the patients to the tcVNS were also noted. In a general linear mixed model, the average power per channel per frequency band was found to be significantly different pre- and post-stimulation in the theta ($p = 0.02$) and alpha bands ($p = 0.04$). The pooled power spectral analysis detected a significant decrease in the alpha ($p < 0.01$), theta ($p = 0.01$) and beta ($p = 0.035$) frequencies post-stimulation. No significant interaction was observed between dog, attitude, and stimulation in the multivariate model, neither within the same dog nor between individuals. There was a significant increase in the HRV measured by the standard deviation of the inter-beat (SDNN) index ($p < 0.01$) and a decrease in mean heart rate ($p < 0.01$) after tcVNS. The tcVNS was found to be well-tolerated. The results of this pilot study suggest that EEG and ECG can detect changes in brain activity and HRV associated with tcVNS in healthy dogs. Larger randomized controlled studies are required to confirm the results of this study and to assess tcVNS potential therapeutic value.

Keywords: vagus nerve stimulation, transcutaneous cervical vagus nerve stimulation, frequency band analysis, power spectral analysis, heart rate variability, SDNN index

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INTRODUCTION

Vagus nerve stimulation (VNS) is a non-pharmacologic treatment option in human patients with epilepsy (1–3). This technique consists of providing controlled electrical stimulation of the vagus nerve, either through a device surgically implanted on the left cervical vagus nerve or through a non-invasive transcutaneous unit (2, 4). Transcutaneous cervical vagus nerve stimulation (tcVNS)

has been reported as an adjunctive treatment in drug resistant epilepsy and other diseases like depression, migraines or pain (5–8).

In veterinary medicine, the estimated prevalence of dogs considered resistant to conventional anti-seizure therapy is ∼25% (9). This high prevalence drives the investigation of non-pharmaceutical therapies like VNS. A surgically implanted VNS device has been studied in dogs (10). Despite the favorable response observed in some dogs, the cost, unreliable functionality and short- and long-term complications make a surgically implanted device inaccessible for many drug-resistant patients (10, 11). The tcVNS apparatus in dogs with drug resistant idiopathic epilepsy is reported to be well-tolerated; although no significant effect on the overall reported seizure frequency was found (12). Objective measures of tcVNS effects on vagus nerve activity remain to be described.

The vagus nerve has an important role in homeostasis functions. Due to its mixed composition of afferent sensory and efferent motor and parasympathetic axons, stimulation of the vagus nerve results in a plethora of different physiologic changes, with those in the brain and heart of particular interest for this study (13–15). The proposed mechanism of action of VNS on the brain is a direct increase in the release of norepinephrine from the nucleus of the solitary tract to the projections sent to the locus coeruleus and, indirectly, increasing the serotonin release by the dorsal raphe nucleus (16, 17). There is evidence that VNS results in an increase in the norepinephrine concentration in healthy Beagles, further suggesting that an increase in this monoamine could play an important role in the mechanism of action of VNS (18). The changes in the concentration of these neurotransmitters presumably influence the synaptic plasticity of the neurons, resulting in an anti-seizure effect (16, 17, 19, 20). Another change that has been identified in healthy dogs undergoing VNS is a decrease in the perfusion of the frontal lobe, which has been hypothesized could contribute to the mechanism of action of VNS (21). While it is safe to stimulate either side of the vagus nerve, stimulation of the right branch and sinoatrial node is more likely to result in bradycardia and asystole (11, 22). Dogs, as opposed to humans, have a more caudal branching of cardiac motor fibers innervating the atrioventricular node on the left side of the heart, therefore stimulation of the left side of the vagus nerve could still result in bradycardia and asystole (11, 23). Notably these cardiovascular complications have been observed with surgically implanted but not with transcutaneous devices in dogs (12).

The effect of VNS and tcVNS on the cardiovascular system has been studied in people and animals (24–29) mainly by means of heart rate variability (HRV). This is defined as the variation in the interval between successive R waves (NN or R-R intervals) in the cardiac cycle, and is considered to be an indicator of the autonomic nervous system activity (30–33). The standard deviation of the inter-beat (NN or R-R) interval in a sinus rhythm is termed SDNN, and the mean of the standard deviations of the normal inter-beat intervals in a given period of time is called the SDNN index (34). This is considered the most reliable way to assess the autonomic function of the heart (34). The decrease in heart rate and the increase in HRV observed with VNS is secondary to the release of acetylcholine at the sinoatrial node (35).

In people, tcVNS has been documented to result in identifiable changes in brain activity. Electrical brain activity is registered by electroencephalography (EEG), recorded via electrodes and then transferred to a computer system (36, 37). Brain activity changes in people after tcVNS include a decrease in abnormal EEG patterns in patients with epileptic seizures, attenuation of the alpha rhythm, and an overall decrease in the theta and alpha frequency bands power (5, 38, 39). Power is a form of quantitative EEG analysis that divides the signal into bands based on their frequencies; this is known as frequency band analysis (40–42). The different frequency bands are historically designated as beta (13–30 Hz), alpha (8–13 Hz), theta (4–8 Hz) and delta (1–4 Hz) (43). Spectral analysis of these frequency bands calculates the power, the relative strength of the frequencies in a signal within a determined time period of the EEG recording, known as an epoch (41, 44, 45). This quantitative analysis is useful in detecting alterations to normal brain activity associated with changes in the resting state or in a disease process.

Based on this research into the effects of tcVNS on the brain and heart in people, both EEG and HRV have been shown to provide objective measures. Objective measurement of these outcomes would support the determination of optimal tcVNS dose and response for dogs. Before establishing tcVNS as a potential supplementary treatment for drug-resistant epilepsy in dogs, it is important to determine if tcVNS results in objective physiologic changes and, if it does so, what are the expected physiologic changes to be registered.

The objectives of this pilot study were: (1) to determine if tcVNS could induce changes in frequency band spectral analysis as assessed by EEG and changes in the HRV as assessed by Holter monitoring, and (2) to assess the tolerability of the tcVNS device in dogs.

MATERIALS AND METHODS

Animals

Six healthy community owned dogs were recruited for this prospective pilot study. As the first study of its kind, the effect size of this intervention has not been established from previous pilot studies nor has the minimum difference considered to be significant been determined. The sample size was proposed based on previous studies that successfully assessed the effect of drugs or the use of different electrodes on EEG recordings (7, 11, 46). The dogs were enrolled from the teaching hospital staff, university staff and referring veterinarian community. Dogs included in the study had to be older than 1 year but under 10 years of age and healthy based on a complete physical and neurological examination performed by one of the authors (GC). The patients were placed in a quiet room under the supervision of GC for the duration of the recording period. The Institutional

Abbreviations: EEG, Electroencephalography; ECG, electrocardiography; HRV, heart rate variability; VNS, vagus nerve stimulation; tcVNS, transcutaneous cervical vagus nerve stimulation; SDNN, standard deviation of the inter-beat N-N interval.

Animal Care and Use Committee of the University of Guelph approved the animal use protocol (# 4265) for this study and the protocol followed the Canadian Council on Animal Care guidelines. Written informed consent was obtained from the owners for the participation of their animals in the study.

Holter Monitor

The Holter monitoring was performed using a digital Lifecard CF 3 channel Holter recorder (Spacelabs Healthcare, Snoqualmie, WA) that was fitted in a snug jacket after securing the leads to the chest with sticky ECG pads and adhesive medical tape right after the physical and neurological examinations were completed. The ECG recording was started immediately after the Holter instrumentation was completed but data was not collected for analysis until the remaining study instrumentation was complete.

After the Holter was placed, a 10 cm in length x 4 cm in width area of fur was clipped over the left jugular groove over the carotid pulse where the tcVNS therapy was to be delivered.

EEG

After the Holter placement, the EEG instrumentation was performed with subdermal wire electrodes loaded in 25-gauge needles. Electrode placement was the same as described in a previous study (47). EEG instrumentation was first attempted without sedation, however for dogs whose temperament prevented the placement of the electrodes, a 22-gauge catheter

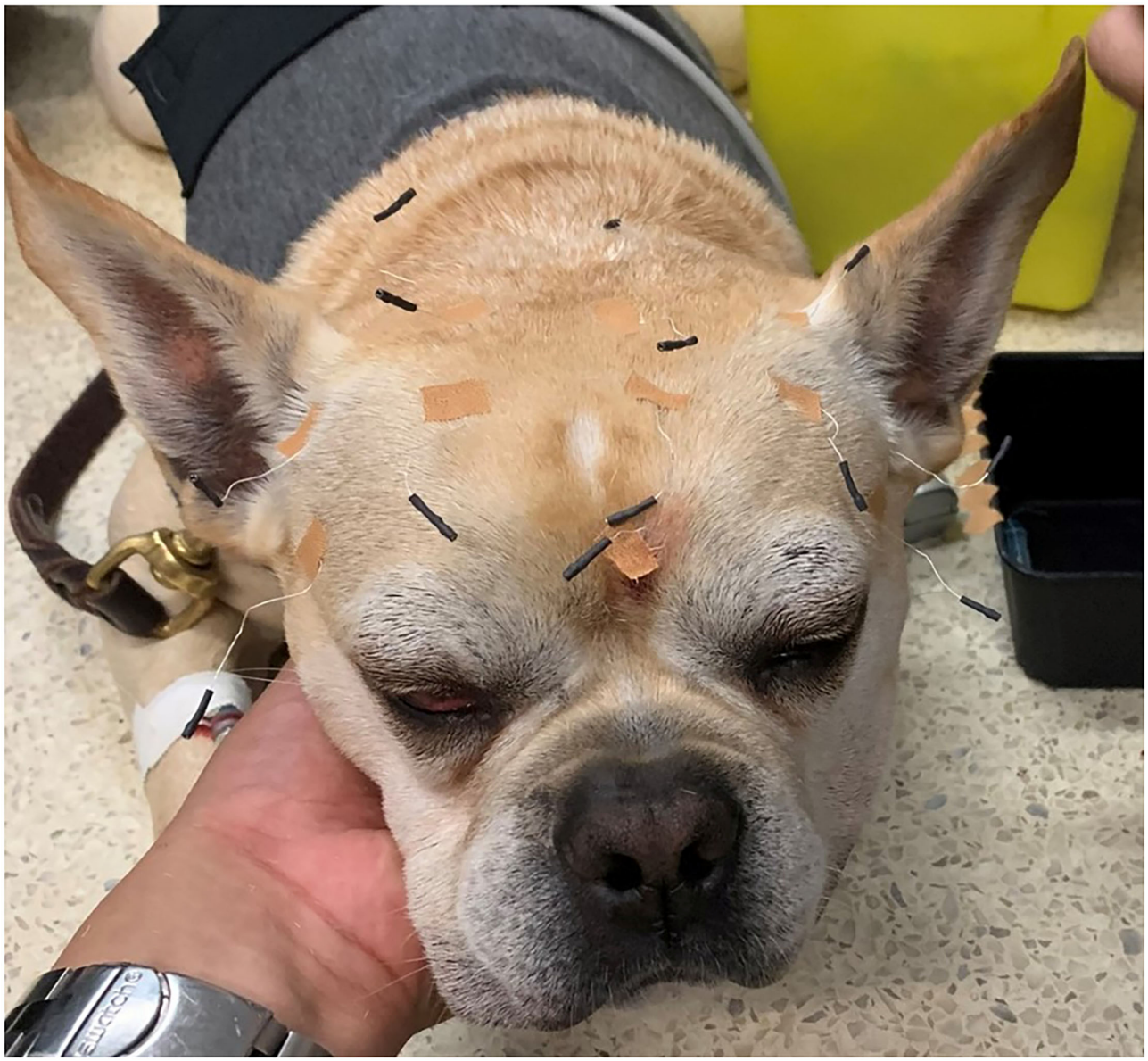

FIGURE 1 | Electrode placing and fixation with sticky tape on a dog sedated with propofol.

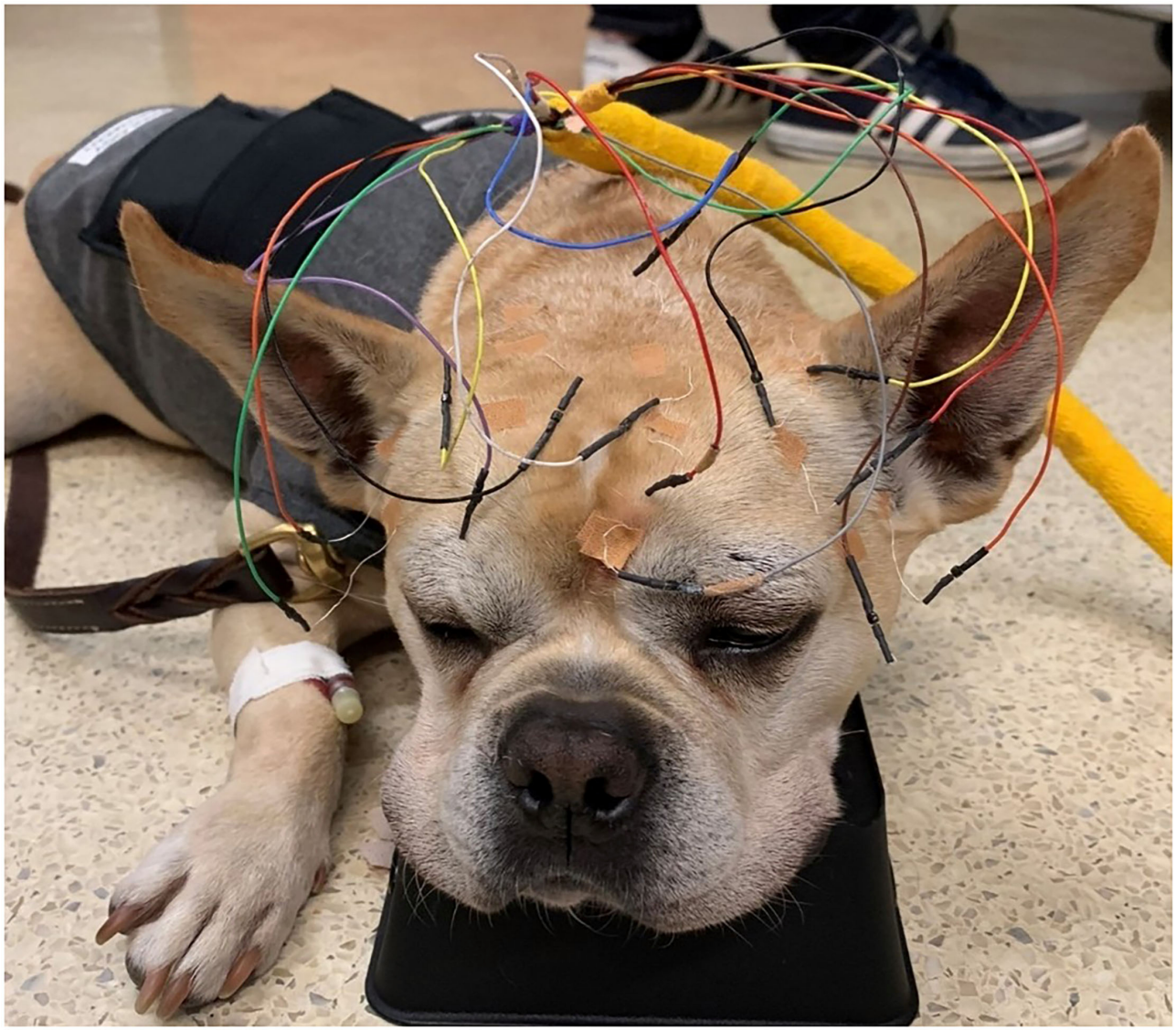

FIGURE 2 | Leads attached to the electrodes prior to the placement of the head bandage.

was placed in a peripheral vein (cephalic or saphenous vein). Following catheter placement, a 6 mg/kg bolus of propofol was slowly administered IV to the level required to achieve calm relaxation while maintaining jaw tone, palpebral reflexes, and spontaneous breathing. Then 2 mg/kg boluses of propofol were given as needed to complete instrumentation. Femoral and pedal pulses along with the respiratory rate were constantly monitored during the 20–40 min of the EEG instrumentation phase. The electrodes were kept in place by using sticky bandage and both electrodes and leads were safely secured with loosely placed elastic sticky bandage around the dog's head and neck. Finally, the wireless transmitter TrackIt MK3 EEG recorder with video (Lifelines Neurodiagnostics Systems, Troy, IL, USA) was placed in the same snug jacket of the dogs (**Figures 1**–**3**). The EEG synchronized video camera recording was started at the same time as the instrumentation and the angle of the camera was kept in a position that allowed the greatest possible visualization of the dog. At the end of instrumentation, impedance was checked, and recording was started only when all the electrodes showed an electrical impedance under 30 kOhms (48). After the sedation for the instrumentation wore off, an e-collar was placed on one of the dogs for the rest of the recording to avoid electrode removal.

Recording and Stimulation

Once that both Holter and EEG devices were safely placed and dogs were completely awake from the sedation and able to stand up on their own, 1-h of pre-stimulation (basal) ECG and EEG were recorded followed by 120 s of stimulation and finally by 1-h of ECG and EEG recording post-stimulation. Even though the EEG and Holter devices had started recording right after

placement, the 1-h pre-stimulation period used for analysis was not counted until after the dogs had recovered from sedation as described above. This was to avoid any potential changes in the EEG and ECG induced by manipulation of the dogs as they were being instrumented (e.g., clipping the fur, placing IV catheter, and fitting the jacket). Thereafter, the dogs were

FIGURE 3 | Head bandage placed after the dog recovered from the sedation with propofol. A snug-fitting jacket has been placed to secure the Holter monitor and the TrackIt device.

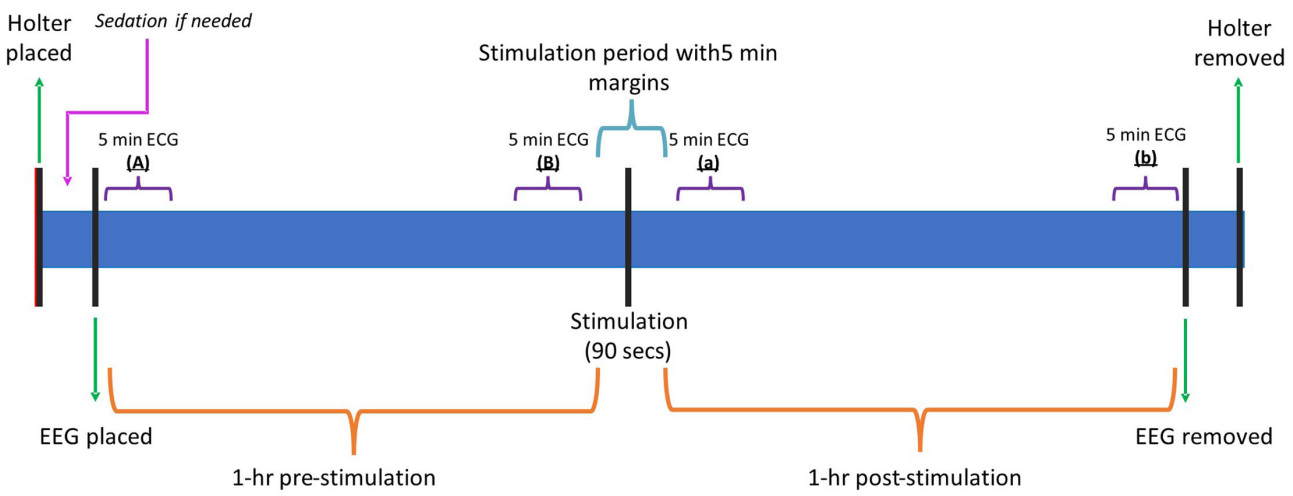

FIGURE 4 | Timeline graphic summarizing the different events and key points during the recording. Min, minutes; Hr, hour; ECG, electrocardiogram; EEG, electroencephalogram.

supervised but undisturbed in the room except for the 120-s period of stimulation. During the entire recording session, the dogs were allowed to explore the room and were provided with a bowl of water and comfortable bedding to lie down if they preferred to.

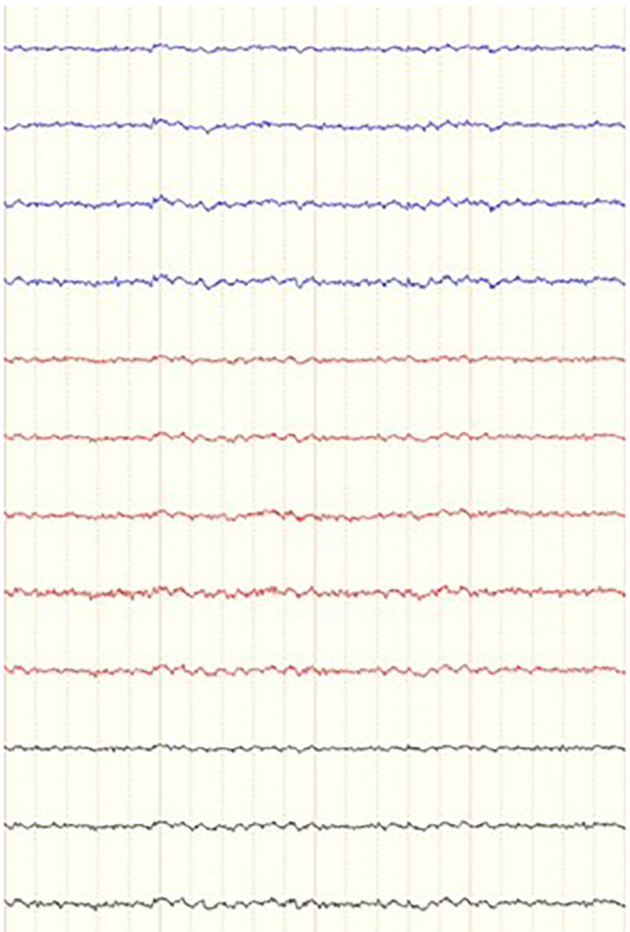

FIGURE 5 | Example of a 4-s epoch of a dog in a during a period of "calm" behavior as it was presented to the blinded investigator. Referential montage, low frequency filter 0.16 ms, high frequency filter 70 Hz, notch filter 60 Hz and sensitivity of 7 uV. Ms, milliseconds; Hz, Hertz.

For the stimulation period, contact gel was placed on the previously clipped spot on the neck and the tcVNS device (gammaCore-VET; ElectroCore, LLC, New Jersey, United States) was placed making sure that both electrodes were in direct contact with the skin by applying light pressure. The gammaCore instrument is programmed by the manufacturer to deliver 120 s of stimulation after which the device automatically turns off. The device delivers an electrical signal consisting of a 5-kHz sine wave burst lasting for 1 ms (5 sine waves, each lasting 200 ms), with such bursts repeated once every 40 ms (25 Hz) for 2 min per stimulation, as described previously (49). During the first 30 s, using the graded thumbwheel on the side of the tcVNS (graded from 1 to 5, 5 being the highest stimulation intensity), the dog's highest tolerable dose was identified. The device was started at 1 and the intensity slowly increased every 5 s. If the dog showed any signs of discomfort (yelp, growl, or movement of the head away from the device) or if marked muscle fasciculations were observed at the site of the stimulation, the intensity was decreased by 0.5 and the stimulation level was recorded as the highest tolerable dose. The reaction of the dogs to the stimulation was recorded with a video camera pointing toward the head of the dogs for the purpose of capturing any behavioral or physiological reaction that could indicate pain or discomfort from the stimulation. The remaining 90 s of the stimulation was delivered at the highest tolerable dose.

After the remaining 90 s of stimulation were completed, another at least 1-h period of ECG and EEG were recorded in the same room under the same conditions as the first hour of recording. **Figure 4** shows the timeline of events during the recording period and the data obtained.

Frequency Band and Power Spectra Analysis

To account as much as possible for the influence of the attitude and activity of the individual dogs during the recordings, the EEG video was reviewed to pair pre- and post-stimulation segments where the patient was considered "calm" or "active" depending on their behavior. If the dog was lying down or sitting comfortably and not engaged in any activity, the segment was categorized as "calm". A segment was labeled as "active" if the dog was standing or exploring the room. A referential montage

TABLE 1 | Signalment, stimulation level and amount of sedation used in the 6 dogs.

Dog	Breed	Age (years)	Sex	Weight (kg)	Highest tolerable dose of tcVNS (1–5)	Propofol (mg/kg)
1	Mix breed	2	MC	44	2	8
2	French bulldog	7	FS	13	4	10
4	Mix breed	2	FS	26.5	4	8
5	Border Collie	8	FS	19	4	None
6	Mix breed	1	FS	20	4.5	9
10	Mix breed	6	FS	22.2	3	None

MC, male castrated; FS, female spayed; tcVNS, transcutaneous vagus nerve stimulation.

was used to retrieve the data using 7 different channels (F3, F4, C3, C4, Cz, Fz and Pz).

For each dog, several 4-second-long epochs were manually selected from the raw EEG data pre- and post- stimulation. One of the researchers (FJ), who was blinded to the signalment of the patients, the time and sequence of the recordings, and behavior, selected 9–12 epochs pre- and post- stimulation that were as artifact-free as possible (**Figure 5**). The 4-s epochs were then analyzed in 2 ways. Firstly, for each individual channel in each selected epoch, the average power and mean frequency values for the different frequency bands were calculated, however the algorithm in this tool (FDA Tables tool) used overlapping frequency bin borders (i.e., delta from 1 to 4 Hz, theta from 4 to 8 Hz). Secondly, for the pooled channels for each epoch, power spectral analysis of the different frequency bands, median frequency (F50) and spectral edge frequency (F95) were calculated via fast Fourier Transformation (FFT tool) with more precise frequency band borders set in the algorithm (e.g., theta from 4.1 to 8.0 Hz). Then, channel frequency and power, pooled power, F50 and F95 were compared between pre- and post-stimulation for each individual dog in both calm and active epochs. All the EEG analysis was performed using specific software (Insight II, Persyst Development Corporation, Prescott, AZ) applying a Hamming window with 256 points per window and 50% overlap in between windows.

HRV Analysis

The data collected with the Holter monitor was analyzed with a specific software (Pathfinder SL 1.9.2 11104 version). An approximately 1-h section of the recording was selected before and after stimulation leaving a 5-min margin before and after the stimulation to account for any potential clock-time discrepancies between the Holter monitor and the registered time of stimulation. Once the 1-h long pre- and post- stimulation segments were selected, the average SDNN index and the mean heart rate values were collected from these segments. Additionally, the averaged mean heart rate of the 5-min margin before and after stimulation (for a total of 10-min-long segment) was registered.

To check whether any changes obtained when comparing the HRV and SDNN between dogs and before/after the stimulation were simply due to the dogs' habituation to the room causing a general decrease in the heart rate over the entire 2-h study, the mean heart rate and maximum heart rate for the first 5-min after the start of the EEG recording ("A") were compared against the last 5-min prior to the stimulation ("B"). Additionally, the first 5-min of the post-stimulation period ("a"), and the 5-min prior to the end of the recording ("b") were compared. **Figure 4** shows the different 5-min intervals selected.

Statistical Analysis

To determine the effect of VNS a general linear mixed model was used for both the individual and pooled channels' quantitative variables listed above. Fixed effects of channel, time, and attitude as well as their interactions were included in the model. Individual dog, dog plus stimulation, and dog plus stimulation plus attitude were included as random effects. When interaction terms were not significant, the models were simplified, and *p*-values were reported for the main effects. Data was checked

TABLE 2 | Average power per channel per frequency band with upper and lower confidence intervals for the pre- and post-stimulation periods and for the calm vs active states.

Frequency band	Average power (μV)	Lower 95% CI	Upper 95% CI	*p*-Value pre vs. post stimulation	*p*-Value calm vs. active
Delta pre-stimulation	3.31	2.45	4.47	0.23	
Delta post-stimulation	2.9	2.15	3.91		
Delta calm	2.72	2.03	3.65		0.03*
Delta active	3.53	2.59	4.80		
Theta pre-stimulation	1.28	0.95	1.73	0.02*	
Theta post-stimulation	1.05	0.78	1.42		
Theta calm	1.04	0.77	1.40		0.01*
Theta active	1.29	0.95	1.75		
Alpha pre-stimulation	0.88	0.67	1.16	0.04*	
Alpha post-stimulation	0.74	0.57	0.98		
Alpha calm	0.74	0.57	0.97		0.04*
Alpha active	0.88	0.67	1.16		
Beta pre-stimulation	0.85	0.62	1.17	0.61	
Beta post-stimulation	0.78	0.57	1.08		
Beta calm	0.60	0.44	0.82		<0.01*
Beta active	1.10	0.79	1.55		

CI, confidence interval.
*An * denotes statistical significance.*

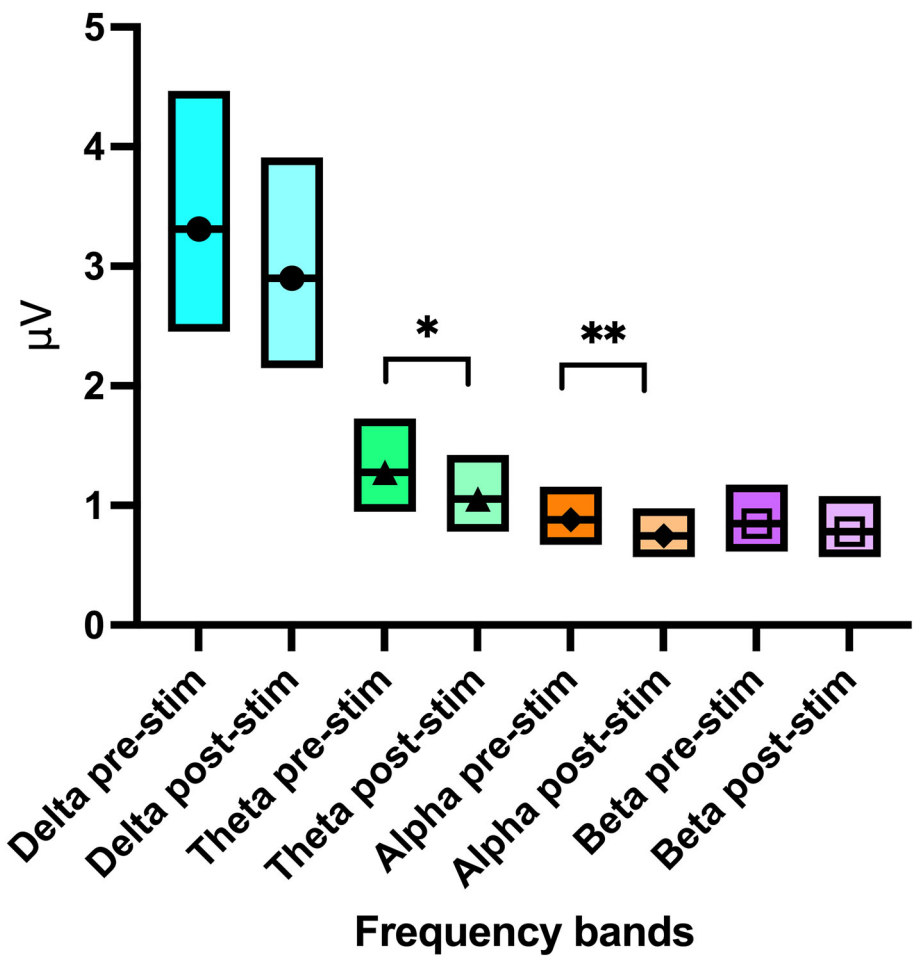

FIGURE 6 | Boxplot graph showing the average power per channel per frequency band with the upper and lower confidence intervals before and after stimulation. The line crossing the boxes indicate the median value obtained in that frequency band. An * denotes statistical significance of $p = 0.02$ and ** denotes a significance of $p = 0.04$.

for normality with a Shapiro Wilk test and examination of the residuals. Data was log transformed to meet the assumptions of normality. Similarly, a general linear model was run for the HRV analysis. Significance was set at $p < 0.05$.

For the 5-min segment comparisons, an ANOVA for repeated measures accounting for the correlation of structure of measures made within animals was used to test for differences in heart rate over time. Residuals were checked for normality to confirm the data met the assumptions of normality and the data was normally distributed.

RESULTS

The signalment of the dogs included in this study, the highest tolerable tcVNS dose and the total mg/kg of propofol used for sedation are summarized in **Table 1**.

EEG Power and Mean Frequency Analysis per Channel

Analysis of the recordings detected a significant difference in all frequency bands (delta $p = 0.03$, theta $p = 0.01$, alpha $p = 0.04$, and beta $p < 0.01$) when examining average power per channel per frequency band in the 2 attitude states (calm vs. active) (**Table 2**). Average power per channel per frequency band was lower when dogs were calm than when they were active. A significant treatment effect (pre- vs. post- stimulation) in average power per channel was observed only in the theta ($p = 0.02$) and alpha ($p = 0.04$) bands (**Table 2** and **Figure 6**), with the average power of both frequency bands decreasing post-stimulation. In the mean frequency analysis, attitude had a significant effect only for the beta and theta bands ($p \leq 0.01$ for both values). Stimulation had no significant effect on the mean frequency in any band (**Supplementary Table 1**). No significant

TABLE 3 | Pooled power spectral analysis per frequency band with upper and lower confidence intervals for the pre- and post-stimulation periods and for the calm vs. active states.

Frequency band	Mean value (μV^2/Hz)	Lower 95% CI values	Upper 95% CI values	*p*-Value pre vs. post stimulation	*p*-Value calm vs. active
Delta pre-stimulation	3.32	2.63	4.2	0.16	
Delta post-stimulation	2.86	2.28	3.60		
Delta calm	2.5	2.01	3.10		<0.01*
Delta active	3.8	2.96	4.88		
Theta pre-stimulation	1.17	0.63	1.2	0.01*	
Theta post-stimulation	0.87	0.85	1.62		
Theta calm	0.89	0.64	1.2		0.01*
Theta active	1.16	0.83	1.62		
Alpha pre-stimulation	0.66	0.44	0.99	< 0.01*	
Alpha post-stimulation	0.46	0.31	0.68		
Alpha calm	0.55	0.37	0.81		0.91
Alpha active	0.55	0.37	0.84		
Beta pre-stimulation	0.83	0.61	1.13	0.03*	
Beta post-stimulation	0.64	0.47	0.88		
Beta calm	0.63	0.47	0.85		0.01*
Beta active	0.85	0.61	1.17		
F50 pre-stimulation	4.69	2.84	7.72	0.21	
F50 post-stimulation	3.28	2.01	5.35		
F50 calm	4.75	3.01	7.49		0.18
F50 active	3.23	1.89	5.54		
F95 pre-stimulation	11.55	9.55	13.55	0.20	
F95 post-stimulation	9.97	8.04	11.9		
F95 calm	10.64	8.92	12.35		0.84
F95 active	10.88	8.67	13.1		

CI, confidence interval.
*An * denotes statistical significance.*

interaction was observed between dog, attitude, and stimulation in the multivariate model, neither within the same dog nor between individuals.

EEG Pooled Power Spectral Analysis

In this parallel analysis of pooled data, attitude also had a statistically significant effect on all frequency bands (delta $p \leq 0.01$, theta $p = 0.01$, and beta $p = 0.01$) except for alpha ($p = 0.91$). Similar to the per channel analysis, the power was lower when "calm" vs. when "active" (**Table 3**). When comparing pre- and post- stimulation mean power values, theta ($p = 0.01$), alpha ($p \leq 0.01$), and beta ($p = 0.03$) frequencies showed a significant decrease post-stimulation (**Table 3** and **Figure 7**). No significant difference was found in the F50 pre- and post-stimulation ($p = 0.21$) and active vs. calm ($p = 0.18$) nor the F95 results pre- and post-stimulation ($p = 0.2$) and active vs. calm ($p = 0.84$). Dog, attitude, and treatment did not have any significant interaction in the multivariate model. The mean, *p*-values and confidence intervals for all the quantitative analysis have been summarized in **Table 3** and **Figure 7**.

Holter Recordings

In the HRV analysis, the SDNN index increased in the post-stimulation period compared to the pre-stimulation period and this difference was statistically significant (pre-stimulation median: 117.26 ms, 95% CI: 71.76–191.61 ms and post-stimulation median 213.96 ms, 95% CI: 130.94–349.61 ms; $p = 0.01$; **Figure 8**). A significant decrease in the mean heart rate after stimulation was also identified ($p = 0.01$, **Table 4** and **Figure 9**). A tachogram from 1 of the dogs as an exemplary representation of the changes in the SDNN index is shown in **Figure 10**. The comparison of the mean heart rate and the maximum heart rate of the different 5-min segments ("A", "B", "a", and "b"; **Figure 4**, **Supplementary Table 2**) did not reveal any significant differences in the mean and maximum heart rate over time ($p = 0.096$ and $p = 0.258$, respectively).

Tolerability

The stimulation was well-tolerated by the dogs. The responses observed when the stimulation intensity was considered to cause discomfort in the dogs were: pulling away from the apparatus (3 dogs), significant muscle twitching in the neck region (1 dog), and vocalization (1 dog). Once these signs were observed, the level of stimulation was immediately decreased by 0.5 grades. No adverse effects were observed during the 90 s stimulation period, during the post-stimulation period, or during the removal of the EEG and Holter. The owners of the dogs were contacted by one

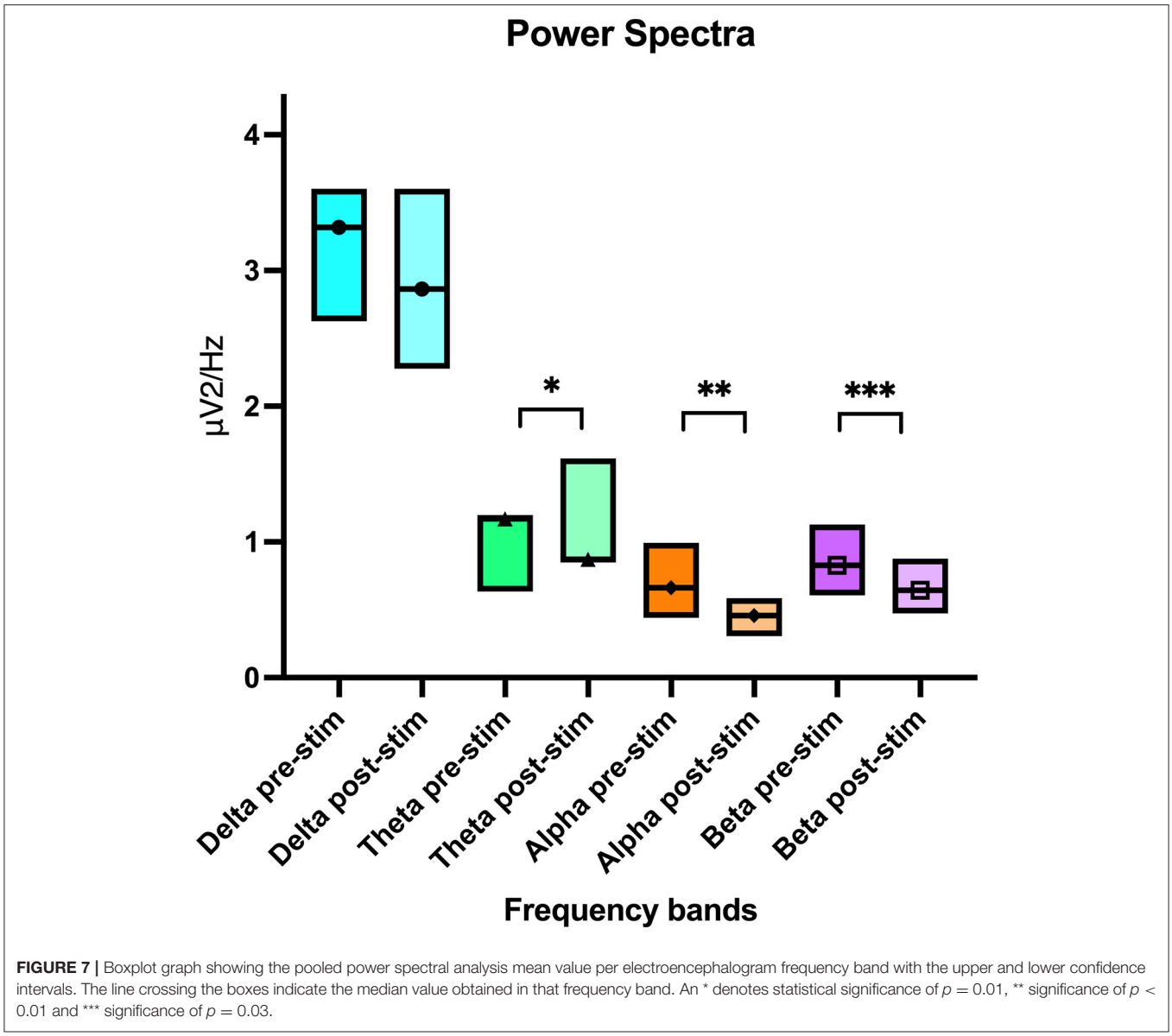

FIGURE 7 | Boxplot graph showing the pooled power spectral analysis mean value per electroencephalogram frequency band with the upper and lower confidence intervals. The line crossing the boxes indicate the median value obtained in that frequency band. An * denotes statistical significance of $p = 0.01$, ** significance of $p < 0.01$ and *** significance of $p = 0.03$.

of the investigators (GC) 1 day after the session and then again 1 week after, and no adverse effects were reported.

DISCUSSION

The EEG and Holter monitoring identified electrophysiologic changes in both brain activity and heart rate and HRV subsequent to tcVNS. The EEG detected decreased power after stimulation in the alpha, beta, and theta frequency bands in line with changes reported in people (38, 39). The Holter monitor identified an increase in the HRV post-stimulation as well as a reduction in the heart rate similar to reports in the human medical literature (50–53).

The decrease in the power of the theta and alpha frequency bands via both methods of power analyses in our study are suggestive of adequate tcVNS, similar to reported EEG changes with VNS/tcVNS in people (38, 39, 54, 55). These acute changes in the EEG are thought to be secondary to the desynchronization of brain activity as afferent vagus nerve signals cause a shift from predominantly low frequency activity (like delta, theta and alpha) to high frequency bands (like beta and gamma) thus promoting a higher arousal/awareness state (38, 39, 54). This shift in the mentation is considered important as seizure activity occurs with synchronized cortical activity, and predominance of slow-waves in delta frequency bands have been observed in interictal and ictal periods (56–58).

The power decrease in the beta frequency band post stimulation has not been reported in people and is in contrast to the mechanism proposed above. It is possible that this finding is a common change observed in the EEG of dogs after tcVNS, although another possibility is habituation of the

dog to the surroundings and decreased exploration ("active") behavior during the second part of the recording, however, this last explanation is considered less likely. Firstly, the epochs for analysis were carefully selected and paired for mental status pre- and post-stimulation; moreover, the multivariate analysis did not find any significant effect of attitude on the power pre- vs. post-stimulation. Additionally, if the decrease in the power of the theta and beta bands was secondary to a more "calmed" mentation, an increase in the delta frequency should also be expected and was not found. A final possibility for the results of this study is that the small sample size of this study skews these findings, thus confirmation with a larger cohort of dogs and a placebo-control group for comparison is required.

In both dogs and people, the resting EEG shows a different prevalence of the various frequency bands depending on the level of alertness of the individual. For instance, a delta or theta rhythm dominates in early sleep or drowsiness while an alpha rhythm predominates in periods of wakefulness when the eyes are closed (36, 41, 59, 60). Ideally, quantitative EEG analysis comparisons are performed on the same mentation state before and after the stimulation, i.e., drowsiness pre- vs. post-stimulation. In dogs, it is not possible to perfectly

TABLE 4 | Pre- and post-stimulation values, along with the lower and upper 95% confidence intervals for the mean heart rate.

	Mean heart rate (beats per minute)	Lower 95% CI	Upper 95% CI	*p*-Value
Pre	99.83	81	118	* <0.01
Post	79	61	98	

CI, confidence interval.
*An * denotes statistical significance.*

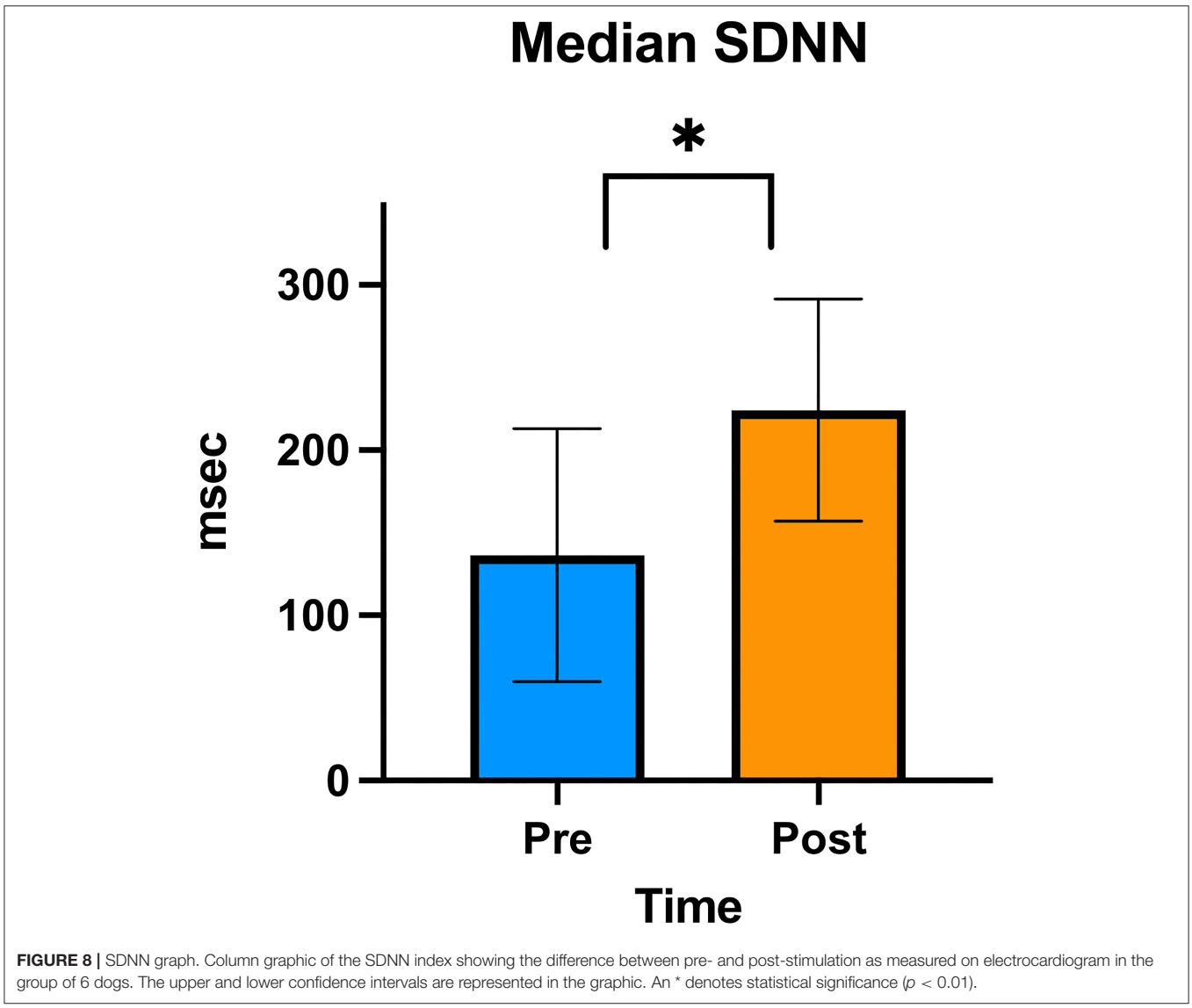

FIGURE 8 | SDNN graph. Column graphic of the SDNN index showing the difference between pre- and post-stimulation as measured on electrocardiogram in the group of 6 dogs. The upper and lower confidence intervals are represented in the graphic. An * denotes statistical significance ($p < 0.01$).

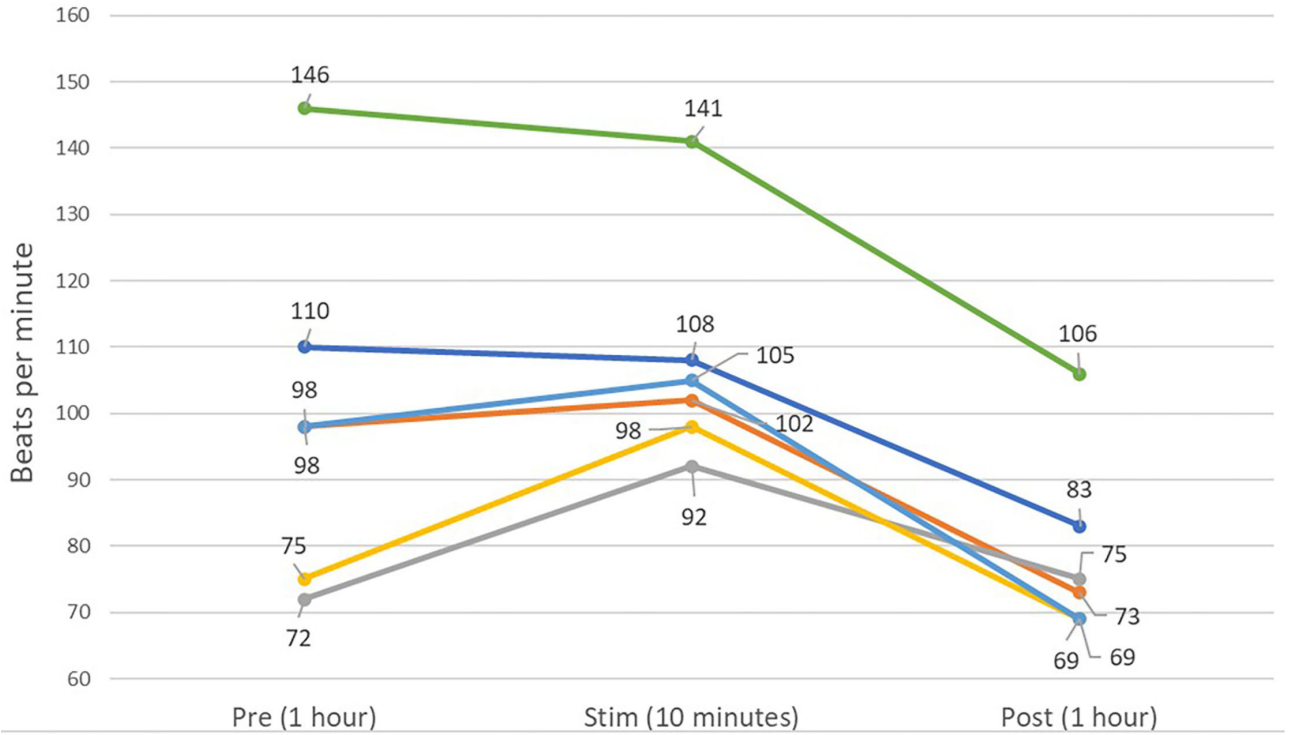

FIGURE 9 | Line graph showing the averaged mean heart rates of the 6 dogs over 1 h of pre-stimulation (Pre) and post-stimulation (Post), as well as the averaged mean heart rate of the 10 min of the peri-stimulation period. A decreased mean heart rate is present in the post-stimulation period for five of six dogs.

match this behavior pre- and post-stimulation. Instead, we grouped the dogs' behavior more broadly based on their level of engagement and activity during the analysis of the EEG recording. By pairing epochs with similar mentation states pre- and post-stimulus we tried to reduce as much as possible the influence of the mentation on the analysis of the results. To make the method of assessing mentation as objective as possible, the criteria used for categorizing an epoch as "calm" or "active" were consistent for every dog. Moreover, the multivariate model accounted for "attitude" as a random effect and did not find any significant interaction with the stimulation in the analysis.

One of the measures included in this study as an internal control for the stimulation was the detection of significant differences between "calm" and "active" mentation states. In the average power analysis, there was an increase in the power across all the frequency bands in the "active" epochs selected, regardless of if it was pre- or post-stimulation. The increase in power across the higher frequency bands (beta) is associated with the high level of alertness signified by the engagement of the dogs with their surroundings. A decrease in the low frequency bands might be anticipated in "active" dogs, however the increase power in the low frequency bands in our study could be explained by the fact that most of the physiologic artifacts associated with activity, like eye movements, respiration, perspiration, and whole body movement, present in the low frequency domain of delta and theta (61, 62).

Another internal control measure used in our study was the HRV analysis. Besides the importance of the objective documentation of the effects of tcVNS on the cortex, objective changes were expected in heart rate and HRV due to the vagus nerve's efferent influence on several organs, including the heart. Similar studies in people found that VNS/tcVNS resulted in measurable changes in heart rate and HRV (26, 27, 63, 64). The changes reported in these studies are in line with the results reported in our study. However, a more recent meta-analysis assessing HRV changes with auricular tcVNS found that there is insufficient evidence to support the expectation of a change in this physiologic parameter after auricular stimulation; this stimulation route may not be sufficiently analogous for comparison (65).

Interestingly, a study using a canine heart failure model found that chronic VNS resulted in an increase in HRV assessed by SDNN, as in our study (24). From this perspective, VNS/tcVNS could offer a novel adjunctive treatment modality for those cardiac diseases affecting dogs where parasympathetic dysfunction might contribute to disease presentation or progression, as in heart failure, arrhythmogenic right ventricular cardiomyopathy and dilated cardiomyopathy (24, 31, 32, 66). A previous HRV study in dogs comparing VNS vs. sham stimulation did not find significant difference in HRV between groups (29). It is unclear if the lack of detectable changes in dogs in the aforementioned paper could have been due to the short period of stimulation, the stimulation parameters or if healthy

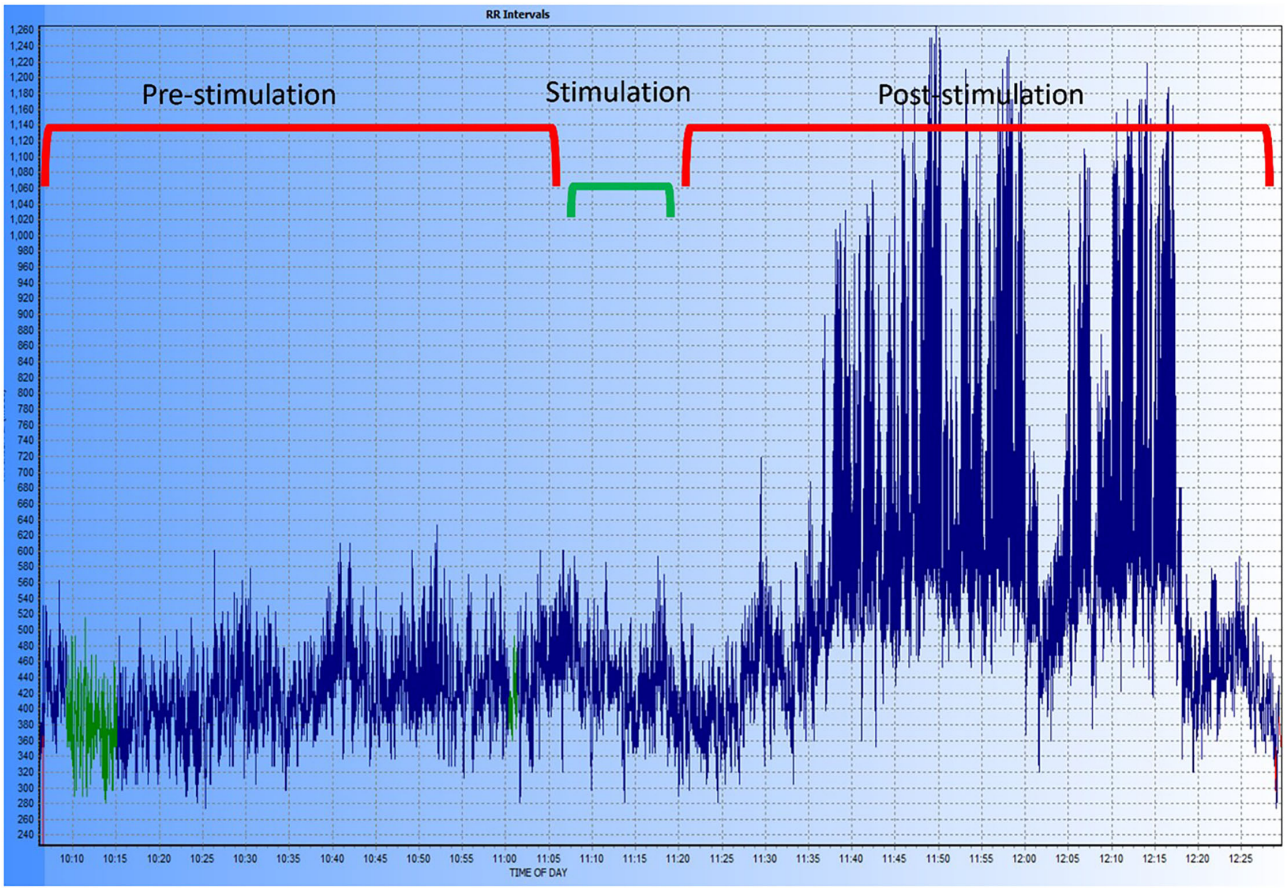

FIGURE 10 | Exemplary tachogram representing the R-R intervals in one of the dogs of the study. The different time periods are shown at the top of the graphic, the period labeled "stimulation" includes the 5-min pre- and post-stimulation margin used for the HRV analysis. A marked increase in HRV can be observed after stimulation and during the entire post-stimulation period recorded.

individuals do not show a response to the physiologic effects of vagus nerve stimulation. These findings are contrary to what was found in our study that also used healthy dogs.

To determine whether the decrease in heart rate and increase in HRV were due to habituation to the room over the 2-h study, mean and maximum heart rate were captured from 5-min segments at the beginning and end of each 1-h period (pre- and post-stimulation). Comparisons between these 4 time periods showed no statistically significant difference, suggesting that the HRV changes are more likely to be secondary to the tcVNS itself rather than habituation of the individuals. A decrease in both, mean and maximum heart rate over the 2-h study period would be expected if the dogs progressively became more relaxed.

A previous canine study using tcVNS in epileptic dogs did not find a significant effect on seizure frequency using similar device settings as our study (12). Our findings support that these settings provided adequate stimulation of the vagus nerve to cause detectable physiologic changes. Further, the two studies agree that the apparatus appears to be relatively innocuous and is well tolerated. These findings are encouraging to continue exploring tcVNS and its potential as an effective adjunctive therapy in drug-resistant epilepsy in dogs.

There are important limitations to consider in our study. The study was designed as a pilot study and, because of this novelty, a sample size calculation was not performed. A larger sample size would have allowed to better characterize the normal resting state of the individuals and would have increased or reduced the significant results.

The selection of artifact-free epochs by a blinded investigator as well as their number and length for analysis were established following the recommended guidelines in human studies (41). There are common morphological EEG features between dogs and people, for instance, an 8–12 Hz predominantly alpha rhythm in the occipital area, rostral to caudal (anterior to posterior) gradient of frequencies and similar sleep-associated waveforms (67). Despite these similarities, differences between the 2 species could exist–this needs more exploration. Most of the recent veterinary publications highlight confluences in the epileptogenic waveforms in clinical epileptic syndromes in the 2 species (47, 68–73). Some of the main challenges in veterinary medicine as compared to human epileptology are the

lack of consensus on the electrode placement array, number of channels used, and the different skull types. This absence of standardization could lead to incorrect classification of normal EEG patterns into changes or alterations thought to be secondary to the intervention. Future studies replicating or testing our study should ideally try to follow a consistent, validated instrumentation protocol to avoid unnecessary variables.

Currently, information regarding the ideal intensity and duration of stimulation in dogs is missing, therefore, the intensity and duration of stimulation used in this study were those recommended by the manufacturer. Despite a recent study having used the suggested intensity in humans (1.50 mA) to describe the side effects of implantable VNS therapy in dogs, it is still unclear what is the recommended intensity and duration needed for chronic treatment in dogs (74). The design of our pilot study was focused on identifying the acute physiologic changes detectable when using tcVNS, therefore, the comparison of different intensities, durations, and periods of treatments was not performed. Despite this limitation, the intensity and duration of stimulation used in this study resulted in changes in brain activity and HRV.

Finally, due to the design of this study and the number of individuals recruited, the inclusion of a control group was not possible. The inclusion of a control group would have helped to confirm that our results were indeed secondary to the use of the tcVNS and not secondary to other external variables. Future studies should consider the incorporation of a sham-control group to prove the efficacy and/or effects of this therapy.

There are important considerations for future studies considering the use of tcVNS. For instance, the individual's seizure type, client education to ensure adequate delivery of the stimulation, habituating the dog to the tcVNS, establishing the therapeutic or highest tolerable dose for each individual, the number and timing of daily stimulations, and the length of the treatment. The length of the treatment is of particular importance because tcVNS appears to be efficacious after several months of treatment based on studies in people (5, 75, 76). Ideally, future studies looking at tcVNS efficacy in dogs should consider several months of randomized, sham-controlled treatment in epileptics with similar seizure types.

This study revealed that the use of a handheld tcVNS device in healthy dogs could result in measurable and recordable physiologic changes in brain activity and HRV detected by EEG and Holter monitoring, respectively. As a pilot study, the impact of confounding factors was minimized but not completely excluded. These results are encouraging and warrant further investigations to confirm these findings in a larger cohort of dogs and to clarify the potential clinical relevance of this treatment modality.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol was reviewed and approved by the institutional Animal Care Committee of the University of Guelph (AUP 4265) and follows the Canadian Council on Animal Care Guidelines. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

GC, FJ, and CC: conception and design. GC, LG, SF, GM, and FJ: acquisition of data and drafting the article. GC, LG, SF, CC, GM, and FJ: analysis and interpretation of data, revising article for intellectual content, and final approval of the completed article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.878962/full#supplementary-material

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Conflict of Interest: CC is a paid consultant to electroCore, Inc. (Rockaway, NJ, United States) and is co-founder and chief scientist of Convergent Medical Technologies, Inc. (Oyster Bay, NY, United States), which is developing wearable closed-loop neuromodulation therapies for the veterinary market.

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REVIEW
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Neurostimulation as a Method of Treatment and a Preventive Measure in Canine Drug-Resistant Epilepsy: Current State and Future Prospects

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Modulation of neuronal activity for seizure control using various methods of neurostimulation is a rapidly developing field in epileptology, especially in treatment of refractory epilepsy. Promising results in human clinical practice, such as diminished seizure burden, reduced incidence of sudden unexplained death in epilepsy, and improved quality of life has brought neurostimulation into the focus of veterinary medicine as a therapeutic option. This article provides a comprehensive review of available neurostimulation methods for seizure management in drug-resistant epilepsy in canine patients. Recent progress in non-invasive modalities, such as repetitive transcranial magnetic stimulation and transcutaneous vagus nerve stimulation is highlighted. We further discuss potential future advances and their plausible application as means for preventing epileptogenesis in dogs.

Keywords: drug-resistant epilepsy, dogs, vagus nerve stimulation, deep brain stimulation, transcranial magnetic stimulation, seizure, epileptogenesis

INTRODUCTION

Epilepsy is the most common neurological brain disorder affecting both humans and non-human animals, with a prevalence in the human population of 0.64% in the active form or 0.76% with cases in remission (lifetime prevalence) (1), and in dogs 0.6–0.75% of the general dog population (2). However, the mere presence of genetically very homogenous purebred populations favors a more frequent occurrence of epilepsy in some canine breeds. Here, the prevalence can range from 3% up to 18% (2) or even 33% as described in a family of Belgian shepherd dogs (3). The high prevalence rates underscore the relevance of this condition for veterinary practice.

Epilepsy poses a significant challenge for veterinary and human medicine, in part because of the high rates of resistance to first and second line anti-seizure medications. The occurrence of drug-resistant epilepsy (DRE) has been reported in 13.7% of the community out-patient and 36.3% of the clinic-based human population (4), and similar numbers are assumed to apply in dogs (5). Many hypotheses exist regarding the pathophysiology of DRE, including alterations in blood

brain barrier's multidrug transporter expression, pharmacokinetics, pharmacodynamics, genetic variability, functional changes of neural networks and intrinsic severity of the disease, as well as involvement of inflammatory processes (6, 7). Most third line therapeutic approaches aim to circumvent some of those challenges. Common treatment approaches in human medicine include dietary approaches, brain surgery and neurostimulation (6, 8). While the first two approaches are relatively easy to implement in veterinary practice (9, 10), surgery and neurostimulation remain problematic because of their cost, time, and high level of skills required. However, the growing body of evidence for efficacy of neurostimulation techniques in human patients is raising awareness and interest in this therapeutic approach among veterinary practitioners. Therefore, it is of interest to know, which techniques have already been applied in canine patients with DRE, to understand their advantages and disadvantages, and to develop a road-map for their further development and assessment in canine patients.

First mentions of neurostimulation as a therapeutic method date back to the first century CE. At that time, electric fish attachment to skin was used to relief pain in patients (11). Advances in understanding of physics of electricity in the late nineteenth and early twentieth century revived interest in neurostimulation, which became a popular topic in the 1950s and 1960s when various devices, including those for epilepsy management, were developed (12–17). However, although significant technological improvements have been made in recent decades, our understanding of the mechanism of action of neurostimulation in the context of many diseases remains vague.

Neurostimulation can be performed both in the peripheral and in the central nervous system. While first one is e.g., performed in cases of neuropathic pain (18, 19), for nerve regeneration after injury (20) and to re-establish sensation in people with prostheses (21), central stimulation serves alleviation of symptoms of e.g., tremor diseases (22–24), neuropsychiatric disorders (25–27), pain (28, 29) and epilepsy (30–32). Vagus nerve stimulation (VNS), deep brain stimulation (DBS), and transcranial magnetic stimulation (TMS) are the current methods applied and described in veterinary medicine. Therefore, the review article has focused on these three therapy options.

Neurostimulation exerts effects on nervous tissue at cellular, molecular and structural levels. Mathematical modeling of high frequency stimulation in neural networks revealed its stabilizing influence on cells (33). Neural circuits showed reduced susceptibility to sudden transitions into oscillations usually marking the onset of a seizure. Moreover, inhibitory cells were recruited more strongly than excitatory cells, putting the system in an "anti-seizure state" (33). This mechanism may be the basis for acute seizure termination after application of high frequency stimulation. Brain stimulation also led to changes in connectivity of the brain inside and outside of epileptic foci and different protocols led to promotion or suppression of circuit synchronicity (34, 35).

Stimulation of neural tissue alters not only its electrical properties but also its chemical microenvironment. Several studies describe its modulatory influence on release and production of neurotransmitters, extracellular vesicles, brain-derived neurotrophic factor (BDNF) and on receptor function (36–40). Similarly, neurostimulation promotes glial cell activation, astrocytic signaling and proliferation of neuronal progenitor cells (41, 42). This might serve as a double-edged sword in the process of epileptogenesis, starting regenerative processes in the brain on one hand, which on the other hand might lead to creation of hyperexcitable networks, when they turn abnormal, as observed in rodent models of epilepsy and epileptogenesis (43, 44). However, early concerns about therapeutic electrical brain stimulation kindling human brain has not been seen in the class-I evidence trials of responsive neural stimulation (RNS) (45) and deep brain stimulation of anterior nucleus of thalamus (DBS) (46) in long-term human trials.

As a matter of course, the main goal of electrical stimulation of the epileptic brain is better seizure control. As can be seen from the neuronal network studies, this can be achieved either by stopping a developing seizure or by preventing its occurrence in the first place. The goal can be achieved either by targeted stimulation ideally before a seizure manifests (using sophisticated prediction algorithms), or by providing a cumulative long-term anti-seizure effect of regular continuous stimulations. Since long-term complete freedom from seizures is rarely achieved with electrical stimulation, therapeutic success can be difficult to define and quantify. Moreover, it often depends on patient's age, sex, and individual variability (47). Particularly important in human epilepsy is the impact of epilepsy on mood, memory, and quality of life. While rarely achieving complete seizure freedom the class-I evidence trials in humans demonstrate improved quality of life.

The need for individualized decisions is also evident when it comes to selection of the optimal method of neurostimulation: not every patient will be eligible for surgery or anesthesia, so electrode implantations might be contraindicated in these cases. Understanding the advantages and disadvantages of the most commonly used neurostimulation methods will certainly be beneficial to many veterinary neurologists. Learning from veterinary researchers conducting pilot studies in canine patients and from experienced human neurologists applying neurostimulation approaches in their clinics will be useful for applying neurostimulation in their veterinary research and practice.

VAGUS NERVE STIMULATION

Vagus nerve stimulation (VNS) as treatment of human epilepsy was first introduced in 1988 (48); however, initial trials of external stimulation of the vagus nerve date more than 100 years earlier (49). Even before the first implantation in humans, Zabara managed to attenuate seizures evoked by injections of strychnine or pentylenetetrazole (PTZ) in dogs (50), which paved the way for further clinical trials. VNS got approval for management of epilepsy in Europe in 1994 and in the USA in 1997 (49) and currently, it is being used by more than 100,000 patients worldwide (51).

TABLE 1 | Current neurostimulation parameters and outcomes in veterinary medicine.

Authors (year)	Intervention	Study design	Participants	Inclusion criteria	Parameters	Main outcomes	Side effects
Muñana et al. (59)	VNS	Double-blinded placebo-controlled crossover study	10 owner-kept dogs, randomized allocation	Onset 1–5 years, at least 1 year seizure history, frequency at least 5 seizures/months, no longer seizure-free than 2 weeks or clusters 1/month; current treatment with ASD (normal serum conc.), at least 15 kg	0.25 to 1.0 mA, 30 Hz, pulse width 500 μs, ON: 30 s, OFF: 5 min	13 week treatment—no difference; last 4 days—decrease in seizure frequency (34.4%)	Intraoperative: bradycardia, asystole, apnea; postoperative: seroma, device migration, Horner's syndrome
Martlé et al. (60)	VNS	Placebo-controlled crossover study, single-blinded for PTZ test	8 experimental Beagle dogs, randomized paradigms	No history of neurological or other diseases	Output current: as high as possible without cough; ON: 7 s, OFF: 18 s; *rapid cycling standard VNS*: 30 Hz, pulse width 500 μs; *microburst VNS*: 300 Hz, pulse width 500 μs, 3 pulses/burst, inter-burst interval: 0.4 s	Increase of CSF norepinephrine conc. 1 h after stim. in standard (67%) and microburst (76%); no difference in dopamine and serotonin conc.; no difference in PTZ threshold	Muscle tremors and spasm of left thoracic limb (one dog)
Martlé et al. (53)	VNS	Single-blinded placebo-controlled crossover study	10 experimental Beagle dogs, randomized paradigms	No history of neurological or other diseases	Output current: as high as possible without cough; ON: 7 s, OFF: 18 s; *rapid cycling standard VNS*: 30 Hz, pulse width 500 μs; *microburst VNS*: 300 Hz, pulse width 500 μs, 3 pulses/burst, inter-burst interval: 0.4 s	Hypoperfusion of left frontal and right parietal cortices in microburst	Seroma, hoarseness, Horner's syndrome (exclusion criteria)
Harcourt-Brown and Carter (61)	VNS	Non-blinded prospective cohort study	16 owner-kept dogs, non-randomized allocation	Tier II diagnosis of idiopathic epilepsy	0.25 to 1.5 mA (*slow ramping*: increase every 1–3 weeks; *fast ramping*: 8–12 h), 30 Hz, pulse width 250 μs, ON: 7 s (30 s), OFF: 1.8 min (5 min)	14 dogs reached 1.5 mA (72 days fast vs. 77 days slow)—no effectiveness of seizure frequency decrease was evaluated	Seroma, coughing, muscle fasciculation, abnormal tongue position and dysphagia (one dog), lead twisting and breaking
Hirashima et al. (62)	VNS	Case report	1 owner-kept Shetland sheepdog	Tier III diagnosis of idiopathic epilepsy	0.25 to 0.75 mA, 20 Hz, pulse width 250 μs, ON: 30 s, OFF: 5 min (1.8 min)	87% reduction of focal to generalized tonic-clonic seizures; 89% reduction of focal to generalized tonic-clonic seizures clusters; 76% reduction of days with a focal to generalized tonic-clonic seizures; no generalization of focal seizures upon magnet use	Cough during stim.
Robinson et al. (63)	Non-invasive VNS	Non-blinded prospective cohort study	14 owner-kept dogs, randomized allocation	Tier I or tier II diagnosis of idiopathic epilepsy	60 mA (at the skin level), 5 5,000 Hz pulses repeated at 25 Hz for 90–120 s 3 times a day	Four dogs with seizure frequency reduction ≥50%, 9/14 reduction, 1/14 no change, 4/14 increase	Hoarseness and trembling of left thoracic limb (one dog), progressive behavioral changes (one dog)
Zamora et al. (64)	DBS	Case report	1 owner-kept mixed-breed dog	Tier II diagnosis of idiopathic epilepsy	*Basal stim. during awakefulness and active*	Prevention of SE and reduction of coherent cluster	Involuntary motion during HF stimulation

(Continued)

TABLE 1 | Continued

Authors (year)	Intervention	Study design	Participants	Inclusion criteria	Parameters	Main outcomes	Side effects
					phases: 13 Hz 0.5 (day) or 0.7 (night) mA; *elevated stim. during sleep phases:* 13 Hz, 1.3 mA; *high-amplitude HF stim. to terminate seizures*: burst of 130 Hz, 1.5 mA	seizures during the follow-up phase of 7 months	
Charalambous et al. (65)	rTMS	*Trial I*: single-blinded placebo-controlled prospective study; *trial II*: open-labeled uncontrolled prospective study	*Trial I*: 12 owner-kept dogs, randomized allocation; *trial II*: 5 owner kept-dogs, non-randomized (dogs from trial I sham group)	Tier I or tier II diagnosis of idiopathic epilepsy	1 Hz, 90 pulses, 18 trains/day, 5 days	*Trial I*: reduction in the monthly frequency of seizures and seizure day; *trial II*: as above, effect lasting 4 months	No treatment-related side effects were reported

ASM, anti-seizure medication; conc., concentration; CSF, cerebrospinal fluid; DBS, deep brain stimulation; HF, high-frequency; PTZ, pentylenetetrazole; rTMS, repeated transcranial magnetic stimulation; SE, status epilepticus; stim., stimulation; VNS, vagus nerve stimulation.

The exact mechanism of action of VNS in epilepsy has not been fully elucidated yet, but anatomy and physiology of the vagus nerve gives insight into possible processes involved. Vagus nerve, the longest cranial nerve, arises in the nucleus ambiguous of the medulla, exits the cranium *via* the jugular foramen and extends into the neck, thorax and abdomen, where it supplies muscles and inner organs. Over 80% of vagal fibers carry sensory information from the viscera toward the brain (afferent fibers), while only around 20% of fibers are responsible for motor signaling (efferent fibers) (49, 52). Afferents terminate in the nucleus of solitary tract, which projects to multiple brain regions, among which the most crucial for the anti-seizure effect seem to be locus coeruleus and raphe nuclei (52). These regions are strongly activated by VNS and they are heavily engaged in production of neurotransmitters, such as noradrenaline and serotonin, which further stimulate interneurons to release gamma-amino butyric acid (GABA), increasing seizure threshold of neurons. Other potential mechanisms of anti-seizure action of VNS include changes in blood flow in regions correlating with seizure reduction (52–54), up-regulation of neurotrophin production (55) and anti-inflammatory effects (56, 57).

Histologically, the vagus nerve is composed of A-, B-, and C-fibers, the first two types being myelinated. Myelination and diameter of fibers (the largest in A-fibers, the smallest in C-fibers) directly translates in their various stimulation thresholds. Recordings from de-sheathed vagus nerves in healthy dogs placed amplitude thresholds to evoke action potentials at 0.4 mA for A-fibers, for fast B-fibers: 1.6 mA, for slow B-fibers: 3.8 mA and for C-fibers: 17 mA (58). Since current amplitude used for invasive VNS in dogs ranges in literature from 0.25 to 1.5 mA (**Table 1**), it can be assumed the effects of stimulation are mostly associated with activation of A- and fast B-fibers, consisting of motor and sensory afferent fibers (58).

In human neurology, VNS is indicated, as a third line treatment of epilepsy, when candidates meet following criteria: medically refractory seizures; adequate trials of at least 2 anti-seizure drugs; exclusion of non-epileptic events; and ineligibility for epileptogenic focus resection surgery (66). Usually, it is applied in cases of intractable focal and secondarily generalized tonic-clonic epilepsy, in epilepsy of generalized onset (including atonic seizures) and in epileptic syndromes (54). The implantable device consists of a helical electrode placed around the cervical part of the vagus nerve, a connective lead and a pulse generator, usually localized in a subclavicular region (57). Usually in epilepsy treatment, VNS is applied to the left vagus nerve due to its innervation of the atrioventricular node of the heart. The right vagus nerve innervates the sinoatrial node, the stimulation of which could lead to severe cardiac adverse effects (67). Additionally, care is taken to place the VNS electrodes distal to the superior and inferior cervical cardiac branches of the vagus nerve.

In dogs, it is impossible to spare the cardiac branches from stimulation, because they leave the nerve more distally in the thoracic cavity. Therefore, the electrodes are wrapped around the left vagosympathetic trunk, as both nerves are fused in the cervical region in this species (68) (**Figure 1**). Consequently, additional sympathetic stimulation and influence on the heart

cannot be excluded. During the surgery, the cathode is placed rostrally, the anode in the middle and anchor tether on the caudal portion (68). This configuration (proximal cathode/distal anode) stimulates predominantly afferent vagal fibers, while proximal anode/distal cathode leads mostly to the stimulation of efferents (69). Simultaneously, it does not influence vagal fibers' threshold to evoke action potentials, which only remain sensitive to the amplitude of current used for stimulation (58, 69). Pulse generator can be located dorsally on the left cervical region (68) or on thorax (61), underneath muscular fascia or muscle. Subcutaneous placing is discouraged to avoid migration and seroma formation at the surgery site (68).

In people, the vagus nerve is mostly stimulated in an open-loop fashion: duty cycle (ON and OFF periods) with additional extra stimulation delivered by an external magnet swipe delivered by the patient or caregiver for acute seizures (57). Available closed-loop stimulators utilize sophisticated algorithms to detect seizure events based on ictal cardiac activity associated with seizures (70, 71). Comparison between open- and closed-loop approaches in one cohort study of pediatric patients suggests a better response to VNS after 2 years of treatment, especially among children with generalized epilepsy (71). In dogs, closed-loop VNS has not been studied yet, the evidence from humans suggests however, it could prove beneficial, especially in long term. Additionally, it could decrease the burden of caretakers and veterinary staff, since they would not have to apply additional stimulation with external magnet swipe at the seizure onset.

Human patients with epilepsy undergoing VNS experience a decrease of seizure intensity, seizure duration and a shortening of the post-ictal period (54, 57, 72, 73). The main outcome crucial for the success of anti-seizure therapy, namely reduction of seizure frequency by ≥50%, is reported in ∼60% of patients (73) and this effect increases with time (49), often requiring more than half a year for maximal effect (74). Long-term studies demonstrated an improvement of seizure frequency reduction after 1 year of treatment as compared to 3 months stimulation (75, 76) and it reached its peak after 2 years of VNS (77). Secondary effects associated with VNS include improvement of mood, cognition and memory (52, 78–80) as well as lowering of anxiety (78, 81). More recently VNS has been shown to reduce the incidence of sudden unexplained death in epilepsy (SUDEP) (82). Evidence of VNS effects in canine epilepsy is much less abundant than of those gathered from human patients, nevertheless this mode of stimulation has already proved beneficial for dogs with DRE. In the first clinical study published in 2002 by Muñana et al. 10 dogs with DRE demonstrated a decrease in mean seizure frequency by 34.4% in the last 4 weeks of 13-week long therapy (59). Four of nine dogs showed a reduction of seizure frequency by ≥50% (so-called good responders) in this period, while two of them responded in that way during the whole study period (59). This study has shown VNS to reduce seizure frequency in a subpopulation of dogs with DRE, but it is unknown if the seizure suppressing effect increases further, like in people, in the first 6 to 8 months or if VNS remains effective long term. Hirashima et al. recently published a case study with a longer follow-up period (62). A 5-year old Shetland sheepdog had focal seizures and generalized seizures with focal onset for 4 years before implantation of the VNS system. The study followed the patient from 3 months before the implantation up to 1 year after the beginning of the stimulation and described in detail protocol adjustments and their outcomes. After a 1-year follow-up the authors noticed 87% reduction in generalized seizures with focal onset, 89% reduction of focal-to-generalized cluster seizures and 76% decrease of days in which focal-to-generalized seizures appeared (62). Moreover, focal seizures did not progress into generalization, when the owner activated the VNS system externally with a magnet at their onset (62). The cognitive effect of VNS has not been described in canine epileptic patients to date. However, the treatment improved their overall quality of life (62), even in cases when the seizure frequency was not reduced (59).

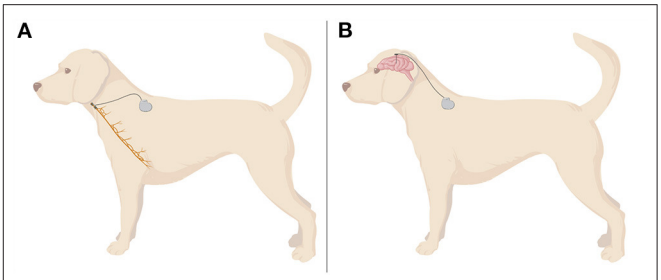

FIGURE 1 | A demonstrative illustration of assembly of invasive VNS **(A)** and DBS **(B)** in a dog. VNS electrodes are mostly wrapped around the cervical portion of left vagus nerve, whereas DBS electrodes are usually placed in thalamic nuclei. Wires and a controlling device are usually located in a dorsal cervical region. Created with BioRender.com.

The most common adverse effects of invasive VNS in humans include postoperative infection (3–6% of cases), vocal cord paresis and lower facial nerve palsy (54, 57, 74). Cardiac side effects such as bradycardia or asystole usually happen during the intraoperative device testing and cease after protocol modification (54). In dogs, side effects associated with VNS include seroma at the site of implantation (53, 59, 68), coughing (62) and muscle twitching during the treatment (60). Cardiac adverse effects such as bradycardia, asystole, and apnea were observed only during intraoperative device testing (59). A prospective cohort study by Harcourt-Brown et al. examined in detail short-term adverse effect in dogs suffering from DRE, reporting cough as the most common one, having developed in 11 out of 14 dogs (61). To eliminate severe coughs (mild and moderate coughing few to several times a day was considered tolerable) the authors introduced protocol modifications based on guidelines published for humans (83). Briefly, when intolerable (harsh or accompanied by retching) coughing was encountered, the authors first changed duty cycle (ON-time: 30 s to 7 s; OFF-time; 5 min to 1.8 min), in the case of no effect they reduced frequency (25 to 20 Hz), and as the last step they reduced current to the highest tolerable level (61). A similar approach was used by Hirashima et al. and proved beneficial for the examined patient (62). Recently, a prospective, double-blind clinical trial aiming to develop new titration protocols has been conducted in human DRE-patients (84). It could lead to better

optimization of stimulation parameters and perhaps offer better adjustment strategy for veterinary patients as well.

In recent years, popularity in human epileptology was gained by transcutaneous non-invasive VNS (nVNS), which can be applied either on skin of pinna (auricular branch of the vagus) or along the nerve trajectory on the neck (57). Transcutaneous approaches require higher current intensity, while other stimulation parameters (pulse width, frequency and duty cycles) remain usually similar to invasive VNS (85). Although extensive clinical evidence regarding nVNS is still lacking, data from preliminary human trials showed that this method engages the same neural pathways as invasive VNS (86) and yields seizure reduction in patients with DRE (87–89). nVNS requires less frequent stimulation schedules, which leads to overall less adverse effects (88). Most frequently reported adverse effects of nVNS are headache, ear/facial pain and skin irritation at the stimulation site (57). nVNS constitutes an attractive alternative approach for veterinary medicine, especially for patients not eligible for surgery. In a study published in 2020 by Robinson et al., 14 dog patients with refractory idiopathic epilepsy underwent 8- or 16-week long VNS treatment with a non-invasive stimulator along the cervical portion of the left vagus nerve (63). Nine dogs showed reduction in seizure frequency compared to baseline, among which four were considered good responders (reduction of seizure frequency by $\geq$50%) (63). Authors also mention that one patient did not show any change and four experienced an increase in seizure frequency (63). More studies would be welcome to elucidate long-term applicability and safety of nVNS in canine epilepsy. Additionally, auricular stimulation could prove beneficial, especially in patients who do not accept manipulations around their neck. However, diverse anatomy of canine ears could negatively influence standardization of such study.

VNS application extends beyond neurological diseases: a growing body of clinical evidence from human patients indicates its suitability for treatment of chronic heart failure (90, 91) or inflammatory diseases such as Crohn's disease (92, 93) or rheumatoid arthritis (94, 95). Recently, nVNS has been proposed and applied to patients with respiratory symptoms of COVID-19 to modulate their inflammatory response (96–98). VNS improved cardiovascular parameters and decreased plasma and heart tissue biomarkers associated with heart failure in a canine model of heart failure (99) and lead to weight loss in dogs (100) and minipigs (101). VNS is undoubtedly a powerful tool, which, if understood and applied properly, could bring a new value to human and veterinary medicine and lead to bidirectional translation of methodology and applications.

DEEP BRAIN STIMULATION

Intracranial deep brain stimulation (DBS) in human patients with epilepsy has been investigated for many targets including: cerebellum, subthalamic nucleus, centromedium thalamus and hippocampus (102). DBS is an approved therapy for human focal epilepsy in Europe, USA, Canada, South America and Australia targeting the anterior nuclei of the thalamus (ANT). Responsive neurostimulation (RNS) of the epileptogenic focus and network is approved in the USA (45, 103). The latter approach is further discussed below detailing the closed-loop approach interfering with ongoing ictal activity.

One major question addressed in experimental studies and clinical pilot studies related to the choice of the optimal anatomical target (104). Several potential target regions have been assessed in experimental and clinical pilot studies. Among these the ANT has been selected for a large double-blind randomized multicenter trial. In this initial trial (the SANTE trial) a gradual increase in efficacy was observed in the group of patients with a high frequency 145 Hz bilateral stimulation (46). In this group, the reduction in seizure frequency at 3 month amounted to 40.4% as compared to 14.5% in the control group without stimulation. However, group differences did not reach significance, when considering the entire 3-month stimulation phase. Trial data resulted in approval of ANT for treatment of drug-resistant epilepsy in patients with focal-onset seizures in Europe, Australia and South America, but was delayed in the US until 2018. Long-term follow-up studies provided evidence that efficacy may further increase with prolonged stimulation (105).

Adverse effects described in the initial clinical trial and subsequent studies comprised surgery-related risks including infection, hemorrhage and pain, and stimulation-related effects including headache, sleep disturbance, increased anxiety, and depression (16).

Despite the growing amount of human clinical data and the increasing interest in ANT deep brain stimulation for management of DRE, there a still various open questions concerning the mechanisms, patient selection, electrode placement techniques, and optimal programming (106, 107). In line with the role of the ANT as a network hub in limbic circuits, evidence exists that patients with temporal lobe epilepsy show a favorable response as compared to patients with frontal lobe epilepsy and epilepsies with other locations. Further clinical factors in patient selection include patient preference, operability, history of psychogenic seizure and of psychiatric disorders (106). According to an expert consensus contraindications for ANT deep brain stimulation comprise progressive etiology, psychiatric disorders, MRI contraindications (e.g., older generations electric implants such as cardiac pacemakers, insulin pumps as well as metal foreign bodies), and incomplete seizure diaries (106).

Considering the impact of high frequency stimulation on ictogenesis different mechanisms are discussed. These comprise preferential activation of inhibitory GABAergic neurons, alterations in extracellular potassium concentrations, desynchronization of neuronal activities, and reduction of the recruitment of neurons to epileptic rhythmic activity (108). Recently, attempts with continuous stimulation paradigms (109–111) and multiple thalamic targets using 4-lead devices (112) have been undertaken.

Recently a first case study has been published reporting deep brain stimulation in a canine patient with a progressive increase in seizure severity with frequent cluster seizures and repeated escalation of seizure activity into status epilepticus (64). Considering evidence that the centromedian nucleus of the thalamus (CMNT) can play a role during the early or late phase

of an epileptic seizure the stimulation electrode was placed in this thalamic nucleus (113). Case reports in human patients with super-refractory status epilepticus have already suggested DBS of the CMNT or ANT as a rescue therapy for super-refractory status epilepticus (114–119).

Building on this clinical experience, Zamora et al. (64) have applied a multi-scale, rhythm entrained stimulation of the CMNT in a 4-year old, mixed breed dog suffering from idiopathic drug-refractory epilepsy with seizure occurrence associated with awake/sleep phases (**Figure 1**). The individualized approach considered circardian and infradian rhythmicity and the modulation of biological rhythms by pathophysiological disease-associated mechanisms. The development of respective approaches is of particular interest considering the detrimental impact of DBS on sleep patterns and quality, and the frequent link between ictogenesis and selected sleep or awakening phases in many patients. Thus, an individualized approach which takes biological rhythms into account can on one hand limit adverse effects of DBS and on the other hand better prevent or stop breakthrough seizures by adjusting stimulation to the situation and vigilance states. The adjusted stimulation algorithm applied in the case study comprised three levels with increasing stimulation intensity: (1) circadian basal stimulation during awakefulness and active phases with a day- and a night-time mode (13 Hz, 0.5 or 0.7 mA, respectively), (2) elevated stimulation during the patient's more seizure-prone sleep phases to protect from sleep-associated breakthrough seizures, controlled by activity/inactivity-assessing accelerometry (13 Hz, 1.3 mA), and (3) high-amplitude, high-frequency stimulation aiming to terminate seizures activity in case of breakthrough seizures (burst of 130 Hz, 1.5 mA) (64). The latter mode can be activated by the carer by a tap on the device on the forehead (detected via accelerometry) or by a tablet computer. Implantation and application of the described stimulation algorithm in the canine patient successfully prevented status epilepticus and reduced coherent cluster seizures during the follow-up phase of 7 months (64). Another closed-loop investigational device, sensing and stimulating both hippocampi and anterior nuclei of the thalamus, was implanted in two dogs with idiopathic epilepsy (120, 121). The authors reported that the device tracked successfully seizure activity, but did not report about how successful the device was in suppressing epileptic seizures. Lessons learned from these case studies in canines have now informed human trials. However, further randomized trials are also needed in veterinary medicine to explore if DBS should be developed as a clinical therapeutic tool despites its significant costs and the need of advanced neurosurgical expertise [a summary of the equipment needed and surgical approach can be found in the Supplementary Material of (64)]. In summary, these case studies provided proof-of-concept for adaptive devices combining physiological sensing of activity and vigilance states with a chronotherapy approach. The findings suggest that it is worthwhile to further explore the therapeutic potential and tolerability of multi-scale rhythmic brain stimulation approaches and highlights the dog's role as a translational model.

REPETITIVE TRANSCRANIAL MAGNETIC STIMULATION

Transcranial magnetic stimulation (TMS) uses alternating magnetic fields to create a secondary electric field allowing for a non-invasive brain stimulation. Over the past decades repetitive TMS (rTMS) have increased clinical use with low frequency stimulation (<1 Hz) to induce reduced excitability or high frequency stimulation (>1 Hz) to achieve increased excitability (122). The principle behind this is mostly attributed to changes in synaptic plasticity in the form of long-term potentiation or depression (123). In epilepsy, rTMS focuses either on a precise epileptogenic zone or diffuse epileptogenic networks (124, 125). Cortical areas are mainly affected by the rTMS as its effect declines with the square of the distance from the coil; this is in contrast to other neurostimulation techniques such as DBS which can directly affect subcortical areas. Hence, epileptogenic networks located deeper than the cortex (e.g., cerebral or in particular thalamic nuclei) are less likely to be stimulated, unless the coil output is strong and/or the tissues between the coil and the brain (i.e., skull, muscles) are thin enough to allow penetration of the focused magnetic field up to these areas (126). However, studies have shown that rTMS can also have an impact on these subcortical areas through altering the function and connectivity of various neural networks (127–129).

Low frequency rTMS targeting a predetermined cortical area has been considered as a supportive therapy for suppression of seizures in refractory status epilepticus unresponsive to the conventional treatment options (130). Ictal rTMS in human patients provided promising results to abort ongoing prolonged seizures (ranging from few to 40–50 seizures per day) of human patients in inpatient or intensive care units. In a case report (131), one patient was treated with rTMS for 8 days (0.5 Hz, 60 min), which resulted in a marked clinical improvement successfully allowing the patient to be weaned off the respirator and sent to a rehabilitation clinic after discharge. In another study (132) similar improvement was achieved with only a single train of stimulation in one of the two patients (1 Hz, 20 min), whilst another patient (1 Hz, 30 min) responded with increased seizure frequency at 72 h post rTMS after a temporary improvement at 48 h. In another patient rTMS resulted in seizure freedom on lower doses anti-seizure medications after 11 days of stimulation (1 Hz, 10 min) (133). It should be noted that the improvements reported show quite heterogeneous periods ranging from hours to months.

Interictal rTMS, on the other hand, is applied at predetermined intervals and in structured sessions. The first pivotal study of interictal rTMS reported a transient improvement of about 38% reduced incidence of seizures per week in 9 patients during the 4 weeks post-treatment (0.33 Hz, 500 pulses of 2 trains per day, 5 consecutive days) (134). A later study reported improvements only in patients with single epileptic focus (2/4 patients) after a treatment that spanned 4 weeks (0.5 Hz, 100 pulses, applied biweekly), but not in patients with multiple foci (135). Whilst such a beneficial effect was not possible to be reproduced in another study with either

single or multiple epileptic foci (136), the heterogeneous results were attributed to the differences in coil type, coil positioning, number and location of the epileptic foci (137, 138). A relatively recent controlled clinical trial (139) reported absence of any improvement after rTMS (0.5 Hz, 1,500 pulses/day, 10 weekdays) in patients with well-defined focal epilepsy during 10 weeks of follow-up period, regardless of the coil type used (8-shaped, circular or sham).

Differences in stimulation frequency (ranging 0.3 to 1 Hz), coil type (8-shaped, cone-shaped or round coils), output (>70% vs. <70%) and positioning (over epileptic focus, vertex or cerebellum) as well as stimulation period (days to weeks) and pattern (consecutive days or intermittent) in addition to the patient heterogeneity and small cohort sizes in clinical studies altogether hinder a direct systematic comparison and deduction of a standardized treatment protocol.

The first report on the use of rTMS in dogs with epilepsy was presented as an abstract during the 60th Annual Meeting of the American-Epilepsy-Society in 2006 (140). Although this study was a non-randomized uncontrolled trial and included only a very small number of subjects ($n = 3$), its preliminary results showed an increased seizure interval after stimulation compared to the baseline; however, further details on the outcome were not reported. Recently, a single-blinded randomized sham-controlled clinical trial was published by Charalambous et al. (65), which involved 12 dogs with drug-resistant idiopathic epilepsy. A round coil was used over the vertex to globally stimulate the cortex (1 Hz, 90 pulses, 18 trains/day, 5 consecutive days). Significant reductions in the monthly seizure frequency and monthly seizure day frequency were observed in the actively stimulated patients (7/12), but not in the sham treated patients (5/12). In a second trial, the sham group received active stimulation using the same parameters, which also resulted in a significant improvement. The positive effects lasted for 4 months, and no treatment-related side effects were reported. These results are quite encouraging compared to the discrepant reports in human studies. Due to practical reasons, canine patients, unlike human patients, invariably require sedation, which attenuates the extent of cortical excitation achieved by TMS (141). Although anesthetic drugs can suppress the neuronal activity (142–144), neuronal effects of rTMS have been shown in anesthetized rats (145). In an experimental study in dogs, an increase in the cerebral blood flow at the stimulation site was detected under both anesthesia and sedation, with higher but shorter increases in dogs under sedation (146). The study showed that, despite the effect of anesthesia and sedation on the neural networks, comparable and clinically relevant increases on the cerebral blood flow can be achieved in dogs when stimulated with rTMS.

SEIZURE DETECTION AND FORECASTING AND ITS APPLICATION IN NEUROSTIMULATION

A fundamental gap in epileptology is the lack of accurate seizure diaries. In fact, all pharmacologic and neurostimulation device studies to date have relied on patient diaries despite their unreliability (147, 148). While NeuroPace RNS and Medtronic Percept have recording capabilities, they do not reliably provide accurate seizure diaries (149, 150).

Recent device advances including continuous intracranial electroencephalography (iEEG) streaming, embedded and off-the-body detection algorithms and increasing on device data storage are poised to overcome this important engineering gap (151–153).

The potential importance of seizure forecasting is widely recognized (154). Evidence from RNS Neuropace Inc. investigations support that seizures are difficult to stop once they are detected on clinical iEEG macroelectrodes. In clinical practice this generally leads to using a highly sensitive detector resulting in >100 responsive electrical stimulations a day for optimal efficacy. Forecasting seizures with relatively good sensitivity has been demonstrated in canines (147, 155) and humans (148, 156) using continuously recorded iEEG. This has opened a potential new therapeutic window where neurostimulation or pharmacological treatments could be adjusted according to the probability of seizure occurrence (157).

The advances in device technology have yielded important insights into the generation of seizures. In particular, it is now well-established that seizures and seizure risk show multidien rhythms (158) in humans (159, 160) and canines (161). This important observation, that was first reported nearly 100 years ago (162), should prove useful for seizure forecasting and intelligent chronotherapy (64, 120).

FUTURE PERSPECTIVES

Neurostimulation (VNS, DBS, and RNS) are established therapies in human DRE. Transcutaneous VNS and TMS appear well-tolerated, but there are currently insufficient data to support the efficacy of any of these modalities for drug-resistant epilepsy (163). Although each of the described approaches possesses its specific advantages and challenges (**Figure 2**), they all proved to reduce seizure frequency and disease burden in both human and veterinary medicine. These methods are mostly associated with mild, often local side effects, therefore should be considered as alternative long-term treatment option of DRE in canine patients. However, the application of brain stimulation is currently rather limited to halting seizures on their onset, either in an open-loop or in a closed-loop manner. A reasonable next step in the research of neurostimulation in epileptology would be exploration of its anti-epileptogenic potential and possibility of disease modification (**Figure 3**).

Empirical evidence of the influence of electrical stimulation on epileptogenic process is already available from animal models. DBS performed in irregular intervals during interictal phases slowed progression of kindling-induced epileptogenesis and decreased generalized seizure duration in rats (164). High frequency DBS applied during 3 months in a macaque model of mesial temporal lobe epilepsy decreased levels of mRNA of genes involved in focal-adhesion and extracellular matrix-receptor interaction pathway (165), known to be up-regulated in epileptogenesis (166). Low frequency stimulation improved

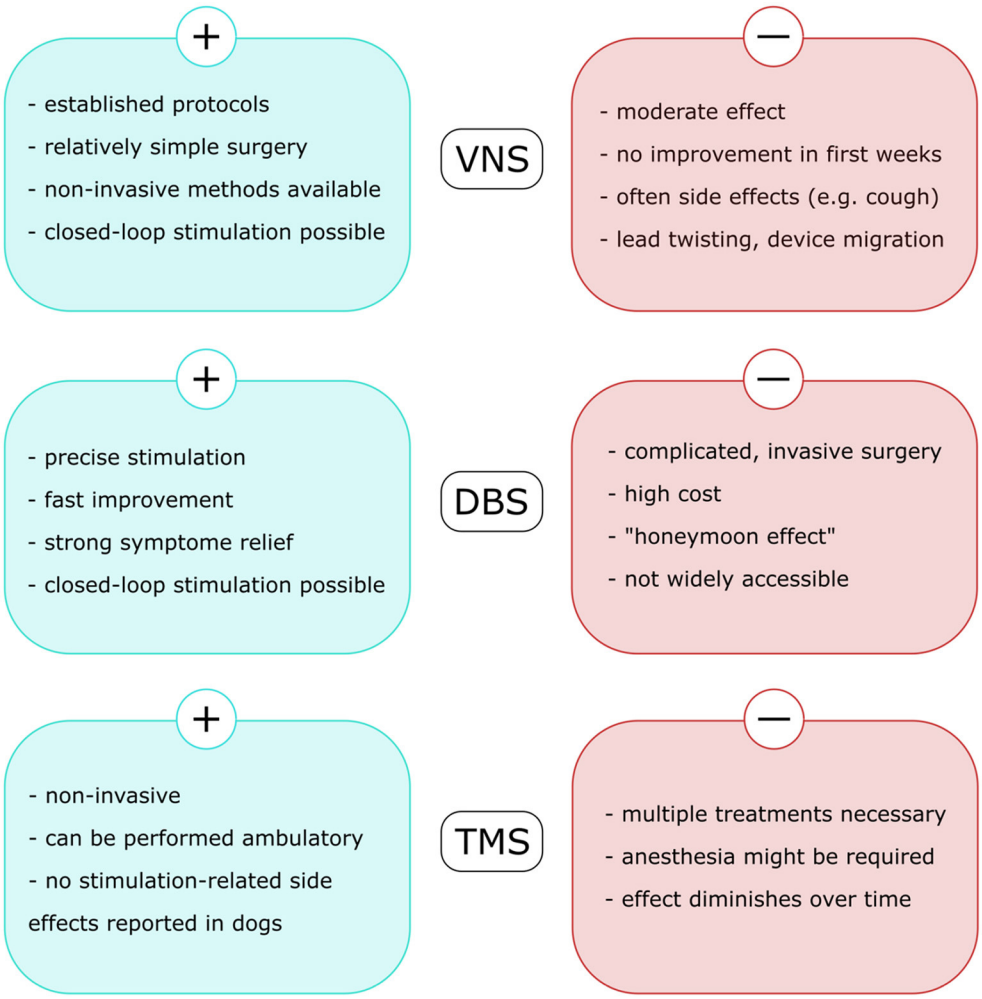

FIGURE 2 | Advantages and challenges related to each of the neurostimulation methods used in veterinary medicine to treat drug-resistant epilepsy in dogs. VNS, vagus nerve stimulation; DBS, deep brain stimulation; TMS, transcranial magnetic stimulation.

cognitive functions and memory during epileptogenesis in a kindling rat model (167), suggesting its influence on vast neuronal networks. In case of confirmation of these processes taking place in a canine brain, this might be of future interest for dogs following epileptogenic insults such as traumatic brain injury or virus encephalitis.

It is difficult to pinpoint, which exact mechanisms are involved in long-term outcomes of neurostimulation in epilepsy. Hypothetically, they could arise due to modifications in epileptic networks, their anti-inflammatory effects or due to other, more elusive processes, such as involvement of gut microbiota or anti-oxidative processes.

The effect on neuronal networks can be explained in the context of prolonged stimulation. The number of applied treatments may exceed the number of actual seizures and occur predominantly in the interictal period. Long-term iEEG recordings in patients with focal epilepsy undergoing chronic responsive neurostimulation system (RNS) therapy revealed reorganization of their brain networks: connectivity was lower between epileptic foci than in brain regions outside the foci (34). This effect was more prominent in patients with a better outcome in seizure reduction, which may suggest that neurostimulation helps disrupt pathological epileptogenic networks. However, epileptic networks could later re-adapt to the stimulation pattern, which might be responsible for the emergence of a "honeymoon phase" after the stimulation—this effect has been observed in patients with Parkinson's disease treated with DBS (168, 169). Therefore, if this change is permanent or why in some patients an alternate epileptogenic network re-organizes does require further research. In this context it needs to be considered that the outcome is also influenced by the parameters of the stimulation: e.g., in patients with Parkinson's disease, DBS performed with low frequency signals promoted circuit synchronization, whereas high frequency DBS suppressed synchronous activity (35). Long-term VNS resulted in changes in neural networks as well. Chronic VNS performed in naïve rats led to long-lasting

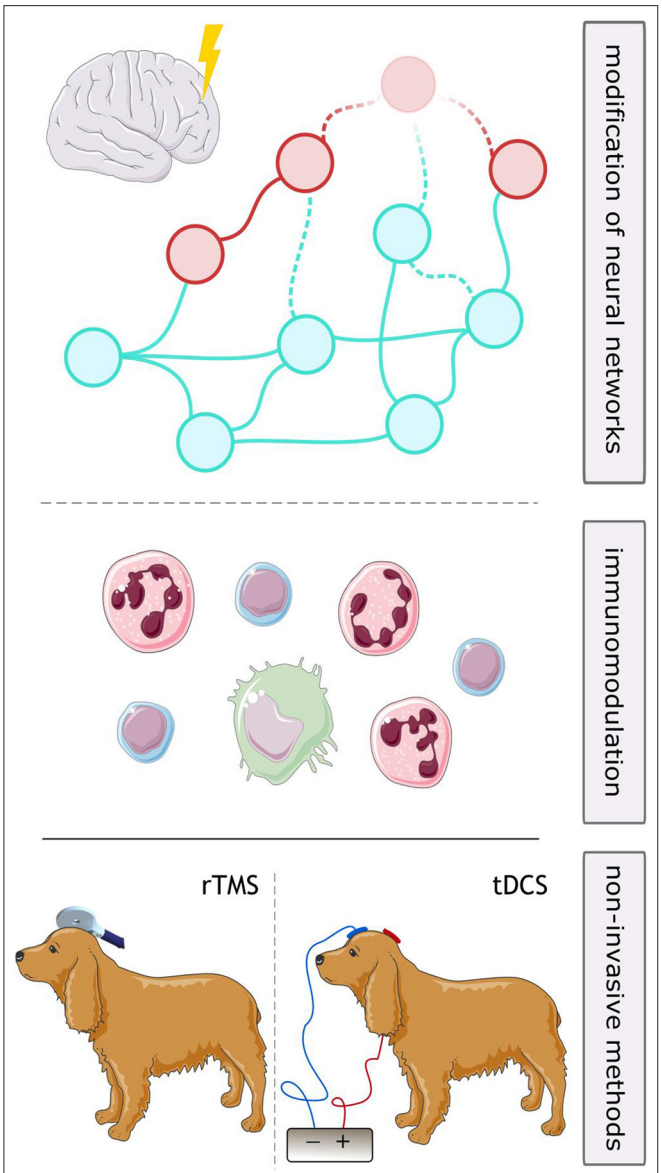

FIGURE 3 | Future perspectives for neurostimulation in drug-resistant epilepsy in dogs. Long-term stimulation might lead to disease modifying effects through alterations in neuronal networks (upper part) or anti-inflammatory effects (middle part), which might be utilized to curb epileptogenic processes. Use of repetitive transcranial magnetic stimulation (rTMS) or transcranial direct current stimulation (tDCS) might both be considered non-invasive strategies for long-term stimulation in patients not eligible for surgery.

increases of doublecortin-positive cells in the hippocampus as well as their dendritic complexity and expression of brain-derived neurotrophic factor (170), all of which are hallmarks of neuroplasticity. The data from patients additionally supports the evidence that VNS modulates neuronal networks into a less epilepsy-prone state (108). Moreover, unlike DBS, VNS does not induce a "honeymoon phase"—on the contrary, its effect seems to improve with time, which could indicate a beneficial influence of this stimulation mode on epileptic networks.

Inflammation is a process inseparably connected to epilepsy. Seizures can provoke production of pro-inflammatory cytokines, prostaglandins and chemokines by glia and neurons, by which they recruit immune cells from peripheral blood and lead to brain inflammation (171). Inversely, activation on innate immunity receptors causes rapid changes in ionic fluxes in neurons, which results in hyperexcitability and leads to onset or progression of a seizure (172). Brain inflammation also modifies expression of genes involved in production of neurotransmitter receptors, in neurogenesis and cell death and survivability (171, 172). This leads to network reorganization and changes in neuronal excitability, which can result in precipitation of the epileptogenic process.

The vagus nerve, as a part of the autonomic nervous system, is heavily involved in modulation of immune response (173). Stimulation of both vagal efferents and afferents has shown anti-inflammatory effects, attributed to cholinergic signaling (174). Experimental data supports positive effect of VNS on neuroinflammation in various animal disease models (175–178). Importantly, chronic VNS decreased levels of pro-inflammatory cytokines in hippocampus of rats with spontaneous recurrent seizures (178). Moreover, in a traumatic brain injury (TBI) rat model, VNS significantly suppressed expression of nuclear factor-kappa B (176), which is critically important for both inflammation and epileptogenesis (171). These findings could prove vital for prevention of disease development after epileptogenic insults.

Anti-inflammatory effects of DBS have also been established in animal models of epilepsy. DBS of ANT reduced blood-brain barrier disruption and albumin extravasation (179) as well as inflammation and apoptosis in rats with chemically induced status epilepticus (179, 180). It might suggest positive influence of stimulation on anti-inflammatory state of the brain is more pronounced than local inflammation caused by electrode insertion.

There might be other processes influencing to lesser extent the onset and progression of epileptogenesis, which might be targeted by brain stimulation. Recently, considerable insight has been gained into the role gastrointestinal microbiota plays in epilepsy (181). Even though short VNS did not alter gut microbiota composition in mice (182), repeated TMS of prefrontal cortex influenced rectal function of human volunteers, supposedly also affecting their microbiota (183). Another important epileptogenic factor is oxidative stress, leading to mitochondrial dysfunction and ionic dysbalance in neurons (184). Anti-oxidative effects have been reported in TMS in humans (185) and described in ischemic myocardiac injury in dogs (186), so it is plausible to assume they might also play a role in epileptic brains.

Canine patients with epilepsy have been included in clinical research involving three brain stimulation methods: VNS, DBS, and TMS. One of non-invasive stimulation methods used as treatment in humans with epilepsy and not researched in dogs as to date is transcranial direct current stimulation (tDCS). It utilizes weak (1–2 mA), constant, unidirectional flow of electrical charge applied to the scalp via electrodes mounted on a skin using an electrolytic contact medium (e.g. conductive gel) (187). The current modulates membrane potentials, leading to alteration

of neuronal excitability. The effect of the stimulation depends on the direction and intensity of the applied current—anodal (positive) tDCS generally leads to increase of cortical excitability, while cathodal (negative) tDCS results in inhibition (187). Several studies in humans showed promising results including suppression of epileptiform discharges and decrease of seizure frequency following tDCS treatment (32, 187). Among adverse effects, minor skin itching and irritation at the stimulation site were reported (187). Considering lack of invasiveness, positive stimulation results, relatively short stimulation sessions (usually 20 min a day) and lack of serious side effects described, tDCS poses an excellent opportunity for canines with epilepsy (**Figure 3**).

To introduce new methodology into veterinary medicine and further establish existing ones, more clinical research in canines is needed. This would allow development of reliable protocols to improve the anti-seizure effect and avoid undesirable side effects, so that the neurostimulation becomes more effective and more safe for the patients. Equally important is further elucidation of the mechanisms governing respective stimulation approaches. So far, thanks to the basic research on dogs, it was possible to identify parameters for VNS in dogs (58) and describe its effect on seizure threshold and monoamine concentration (60). Nevertheless, further research is vital to better understand methods applied to the patients and ascertain the best possible management of refractory epilepsy.

AUTHOR CONTRIBUTIONS

MN, HV, HP, GW, TD, and MÜ: outline of the review. MN, HV, HP, GW, MÜ, MC, SB, and SM: writing of the manuscript. TD and HV: supervision. All authors contributed to the article and approved the submitted version.

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Cross Sectional Survey of Canine Idiopathic Epilepsy Management in Primary Care in the United Kingdom

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The aims of this study are to gain insight on how primary care veterinarians in the UK diagnose and treat canine idiopathic epilepsy (IE) and what they perceive as challenges in the management of canine IE. Two hundred and thirty-five primary care veterinarians took part in this survey. The questionnaire asked about the type of practice the respondent worked in, any relevant post-graduate qualifications, how many years' experience they had in practice and the participant's canine IE caseload. Participants were asked how they diagnose canine IE, how they select antiseizure drugs (ASDs) and how they assess outcome. The questionnaire also explored which information sources they have access to for deciding on canine IE treatment, challenges that may be faced when managing these cases and areas in which more support can be provided. 94.5% of participants (n = 222/235) managed <10 canine IE cases in a year and 87.8% (n = 206/235) used phenobarbital as their first line ASD. The reported mean initial phenobarbital dose was 2.1 mg/kg (standard deviation = 0.71) every 12 h. When considering how closely participants aligned with IVETF guidelines on the topics of diagnosis, ASD initiation and outcome assessment, on average participants would score around half of the available points. 53.2% (n = 125/235) of respondents recommended neutering in canine IE and 46.8% (n = 110/235) did not. 53.2% (n = 125/235) did not recommend any additional treatments for canine IE beyond use of ASDs. 23.4% recommended Purina Neurocare diet (n = 55/235), 12.8% recommended environmental modification (n = 30/235), and 6.8% (n = 16/235) recommend medium chain triglyceride supplements. In this study participants found managing client expectations to be most challenging alongside canine IE emergency management. The main limitation of this study is the relatively low response rate and therefore the results may not reflect the entire small animal veterinary profession in the UK. However, the results of this study represent a starting point to inform educational resources and support strategies to improve quality care of canine IE in primary care.

Keywords: dog, first opinion, veterinary, antiepileptic drugs, epileptic seizures

INTRODUCTION

Canine idiopathic epilepsy (IE) is the most common chronic neurologic disease in dogs, with an estimated prevalence of 0.6–0.75% in the general canine population (1, 2). Breed specific prevalence is often much higher, being as high as 20% in Belgian shepherds in the US and 33% in certain breeding lines in Denmark (3).

Cases of canine IE are diagnosed at a young age and the disorder is often lifelong, with remission rarely achieved (4, 5). Canine IE is a complex brain disease with a broad range of impacts upon quality of life of affected dogs as well as their caregivers (6–10). Dogs with IE not only suffer from recurrent epileptic seizures but can also develop behavioral and cognitive co-morbidities (6–9). In addition, canine IE had been shown to threaten the lifespan of affected dogs (11).

Consensus statements on the diagnosis, treatment and treatment outcome of canine IE based on available scientific evidence were published in 2015, by the International Veterinary Epilepsy Task Force (IVETF) (12–14). The diagnosis of canine IE can be achieved according to 3 consequential tiers of confidence level, from lower (Tier I) to higher (Tier III), depending on available historical information, such as the age at epileptic seizure onset, normal interictal general and neurological examinations, and results of diagnostic investigations to exclude toxic, metabolic, and structural brain disease. The knowledge of genetically related dogs also affected by IE would support the confidence of the diagnosis further (12). The administration of anti-seizure drugs (ASDs) is currently the pillar of the treatment of canine IE supported by administration of a balanced consistent diet, environmental modifications, and trigger avoidance (should trigger be identified) (13). Systematic reviews and metanalyses of tolerability and efficacy of ASDs for canine IE have been published in 2014 and 2016 (15, 16). Treatment success has been defined as seizure freedom for a time span exceeding three times the longest pre-treatment inter-seizure interval and for a minimum of 3 months. Partial therapeutic success is defined as one or more of the following: prevention of cluster seizures or status epilepticus; a relevant reduction of seizure frequency based on the pretreatment seizure frequency; a reduction in seizure severity (14).

Historically the first line ASDs for canine IE included phenobarbital (authorized for use in dogs in the UK in 2001[1] and/or potassium bromide (authorized for use in dogs in the UK in 2010[2] due to their widespread availability and low cost. In 2013 imepitoin was introduced to the UK market[3]for management of recurrent single seizures in canine IE (17). Whilst yet not licensed for the treatment of canine IE, other ASDs with varying efficacy such as levetiracetam, zonisamide, felbamate, topiramate, gabapentin and pregabalin appear to be safe for use in canine IE and may be used as a second- or third-line ASDs under the Cascade in the UK (15, 18, 19).

It is unknown if and how much primary care veterinarians are aware of the currently published guidelines on diagnosis and treatment of canine IE and if this knowledge translates into clinical practice. Currently no study had investigated the prescribing preferences of ASDs by primary care veterinarians in the UK. Information about the use of ASDs has been investigated in other country-specific studies in Australia (18), Florida (19) and the Netherlands (20), providing useful insight in the use of ASDs for canine IE. The prescribing regulations in these countries differ from those in the UK.

The aims of this study are to gain insight on how primary care veterinarians in the UK diagnose and treat canine IE. This study investigates the challenges encountered by the primary care veterinarians in the treatment of canine IE to determine the need for support/educational material to improve patient and customer care as well as veterinarian confidence in the management of this condition.

MATERIALS AND METHODS

This is a prospective cross-sectional survey-based study into the management of canine IE and prescribing of ASDs in the UK by primary care veterinarians. The study (including the participants' information sheet and questionnaire) was approved by the Ethics Review Panel of the Royal College of Veterinary Surgeons (2021-75-GRIFFIN, approval granted 1st of October 2021).

The study used an online 18 question questionnaire plus one question to gain informed consent and one question to ascertain eligibility (**Table 1**). Responses were anonymous and only one response per participant was allowed. The questionnaire was developed by two experienced neurologists (one of which is a founding member of the IVETF), a primary care veterinarian and an experienced biostatistician. The questionnaire was piloted on four primary care veterinarians (not included as respondents or authors in this study) with various level of experience and expertise, including one with expertise in education and one with expertise in psychology.

The inclusion criteria for participation in this study were: being a member of the Royal College of Veterinary Surgeons (i.e., being licensed to practice veterinary medicine in the UK), working in primary care veterinary practice in the UK, and having treated at least one case of canine IE in the previous 12 months before completing the questionnaire.

Participants were recruited via internal advertisement within Linnaeus Veterinary Ltd, other veterinary groups in the UK, and large charities providing veterinary care to dogs in the UK, as well as via social media advertisement on Twitter, Facebook, and LinkedIn. Articles inviting UK registered veterinarians to complete the questionnaire were published in various UK veterinary journals (online and/ or in print) including, Veterinary records, VetTimes, Vet Report, Vet Click, vetsurgeon.org,

[1]Vmd.defra.gov.uk. 2021. Available at: https://www.vmd.defra.gov.uk/productinformationdatabase/files/SPC_Documents/SPC_141082.PDF (accessed 30 September 2021).

[2]Noahcompendium.co.uk. 2021. *Libromide® 325 mg Tablets for Dogs*. Available at: https://www.noahcompendium.co.uk/?id=-449908&template=template_printview (accessed 30 September 2021).

[3]European Medicines Agency. 2021. *Pexion - European Medicines Agency*. Available at: https://www.ema.europa.eu/en/medicines/veterinary/EPAR/pexion (accessed 30 September 2021).

TABLE 1 | Questionnaire used in the cross-sectional survey of canine idiopathic epilepsy management in primary care in the United Kingdom.

Question number	Question	Answer options
1	I understand participation in this study is voluntary and anonymous, by completing and submitting this questionnaire I consent to participate in this study.	Tick box for consent
2	I confirm that I am a Member of the Royal College of Veterinary Surgeons (MRCVS) working in primary care in the United Kingdom (UK) and I have managed at least one dog with idiopathic epilepsy in the past 12 months.	Tick box to confirm eligibility
3	Do you currently work in corporate owned or independent owned practice?	a) Corporate b) Independent c) Prefer not to say
4	Which type of primary care practice do you work in most of the time?	a) Small animal first opinion—with 24 h care b) Small animal first opinion—without 24 h care c) Mixed practice—with 24 h care d) Mixed practice—without 24 h care e) Other (open text field to complete)
5	Do you hold any postgraduate qualification in neurology or neuroscience? (Select all that apply).	a) No, I do not hold any postgraduate qualification in neurology or neuroscience b) PGCert c) CertAVP d) Master of philosophy e) Master of research f) Master of science g) PhD h) Other, please specify
6	How many years have you been working as a vet in clinical practice?	(open text field to complete)
7	Based on which of the following criteria do you diagnose canine idiopathic epilepsy most commonly (Select all that apply)?	a) Age at the time of the first observed epileptic seizure in the following range a. 6 months–6 years b. 1–5 years c. 1–10 years b) History of one or more epileptic seizures c) No significant abnormalities on haematology and serum biochemistry d) No significant abnormalities on urinalysis e) Unremarkable physical and neurologic examination between epileptic seizures f) Unremarkable magnetic resonance imaging of the brain g) Unremarkable cerebrospinal fluid analysis h) Other (open text field to complete)
8	Approximately how many new cases of canine idiopathic epilepsy have you treated in the last 12 months?	a) < 10 b) 11–25 c) 26–50 d) > 50
9	When do you initiate antiepileptic treatment in canine idiopathic epilepsy (most commonly)? (Select all that apply).	a) After 1 epileptic seizure lasting < 5 min b) After 1 epileptic seizure lasting > 5 min (status epilepticus) c) After 2 or more epileptic seizures in 24 h (cluster seizures) d) After 2 or more epileptic seizures in the last month e) After 2 or more epileptic seizures in the last 3 months f) After 2 or more epileptic seizures within the last 6 months g) Other (open text field to complete)
10	Which ONE of the following anti-epileptic medications do you most commonly prescribe as a first line long term maintenance treatment in canine idiopathic epilepsy?	a) Imepitoin (Pexion) b) Levetiracetam (Desitrend, Keppra) c) Phenobarbital (Epiphen, Epityl, Phenoleptil, Soliphen) d) Potassium Bromide (Bromilep, Epilease, Libromide) e) Zonisamide (Zonegran) f) Other (open text field to complete)
11	Which initial dosage (dose in mg/kg and administration frequency in hours) do you commonly prescribe of the long-term maintenance anti-epileptic medication selected in the above question?	(open text field to complete)

(Continued)

TABLE 1 | Continued

Question number	Question	Answer options
12	Do you use intermittent pulsed treatment with Levetiracetam (e.g., 60 mg/kg once followed by 20 mg/kg every 8 h for 2–3 days or similar protocol) alongside one of the long-term maintenance anti-epileptic medications selected in question 10?	a) No, I do not use pulsed treatment with Levetiracetam b) Yes, I often use pulsed treatment with Levetiracetam in dogs with idiopathic epilepsy that tend to have cluster seizures c) Yes, I occasionally use pulsed treatment with Levetiracetam in dogs with idiopathic epilepsy that tend to have cluster seizures d) Other (open text field to complete)
13	Where do you most commonly find information about canine idiopathic epilepsy treatment? (Select all that apply).	a) Advice from other primary care veterinarians b) Advice from a neurology specialist c) Advice from technical advisors of the relevant pharmaceutical company d) Internet search engine (specify which one) e) Neurology book/s f) Non-peer-reviewed veterinary literature (e.g., Veterinary Times, etc) g) Peer-reviewed veterinary literature (e.g., BMC Vet Research, JASAP, Vet Rec, JVIM, etc) h) Practice policy i) Social media j) University education k) Other (open text field to complete)
14	Based on which criteria do you establish if the antiepileptic treatment is successful? (Select all that apply).	a) ≥ 50% decrease in epileptic seizure frequency and/ or decreased severity of epileptic seizures after 6 months of treatment b) ≥ 50% decrease in seizure frequency and/ or decreased severity of epileptic seizure after 3 months of treatment c) ≥ 50% decrease in seizure frequency and/ or a decreased severity of epileptic seizure, following treatment for 2–3 times the longest pre-treatment epileptic seizure free interval (e.g., for a dog with a pre-treatment longest seizure-free interval of 6 weeks, the initial post-treatment follow-up duration will be 18 weeks) d) Antiepileptic medication serum concentration within the reference range e) Client satisfaction f) Good quality of life for the dog based on assessment of its owner/ caregiver g) Lack of or minimal adverse effects h) Epileptic seizure freedom for 3 months or 3 times the longest pre-treatment epileptic seizure free interval (whichever is the longer period) i) Other (open text field to complete)
15	Which ONE of the following anti-epileptic medications do you most commonly prescribe as a second line long term treatment in canine idiopathic epilepsy?	a) Imepitoin (Pexion) b) Levetiracetam (Desitrend, Keppra) c) Phenobarbital (Epiphen, Epityl, Phenoleptil, Soliphen) d) Potassium Bromide (Bromilep, Epilease, Libromide) e) Zonisamide (Zonegran) f) Other (open text field to complete)
16	Do you recommend any other treatments for canine idiopathic epilepsy? (Select all that apply).	a) None b) Acupuncture c) Cannabinoids d) Herbal Medicine (Please specify which product below) e) Environmental modification f) Homeopathy (Please specify which product below) g) Medium chain fatty acid (MCT) supplement (Please specify which product below) h) Nutraceuticals (Please specify which product below) i) Purina Neurocare diet j) Other diet, please specify which diet k) Physical therapy l) Omega 3 Fatty Acids m) Skullcap and valerian n) Other (open text field to complete)
17	Do you recommend neutering dogs with idiopathic epilepsy?	a) Yes b) No

(Continued)

TABLE 1 | Continued

Question number	Question	Answer options
18	Which aspects of management of canine idiopathic epilepsy do you find the most challenging? (Select all that apply).	a) Diagnosis b) Emergency treatment of cluster epileptic seizures and status epilepticus c) Long term routine antiepileptic treatment administration, monitoring and modulation d) Knowing when to refer e) Management of client expectations f) Idiopathic epilepsy-associated behavioral changes and/or cognitive dysfunction (which can occur in some dogs during the interictal period) g) Other (open text field to complete)
19	In which area/s of canine idiopathic epilepsy would you like more educational support? (Select all that apply).	a) Diagnosis b) Emergency treatment of cluster epileptic seizures and status epilepticus c) Long term routine antiepileptic treatment administration, monitoring and modulation d) Managing client expectations e) Management of idiopathic epilepsy-associated behavioral changes and/or cognitive dysfunction (which can occur in some dogs during the interictal period f) Educational resources for owners/ carers of dogs with idiopathic epilepsy g) Emotional support for owners/ carers of dogs with idiopathic epilepsy h) Other (open text field to complete)
20	On a scale from 1 (not needed) to 7 (extremely beneficial) how beneficial would it be for you to be able to discuss canine epilepsy cases with a specialist in veterinary neurology?	Open text field to complete with selected score (1–7)

Improve International and Vetpol. The study was advertised repeatedly between 6th October 2021 and 30th January 2022.

The questionnaire explored the type of practice the participant worked in (i.e., corporate vs. independent, small animal vs. mixed practice, 24-h care or not), any relevant post-graduate qualifications, how many years' experience they had in practice and the participant's canine IE caseload in the 12 months prior to completing the questionnaire. This was categorized as <10, 11–25, 26–50, 50+ cases. This demographic information was used to create categories to compare against responses to the other questions.

The participants could select multiple answers to questions which related to aspects of IE diagnosis, ASD initiation and assessment of treatment success. To enable analysis of responses to questions 7, 9,14, the answers to each option were scored (from −1 to +1) based on how accurately the selected answer aligned with IVETF consensus statements (12–14). A score of +1 indicated the selection of an answer as indicated in IVETF consensus statements, a score of +0.5 indicated the selection of an answer partially aligned with what indicated in IVETF consensus statements, a score of 0 indicated an answer neither aligned or opposite to the IVETF consensus. A score of −1 indicated the selection of an answer in contradiction with IVETF consensus statements. A combined score was then calculated for each respondent reflecting how closely their answers reflected IVETF guidelines.

The scores were assigned as follows (indicated by the number in parenthesis after each possible answer):

Question 7:

Based on which of the following criteria do you diagnose canine idiopathic epilepsy most commonly (Select all that apply)?

1. Age at the time of the first observed epileptic seizure in the following range 6 months−6 years (1).
2. 1–5 years (0.5).
3. 1–10 years (-1).
4. History of one or more epileptic seizures (1).
5. No significant abnormalities on haematology and serum biochemistry (1).
6. No significant abnormalities on urinalysis (1).
7. Unremarkable physical and neurologic examination between epileptic seizures (1).
8. Unremarkable magnetic resonance imaging of the brain (1).
9. Unremarkable cerebrospinal fluid analysis (1).

Question 9:

When do you initiate antiepileptic treatment in canine idiopathic epilepsy (most commonly)? (Select all that apply)

1. After 1 epileptic seizure lasting < 5 minutes (0).
2. After 1 epileptic seizure lasting > 5 minutes (status epilepticus) (1).
3. After 2 or more epileptic seizures in 24 hours (cluster seizures) (1).
4. After 2 or more epileptic seizures in the last month (0.5).
5. After 2 or more epileptic seizures in the last 3 months (0.5).
6. After 2 or more epileptic seizures within the last 6 months (1).

Question 14:

Based on which criteria do you establish if the antiepileptic treatment is successful? (Select all that apply)

1. ≥50% decrease in epileptic seizure frequency and/ or decreased severity of epileptic seizures after 6 months of treatment (0).
2. ≥50% decrease in seizure frequency and/ or decreased severity of epileptic seizure after 3 months oftreatment (1).
3. ≥50% decrease in seizure frequency and/ or a decreased severity of epileptic seizure, following treatment for 3 times the longest pre-treatment epileptic seizure free interval (e.g., for a dog with a pre-treatment longest seizure-free interval of 6 weeks, the initial post-treatment follow-up duration will be 18 weeks) (1).
4. Antiepileptic medication serum concentration within the reference range (1).
5. Client satisfaction (0.5).
6. Good quality of life for the dog based on assessment of its owner/caregiver (0.5).
7. Lack of or minimal adverse effects (1).
8. Epileptic seizure freedom for 3 months or 3 times the longest pre-treatment epileptic seizure free interval (whichever is the longer period) (1).

The scoring technique based on IVETF guidelines was used to score the questions about which criteria participants used to diagnose canine IE (maximum score 7), criteria used to decide to initiate ASDs (maximum score 4), and criteria to establish if treatment with ASDs has been successful (maximum score 6). The questionnaire also explored which information sources they have access to for deciding on canine IE treatment, challenges that may be faced when managing these cases and areas in which more support can be provided.

Participants were asked on a scale from 1 (not needed) to 7 (extremely beneficial) how beneficial would it be for them to be able to discuss canine IE cases with a specialist in veterinary neurology.

Whenever applicable, multiple-choice questions included a free text field available as "Other" to complete if a participant felt more information was required or the available options did not reflect their choices.

Because of the nature of the responses, non-parametric statistical methods were used throughout. Kruskal Wallis tests were used to assess statistical significance between categorical variables where the response was continuous or ordinal. Spearman Rank Correlation was used to assess statistical significance where both variables were continuous or ordinal. Fisher's Exact Test was used to assess statistical significance where both variables were categorical.

RESULTS

Demographic Details

Two hundred and 35 veterinarians who worked in primary care in the UK took part in this survey. 72.8% ($n = 171/235$) worked in corporate practice, 26.4% ($n = 62/235$) worked in independent practice and 0.8% ($n = 2/235$) preferred not to say. 36.6% ($n = 86/235$) worked in small animal first opinion practice with 24-h care, 54.9% ($n = 129/235$) worked in small animal first opinion practice without 24-h care and 8.5% ($n = 20/235$) worked in mixed practice (with or without 24-h care).

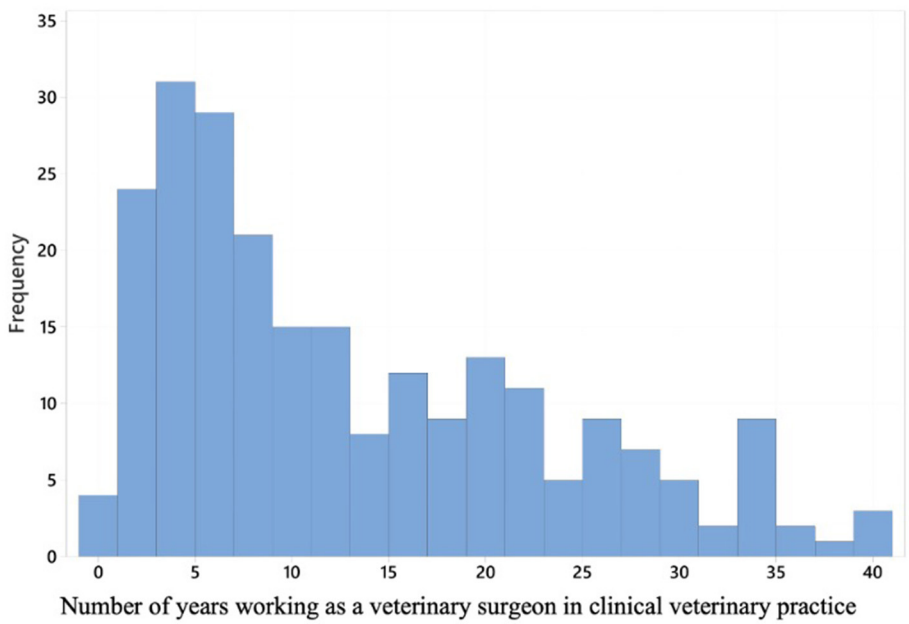

FIGURE 1 | A histogram displaying years' experience working in clinical veterinary practice for the 235 respondents.

98.3% of participants (n = 231/235) did not hold any post-graduate qualification in neurology or neuroscience. Therefore, this variable was not assessed statistically.

The mean number of years working as a veterinarian in clinical practice was 12.8 (standard deviation of 10.0) (**Figure 1**). When comparing small animal first opinion practice with 24-h care, small animal first opinion practice without 24-h care and mixed practice there was no statistically significant difference in numbers of years working as a veterinarian in clinical practice ($p = 0.67$).

94.5% of participants (n = 222/235) treated < 10 new cases of canine IE and 5.5% (n = 13/235) had treated 11–25 new cases in the 12 months prior to undertaking the study survey. Of veterinarians working in independent practice 11.3% ($n = 7/62$) treated 11–25 new cases of canine IE compared to 3.5% (n = 6/171) of veterinarians in corporate practice. This difference was statistically significant ($p = 0.05$).

Canine Idiopathic Epilepsy Diagnosis and Management

Participants from independent practices scored lower on the question regarding criteria used to diagnose canine IE (mean = 4.0 out of 7) when compared to corporate practice (mean = 4.5 out of 7). This difference was statistically significant ($p = 0.028$).

The mean score for the question regarding when a participant initiates use of ASDs in canine IE was 2.1 out of 4. No statistically significant differences were found between when a participant initiates use of ASDs and the demographic categories.

87.8% of participants (n = 206/235) prescribe phenobarbital as their first line long term maintenance ASD. Eleven percent (n = 26/235) prescribe imepitoin, 0.8% (n = 2/235) prescribed levetiracetam and 0.4% (n = 1/235) prescribe diazepam. No statistically significant differences were found between first line long term maintenance ASD and the demographic categories.

As the majority (88%) of participants selected phenobarbital as the preferred first line maintenance ASD, further data analysis was conducted in this subgroup. Of the 206 veterinarians who selected phenobarbital as their preferred first line ASD, 90.2% (n = 186/206) provided the initial dosage (dose in mg/kg and administration frequency in hours) that they commonly prescribed in long term maintenance when initiating ASDs. This was an open question where respondents could write the dose in mg/kg and administration frequency in h. The reported mean phenobarbital dosage was 2.1 mg/kg (standard deviation = 0.71) every 12 h (**Figure 2**). An initial phenobarbital dosage between 2.5 and 3 mg/kg every 12 h (which is recommended by the IVETF) (13) was used by 28.5% (n = 53/186) of participants who selected phenobarbital and provided a dose. No statistically significant differences were found between reported phenobarbital dose and the demographic categories.

When questioned about the use of intermittent pulsed treatment with levetiracetam alongside the preferred first-line long-term maintenance ASD, 33.2% ($n = 78/235$) of participants did not use intermittent pulsed treatment with levetiracetam, 26.0% (n = 61/235) often used this, and 39.6% (n = 93/235) occasionally used this. 1.2% ($n = 3/235$) of participants declined to answer this question. No statistically significant differences were found between demographic categories and the use of intermittent pulsed treatment with levetiracetam.

Forty-six percent of participants (n = 107/235) prescribed potassium bromide as a second line long term ASD, 32.4% (n = 76/235) prescribed levetiracetam and 11.0% (n = 26/235)

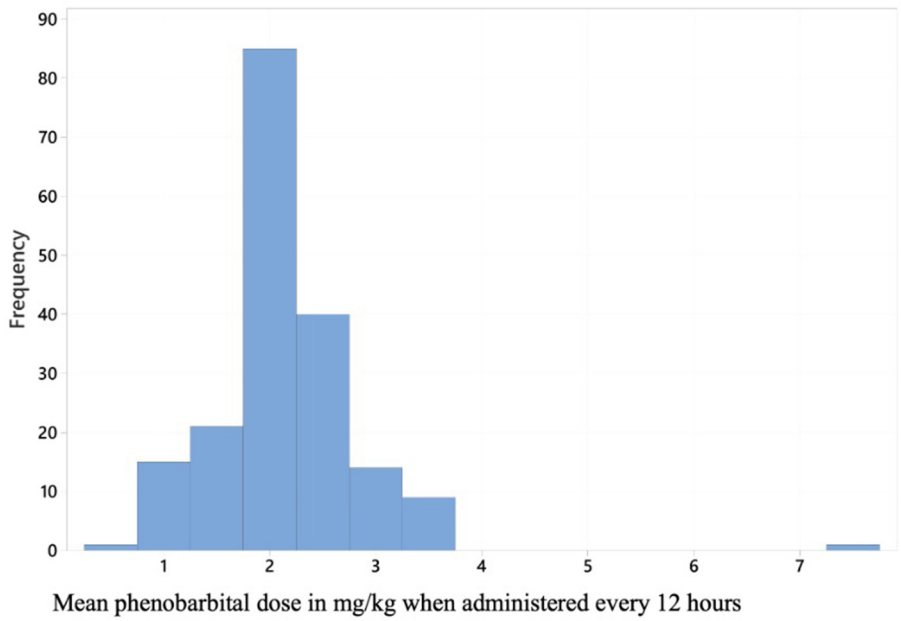

FIGURE 2 | A histogram displaying initial phenobarbital dose reported by the 206 respondents who selected phenobarbital as their preferred first line antiseizure drug for canine idiopathic epilepsy.

prescribed imepitoin. 10.6% (n = 25/235) of participants who did not use phenobarbital as their first line long term ASD used phenobarbital as their second line long term ASD. There was a difference when comparing second line ASD choice with years working as a veterinarian in clinical practice, with those with more years in practice being statistically significantly more likely to select potassium bromide ($p = 0.001$).

The mean score for the question regarding which criteria veterinarians use to establish if treatment with ASDs has been successful in dogs with IE was 2.98 out of 6. No statistically significant differences were found between which criteria are used to establish if treatment with ASDs has been successful and the demographic categories.

Fifty-three percent of participants (n = 125/235) did not recommend any additional treatments for canine IE. Of the remaining 47.0% (n = 110/235) recommendations included Purina Neurocare diet (23.4%, n = 55/235), environmental modification (12.8%, n = 30/235), medium chain triglyceride supplement (6.8%, n = 16/235), omega 3 fatty acids (6.4%, n = 15/235), other nutraceuticals (3.4%, n = 8/235), cannabinoids (2.9%, n = 7/235), acupuncture (1.7%, n = 4/235), homeopathy (0.8%, n = 2/235), physical therapy (0.8%, n = 2/235), and skullcap and valerian (0.8%, n = 2/235). Participants from small animal practice were statistically significantly more likely to recommend medium chain triglyceride supplements ($p = 0.034$) and those from mixed practice were statistically significantly more likely to recommend Purina Neurocare diet ($p = 0.037$). Environmental modification was statistically significantly more likely to be recommended by participants who had fewer years working in clinical practice ($p = 0.009$).

Neutering in canine IE was recommended by 53.2% (n = 125/235) of participants and was not recommended by 46.8% (n = 110/235). No statistically significant differences were found between neutering recommendation and demographic categories.

Perceived Challenges and Educational Needs

Forty-nine percent of participants selected managing client expectations (n = 115/235), and 45.1% (n = 109/235) selected emergency treatment of cluster epileptic seizures and status epilepticus (**Figure 3**) as the most challenging aspects of canine IE management. Participants from small animal practice with 24-h care were statistically significantly less likely to select emergency treatment of cluster epileptic seizures and status epilepticus as the most challenging aspect of canine IE management ($p = 0.039$). Participants with fewer years' experience were statistically significantly more likely to select long term routine ASD treatment monitoring and modulation as the most challenging aspect of canine IE management ($p = 0.003$).

Respondents sought information about canine IE from multiple sources, including neurology specialists (73.6%, n = 173/235), other primary care veterinarians (51.9%, n = 122/235), peer reviewed literature (51.9%, n = 122/235), neurology books (35.7%, n = 84/235), university education (35.7%, n = 84/235), advice from technical advisors of the relevant pharmaceutical company (25.1%, n = 59/235), non-peer-reviewed veterinary literature (28.5%, n = 67/235), internet search engines (14.0%, n = 33/235), practice policy (12.3%, n = 29/235) and social media (2.1%, n = 5/235). Participants from corporate practice were statistically significantly more likely to seek information from neurology specialists ($p = 0.02$) and peer reviewed veterinary literature ($p = 0.03$). Participants from small animal practices with 24-h care were least likely to seek information from social media and this was statistically significant ($p = 0.03$). Participants with fewer years' experience were statistically significantly less likely to seek information from other primary care veterinarians ($p < 0.001$), social media ($p < 0.001$) or use university education ($p = 0.002$). Participants with more years' experience were statistically significantly

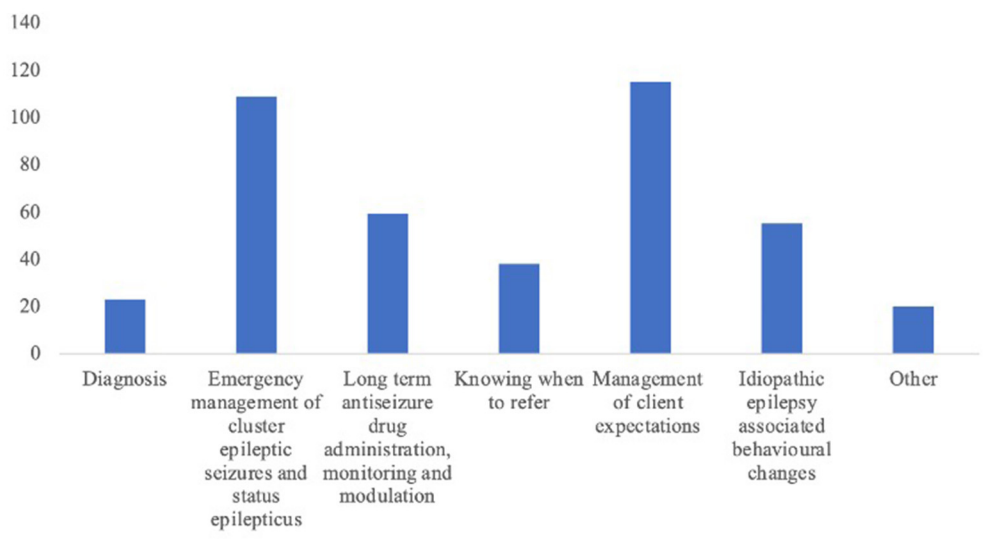

FIGURE 3 | A bar chart displaying which aspects of canine idiopathic epilepsy the 235 respondents felt most challenging.

more likely to seek information from technical advisors of pharmaceutical companies.

Sixty percent of participants (n = 142/235) selected educational resources for owners/carers of dogs with IE as the area where they would like more educational support, 52.3% (n = 123/235) selected long term ASD treatment, 48.1% (n = 113/235) would like more support in the management of neurobehavioral co-morbidities of canine IE, and 46.0% (n = 108/235) would like more support in the management of epilepsy emergency circumstances (**Figure 4**). Participants with fewer years' experience were statistically significantly more likely to want more educational support in the diagnosis of canine IE (p = 0.018) and long-term routine ASD treatment monitoring and modulation (p = 0.047).

The mean score on a scale from 1 (not needed) to 7 (extremely beneficial) for how beneficial it would be for participants to be able to discuss canine epilepsy cases with a specialist in veterinary neurology was 5.3 (standard deviation = 1.5) (**Figure 5**). This was statistically significantly negatively correlated (p = 0.014) with number of years in clinical practice. The more years in practice a respondent had spent, the less beneficial they scored discussion of canine IE cases with a specialist in veterinary neurology.

DISCUSSION

This is the first study investigating how primary care veterinarians in the UK manage canine IE and which resources and support they feel would be beneficial to them.

Phenobarbital is a widely used ASD in veterinary medicine due its efficacy, low cost, safety, and variety of formulations (13, 15, 16). In our study, 87.8% (n = 206/235) of participants reported that phenobarbital was the first line ASD most frequently used for long term treatment of canine IE. A cross-sectional survey investigating ASD preferences amongst Australasian veterinarians in 2009 (before imepitoin was introduced to the Australasian market)[4], reported that phenobarbital was used by 99% (n = 177/178) of participants for managing canine epilepsy and by 94% (n = 119/127) of participants for feline epilepsy (18). In a cross-sectional survey on ASD use in dogs with suspected IE among board-certified emergency (n = 128) and neurology specialists in North America (where imepitoin is not licensed), phenobarbital was the initial ASD of choice for 66% (n = 114/172) of neurologists and 64% (n = 82/128) of emergency and critical care specialists (19). In

[4]https://www.tga.gov.au/sites/default/files/scheduling-decisions-1407-final.pdf

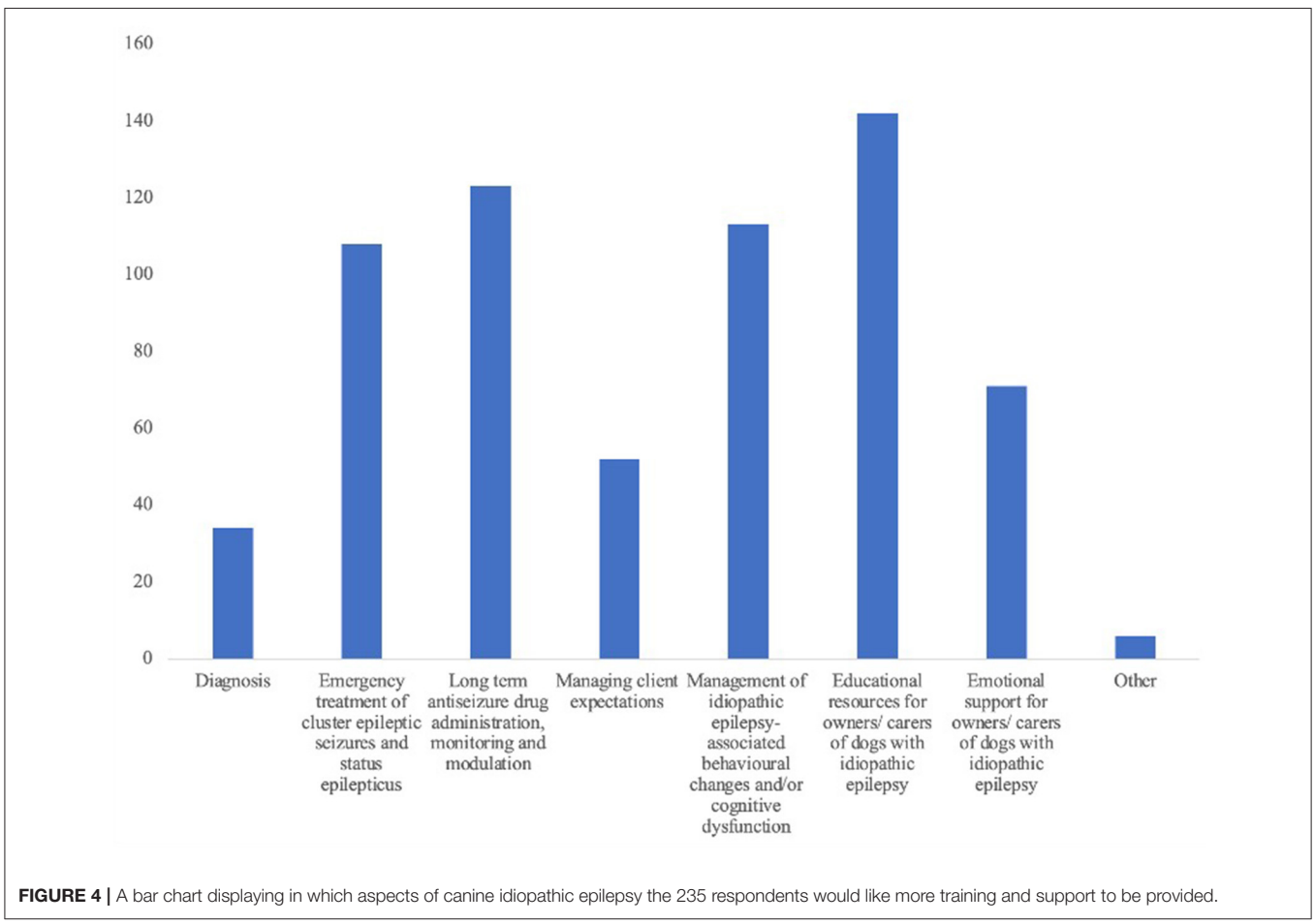

FIGURE 4 | A bar chart displaying in which aspects of canine idiopathic epilepsy the 235 respondents would like more training and support to be provided.

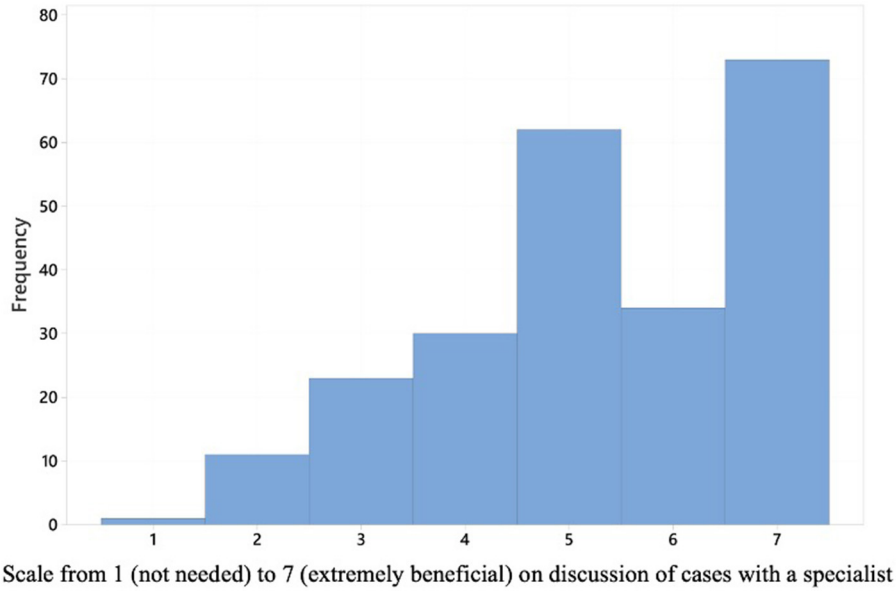

FIGURE 5 | A histogram displaying how the 235 respondents scored on a scale from 1 (not needed) to 7 (extremely beneficial) how beneficial would it be for them to be able to discuss canine idiopathic epilepsy cases with a specialist in veterinary neurology.

another web-based, cross-sectional survey (20) of Dutch primary care veterinarians working in small animal or mixed-practice in 2016, phenobarbital was used by 89% ($n = 91/102$) and imepitoin was used by 75% ($n = 77/102$) respondents in the management of cases of canine epilepsy. This survey (20) did not ask about preferred first line ASD for long term treatment of canine IE.

The reported efficacy of phenobarbital monotherapy in reducing or leading to remission of epileptic seizure occurrence in dogs with serum phenobarbital concentration within the recommended target range (20–30 mg/L) varies between 60 and 93% (13, 21–25). The mean dose of phenobarbital used by respondents was 2.1 mg/kg every 12 h which is consistent with recommendations by phenobarbital manufacturers and the BSAVA Small Animal Formulary (1–2.5 mg/kg/every 12 h) (26, 27). However, the IVETF recommend a starting dose of 2.5–3 mg/kg every 12 h based on phenobarbital pharmacokinetic studies and serum phenobarbital concentrations required to achieve seizure control in most dogs (13). Only 22.6% ($n = 53/235$) participants in our study reported 2.5–3 mg/kg every 12 h as the most used initial phenobarbital dose. This suggests the common point of reference are the product data sheets rather than IVETF consensus guidelines on treatment. More education on initial phenobarbital dosage and target serum phenobarbital concentrations may be beneficial in primary care.

IVETF guidelines were published in 2015 to provide guidance on how to diagnose canine IE, when to start treatment with ASDs, which ASDs are safe and efficacious, recommended ASDs' dosage and monitoring as well as definitions of treatment success (12–14, 28). These guidelines are based on evidence-based literature, the cascade, and the authors experiences. When considering how closely participants aligned with IVETF guidelines on the topics of diagnosis, ASD initiation and outcome assessment, on average participants would score around half of the available points. This could suggest that primary care veterinarians may have only moderate awareness of IVETF guidelines or that they find it challenging to implement them in their work setting. The guidelines themselves form detailed, lengthy documents which may seem inaccessible to read and use in a busy primary care practice. Respondents from independent veterinary practices scored significantly lower than respondents from corporate veterinary practices about diagnosis of canine IE. This may be due to structured continued professional development programs or facilitated access to veterinary neurologist specialists working in referral centers. There was no difference in ability to diagnose and treat cases based on the number of years working in clinical practice, whether the participants had access to 24-h care and whether they worked in small animal or mixed practice.

The topic of neutering in the management of canine IE remains controversial (29) and this may be the reason why 53.2% ($n = 125/235$) of participants recommended neutering and 46.8% ($n = 110/235$) did not. No statistically significant associations were found between neutering recommendation and the different demographic categories. Two studies suggest that survival is longer in dogs with IE that are not neutered (30, 31). A VetCompass study in the UK investigated associations of neutering with IE in 117 Labrador retrievers and 57 Border collies diagnosed with IE in primary care. The majority (74.2%) of neutered dogs were neutered prior to the onset of IE. Dogs intact at IE onset had longer median survival times than dogs neutered before IE onset (males, 1,436 days vs. 1,234 days: females, 1,778.5 days vs. 1,261 days) (30). In a retrospective study in Denmark, survival was significantly shorter in neutered male dogs with IE (median survival: 38.5 months) compared to intact male dogs with IE (median survival: 71 months) (31). Cluster seizures in

dogs with IE have been associated with a reduced likelihood of achieving seizure freedom, decreased survival time and increased likelihood of euthanasia. A retrospective study including 384 dogs with IE treated at a multi-breed canine specific epilepsy clinic, did not identify any association between occurrence of cluster epileptic seizures and sex or neuter status (32). In contrast, in a previous retrospective study (33) including 407 dogs with IE, intact males were twice as likely than neutered males to suffer from cluster seizures and intact females had significantly more frequent cluster seizures than neutered females. The lack of a clear indication from the evidence-based literature either in favor or opposing neutering in dogs with IE may be one of the reasons why the population of primary care veterinarians in this study is split down the middle when it comes to this difficult to navigate topic.

53.2% (n = 125/235) of participants did not recommend any additional treatments for canine IE beyond use of ASDs. When additional treatments were recommended environmental modification, Purina Neurocare diet and medium chain triglyceride supplements were the most frequently selected. Participants with fewer years' experience working as a veterinarian were more likely to recommend environmental modification. Dogs with IE can present with concurrent behavioral and cognitive abnormalities, which in some cases can become severe following the onset of IE (7–11). Dogs can become more anxious and fearful in nature (7). The cognitive and behavioral abnormalities can be present every day and may have a significant impact on the dog's and caregiver's quality of life. While changing the environment will not directly benefit seizure control (unless stress is a trigger for the seizures) it can be used to address the IE-associated co-morbidities and significantly enhance the care delivered to dogs with IE (9, 34). An online questionnaire study investigated how owners of dogs with IE manage their dog's interictal anxiety (34). This cross-sectional study showed that while 83.6% of owners reported to be using a range of approaches to manage interictal anxiety there is a lack of consistent information on how to do this and lack of support from veterinary professionals (34). There is scope that by providing better education to veterinarians on this topic that better support could be provided to the owners of dogs with IE which could lead to significant advancements in the care of these patients.

Participants from small animal primary care were more likely to recommend medium chain triglyceride supplements than those from mixed primary care practice. There is some evidence to support the use of medium chain triglycerides as a dietary supplement in drug resistant cases of canine IE (35–38). There was a small decrease in seizure frequency of some dogs in a recent randomized controlled trial (37). Currently the most effective dose of medium chain triglycerides and their ideal formulation for use in canine IE is unknown.

While dietary manipulation is commonplace by owners of dogs with IE (two thirds of owners changed the diet following IE diagnosis in one study (37) it is important that dogs with IE are fed a high-quality diet due to the long-term nature of management (37–39). A low-calorie diet is often necessary to maintain a healthy body weight in dogs with IE and long-term ASD treatment (38). Medium chain triglyceride oils may be high in fat and powders may be high in carbohydrates. While cheap and widely available products which contain medium chain triglyceride may vary greatly in the quality and quantity found within. Factors to bear in mind include that restriction of protein or fat can increase clearance and elimination of phenobarbital (39). While most dietary supplements are considered safe products, administering large quantities of a medium chain triglyceride oil may lead to gastrointestinal adverse reactions (40, 41). When administering a medium chain triglyceride oil the total diet composition should be carefully considered as there may be risk of pancreatitis associated with hypertriglyceridemia (42). Therefore, this should be done in consultation with a veterinary nutrition specialist to feed a complete and balanced diet with adequate quantities of proteins, fats, vitamins, and minerals. Participants from mixed primary care were more likely to recommend the Purina Neurocare diet. While the Purina Neurocare diet does not have a label claim for the management of canine IE, it does contain medium chain triglycerides (42).

It is promising that veterinary neurology specialists are the most common source of information about management of canine IE. Participants from corporate practices were more likely to seek information from veterinary neurology specialists and this may arise from greater access to veterinary neurology specialists if, for example, the corporate practice owns both primary care and referral care practices. This may provide an opportunity to pass on invaluable information about evidence-based case management, including the IVETF consensus guidelines and recent scientific publications. Concerningly, as the mean years' experience in practice increased participants were more likely to seek information from pharmaceutical companies which may differ from advice provided by veterinary neurology specialists.

In this study, participants found managing client expectations to be most challenging alongside emergency management of cluster seizures and status epilepticus. Participants from small animal practice with 24-h care were statistically significantly less likely to select emergency management as the most challenging aspect of canine IE management. Participants with fewer years' experience in practice were more likely to select long term routine ASD treatment monitoring and modulation as the most challenging aspect of canine IE management. This may reflect the lack of experience gained from case continuity and following a case of canine IE over several years and learning from the management of these cases. These participants also wanted more educational support in the diagnosis of canine IE and long-term routine ASD treatment monitoring and modulation. The participants overall indicated they would like more educational support in the management of cluster seizures and status epilepticus, as well as in the long-term treatment of canine IE and in the management of canine IE related co-morbidities. It is the authors' opinion that the provision of better educational resources and of support in the long-term management of canine IE will allow better management of client expectations.

The main limitation of this study is the relatively low response rate and therefore the results may not reflect the entire small animal veterinary profession in the UK. There are an estimated 27,200 veterinarians employed in the UK (43), of which, 53% work exclusively in small animal practice and 12% work in mixed practice (44). The invite to participate in the study was shared in the electronic newsletter received by 655 primary care veterinary surgeons within Linnaeus as well as widely and repeatedly promoted by various UK veterinary media during nearly 4 months. The relatively low response rate may be due challenges in practice to find free time to spend 5–10 min to undertake a survey. Primary care veterinarians in the UK are under immense pressure on a day-to-day basis with the number of pet dogs in the UK increasing from 7.6 million in 2010–2011 to 12.5 million in 2020–2021 (45). This increase applies significant time pressures in primary care practices. During the study period, the veterinary profession in the UK has also been under exceptional stress (46) due to staff shortages from COVID-19 combined with the increased workload (47). However, despite this, the results of this study provide insight into how cases of canine IE are managed in primary care and represent a starting point to inform educational resources and support strategies to improve quality care of canine IE in primary care.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Panel of the Royal College of Veterinary Surgeons (2021-75-GRIFFIN, approval granted 1st of October 2021). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD, SG, and FS contributed to conception and design of the study. LD and SG conducted data analysis with the support of a statistician. SG set up the online survey, revised and coded responses, and wrote the first draft of the manuscript with LD's support. All authors contributed to manuscript revision, read, and approved the submitted version.

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CASE REPORT
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Case Report: Anti-$GABA_A$ Receptor Encephalitis in a Dog

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Autoantibodies against neurotransmitter receptors detected in cerebrospinal fluid (CSF) and serum are increasingly recognized in people with human autoimmune encephalitis causing severe neurological deficits, such as seizures and behavioral abnormalities. This case report describes the first encephalitis associated with antibodies against the γ-aminobutyric acid-A receptor ($GABA_AR$) in a dog. A young male intact Cavalier King Charles Spaniel was presented with recent onset of initial multiple generalized tonic-clonic seizures progressing into a status epilepticus. Interictally, he showed alternating stupor and hyperexcitability, ataxia, pleurothotonus and circling behavior to the left side. Magnetic resonance imaging (MRI) of the brain showed breed-specific anatomical abnormalities. Standard CSF analysis was unremarkable. Despite treatment with multiple antiseizure medications (ASMs) seizures and behavior abnormalities sustained. Immunotherapy with dexamethasone was started on the fifth day after disease manifestation. This led to rapid improvement of clinical signs. An extensive antibody search in CSF and serum demonstrated a neuropil staining pattern on a tissue-based assay compatible with $GABA_AR$ antibodies. The diagnosis was confirmed by binding of serum and CSF antibodies to $GABA_AR$ transfected Human Embryonic Kidney cells. The serum titer was 1:320, the CSF titer 1:2. At the control visit 4.5 weeks after start of immunotherapy, the dog was clinically normal. The $GABA_AR$ antibody titer in serum had strongly decreased. The antibodies were no longer detectable in CSF. Based on clinical presentation and testing for $GABA_AR$ binding antibodies, this describes the first veterinary patient with an anti-$GABA_AR$ encephalitis with a good outcome following ASM and corticosteroid treatment.

Keywords: autoimmune encephalitis, $GABA_A$ receptor encephalitis, seizures, epilepsy, dog

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INTRODUCTION

Inflammatory autoimmune encephalopathies play a major role in canine neurology (1). They are described with the umbrella term meningoencephalitis of unknown origin (MUO), referring to a group of heterogenous sterile autoimmune encephalitides including the main histopathologically distinguishable subtypes: granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME) and necrotizing leucoencephalitis (NLE) (2, 3). Initiating trigger factors or underlying etiologies are largely unknown and a definite diagnosis is only possible with histopathological investigations of brain tissue (3).

In contrast, there is growing knowledge on autoimmune encephalitis in humans: In the last 40 years, a steadily increasing number of antibodies against neural intracellular or surface antigens in human patients with autoimmune encephalitis, often presenting with seizures and also psychiatric or behavior symptoms, have been described (1, 4, 5). Different subtypes of autoantibody associated encephalitis tend to differ in their initial clinical signs (6–8). As such autoantibodies bind to these antigens and reduce the number of accessible target receptors, a direct pathogenic influence is strongly suspected (9, 10). This increasing number of autoimmune encephalitis in Western countries is comparable to that of infectious ones (11, 12). In dogs as well, autoimmune inflammatory diseases have recently been described to outnumber infectious causes of CNS inflammation in the UK (13).

γ-aminobutyric acid-A receptors (GABA$_A$R) are pentameric intrinsic or synaptic chloride ion channels and function as an inhibitory system of the postsynaptic potential in the brain (8, 10). Genetic mutations or aberrations due to antibodies binding to subunits (α1, β3, γ2) lead to a specific reduction of synaptic GABA$_A$R, possibly inducing hyperexcitability and risk of seizures up to status epilepticus (6, 8, 14, 15). The disease appearance can depend on the affected subunit and the number of antibodies (6, 8, 16). Further symptoms described in humans can be personality and behavior changes, reduced consciousness, memory deficits or asymmetric signs like hemibody paresthesia (14, 16).

Case numbers of antibody associated encephalopathies are overall still low in human medicine and even more scarce in veterinary medicine (1, 17). Baulac et al. (18) already provided evidence of an association between GABA$_A$R dysfunction and epilepsy in humans due to a mutation in the γ2-subunit gene in 2001. Nonetheless, GABA$_A$R encephalitis is an only very recently reported disease in humans (19). In humans, a partial or complete recovery occurs in around 80% of cases with immunotherapy and early start of therapy improves prognosis (1, 8, 14). In dogs, so far only anti-*N*-methyl-D-aspartate receptor (NMDAR) 1 antibodies but no anti-GABA$_A$ antibodies were detected in two dogs with MUO (17).

This case report describes the clinical signs, diagnostic work up and outcome of the first canine anti-GABA$_A$R encephalitis in veterinary medicine.

CASE DESCRIPTION

An 1-year-old male intact Cavalier King Charles Spaniel (CKCS) was presented to the emergency service with a history of acute generalized tonic-clonic epileptic seizures progressing to status epilepticus. The owners reported acute onset of behavioral changes including unusual reactivity toward other dogs, restlessness, hyperesthesia when touched and excessive circling to the left, starting 2 days prior to presentation. The hyperexcitability was associated with anxiety and defensive aggression behavior when the dog was touched on different body parts like tail or pinnae. The dog was reported to be otherwise healthy except for a suspected food intolerance with recurrent episodes of diarrhea. He was fed with a hypoallergenic commercial diet. There was no previous travel history or a recent vaccination. The owner excluded the possibility of toxin ingestion as the dog was kept indoors and on the leash during walks.

On general examination immediately after the seizure events, the dog had an elevated body temperature of 39.7°C and a systolic cardiac murmur grade II of VI. The scrotal and inguinal skin was hyperemic.

The dog had severely reddened, moderately swollen conjunctives with unilateral subconjunctival hemorrhage and bilateral prolapse of the nictitating membranes. Ophthalmic examination revealed diffuse conjunctival chemosis in both eyes and a diffuse subconjunctival bleeding of the left eye.

The neurological examination was performed postictally. The dog showed a reduced level of mentation, a moderate head turn to the left, generalized ataxia and circling to the left. The dog reacted to touch with hyperexcitability and dysphoric vocalization, as well as compulsive circling to the left. Conscious proprioceptive placing was reduced on all four limbs. The menace response was absent and visual function was reduced. In the neuroanatomical localization a diffuse forebrain lesion potentially lateralized toward the left was suspected because of the asymmetrical clinical signs.

Hematology revealed a mild to moderate leukocytosis and a macrocytic thrombocytopenia. Serum biochemistry, glucose, clotting times, dynamic bile acid levels, urine analysis and blood pressure measurements were unremarkable.

Further examination was performed under general anesthesia. Premedication consisted of 0.15 mg/kg intravenous (IV) midazolam (Midazolam ratio 5 mg/ml, Ratiopharm GmbH, Ulm, Germany) and 0.3 mg/kg IV butorphanol (Butorgesic 10 mg/ml, CP-Pharma, Germany) as well as 0.6 mg/kg IV narcofol (Propofol 10 mg/ml, CP-Pharma, Germany). After endotracheal intubation, isoflurane E_T 0.9–1.3 Vol% (Isofluran CP, CP-Pharma, Germany) in oxygen was applied to maintain general anesthesia.

Magnetic resonance imaging (MRI, Achieva SmartParth to dStream for XR, a 3.0 T scanner, Philips Medical Systems, Best, The Netherlands) of the head depicted a mild Chiari-like malformation with flattening of frontal lobe with increased height of occipital lobe, suspected reduced volume of the caudal cranial fossa and caudal displacement of the cerebellum through the foramen magnum as well as a kinking of the craniocervical junction (20). No abnormal findings regarding inflammatory diseases or reversible postictal hyperintensities could be found in T2-weighted (w) Dixon or Fluid-attenuating inversion recovery (FLAIR), nor in T1w pre-and post-contrast, and susceptibility-weighted imaging sequences (17, 21).

The results of the cerebrospinal fluid (CSF) analysis obtained by cisternal puncture (nucleated cell count, total protein, cytology) were within normal reference ranges. The computed tomography (CT (IQon/Spectral CT, Philips GmbH, Hamburg,

Abbreviations: ASM, antiseizure drugs medication; CKCS, Cavalier King Charles Spaniel; CSF, cerebrospinal fluid; FLAIR, fluid-attenuated inversion recovery; GABA$_A$R, γ-aminobutyric acid-A receptors; GABA$_B$R, γ-aminobutyric acid-B receptor; IgG, immunoglobulin G; IV, intravenously; LGI1, leucine-rich glioma-inactivated protein 1; MUO, meningoencephalitis of unknown origin; NMDAR, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; w, weighted.

Germany) of the thorax revealed a markedly hypoattenuating area next to the tricuspid valve without contrast enhancement. An artifact, turbulence, injected gas or a thrombus was suspected. Repeated echocardiography (first under anesthesia and later when awake) did not confirm any cardiac abnormalities except for mitral regurgitation.

In the 2 days after general anesthesia, the dog remained stuporous with episodes of hyperexcitability. Seizure activity and transient obsessive circling to the right continued despite treatment with ASMs such as diazepam followed by phenobarbital. For seizure management, 2 mg/kg IV phenobarbital (Luminal, Sanacorp Langenhagen, Germany) was administered twice daily and during cluster seizures 2 mg/kg IV diazepam (Diazepam 10 mg/2 ml, B. Braun, Germany) and additional boli of 2 mg/kg IV phenobarbital. It was noticed that the epileptic seizures were triggered by stress during handling or touch.

To ensure nutritional supply, a parenteral gastrostomy tube was placed under general anesthesia with a premedication consisting of 4.8 μg/kg IV fentanyl (fentanyl 0.1 mg/2 ml, Sanacorp Langenhagen, Germany) and 0.5 mg/kg IV narcofol (Propofol 10 mg/ml, CP-Pharma, Germany) and isoflurane E_T 0.9–1.3 Vol % (Isofluran CP, CP-Pharma, Germany) in oxygen via an oxygen mask was applied to maintain general anesthesia.

Due to the lack of improvement, intravenous therapy with 0.5 mg/kg IV dexamethasone (Dexamethason, CP-Pharma, Germany) was started 5 days after clinical manifestation followed by 0.2 mg/kg IV. Gradual clinical improvement occurred over 3 days after start of dexamethasone administration. Then, the level of mentation and the behavior had improved significantly and were close to normal. His responsiveness to stimuli was still limited but he was aware of his surroundings and was interacting. At this timepoint, the dog showed a normal response to touch and handling rather than hyperexcitability or stupor as seen at presentation. The menace response deficits resolved. However, the head turn to the left and intermittent circling were still present. After 10 days of hospitalization, the dog was discharged with normal mentation, but still recurring intermittent circling to the left and bilateral proprioceptive deficits in the pelvic limbs.

Further examinations for possible pathogens such as tick borne encephalitis immunoglobulin G (IgG) antibodies enzyme-linked immunosorbent assay in CSF, Borna virus polymerase chain reaction (PCR) in ethylenediaminetetraacetic acid blood, fecal examination for parasites by flotation and emigration method according to Baermann, Angiostrongylus vasorum antigen and Anaplasma phagozytophilum PCR in serum were unremarkable.

Due to acute onset of epileptic seizures associated with marked behavioral changes resembling a psychosis in people, panels of neural antibodies were measured in serum and CSF by coauthors CGB, CB. An extensive antibody profile demonstrated a neuropil staining pattern on a tissue-based assay compatible with $GABA_AR$ antibodies (**Figure 1**); the diagnosis was confirmed by binding of serum and CSF to $GABA_AR$ (α1β3 subunits) transfected Human Embryonic Kidney cells [all assays from Euroimmun, Lübeck, Germany, used with in-house-protocols; for the canine material, secondary anti-dog Immunoglobulin G (IgG) was used]. The serum titer was 1:320, the CSF titer 1:2. Antibodies against NMDAR, leucine-rich glioma inactivated protein 1 (LGI1), glutamic acid decarboxylase like γ-aminobutyric acid-B receptor ($GABA_BR$), IgLON family member 5, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, dipeptidyl-peptidase-like protein-6, contactin-associated protein-2, glycin receptor, metabotropic glutamate receptor 5 and metabotropic glutamate receptor 1 tested negative.

The dog showed continuous improvement with ASMs and 1 mg/kg oral corticosteroids (Prednitab vet. 5 mg, CP-Pharma, Germany) once daily. He remained seizure free, and behavior normalized. Four and a half weeks after discharge, the dog was neurologically normal at physical examination. Serum $GABA_AR$ antibodies were found at 1:20, and CSF was negative.

At a follow-up telephone conversation 8 months post diagnosis, owners reported the dog to be clinically normal without recurrence of epileptic seizures or behavioral abnormalities except for separation anxiety. The dog remained treated with 1.7 mg/kg phenobarbital twice daily. Corticosteroid therapy was tapered after 7 months to 0.7mg/kg daily, but then the dog developed a bacterial urinary tract infection and the local veterinarian tapered the corticosteroid dosage further down to 0,1mg/kg daily without a relapse of clinical signs.

DISCUSSION

This case report describes a 1-year-old CKCS presented with a history of acute progressive forebrain signs. He showed generalized tonic-clonic epileptic seizures, episodic hyperexcitability alternating with episode of stupor, and intermittent left circling behavior. Examination for neural autoantibodies in serum and CSF revealed $GABA_AR$ antibodies. The dog improved on immunotherapy with corticosteroids, after the initial ASM treatment failed.

In the last years, the number of cases being affected by neural auto-antibodies has increased in veterinary medicine (12). A very famous case was Knut the polar bear from the Berlin Zoo who was post mortem diagnosed with anti-NMDAR encephalitis (22). This led to further investigations. An age dependence of NMDAR antibody seroprevalence was found in dogs, cats, rats, baboons, and rhesus macaques (23). Furthermore, a direct link between autoimmune limbic encephalitis in cats with seizures and LGI1 autoantibodies has been suspected for years (24, 25). Stafford et al. (17) detected NMDAR antibodies in the CSF of two dogs with MUO. In addition, in dogs with necrotizing encephalitis, antibodies against astrocytic glial fibrillary acidic protein were recognized (26, 27). However, encephalitis associated with $GABA_AR$ has not been described in dogs until now.

In contrast to anti-$GABA_BR$ encephalitis, in which mainly middle-aged human patients with epileptic seizures as core symptoms are affected (28), $GABA_AR$ encephalitis is seen in a broad age range, even having been diagnosed in babies with a few months of age (14, 19). In children, nearly all patients suffer from generalized epileptic seizures, some also have a movement disorder. A neoplasm can usually not be found in affected

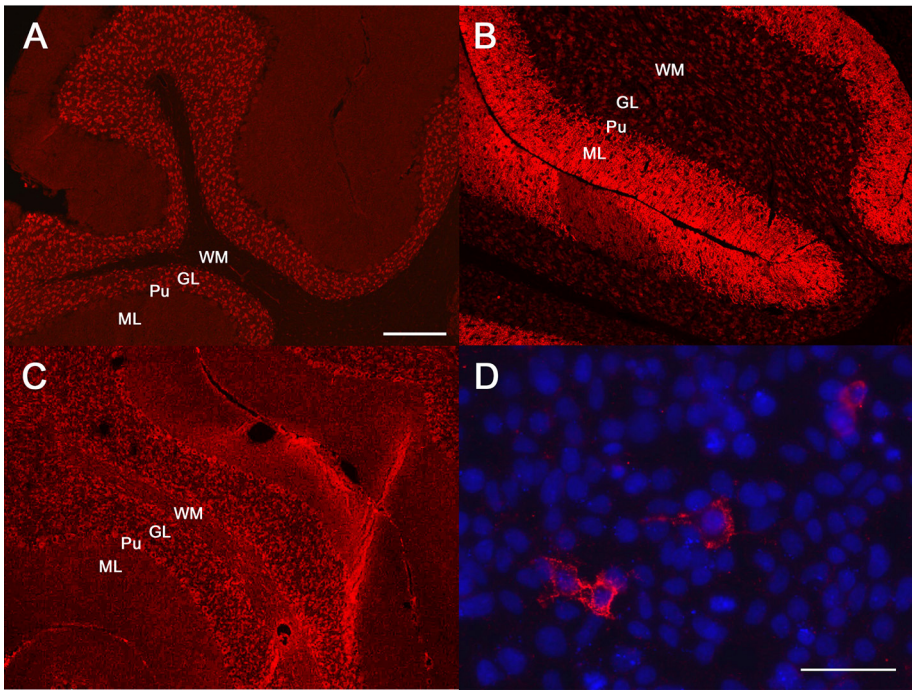

FIGURE 1 | Antibody diagnostics. Immunofluorescence studies: diluted patient material is incubated with mouse brain sections or human embryonic kidney (HEK) cells transfected with the antigens of interest. Binding of patient antibodies to the respective matrix is visualized by a secondary anti-human/canine antibody coupled with an immunofluorescence dye resulting in a red signal. **(A–C)** Tissue-based assays, mouse cerebellum. **(D)** cell-based assay with HEK cells which were transfected with the γ-aminobutyric acid (GABA)-A receptor (GABA$_A$R) subunits α1β3 (all assays from Euroimmun, Lübeck, Germany). Molecular layer (ML), purkinje cell layer (Pu), granular cell layer (GL), white matter (WM). **(A)** Human antibodies against the GABA$_A$R. **(B)** Human antibodies against the GABA$_B$R. **(C)** The canine antibodies against the GABA$_A$R. Please note that the binding pattern is similar to **(A)** and not to **(B)**. **(D)** The canine serum binds to GABA$_A$R transfected HEK cells. Nuclear counterstaining with Hoechst 33342 in blue. Bar in **(A)**, valid also for **(B,C)**: 100 μm. Bar in **(D)**: 25 μm.

children. Elderly patients often have a neoplastic condition (14). The most common associated tumor is a thymoma (8, 14). Other predisposing diseases, which have been associated with anti-GABA$_A$R encephalitis, are a herpes virus infection, myasthenia gravis or other immunocompromising disorders (8, 10, 14, 29). No underlying viral infection (10), inflammatory condition or malignant disease was found in the dog, which underwent extensive tumor staging.

Clinical signs with acute onset seizures progressing to status epilepticus paired with erratic behavioral changes match the typical clinical signs in humans (10, 15). The post-ictal presentation of the CKCS was atypical and exceeded behavioral changes and aggression known from dogs after seizures (24, 30, 31). The clinical presentation and the lack of response to ASMs with rather unremarkable diagnostics raised our suspicion for an antibody associated encephalopathy.

The MRI in the canine case was performed within 48 h after onset of clinical signs and only showed abnormal brain anatomy which is typical for the breed. The finding of Chiari-like malformation in this dog is considered incidental. The CKCS did not have clinical signs typical for Chiari-like malformation, such as recurring vocalization, chronic recurrent spinal pain and reduced activity (20, 32). Nearly 92% of the CKCS population are affected by Chiari-like malformation (33).

In people with GABA$_A$R encephalopathy, T2-w (FLAIR) sequences often reveal multifocal uni- or bilateral cortical and subcortical hyperintensities mainly of the temporal lobes without contrast enhancement (14, 19). Less frequently, a subcortical oedema can be seen (10). In 11.5% of the cases, the MRI is unremarkable (14). MRI abnormalities are more common in GABA$_A$R encephalitis patients with a high serum antibody titer (6). On average, a diagnosis in humans is made 2 months after symptom onset or in a time span of 1 week to 5 years (14). An unremarkable MRI has been reported in 26.6% of patients with GABA$_B$R encephalitis (34, 35). Interestingly, in anti-NMDA-R encephalitis, unremarkable MRIs are described in 45% of patients (36, 37). The MRI in our case was undertaken early after onset of clinical signs and functional impairment of receptors leading to interruption of neurotransmission and therefore seizure activity might have preceded visible edema or increased vascularization and leakage of the blood brain barrier. A follow-up MRI was not performed due to financial constraints. Standard CSF studies were unremarkable in our case. CSF analysis in human patients with GABA$_A$R encephalopathy can present with pleocytosis and

increased protein but also can be inconspicuous in up to 40% of the cases (6, 14).

A possible cause for an unremarkable MRI and CSF examination could be pretreatment with anti-inflammatory drugs such as corticosteroids or non-steroidal anti-inflammatory drugs. This was, however, not reported in the current case. Another explanation could be lack of sensitivity of the MRI or CSF to detect subtle changes. In cases of a mild leptomeningitis, ependymitits or encephalitis MRI can be normal (38, 39). In meningoencephalomyelitis of unknown origin up to 25% of dogs can have an unremarkable MRI and CSF analysis (40–42). In cases of autoantibody encephalitis, clinical manifestations could be due to antibody-mediated dysfunction of receptors (43). The MRI may not be sensitive enough to detect the initial inflammatory process. At a later stage an advanced demyelination and abnormal vascularization could be depicted with an MRI. To investigate this theory, MRI could be repeated later in the disease process, adding diffusion tensor imaging sequences that have been shown to detect abnormalities in human NMDAR encephalitis (44, 45) when there are no T2w FLAIR changes. Another theory is that changes are not seen on MRI as they are purely functional and do not cause changes in structure.

In man, a combination of clinical signs and examination of the autoantibodies in the CSF and serum is recommended for a definite diagnosis, as well as an extended screening for other neural antibodies (8, 14). The therapy consists of ASMs and an immunosuppressive dose of corticosteroids, which is tapered down gradually after 4 weeks, and then in weekly steps for 6–12 months if there is constant clinical improvement (1). In human medicine, additional immunosuppressive medication, intravenous immunoglobulin, plasma exchange and rituximab are used (1, 6, 14).

After the start of the therapy with corticosteroids, the CKCS improved continuously. At the control visit 1 month later, $GABA_AR$ auto-antibodies have decreased significantly. When the antibody production can be stopped, in man, the titer decreases by 50% (one titration level) every 4 weeks (46). In our case, the titer decreased by four levels. Such a rapid decrease could be explained by a shorter half-life of IgG in dogs compared to humans; in fact, the canine IgG half-life is unknown (47). A laboratory inaccuracy [inadvertent deviations by 1 titer level are occasionally hard to avoid (48)] may have contributed to the rapid titer decline.

Interestingly, it was reported by the breeder that a relative of the dog from a previous litter also suffered of epileptic seizures and acute onset circling. Because of a fast and progressive course, the puppy died within hours. No diagnosis was available in this dog, but a familial predisposition for autoimmune brain disease could be possible (49, 50). Nevertheless, heritable basis has also been described for idiopathic epilepsy in CKCS (51).

Even if many parallels can be drawn between human and veterinary medicine with regard to the clinical signs and the corresponding auto-antibodies, it must still be questioned in principle whether the respective antibodies are causative. The syndrome was similar to that of affected humans. The antibody titer decreased in parallel to the clinical improvement. Finally, tests for other neural auto-antibodies gave negative results. All these arguments speak in favor of a pathogenic relevance of the $GABA_AR$ antibodies.

CONCLUSION

We describe the first case of a dog with an anti-$GABA_AR$ encephalitis. Based on the clinical signs and presentation (epileptic seizures lacking any response to ASMs, erratic behavioral changes) and the results of the initial diagnostics (lack of abnormal findings on conventional MRI and CSF examination), an autoimmune encephalitis was suspected, proven, and successfully treated. Clinicians should consider to test for autoantibodies and start immunotherapy in cases with a similar clinical presentation and lack of response to anti-seizure medication even if an inflammatory/infectious or neoplastic cause was clinically excluded on MRI and CSF.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the case report describes normal routine clinical workup. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

EH saw the case with input from NM and HV. ChB and CoB performed the autoantibodies analysis. ChB provided the figure. EH wrote the initial draft. All authors reviewed, revised, and approved the submitted version.

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Pregabalin Add-On vs. Dose Increase in Levetiracetam Add-On Treatment: A Real-Life Trial in Dogs With Drug-Resistant Epilepsy

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Epilepsy is a common neurological disorder affecting 0.6–0.75% of dogs in veterinary practice. Treatment is frequently complicated by the occurrence of drug-resistant epilepsy and cluster seizures in dogs with idiopathic epilepsy. Only few studies are available to guide treatment choices beyond licensed veterinary drugs. The aim of the study was to compare antiseizure efficacy and tolerability of two add-on treatment strategies in dogs with drug-resistant idiopathic epilepsy. The study design was a prospective, open-label, non-blinded, comparative treatment trial. Treatment success was defined as a 3-fold extension of the longest baseline interseizure interval and to a minimum of 3 months. To avoid prolonged adherence to a presumably ineffective treatment strategy, dog owners could leave the study after the third day with generalized seizures if the interseizure interval failed to show a relevant increase. Twenty-six dogs (mean age 5.5 years, mean seizure frequency 4/month) with drug-resistant idiopathic epilepsy and a history of cluster seizures were included. Dogs received either add-on treatment with pregabalin (PGB) 4 mg/kg twice daily (14 dogs) or a dose increase in levetiracetam (LEV) add-on treatment (12 dogs). Thirteen dogs in the PGB group had drug levels within the therapeutic range for humans. Two dogs in the PGB group (14.3%; 2/14) and one dog in the LEV group (8.3%; 1/12) achieved treatment success with long seizure-free intervals from 122 to 219 days but then relapsed to their early seizure frequency 10 months after the study inclusion. The overall low success rates with both treatment strategies likely reflect a real-life situation in canine drug-resistant idiopathic epilepsy in everyday veterinary practice. These results delineate the need for research on better pharmacologic and non-pharmacologic treatment strategies in dogs with drug-resistant epilepsy.

Keywords: epilepsy, seizures, dog, drug-resistance, pregabalin, levetiracetam, drug level

INTRODUCTION

Epilepsy is a common neurological disorder affecting 0.6–0.75% of dogs in veterinary practice (1, 2). Treatment is complicated by the frequent occurrence of drug-resistant epilepsy in dogs with idiopathic epilepsy (3, 4).

In clinical practice in Europe, legal regulations define the use of phenobarbital (PB), potassium bromide (KBr), and imepitoin as first-line antiseizure medications (ASMs) in dogs with idiopathic epilepsy. Imepitoin is only licensed for the treatment of single seizures. Its efficacy for cluster seizures is a matter of ongoing debate (5–8). Dog owners and veterinarians considered the development of new ASMs among the three most important research topics for the future (9). Evidence for the efficacy of non-licensed ASMs in dogs is poor as there are only few prospective controlled studies evaluating treatment strategies in dogs with drug-resistant epilepsy (10–13). Considering that applying a placebo to individuals with drug-resistant epilepsy may be unethical, there is a trend toward comparative active-controlled studies in epilepsy research (4, 14–20).

We, therefore, designed a prospective clinical trial comparing two treatment strategies for dogs with drug-resistant idiopathic epilepsy. The first treatment strategy was pregabalin (PGB) add-on therapy with 4 mg/kg q12 h PO (BID). The second treatment strategy was a 30% increase in the dose of levetiracetam (LEV) add-on treatment given q8 hours (TID). Both LEV and PGB target non-GABAergic pathways and therefore likely exhibit additive antiseizure efficacy to first-line GABAergic drugs (5, 17). PGB showed promising antiseizure efficacy TID dosing in a previous uncontrolled pilot study (21) and had a favorable pharmacokinetic profile suggesting that even BID dosing may be sufficient to achieve effective serum concentrations (22). The American College of Veterinary Internal Medicine (ACVIM) consensus statement on seizure management in dogs recommended LEV as an add-on medication based on evidence and risk profile (23). Both ACVIM and International Veterinary Epilepsy Task Force (IVETF) recommendations suggested an increase in dosage or more frequent application of LEV add-on treatment to overcome tolerance issues (5, 23–25). Little is known about the efficacy of these two treatment strategies in canine drug-resistant idiopathic epilepsy in real life.

Our clinical trial aimed to evaluate and compare these two treatment strategies in dogs with drug-resistant epilepsy. Furthermore, we hypothesized that add-on treatment with 4 mg/kg PGB given BID to dogs would achieve serum concentrations within the therapeutic range for humans.

Abbreviations: PB, phenobarbital; KBr, potassium bromide; ASM, antiseizure medication; BID, every 12 h; TID, every 8 h; ACVIM, American College of Veterinary Internal Medicine; IVETF, International Veterinary Epilepsy Task Force; GTC, generalized tonic–clonic; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; T1, longest baseline interseizure interval; MSF, monthly seizure frequency; MSDF, monthly seizure day frequency; MCDF, monthly cluster seizure day frequency; longest ISI, longest interseizure interval during treatment phase; SD, standard deviation; PGB, pregabalin; LEV, levetiracetam.

MATERIALS AND METHODS

The study design was a prospective, open-label, non-blinded, comparative treatment trial for the evaluation of two add-on treatment strategies.

Dogs with drug-resistant idiopathic epilepsy and monthly generalized tonic–clonic (GTC) seizures (interseizure intervals $\leq$40 days) were recruited at study centers and with study calls placed on websites. Idiopathic epilepsy was diagnosed based on a history of epileptic seizure onset between 6 months and 6 years, review of video footage, documentation of unremarkable physical and neurologic examination, unremarkable blood tests (hematology, serum biochemistry), and bile acid stimulation test. Brain imaging with magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis (tier II) was encouraged but not mandatory (26). Only dogs with drug-resistant idiopathic epilepsy as defined previously were included (4), i.e., failure to achieve seizure freedom with $\geq$2 ASMs. This required documentation of serum concentrations within the therapeutic range (PB > 20 mg/L, KBr > 1,000 mg/L) and/or treatment with adequate dosages (imepitoin 20–30 mg/kg BID; LEV, 15–20 mg/kg TID) unless these were not tolerated. Study participation was denied for the following criteria: dogs younger than 1 year or older than 12 years of age, dogs with post-traumatic epilepsy, ASMs or their dosages modified in the 2 months preceding patient enrolment, dogs treated with immunosuppressants or anti-inflammatory drugs, or in case of any other concurrent relevant metabolic, endocrine, neoplastic, immune, or cardiac disease. Four months (112 days) were selected as the baseline period to provide information on a minimum of three seizure cycles in dogs with monthly seizures. Baseline seizure data were collected from an online questionnaire and written seizure logs provided by the dog owners. Only GTC seizures with and without focal onset were considered. Cluster seizures and status epilepticus were identified as previously described (27). The following baseline parameters were calculated: longest interseizure interval during baseline (T1), and monthly seizure frequency (MSF) during baseline. For T1, the longest seizure-free period between two GTC seizures during baseline was extracted from written seizure logs. For MSF, GTC seizure counts during the 112 days baseline period were divided by four (28 days/month), and each GTC seizure of a cluster event was counted. Furthermore, monthly seizure day frequency (MSDF) and cluster day frequency (MCDF) were calculated to compare baseline characteristics between treatment groups. Owners provided details regarding seizure onset and semiology, triggers, prodromal signs, duration of seizures, postictal signs, and suspicious focal seizure signs. Owners were asked to assign numerical scores for seizure severity (score 1–5; 1 mild, 5 severe), quality of life of their dogs (0–10; 0, poor; 10, excellent), behavioral changes (playfulness, activity level, 0–10; 0, very low; 10, excellent), and observations on possible side effects of ASMs (weakness, ataxia, disorientation, sedation, restlessness, and increased appetite; 0–10; 0, none; 10, severe). Owners were also asked to provide home videos if severe side effects were reported.

TABLE 1 | Matching criteria.

Prioritization	Definition of criteria	Subgroup
C1	Longest interval between two seizure days during 4 months baseline period (T1)	S1: ≤ 14 days S2: > 14 days
C2	Monthly seizure day frequency (MSDF)	S1: > 2 S2: ≤ 2
C3	Cluster seizures	S1: yes S2: no
C4	Age at onset of epilepsy	S1: ≤ 2 years of age S2: > 2 years of age
C5	Predisposed breed	S1: yes S2: no

Matching criteria were defined according to potential impact on study outcome with C1 being most and C5 being least influencing. Matched pair partners had to agree in ≥3 criteria subgroups in ascending order. C, criterion; S, subgroup.

Treatment

Dogs were assigned to either PGB add-on treatment (PGB group) or treatment with increased dosages of LEV add-on treatment (LEV group, only for dogs already treated with LEV). Allocation to treatment groups followed a stratified randomization approach. Matching criteria aimed to establish pairs of dogs with similar disease severity and characteristics (**Table 1**). Dogs were randomized to treatment groups (https://www.random.org) if there was no matching partner available. Dogs in the PGB group received add-on treatment with PGB 4 mg/kg BID. PGB was supplied in different tablet sizes, e.g., 100, 75, 50, and 25 mg, which could be divided into halves (PregaTab®, Neuraxpharm, Germany). In order to avoid excessive sedation, the starting dose was 1 mg/kg BID, the dosage was increased every 4th day by 1 mg/kg BID until the target dose of 4 mg/kg BID was reached resulting in a 2-week titration phase. For dogs in the LEV group, the baseline dosage of LEV add-on treatment given TID was increased by 30% without a change in brand or manufacturer, and a 1-week titration phase was considered. There was no change in emergency treatment protocols. Dog owners were able to leave the study after the 3rd GTC seizure day during the treatment phase (individual endpoint) if there was no relevant short-term effect, which was defined as ≥1.5-fold extension of the longest interseizure interval of the 4-month baseline period (T1). Seizures during the titration phase were not counted. Study exit was also offered if status epilepticus or severe side effects occurred.

Voluntary Study Extension

Owners were offered to extend study participation beyond the individual endpoint (3rd GTC seizure day) and remain in the study for up to 6 months or even longer. In case of treatment failure, owners in the PGB group were offered to increase the daily PGB dose and apply PGB add-on treatment TID. Likewise, in the LEV group, owners were offered to increase the dose of LEV TID add-on treatment by another 30%.

Evaluation of Efficacy

Only GTC seizures and the longest interseizure interval during the treatment phase (longest ISI) were considered. Treatment success was defined as seizure freedom or three-fold extension of the longest baseline interseizure interval (longest ISI ≥ 3 T1; minimum 3 months) (4, 5, 28). Furthermore, time (days) to the 3rd seizure day in the treatment phase was calculated as an additional outcome parameter for all dogs (14, 29–32). For the dogs, which remained in the study for ≥56 days, mean MSF was calculated for the first 56 days of the treatment phase and compared to the 4-month baseline period. Drug retention rates 6 months after treatment initiation and long-term follow-up data were obtained from all dogs.

Statistical Analysis

The sample size was calculated taking an alpha error probability of $p < 0.05$, a power > 80%, and a postulated large effect (Cohen's d 0.8) as a basis. Power-based sample size calculation resulted in a minimum of 12 dogs in each treatment arm using a stratified matched pair design. The generalized mixed linear model was used to compare the PGB group to the LEV group. The matched pairs were defined as subjects and the grouping variable as the repeated measure and fixed effect. The individual dogs were added as a random effect. Depending on the kind of target variable, the target distribution was defined as normal distributed (tested by visualization of Q–Q plots and the Kolmogorov–Smirnov test), gamma distributed with log link, or binomial. In the case of a multinomial target variable (scores), we used the generalized linear model without considering the random effect of the individual dogs. The reason is the technical restrictions of SPSS in the case of multinomial data. Correlations between drug serum concentrations and extension of the interseizure interval were assessed for both treatment groups by calculating the correlation coefficient Spearman's rho. Time to the 3rd GTC seizure day was evaluated with Kaplan–Meier curves and log-rank test (bivariate) and Cox regression analysis in a multivariate setting. Changes in MSF compared to baseline were only assessed for dogs with ≥56 days of study participation. The analysis was performed by using the statistical software package SPSS 28.0.1.0 (IBM, Ehningen, Germany).

RESULTS

Study Population

In total, 142 dogs were screened for eligibility and 26 dogs (18 male, eight female; mean age 5.5 years, range 1.9–11.3) with idiopathic epilepsy were enrolled in the study (**Table 2**; **Supplementary Table S1**). Breeds were as follows: mixed breed (10 dogs), Australian Shepherd (two dogs), Golden Retriever (two dogs), Beagle, Border Terrier, Boxer, Cane Corso Italiano, Elo, Labrador Retriever, Magyar Vizsla, Old German Shepherd, Old English Bulldog, Rhodesian Ridgeback (wild-type JME gene variant *DIRAS1*), Siberian Husky, and White German Shepherd (one dog each). Age at seizure onset was 2.3 years (mean; range 8 months−4.8 years). All dogs experienced generalized seizures with or without a focal onset; all dogs had a history of cluster seizures (24 dogs during a 4-month baseline period, 2 dogs before baseline). The focal onset of generalized seizures was suspected in 46.2% of the dogs (12/26), and this was the predominant seizure type in 26.9% of the dogs (7/26).

TABLE 2 | Baseline characteristics of enrolled dogs with drug-resistant idiopathic epilepsy.

Parameter	All dogs $n = 26$	Pregabalin $n = 14$	Levetiracetam $n = 12$	*p*-value
Predisposed breed	15 dogs (58%)	9 dogs (64%)	6 dogs (50%)	0.805
Body weight, mean (range)	29.5 kg (9–50)	29.2 kg (9–50)	29.8 kg (20–38)	0.932
Sex	18 males (69%)	9 male (64%)	9 male (75%)	1.000
Age at onset of IE	2.3 y (0.7–4.8)	2.2 y (0.7–4.8)	2.5 y (0.9–4.6)	0.599
Age at study inclusion	5.5 y (1.9 –11.3)	4.8 y (1.9–9.6)	6.1 y (4.0–11.3)	0.367
GTC seizures	26 (100%)	14 (100%)	12 (100%)	1.000
- frequent focal onset	5 (19.2%)	2 (14.3%)	3 (25.0%)	1.000
- rare focal onset	7 (26.9%)	4 (28.6%)	3 (25.0%)	1.000
Susp. focal seizure signs	16 (61.5%)	8 (57.1%)	8 (66.7%)	1.000
T1, mean (range)	26.0 d (6–39)	26.4 d (10–38)	25.5 d (6–39)	0.847
MSF, mean (range)	4.0 (1.3–9.8)	4.0 (1.3–9.8)	3.9 (1.5–9.5)	0.923
MSDF, mean (range)	2.6 (1.0–9.3)	2.6 (1.0–4.3)	2.5 (1.0–9.3)	0.872
MCDF, mean (range)	1.0 (0.3–3.5)	1.1 (0.3–3.5)	0.9 (0.0–2.3)	0.607
Duration of postictal signs, mean (range)	38 min (0.5–120)	42 min (5–90)	35 min (0.5–120)	0.746
Seizure severity score, mean (range 1–5)	3.7 (2–5)	3.8 (3–5)	3.6 (2–5)	0.713
Quality of life score, mean (range 1–10)	6.6 (2–10)	6.4 (2–10)	6.7 (3.5–9)	0.876
Baseline treatment				
PB concentration, mean	26.6 mg/l	27.2 mg/l	25.9 mg/l	0.576
KBr concentration, mean	1,493.3 mg/l	1,503.8 mg/l	1,482.8 mg/l	0.950
No. ASMs, mean (range)	2.5 (1–3)	2.2 (1–3)	2.8 (1–3)	0.077
- phenobarbital	24 dogs	14 dogs	10 dogs	
- potassium bromide	17 dogs	11 dogs	6 dogs	
- levetiracetam	17 dogs	5 dogs	12 dogs	
- pregabalin	4 dogs	0 dogs	4 dogs	
- other (topiramate, gabapentin, amantadine)	3 dogs	1 dog	2 dogs	
Levetiracetam pulse therapy	15 dogs (57.7%)	8 dogs (57.1%)	7 dogs (58.3%)	1.000

IE, idiopathic epilepsy; d, days; y, years; min, minutes; T1, longest interseizure interval during 4 months baseline period (days); MSF, monthly seizure frequency; MSDF, monthly seizure day frequency; MCDF, monthly cluster day frequency; GTC, generalized tonic–clonic; PB, phenobarbital; KBr, potassium bromide; No., number; ASM, antiseizure medication.

Additional episodes suspicious for focal epileptic seizures (e.g., twitches and jerks) occurred in 61.5% (16/26) of dogs. Diagnosis of idiopathic epilepsy was supported by unremarkable brain imaging and CSF analysis in 11 dogs (eight MRI, two CT) and unremarkable brain imaging only in three more dogs (two MRI, one CT). EEG was performed on three dogs. Dogs in the PGB and LEV groups did not differ regarding seizure frequency, seizure day frequency, cluster seizures, drug serum concentrations (PB and KBr), and other parameters ($p < 0.05$; **Table 2**). All dogs had minimum two failed adequate ASM trials. At study inclusion, 13 dogs were treated with two ASMs, seven dogs with three ASMs, four dogs with four ASMs, and two dogs with one ASM. Detailed information on the current and previous ASMs is provided in **Supplementary Table S1** for each dog. All dogs were treated with PB and KBr at study inclusion or before, except four dogs (one PGB, three LEV), which had yet not received KBr due to lack of drug supply in 2020/2021. Thirteen dogs were previously treated with imepitoin, but treatment was discontinued due to lack of efficacy in twelve dogs and side effects in one dog. Thirteen dogs had yet not received imepitoin because of a history of cluster seizures.

In the LEV group, the mean dose of LEV at study inclusion was 23.8 mg/kg TID (range 17.2–37.8 mg/kg), which had been provided for a mean of 8.3 months. The mean baseline LEV serum concentration was 10.6 mg/L (0–27.6 mg/L, therapeutic range 10–40 mg/L; MVZ Labor Krone GmbH; Bad Salzuflen, Germany), whereas 50% of dogs had serum concentrations below the therapeutic range for humans. Behavioral changes and side effects of ASMs were commonly reported in both groups (**Supplementary Table S2**).

Treatment Phase

All 26 dogs completed the titration phase and entered the treatment phase: 14 in the PGB group and 12 in the LEV group (**Figure 1**). Mean PGB dosage was 3.96 mg/kg BID (3.66–4.10 mg/kg). Dosage of LEV add-on treatment was increased from 30.4% (mean, range 25–34%) to 31.1 mg/kg TID (mean; range 23.0–50.3 mg/kg). All dogs were treated according to the study protocol until the 3rd day with generalized epileptic seizures or longer (**Figure 2**). The owners of 16 dogs (nine PGB and seven LEV) decided to extend the treatment phase beyond the 3rd GTC seizure day. Overall, dogs from the PGB group participated in the BID treatment phase of the study between 9 and 205

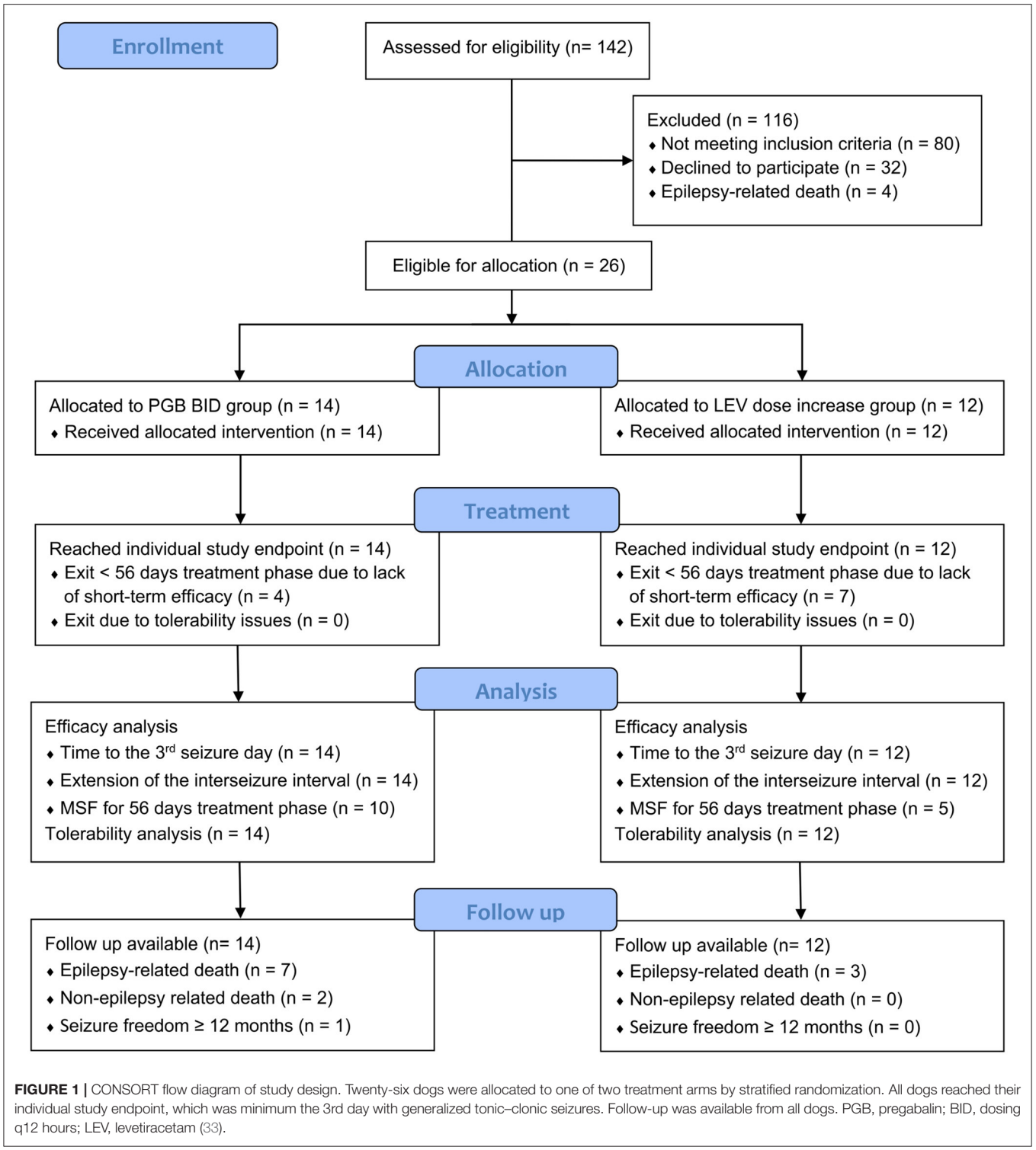

FIGURE 1 | CONSORT flow diagram of study design. Twenty-six dogs were allocated to one of two treatment arms by stratified randomization. All dogs reached their individual study endpoint, which was minimum the 3rd day with generalized tonic–clonic seizures. Follow-up was available from all dogs. PGB, pregabalin; BID, dosing q12 hours; LEV, levetiracetam (33).

days (median 80; mean 97 days), and dogs from the LEV group between 22 and 245 days (median 49; mean 74 days).

Efficacy

Overall success rates were low with no obvious difference in treatment success between the two treatment strategies (14.3 vs. 8.3%; $p > 0.05$). There was also no significant difference between the two treatment groups in time to the 3rd GTC seizure day ($p = 0.334$; **Figure 2**), duration of study participation, and proportions of dogs with ≥50% decrease in MSF in the first 56 treatment days (**Table 3**). The 3rd GTC seizure day occurred within 3 months in 77% of the dogs (20/26; 10 PGB, 10 LEV),

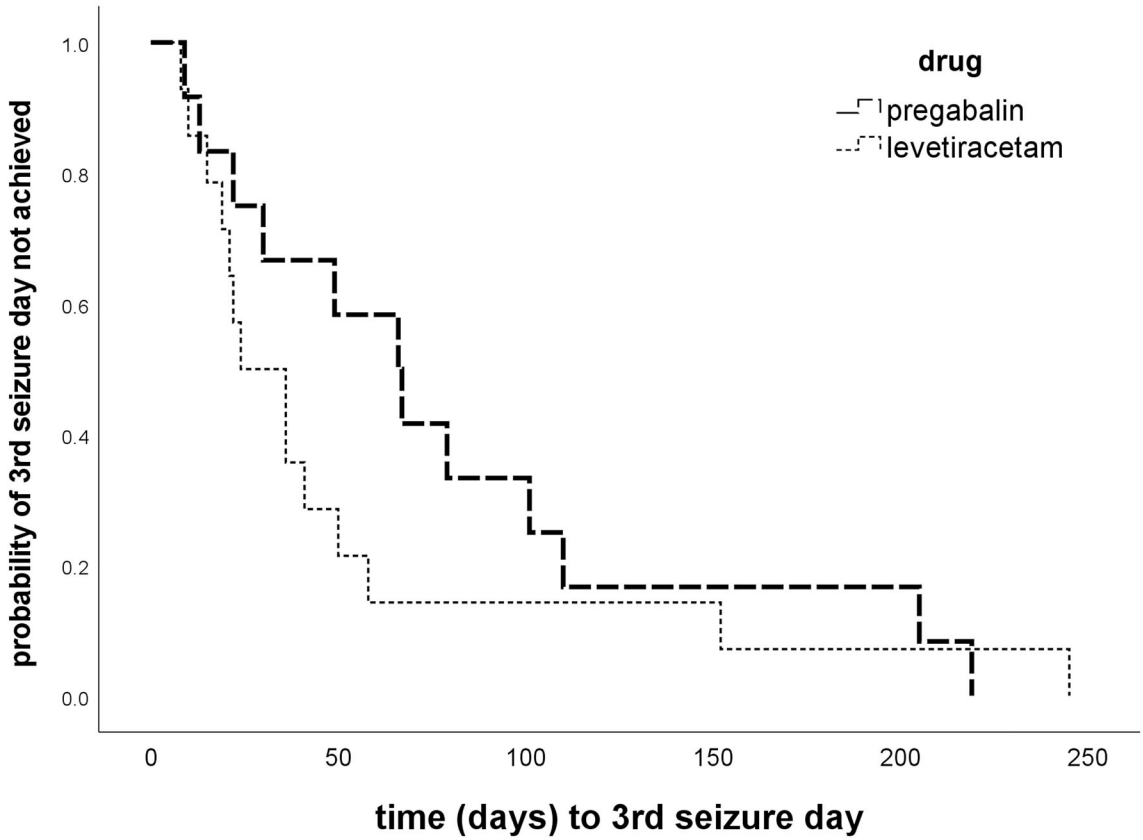

FIGURE 2 | Time to 3rd GTC seizure day. Kaplan–Meier plots of time (days) to 3rd generalized tonic–clonic seizure day in the treatment phase show no difference between the pregabalin BID and levetiracetam dose increase group ($p = 0.334$).

between 3 and 6 months in 11.5% (3/26; 2 PGB, 1 LEV), and after 6 months of treatment in another 11.5% (3/26; 2 PGB, 1 LEV; **Figure 2**). The interseizure interval increased 1.61-fold (mean) in the PGB group and 1.49-fold in the LEV group ($p = 0.681$) compared to baseline (**Table 3**).

Pregabalin group ($n = 14$): Two dogs (14.3%; 2/14) achieved treatment success with a 3.3-fold and 6.4-fold extension of T1 corresponding to seizure-free intervals of 122 and 180 days (longest ISI). Time to the 3rd GTC seizure day was 205 and 219 days, respectively. Both dogs had GTC seizures with suspected focal onset (one rare motor, one frequent autonomic). In one of these dogs, bromide concentration increased from 1,075.0 mg/L at baseline to 1,779.0 mg/L at study completion, presumably after a change in diet due to a suspected allergic food reaction. All dogs but one achieved PGB serum concentrations within the human therapeutic range of the laboratory (2–5 mg/L; MVZ Labor Krone GmbH). Mean PGB serum concentration was 3.8 mg/L (range 1.4–7.8 mg/L; 14 dogs). There was a moderate positive correlation (rho = 0,515, $p = 0.060$) between PGB serum concentration and extension of the interseizure interval. Mean PGB serum concentration was 5.2 mg/L in the two dogs with treatment success compared to 3.6 mg/L (±1.76 SD; $p = 0,144$) in the other dogs. MSF, MSDF, and MCDF during baseline were negatively correlated with extension of the interseizure interval and time to the 3rd GTC seizure day ($p < 0.05$). Owners of four dogs (28.6%; 4/14) chose to leave the study before 56 days of treatment due to a perceived lack of short-term efficacy. Ten dogs adhered to the study protocol for ≥56 days. Of these, five dogs (5/14; 37.5%) experienced ≥50% decrease in MSF during the first 56 days of treatment when compared to baseline (4 months) but a decrease in MSF was only maintained in 21.4% (3 dogs) after 3 months and 14.3% (two dogs) after 6 months of treatment, respectively. The 6-month drug retention rate was 43% (6/14) in the PGB group.

Levetiracetam group ($n = 12$): One dog (8.3%) achieved treatment success with an 8.7-fold extension of T1 corresponding to seizure-free intervals of 218 days (longest ISI). The time to the 3rd GTC seizure day was 245 days in this dog. This dog had GTC seizures with rare focal onset with motor signs. LEV serum concentration was 11.6 mg/L in the dog with treatment success. Considering all dogs, the mean LEV serum concentration was 15.5 mg/L (5.5–52.2 mg/L; 10 dogs). Four dogs failed to achieve LEV serum concentrations within the human therapeutic range (10–40 mg/L; MVZ Labor Krone GmbH; Germany). Serum concentrations were unavailable from two other dogs, which died unexpectedly due to status epilepticus. There was no correlation between LEV serum concentration and extension of the interseizure interval ($p = 0.173$). Duration of postictal signs was negatively correlated with extension of the interseizure

TABLE 3 | Comparative evaluation of efficacy and tolerability of pregabalin add-on vs. dose increase in levetiracetam add-on treatment.

All dogs (*n* = 26)	**Pregabalin BID** ***n* = 14**	**Levetiracetam** ***n* = 12**	***p*-value**
Dogs with treatment success (longest ISI ≥ 3 T1)	2 (14.3%)	1 (8.3%)	1.000
Longest ISI (treatment phase), mean (median)	44.8 d (29.5)	36.7 d (15)	0.527
Extension of the interseizure interval, mean	1.61 T1	1.49 T1	0.681
Time to 3rd seizure day, mean (median)	75 d (62)	55 d (30)	0.500
Dogs exiting study <56 days of treatment	4 (28.6%)	7 (58.3%)	0.233
Only dogs which participated ≥ 56 days	***n* = 10**	***n* = 5**	0.233
MSF 56 days treatment, mean (median)	2.4 (1.5)	2.6 (1.5)	0.841
MSF baseline, mean	3.4	5.5	0.145
% change in MSF, mean	−29.9%	−52.7%*	0.898
≥50% decrease in MSF	5 dogs (35.7%#)	2 dogs (16.7%#)	1.000
Cease of cluster seizures for ≥6 months	2 dogs (14.3%#)	1 dogs (8.3%#)	1.000
Other parameters (all dogs)	***n* = 14**	***n* = 12**	
Study participation, mean (median)	97 d (80)	74 d (49)	0.323
Duration of postictal signs, mean (range)	25 min (0.5–80)	22 min (10–45)	0.768
Seizure severity score, mean (range 1–5)	3.6	3.3	0.652
Suspicious focal seizure signs, no. dogs	6 (42.9%)	7 (58.3%)	0.515
Body weight at study completion, mean (range)	29.3 kg (9–48)	30.2 kg (19–38)	0.966
PB serum concentration, mean (% change)	25.9 mg/l (−4.8%)	24.9 mg/l (−3.9%)	0.721
KBr serum concentration, mean (% change)	1,541.1 mg/l (−2.4%)	1,873.2 mg/l (+26.3%)	0.414
Dogs with drug adaption because of side effects	5 (35.7%)	2 (16.7%)	1.000
Quality of life score, mean (range 0–10)	7.3 (2–10)	6.3 (0–9.5)	0.383
% change in quality of life score, mean	+12.8%	−6.3%	0.243

Longest ISI, longest interseizure interval during treatment phase; T1, longest interseizure interval during 4 months baseline; d, days; y, years; min, minutes; MSF, monthly seizure frequency.
**The decrease of MSF in the levetiracetam group is biased by one dog with high cluster seizure burden during baseline and no cluster seizures during treatment phase.*
#Intention-to-treat analysis, percentages were calculated considering all dogs within the group (14 pregabalin BID, 12 levetiracetam).

interval ($p = 0.027$), and KBr serum concentration was positively correlated with time to the 3rd GTC seizure day (0.05). Owners of seven dogs (58.3%; 7/12) chose to leave the study before 56 days of treatment due to a lack of perceived short-term efficacy of the treatment strategy. Five dogs adhered to the study protocol for ≥56 days. From these, two dogs experienced a ≥50% decrease in MSF compared to baseline in the first 56 days (2/12; 16.7%), but after 6 months of treatment, a decrease in MSF was only maintained in one dog (8.3%; 1/12). The 6 months drug retention rate was 25% (3/12) in the LEV group.

Effect on cluster seizures and focal seizures: cluster seizures stopped for ≥6 months in the three dogs with treatment success (11.5%; 3/26). Cluster seizures were the reason for study exit after the 3rd GTC day (early individual study endpoint) in two dogs from the PGB group and four dogs from the LEV group. Owners reported that twitches and jerks resembling focal motor seizures disappeared in three dogs from the PGB group and three dogs from the LEV group. One dog from the PGB group and two dogs from the LEV group newly developed signs resembling focal motor seizures.

Tolerability

Side effects of the add-on treatment were more frequently reported when PGB was added to the treatment regimen. For the PGB group, mild-to-moderate increase in sedation (seven dogs), weakness (seven dogs), ataxia (six dogs), disorientation scores (five dogs), increased water uptake (three dogs), and flatulence (one dog) were reported. For the LEV group, an increase in ataxia (three dogs), disorientation (two dogs), restlessness scores (two dogs), vomiting (one dog), and flatulence (one dog) were reported. Dogs in the PGB group showed a significant increase in sedation score compared to baseline ($p = 0.011$) and to dogs in the LEV group ($p = 0.041$) (**Supplementary Table S2**). If necessary, side effects were managed with a stepwise 25% dosage decrease of PGB (two dogs, PGB group) or one baseline drug (three dogs, PGB group; two dogs, LEV group). No severe adverse events occurred and no relevant changes in laboratory parameters were observed.

Follow-Up

Five dogs, which had failed to respond to PGB BID, entered treatment with PGB TID for 37–92 days (range; mean 68 days). Three of these five dogs required a subsequent decrease in PGB dose due to weakness and sedation. Yet, the mean PGB serum concentration increased from 2.9 to 4.6 mg/L in four dogs (unavailable from 1 dog). From the LEV group, two dogs entered treatment with a further increase in LEV dose by another 25% for 27 and 94 days, respectively. No further relevant extension of the interseizure interval or decrease in MSF compared to baseline was observed in either group.

Follow-up information was available for all dogs (**Supplementary Table S1**). PGB group: one of the two dogs with treatment success maintained a sustained decrease in seizures for 10 months without any other changes in ASMs or diet, but then relapsed with monthly single seizures and later on also cluster seizures. The other dog experienced 6 months of sustained seizure freedom, but then had a sudden drastic increase in seizure frequency with weekly seizures and was euthanized in status epilepticus 4 months later. One dog, which had not been treated with KBr yet due to lack of drug supply, was started on KBr despite a previous episode of pancreatitis, and PGB was tapered. This dog experienced one more episode of pancreatitis but thereafter remained seizure-free for 15 months until now. One dog became seizure-free after a change to a different commercial pet food diet without any further alteration of ASMs but then was euthanized 15 months after study completion due to a reason unrelated to epilepsy. One dog with ≥50% reduction in MSF in the first 56 days of PGB treatment died due to cluster seizures on day 60. LEV group: the dog with treatment success in the LEV group relapsed to monthly seizures after 218 seizure-free days, but with less severe and less frequent cluster seizures. One dog, which had not been treated with KBr yet due to lack of drug supply, was started on KBr despite a history of gastrointestinal disease and pancreatitis and thereafter experienced a seizure-free period of 6 months, then seizures recurred, but with ≥50% decrease in the frequency compared to study period. One dog with ≥50% reduction in MSF in the first 56 days died on the third seizure day (day 152).

In summary, at the time of writing, 10 dogs, seven dogs from the PGB group, and three dogs from the LEV group knowingly died or were euthanized due to their epileptic seizures. Two dogs from the PGB group were euthanized due to reasons unrelated to epileptic seizures. One dog in the PGB group became seizure-free >12 months and one dog in the LEV group had a significant reduction in seizures after the start with KBr. Seizures continued in the other 12 dogs despite continued treatment with ASMs.

DISCUSSION

Drug-resistance is a serious problem in dogs with idiopathic epilepsy. In this prospective treatment trial, we compared two treatment strategies, PGB add-on treatment with 4 mg/kg BID and a 30% increase in LEV add-on treatment in dogs with drug-resistant epilepsy. The study population was dogs with idiopathic epilepsy and frequent monthly seizures despite adequate treatment with licensed ASMs. All dogs had a history of cluster seizures.

Both drugs have different mechanisms of action than licensed veterinary drugs, which all modulate GABAergic mechanisms. PGB's major mechanism of action is the modulation of excitatory neurotransmitter release *via* binding to the alpha-2-delta subunit of neuronal voltage-gated calcium channels (34–41). LEV binds to the synaptic vesicle protein SV2A also modulating neurotransmitter release (42, 43). In a previous pilot study, PGB resulted in a favorable response in drug-resistant canine epilepsy and was well-tolerated (21). Furthermore, pharmacokinetic data in dogs suggest that therapeutic drug concentrations may be achieved if given BID (22), a fact that could significantly enhance owner compliance. A dose increase in LEV add-on treatment was chosen since the IVETF and ACVIM consensus guidelines advise to increase the LEV dosage or application interval in case of treatment failure with concurrent treatment with PB (5, 23).

Overall success rates were low with both strategies in this drug-resistant population. Threefold extension of the interseizure interval and a seizure-free period ≥3 months occurred only in 14.3% (two dogs) and 8.7% (one dog) in the PGB and LEV groups, respectively. Treatment success lasted 6, 10 (PGB), and 8 months (LEV) in these dogs; thereafter, monthly seizures reoccurred while ASM treatment was not changed. In one dog from the PGB group, treatment success was questionable because bromide serum concentration increased considerably during the study after a change in diet, presumably due to decreased chloride content in the new diet. Tubular reabsorption of bromide competes with chloride; thus, a diet low in chloride may lead to an increase in bromide serum concentrations. This observation underlines the need for long-term follow-up and strict control of diet and drug concentrations when conducting clinical studies on drug-resistant epilepsy. Results and low-response rates could also be attributed to long-term fluctuations and the waxing and waning patterns of seizure occurrence in canine epilepsy (44, 45). Placebo rates as high as 30% were described in other studies previously and may also be relevant for the interpretation of head-to-head trials (44). Nevertheless, the present study protocol and outcome parameters, i.e., minimum of 3-fold extension of the longest baseline interseizure interval appear less sensitive to placebo and regression to the mean effects.

Comparing our results with those of Dewey et al. (21) is challenging due to the different types of analysis and primary outcome parameters. Dewey et al. (21) treated a similar cohort of dogs with idiopathic epilepsy, which was pharmaco-resistant to PB or KBr and had similar seizure frequencies (MSF 4.2 vs. 3.8 in this study), with PGB TID as an add-on medication. The authors reported 7/11 dogs (63.6%) with ≥50% decrease in MSF within the first 3 months of treatment and a mean reduction in MSF of 57% in nine dogs. Defining responders by a ≥50% decrease in seizure frequency may be more prone to variations and a placebo effect than the 3-fold extension of the interseizure interval [as shown in a previous study from our group investigating imepitoin in a placebo-controlled trial in head tremor, (46)]. It should be noted that long-term follow-up was not evaluated in the study by Dewey et al. (21), whereas our follow-up data showed that responder rates with ≥50% decrease in MSF rapidly declined. Furthermore, we failed to observe an additional positive effect on seizure frequency or interseizure intervals in five dogs that underwent dose escalation with PGB TID dosing.

There are several possibilities for the lack of efficacy of PGB BID application in this cohort: insufficient drug serum concentrations, restriction of the efficacy of PGB to focal-onset seizures, or changes in target structures and seizure propagating mechanisms in dogs with chronic epilepsy. PGB 4 mg/kg BID led to serum concentrations within the human therapeutic range in all but one dog. In this regard, it should be mentioned

that previously reported mean PGB serum concentrations were higher (21) (6.8 mg/L) than in the present investigation with BID (3.8 mg/L) or TID (4.6 mg/L) application of respective dosages. A linear relationship between PGB dose and serum concentration, and PGB dose and treatment efficacy exists in humans for the treatment of focal-onset seizures (47–50). In line with these observations in human medicine, there was a trend toward a moderate positive correlation between PGB drug serum concentration and extension of the longest ISI in our cohort of dogs (rho = 0.515, $p = 0.060$).

Pregabalin is only licensed for adjunctive therapy of focal and focal-onset seizures evolving into bilateral tonic–clonic seizures in humans besides neuropathic pain and generalized anxiety disorder (51). In this aspect, anti-seizure efficacy for focal and focal-onset seizures has been well-documented with ≥50% seizure reduction in >40% of human patients (18, 49, 50, 52). Recent investigations in children and adults failed to demonstrate a significant effect of PGB on generalized-onset tonic–clonic seizures compared to placebo (53). In dogs with idiopathic epilepsy, the most frequent seizure type is "focal epileptic seizure" evolving into generalized epileptic seizures (27), which is the equivalent of "focal to bilateral tonic–clonic seizure" in humans (54). Classification of epilepsies by predominant seizure types in dogs analogous to human medicine faces specific challenges, relying on owner observations, difficulties to recognize non-motor signs at seizure onset, and dependence on the interpretation of the investigator without routine support by electroencephalography. Focal epileptic seizure onset was suspected in 46.2% (12/26; six PGB; six LEV) of dogs in the study and was the predominant seizure type in 26.9% (7/26; 4 PGB; 3 LEV). The two dogs with treatment success in the PGB group had suspected focal-onset seizures (one rare motor, one frequent autonomic).

Treatment with increased dosages of LEV add-on treatment is a popular treatment strategy in canine drug-resistant epilepsy and aims at overcoming tolerance issues. In our study, only one dog showed treatment success indicating poor efficacy of this treatment strategy in our cohort of dogs. However, it should be considered that the recruitment process of our study may have already been selected for LEV non-responders. Thus, treatment failure could result from genetic or molecular factors of these dogs' epilepsy not being responsive to LEV's mechanism of action (55). Alternatively, a honeymoon effect may have occurred, i.e., a decrease or loss of efficacy of LEV when used chronically as previously described (55–58). Decrease or lack of efficacy could be related to functional tolerance issues, i.e., pharmacodynamic tolerance due to reduced effects at target structures, or pharmacokinetic tolerance due to increased metabolism of LEV, especially when used as an add-on to PB. The 30% dosage increase of LEV failed to achieve drug concentrations within the human therapeutic range in four dogs in this study Therefore, the LEV dosage increase of 30% in our study might have been insufficient to achieve further treatment effect. In this context, it should be noted that serum concentrations as a guide for LEV add-on treatment are still controversial due to an equivocal relationship between efficacy and drug serum concentrations in humans (23, 59, 60). So far, there is no established therapeutic range for LEV serum concentrations in dogs. Nevertheless, there are concerns that LEV serum concentrations might decrease and be too low with time and when concurrent treatment with PB is applied (24). Measurement of LEV serum concentrations could at least ascertain that drug concentrations within the human therapeutic range are achieved (5, 23). A strategy with continued add-on treatment with LEV at increased dosages may be inappropriate to overcome tolerance issues. Considering previously published results (57), LEV pulse therapy might be the preferred therapeutic strategy for dogs with cluster seizures to address drug tolerance issues and avoid a honeymoon effect (57).

The study protocol allowed for an individual early study end if no relevant short-term success was obtained, with the endpoint being the 3rd GTC seizure day during the treatment phase. This avoided prolonged adherence to a presumably ineffective treatment protocol and aimed at increasing owner compliance. It may be of interest that most dogs remained in the study beyond this early individual endpoint. But finally, five dogs in the PGB and seven dogs in the LEV group exited the study before 56 days of treatment due to persistent GTC seizures. For future studies, a later individual study endpoint, e.g., time to the 4th or 5th seizure day could be discussed (4, 31, 32). Since the current study protocol was unintendedly selected for short-term treatment success, disease modifying effects might have been missed, and the antiseizure effects of these therapeutic strategies were underestimated. On the other hand, short-term treatment success correlated with long-term treatment success in the case of imepitoin (8). In general, longer treatment periods with a supposedly ineffective ASM may also result in higher drop-out rates or lower owner compliance including failure to record seizures precisely and thus contributing to a placebo effect from the inclusion of pseudo-responders. Furthermore, the inclusion of all dogs that entered the treatment protocol into the final analysis (an intention-to-treat analysis) avoids reporting false high efficacy rates, which may occur if only dogs improving during the therapeutic intervention are analyzed. We, therefore, suggest that our results and this treatment protocol reflect a real-life situation.

The dogs in this study represent canine patients in veterinary practice with a need for treatment strategies beyond the drugs licensed for use in dogs with idiopathic epilepsy (9). In humans, older studies reported a chance of ≥50% decrease in seizure frequency in 19–29% of patients after two previously failed ASM trials (61, 62). Newer studies applying the current International League Against Epilepsy definitions of seizure freedom report that 4.4–27% may become seizure-free with the 3rd ASM (28, 63–66). The short-term response rates in our study are in line with these assumptions; however, observations on long-term outcomes revealed recurrence of monthly seizures after 6–10 months.

There were multiple limitations to the study. Only 50% of the dogs had diagnostic imaging of the brain performed; thus, subtle structural brain lesions contributing to drug-resistance may have been overlooked. Dogs neither were primarily randomized to the study groups nor were the investigators blinded. However, in randomized trials, a very high number of participants is

warranted to assure equal groups, which is addressed by the matched pair design of the study.

The strict inclusion criteria selected for a rather chronic, difficult-to-treat group of dogs with a high seizure burden. This is also reflected in the fact that all participating dogs suffered from cluster seizures although this was not an inclusion criterion. It remains undefined whether the response rates of the study would have been higher in a less severely affected population of dogs with idiopathic epilepsy.

The long baseline period of 4 months aimed to compensate for variations in seizure frequencies; however, it is still possible that dogs were enrolled at a state of disease progression or natural fluctuation of disease (4, 44, 45). Furthermore, in the PGB group, side effects made drug adaptions necessary in a considerable proportion of dogs (35.7%; three baseline drugs, two PGB dose, **Supplementary Table S1**). These adaptions of the baseline ASMs reflect daily clinical practice but may have led to some decrease in the antiseizure efficacy of the baseline drug, which had to be compensated by the PGB add-on therapy.

In conclusion, this study design with an early individual study endpoint was associated with high compliance of dog owners and enabled analysis of all study participants. The overall low success rates with both treatment strategies likely represent a real-life situation in canine drug-resistant idiopathic epilepsy in daily veterinary practice. The occurrence of epilepsy-related deaths, even in dogs with a favorable response, prompts the need for investigation of better pharmacologic and non-pharmacologic treatment strategies in dogs with drug-resistant idiopathic epilepsy. Future studies in PGB treatment may imply dose escalations guided by drug serum concentrations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee (AZ 168-02-05-2019) of the veterinary faculty of the Ludwig-Maximilians-Universität Munich and conducted in accordance with the German Animal Welfare Act. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AF, FW, KJ, and SK designed the study. SK conducted the experiments and wrote the first draft of the manuscript. TS, GB, SD, HS, and KJ contributed cases and collected data. AF and KJ supervised case collections. SR, AF, and HP provided input into statistics. AF, KJ, and HP reviewed the first draft of the manuscript. All authors provided input into the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Single-Voxel Proton Magnetic Resonance Spectroscopy of the Thalamus in Idiopathic Epileptic Dogs and in Healthy Control Dogs

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The role of magnetic resonance spectroscopy (MRS) in the investigation of brain metabolites in epileptic syndromes in dogs has not been explored systematically to date. The aim of this study was to investigate metabolites in the thalamus in dogs affected by idiopathic epilepsy (IE) with and without antiepileptic drug treatment (AEDT) and to compare them to unaffected controls. Our hypothesis is that similar to humans with generalized epilepsy and loss of consciousness, N-acetyl aspartate (NAA) would be reduced, and glutamate–glutamine (Glx) would be increased in treated and untreated IE in comparison with the control group. In this prospective case–control study, Border Collie (BC) and Greater Swiss Mountain dog (GSMD) were divided into three groups: (1) healthy controls, IE with generalized tonic–clonic seizures with (2) and without (3) AEDT. A total of 41 BC and GSMD were included using 3 Tesla single-voxel proton MRS of the thalamus (PRESS localization, shortest TE, TR = 2000 ms, NSA = 240). After exclusion of 11 dogs, 30 dogs (18 IE and 12 healthy controls) remained available for analysis. Metabolite concentrations were estimated with LCModel using creatine as reference and compared using Kruskal–Wallis and Wilcoxon rank-sum tests. The Kruskal–Wallis test revealed significant differences in the NAA-to-creatine ($p = 0.04$) and Glx-to-creatine ($p = 0.03$) ratios between the three groups. The Wilcoxon rank-sum test further showed significant reduction in the NAA/creatine ratio in idiopathic epileptic dogs under AEDT compared to epileptic dogs without AEDT ($p = 0.03$) and compared to healthy controls ($p = 0.03$). In opposite to humans, Glx/creatine ratio was significantly reduced in dogs with IE under AEDT compared to epileptic dogs without AEDT ($p = 0.03$) and controls ($p = 0.02$). IE without AEDT and healthy controls did not show significant difference, neither in NAA/creatine ($p = 0.60$), nor in Glx-to-creatine ($p = 0.55$) ratio. In conclusion, MRS showed changes in dogs with IE and generalized seizures under AEDT, but not in those without AEDT. Based upon these results, MRS can be considered a useful advanced imaging technique for the evaluation of dogs with IE in the clinical and research settings.

Keywords: MRS—^{1}H nuclear magnetic resonance spectra, N-acetyl aspartate (NAA), glutamine (Gln), glutamate (Glu), glutamate-glutamine (Glx), canine, generalized seizures

INTRODUCTION

Advanced neuroimaging has been recently introduced in the diagnostic work-up and research of canine epilepsy (1, 2). One of these advanced neuroimaging techniques is magnetic resonance spectroscopy (MRS). In human epilepsy, MRS is, among other techniques, widely used to rule out metabolic diseases in structural negative epilepsy (3), for presurgical evaluation (3), and to study the biochemical composition during drug treatments (4, 5). MRS studies investigating canine epilepsy are sparse. Few case reports and studies showed the utility of MRS in genetic (6) and in structural epilepsy (7, 8). However, only one study examined MRS in idiopathic epileptic dogs (9).

MRS allows non-invasive estimation of metabolite concentrations within a selected volume of interest (VOI) by exploiting slight changes in the magnetic field sensed by nuclei, usually ^{1}H protons, in different metabolites. This so-called chemical shift results in specific peaks for different metabolites in a frequency spectrum (10). Metabolites that can be identified with MRS at 3 Tesla (3T) and are of specific interest in epilepsy include excitatory and inhibitory neurotransmitters, such as glutamate and gamma-amino-butyrate (GABA), but also markers for neuronal integrity, such as N-acetyl aspartate (NAA) (11).

Glutamate is the main excitatory neurotransmitter in the brain. Due to the overlap of glutamate and glutamine spectra and the difficulties in separating these two spectra at field strength lower than 3T, glutamate is commonly reported together with glutamine as glutamate–glutamine complex (Glx) (12). Excessive glutamate release is observed in chronic epilepsy and associated with recurrent seizures (13). In human epilepsy, significant changes in Glx have been identified in different brain regions depending on the underlying epileptic syndrome (11). While for temporal lobe epilepsy with hippocampal necrosis, reduced Glx was found in the ipsilateral hippocampus (14), and increased levels of Glx were detected in the medial prefrontal cortex in juvenile myoclonus epilepsy (15).

GABA is the major inhibitory neurotransmitter of the central nervous system. Furthermore, many antiepileptic drugs target the GABA system and cause increased GABA concentration in the brain (4). However, MRS evaluation of GABA is challenging. GABA concentration compared to other brain metabolites is much lower, and the peaks of GABA in MRS overlap with those of other metabolites (16). Because advanced techniques, such as spectral editing seem unavoidable to obtain reliable results at 3T, information regarding GABA levels in epilepsy is still scarce (11, 17).

NAA is one of the molecules with the highest concentration in the brain (18). Its function in the brain is still controversial and under investigation (18). NAA is mostly reported together with the neurotransmitter N-acetylaspartylglutamate (NAAG) as total NAA (tNAA) because these signals cannot be reliably separated under common conditions (19). NAA is an intraneuronal metabolite synthetized in the mitochondria. Although NAA is not only present in neurons but also in oligodendrocytes/myelin, NAA in MRS is considered the key neuronal marker for brain neuronal health, viability, and number of neurons (18). In human epilepsy, focal reductions in NAA were found in different forms of temporal lobe epilepsy (20). Furthermore, recovery of the NAA levels after successful epilepsy surgery was reported (21).

The presented clinical applications in human medicine demonstrate that alterations in brain metabolites can be detected with MRS. However, one should notice that the brain regions where these alterations can be detected, as well as the magnitude of the alterations strongly depends on the specific underlying epileptic syndrome are not uniform.

In single-voxel MRS, a VOI has to be selected. This represents a major challenge in veterinary medicine. First, because epileptic syndromes are not as well characterized in dogs as in humans (22). Second, as the main target area within the brain in canine epilepsy remains undefined and may be variable in subpopulations of dogs diagnosed with idiopathic epilepsy (IE).

To overcome the issue of the heterogenicity in canine epileptic syndromes, we decided to select breeds with rather well-characterized epileptic syndrome and familiar history of epilepsy, suggesting a common genetic background for the diseases in this breed (23). From those dog breeds presented to our hospital more commonly with IE, Border Collie (BC) and Greater Swiss Mountain Dog (GSMD) met these criteria (24, 25).

Target volume definition is more difficult to solve. One could argue that multivoxel MRS assessing the whole brain could be an alternative. However, standard multivoxel MRS at 3T suffers from long acquisition times, low spatial resolutions, and variable quality (26, 27). With regard to these problems, we decided to use single-voxel MRS, thus, facing the challenge of selecting a specific target VOI. In a recent study, Olszewska at al. selected the temporal lobe as target area in canine IE (9). However, the involvement of the temporal lobe in canine epilepsy is still controversial (9). In humans, a recent MRS study demonstrated alteration of brain metabolites in the thalamus in people affected by the loss of consciousness during seizures (28). Also, BC and GSMD are affected by generalized tonic–clonic seizures which are associated with the loss of consciousness (22). The thalamus is one of the most important center associated with the loss of consciousness in epilepsy in people (29). This together with the thalamic MRS changes detected in humans with the loss of consciousness during epileptic seizures makes the thalamus an appropriate target area for MRS evaluation also in dogs with seizures accompanied by the loss of consciousness.

The purpose of our study was therefore to assess and compare thalamic MRS spectra in healthy control dogs and in IE dogs affected by generalized seizures with a focus on NAA and Glx. Another aim of this study was to assess possible differences between IE dogs with and without antiepileptic drug treatment (IE with AEDT and IE without AEDT, respectively). Our hypothesis was that IE-affected dogs, similar to humans

Abbreviations: BC, Border Collie; Choline, Cho; CRLBs, Cramér Rao lower bounds; GABA, Gamma-aminobutyric acid; Glx, glutamate–glutamine complex; GSMD, Greater Swiss Mountain Dog; Hz, Hertz; IE, idiopathic epilepsy or idiopathic epileptic; LCModel, linear combination model; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAA, N-acetyl aspartate; NAAG, N-acetylaspartylglutamate; NSA, number of signal averages; PRESS, point-resolved spectroscopy; SNR, signal-to-noise ratio; T, Tesla; VOI, volume of interest.

with seizures and loss of consciousness, show elevated Glx and reduced NAA concentration in the thalamus compared to control dogs and that significant differences in NAA and Glx could be detected between treated and untreated dogs with IE.

MATERIALS AND METHODS

Study Population

This prospective study was performed between 2017 and 2021 at the Veterinary hospital of the University of Zurich after approval by the cantonal authorities according to Swiss law under animal license no. ZH272/16 and ZH046/20.

We recruited GSMD and BC dogs with presumptive diagnosis of IE (cases) and clinically healthy relatives, GSMD and BC (controls) of the prospectively enrolled cases. Dogs affected by IE had to fulfill the TIER II criteria for IE of the veterinary epilepsy task force (suspected genetic epilepsy) (30) and had to show generalized tonic–clonic seizures.

All dogs underwent complete clinical and neurological examination performed by a board-certified veterinary neurologist, blood screening for metabolic epilepsy, magnetic resonance imaging (MRI) of the brain, and MRS of the thalamus. Dogs with IE had additionally cerebrospinal fluid (CSF) analyses performed.

The IE dogs were divided into two groups: one group of IE dog was drug-naïve (IE without AEDT), and the other one already received AEDT (IE with AEDT).

Exclusion criteria were MRS of nondiagnostic quality, voxel placement outside of the thalamus, abnormal clinical or neurological examination, abnormal CSF analyses, or identification of an underlying cause for the epilepsy.

Procedures

All dogs underwent MRI and MRS under general anesthesia with a standardized anesthesia protocol previously described (31). A part of the dogs included in this study has also been included in the aforementioned study.

MRI and MRS were performed with a 3T MRI (Philips Ingenia scanner, Philips AG, Zurich, Switzerland) with a 15-channel receive–transmit head coil (Stream Head-Spine coil solution, Philips AG, Zurich, Switzerland) in all patients.

Conventional morphological MR images included T2-weighted (W) turbo spin-echo sequences in transverse, dorsal, and sagittal planes, a fluid-attenuated inversion recovery (FLAIR), a T2* or a susceptibility-weighted sequence, and diffusion-weighted images in transverse, as well as a 3D T1-W sequence before and after intravenous injection of contrast media [gadodiamide (Omniscan) 0.3 mmol/kg, GE Healthcare AG, Glattbrugg, Switzerland, or gadoteric acid (Dotarem) 0.3 mmol/kg, Guerbet AG, Zürich, Switzerland].

Single-voxel MRS of the thalamus was performed with a previously described optimized protocol (32). In brief, transverse, dorsal, and sagittal T2-W images were used to graphically place the single voxel in the thalamus, preferably in the right thalamus. Voxel size was 1.8 cm^3 (10 x 12 x 15 mm), and care was taken to avoid CSF, as well as peripheral soft and bony tissues adjacent to the thalamus to prevent lipid contamination. Before MRS acquisition with point-resolved spectroscopy (PRESS) localization and water suppression using the excitation technique, field homogeneity was optimized with a second-order automatic pencil-beam shim. In addition, a water-unsuppressed spectrum was obtained as concentration reference to estimate metabolite concentrations. Spectra were obtained using the following parameters: shortest possible echo time (TE): 29 to 31 ms; repetition time (TR): 2,000 ms; number of signal averages (NSA): 240; bandwidth: 2,000 Hz. Spectra outside the thalamus and spectra with the presence of artifacts (e.g., presence of strong lipid contamination) were excluded based on visual inspection.

MRI image evaluation and visual MRS analyses were performed on each dog by a board-certified veterinary radiologist or a resident in diagnostic imaging under direct supervision of a board-certified veterinary radiologist.

Data Processing

Metabolite concentrations were estimated, as described before (32) with an automated data processing spectral fitting algorithm (linear combination model, LCModel, version 6.3, S Provencher, Oakville, ON, Canada) using a simulated basis set (details can be found in **Supplementary Table S1**).

To translate the fitted MRS signals into estimates of the metabolite concentrations, an external or internal reference is needed (33). The most common internal reference used in human medicine is tissue water signal (33). Tissue water signal has the advantage of being about 10,000 times higher than the signal of the metabolites. In addition, water signal does not have to be resolved from overlapping metabolites. Nevertheless, to use reliably tissue water signal, segmentation of voxel into gray, white matter, and CSF is recommended, especially to correct for the CSF contamination in the measured voxel (33). Because tissue segmentation was not available in our study, we initially opted for total creatine (tCr, sum of creatine and phosphocreatine) as an internal reference. However, as creatine is a marker for energy metabolism, creatine ratios have the disadvantage that this metabolite can also be affected by some diseases and by the process of aging (33). Successively, we also used the water signal as reference in order to check the results obtained with the creatine ratios.

Metabolite-to-water ratios expressed in institutional units were derived within LCModel using the unsuppressed water signal with a global correction for the relaxation attenuation of the water signal (ATTH20 = 0.7) and estimation of the water concentration in the measured voxel of WCONC = 43300. Metabolite signals were not corrected for relaxation attenuation.

To assess the quality of the spectra, signal-to-noise ratio (SNR), linewidth in form of full width at half maximum (FWHM), and relative Cramér Rao lower bounds (%CRLBs) as estimation of the lower bounds of fitting error (34) were collected from the LCModel output. In addition, the FWHM of the unsuppressed water scan was measured.

Comparison of metabolite ratios and MRS spectra in the thalamus was performed between dogs affected by IE with and without AEDT and healthy control dogs.

TABLE 1 | Population characteristics.

	Healthy controls	IE without AEDT	IE with AEDT
Breed			
BC	2	2	6
GSMD	10	7	3
Sex			
Male	8	6	4
Male castrated	-	-	1
Female	4	3	-
Female spayed	-	-	4
Bodyweight			
kg (median, range)	47.5, 20–55	48, 14–70	21, 15–60
Age			
Years (median, range)	5.4, 1.3–8.1	4.7, 1–7, 4	5.3, 3.3–7

IE, idiopathic epilepsy; AEDT, antiepileptic drug treatment; BC, Border Collie; GSMD, Great Swiss Mountain Dog.

Statistical Analysis

Statistical analysis was performed using R (version 4.1.2 in RStudio) (35). All groups (IE without AEDT, IE with AEDT, and healthy control dogs) were compared using a non-parametric Kruskal–Wallis test on all metabolite ratios to total creatine and on all metabolite ratios to water, respectively. Pairwise comparison between groups was performed using the Wilcoxon rank-sum test. Overall, $p < 0.05$ was considered to be statistically significant.

RESULTS

Study Population

Forty-one dogs fulfilled the inclusion criteria. Nine of these 41 dogs were excluded due to mispositioning of the VOI, one dog was excluded due to incorrectly set echo time during the MRS examination, and one dog was excluded due to visual identification of a broad and abnormal peak in the lipid region. Of the remaining 30 dogs, breeds were represented as follows: 20 GSMD (66.7%) and 10 BC (33.3 %). Population characteristics and seizure semiology are listed in **Tables 1**, **2**, respectively. In the majority of the cases, the VOI for the MRS was placed in the right part of the thalamus (25; 83%) and in the remaining dogs in the left part (5; 17%).

Spectral Quality

All spectra obtained were of good quality, with no spectra being rejected (**Figure 1**). The SNR in the study was between 19 and 8, and the FWHM of the fit (LCModel Output) was 2.9–5.9 Hz (**Figure 2**). No significant differences between the groups were found for these quality parameters. However, the FWHM of the water peak (between 5.6 and 7.8 Hz) was slightly higher (0.5 Hz) in the treated group.

TABLE 2 | Semiology of epileptic events in the affected population.

	IE without AEDT	IE with AEDT
Seizures		
Status epilepticus	-	1
Cluster seizures	-	5
Seizure semiology		
Tonic-clonic	9	9
Tonic	-	-
Focal onset secondary generalization	3	3
Unknown onset	6	6
Additional focal seizures	-	-
Autonomic signs	4	5
Time between first seizure to MRI		
<1 month	-	-
>1–3 month	3	1
>3–12 month	4	3
>12 months	-	2
>24 months	2	3
Time between last reported seizure to MRI		
2–7 days	2	2
8–31 days	5	5
>1 month	2	2
Medical treatment at the timepoint of MRI		
Phenobarbital	-	9
Potassium bromide	-	2
Levetiracetam	-	3
Imepitoin	-	3
Gabapentin	-	1
Special diet or dietary supplement before MRI		
Medium chain triglycerides	1	1
Cannabidiol	1	1

IE, idiopathic epilepsy; AEDT, antiepileptic drug treatment.

MRS Results

The metabolite-to-total creatine ratios of all measured metabolites and the %CRLB are listed in the **Supplementary Table S2**.

The Kruskal–Wallis test revealed significant differences in the NAA-to-creatine ($p = 0.04$) and Glx-to-creatine ($p = 0.03$) ratios between the three groups. Significant differences were also found in the tNAA-to-water ratios ($p = 0.04$) (**Figure 3**).

Pairwise comparisons showed significant decrease in NAA-to-creatine ratios in IE dogs under AEDT compared to IE dogs without AEDT ($p = 0.03$) and compared to healthy control dogs ($p = 0.03$). However, tNAA-to-creatine ratio was not significantly reduced between the IE dogs with AEDT compared to IE without AEDT ($p = 0.06$) nor compared to healthy control dogs ($p = 0.17$).

Glx-to-creatine ratio was significantly reduced in IE dogs under AEDT compared to IE dogs without AEDT ($p = 0.03$) and compared to healthy dogs ($p = 0.02$).

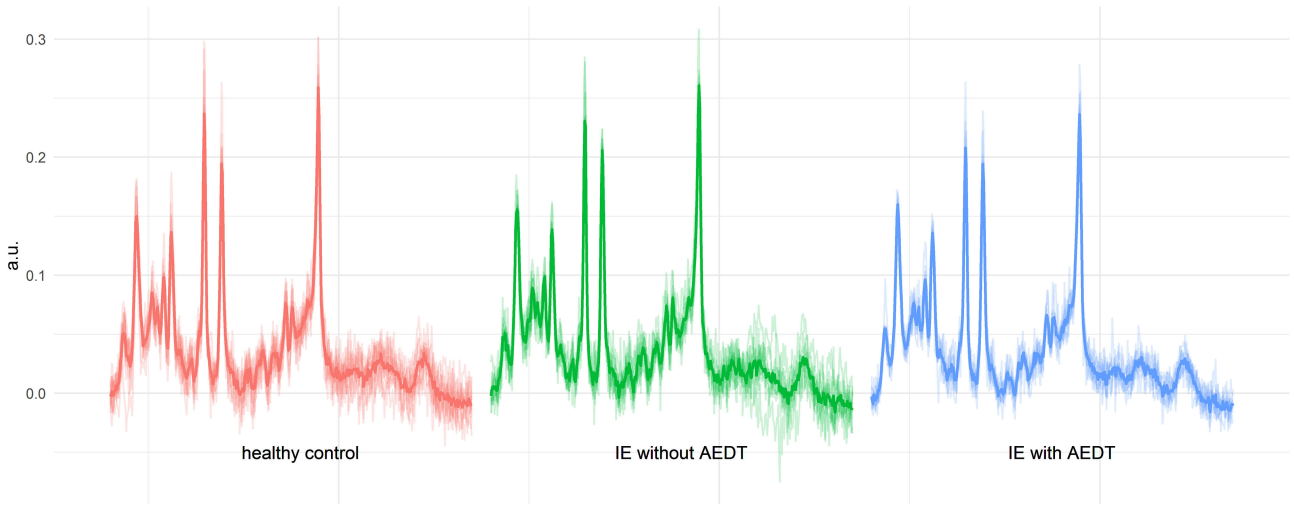

FIGURE 1 | All measured spectra of the three investigated groups. In the background, the individual spectra of all dogs are plotted as outputted from LCModel (.coord files). The thicker line shows the calculated mean spectrum in each group. The spectra show a consistent quality, and the observed differences in N-acetyl aspartate (NAA) and glutamate–glutamine (Glx) in idiopathic epilepsy (IE) with antiepileptic drug treatment (AEDT) are already indicated in this visual overview.

Pairwise comparison of IE without AEDT and healthy controls did not show significant difference, neither in NAA-to-creatine ($p = 0.60$), nor for tNAA-to-creatine ($p = 0.81$) or for Glx-to-creatine ($p = 0.55$) ratio.

NAA and tNAA-to-water ratios were significantly reduced in IE dogs with AEDT compared to IE dogs without AEDT ($p = 0.04$; $p = 0.01$, respectively) but not compared to healthy dogs. Glx to water was not significantly reduced between the compared latter groups of IE dogs ($p = 0.08$).

DISCUSSION

In human epilepsy, MRS has been introduced into the work-up since more than two decades. In contrast, MRS application in canine epilepsy is still in its early days. We investigated thalamic metabolite concentrations with MRS in healthy control dogs and IE dogs with and without AEDT. It was hypothesized that dogs affected by IE showed elevated Glx and reduced NAA concentration in the thalamus compared to control dogs, similar to humans with seizures and loss of consciousness (28). In this study, dogs with IE under AEDT, but not those without AEDT, had significantly lower NAA-to-creatine ratios compared to healthy controls, as well as compared to IE dogs without AEDT. With also reduced tNAA in IE with AEDT, this would convey a relatively clear picture. However, although the tNAA-to-creatine ratio is also generally lower in dogs with IE on AEDT, the differences are not significant. NAA and NAAG are spectroscopically very similar (19), as can also be seen in **Figure 2**. Thus, a distinction at 3T is difficult and strongly dependent on the spectral quality. With linewidths of <8 Hz obtained herein, which is considered excellent for measurements in humans (36), a reliable assignment seems at least plausible. In this case, the absence of differences in tNAA could be explained by changes in NAAG. However, given the observed differences in water linewidth, we must at least consider that the observed difference in NAA could reflect the difference in linewidth and thus a biased fitting. The measured ratios to water confirm the differences for NAA between IE dogs under AEDT compared to IE dogs without AEDT, but there are no significant differences in NAA or tNAA water ratios to the healthy dogs, which do not simplify the interpretation of the results.

Reduction in NAA in MRS is considered a sign for neuronal loss (18), and several factors can contribute to decreased NAA levels in human epilepsy. Some of these factors may also explain the lower NAA-to-creatine ratio found in IE dogs under AEDT compared to those without AEDT.

The number of seizures is an important reason for reduced NAA levels. In humans with generalized tonic–clonic seizures, reduction in NAA was more severe in patients who experienced more than ten generalized tonic–clonic seizures during their lifetime, than in patients with less seizure episodes (37–39). In our study, the total number of seizures per dog was not available, but overall longer duration of epilepsy and more common occurrence of cluster seizures and status epilepticus in dogs with AEDT compared to those without AEDT, suggests a higher number of seizures in this group. Therefore, the NAA reduction in IE dogs with AEDT might reflect the findings in humans, where patients with more seizure episodes showed severer NAA diminution (37–39).

Another reason for reduced NAA levels is poor seizure control. In humans, lower NAA levels were found in the mesial temporal lobe in patients failing to respond to the first AEDT compared to those with seizure freedom after first AEDT (40). Of the nine dogs in our study with AEDT, six had more than one antiepileptic drug. The high number of cases with multidrug treatment suggests a high level of resistance to the first antiepileptic drug in this group. One possible explanation in humans for lower NAA in non-responders is more severe

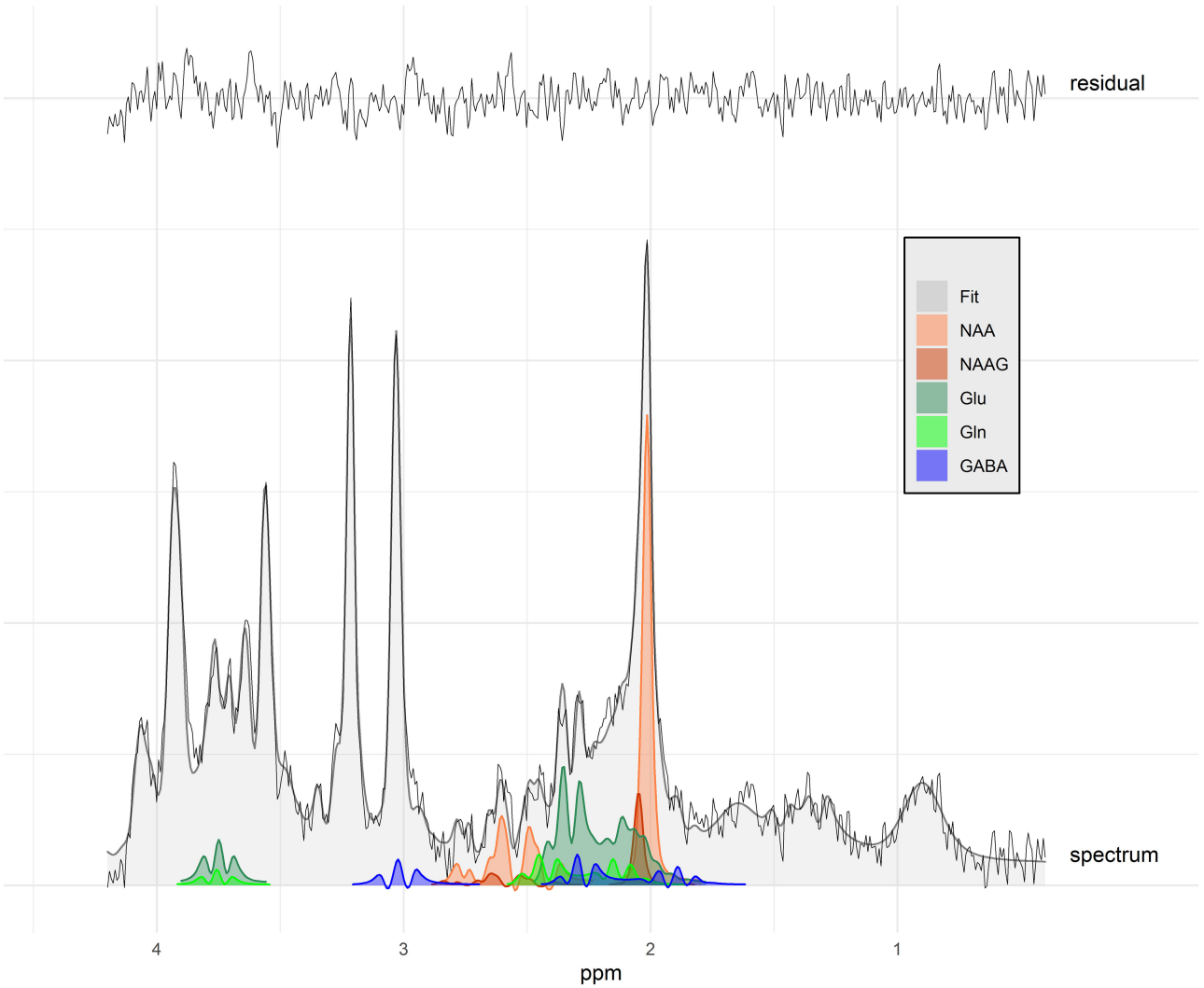

FIGURE 2 | Spectrum with signal-to-noise ratio of 16 (study mean: 12.9) and water peak full width at half maximum of 6.8 Hz (study mean: 6.79 Hz) of an idiopathic epileptic dog with antiepileptic drug treatment. The black line shows the acquired data, the gray area the overall fit, and the colors the individual fit components displayed for selected metabolites. Fit residuals are shown at the top. NAA, N-acetyl aspartate; NAAG, N-acetylaspartylglutamate; Glu, glutamate; Gln, glutamine; GABA, gamma-amino-butyrate.

neuronal damage in this group compared to responders (41). We may speculate that this is also the case in IE dogs with multidrug AEDT in our study. Longitudinal MRS studies during the course of epilepsy with comparison of MRS results between first drug responders and non-responders could help to answer this question.

The third factor resulting in decreased NAA levels is the disease duration and aging. Decreased NAA in humans has been reported with aging in medical resident temporal lobe epilepsy and with increased duration of epilepsy in secondary generalized tonic–clonic seizures bilaterally in the thalamus (28, 42). In our population, dogs under AEDT had slightly higher median age and longer disease duration compared to IE dogs without AEDT. This may also have contributed to the NAA changes, similarly as in human medicine.

Little is known about NAA ratios in dogs suffering from IE. The only study investigating MRS in canine IE was performed with magnetic field strength of 1.5T, MRS VOI placement in the temporal lobe, and included only drug-naïve dogs, without reporting seizure frequency or severity (9). Similar to our findings in IE dogs without AEDT, in the study of Olszewska et al., no significant differences were identified in either NAA/Cr, NAA/choline (Cho), or Cho/Cr between drug-naïve IE dogs and control dogs (9). Interestingly, Olszewska and colleagues found a temporal correlation between NAA/Cho and Cho/NAA ratios and time elapsed between MRS scan and the last reported seizure episode. Higher NAA/Cho ratios were present in dogs with very recent seizure activity, in contrast to lower ratios in dogs with less recent seizure episodes (9). In our study, we included only dogs with a gap of at least 2 days between last reported seizure event

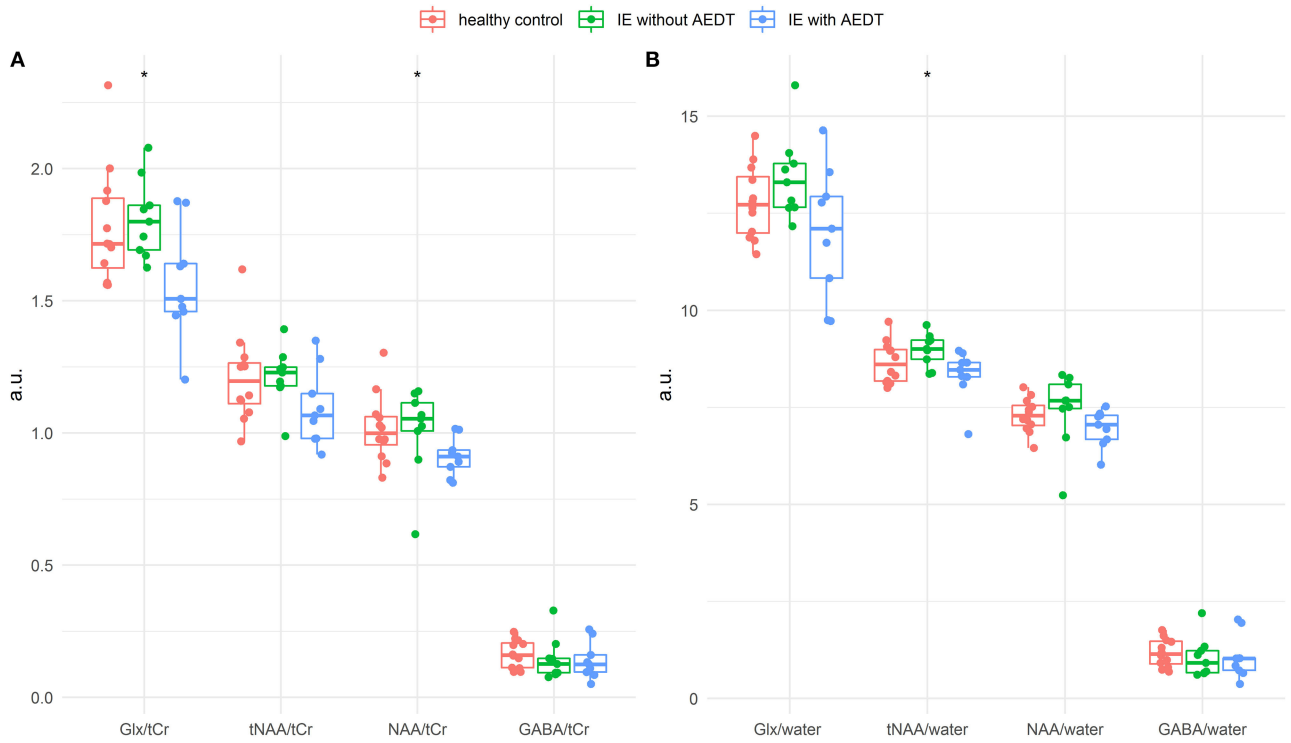

FIGURE 3 | Box plot of thalamic metabolite concentrations relative to creatine **(A)** and water **(B)** in healthy control dogs (red), dogs affected by idiopathic epilepsy with antiepileptic drug treatment (IE with AEDT in blue), and non-treated epileptic dogs (IE without AEDT in green). The Kruskal–Wallis test revealed statistically significant difference in the metabolites NAA/creatine and Glx/creatine, as well as tNAA/water (asterisk). Glx, glutamate–glutamine complex; tCr, total creatine; tNAA, total NAA; NAA, N-acetyl aspartate; GABA, gamma-amino-butyrate.

and MRI/MRS scan. In addition, interval between last reported seizure and MRI/MRS was similar in IE dogs with and without AEDT. Thus, it seems unlikely that the lower NAA ratios detected in our study were influenced by the time interval between the last seizure episode and the time of scanning. It is beyond the scope of this study to draw definitive conclusions about the cause of reduced NAA. Pathological studies systematically proving evidence for neuronal loss in canine epilepsy are lacking (43, 44), but early onset of mental decline/cognitive dysfunction supports impaired neuronal integrity in dogs suffering from IE (45).

In contrast to our hypothesis, we did not find an increase, but rather a decrease in thalamic Glx-to-creatine ratios in dogs with IE under AEDT compared to healthy controls and cases without AEDT.

Even relatively small differences in linewidth have been shown to bias estimates of Glx (46). The linewidth of the water peak was 0.5 Hz higher in IE dogs under AEDT, than in the other groups, and we cannot exclude an influence of the different linewidths on the Glx results. The Glx ratios to water show a similar picture as in the case for NAA. While the difference between IE dogs with and without AEDT stays visible, the difference in the healthy control disappears. This indicates possible differences in the reference signals total creatine or water between the healthy and the IE dogs, which weaken or amplify the differences.

Glx is the sum of glutamine and glutamate, which are present in both neuronal and glial cells. Concentrations of glutamate and glutamine are coupled *via* glutamate–glutamine cycling. Slow rates of glutamate–glutamine cycling, reduced glutamine levels, and a relative increase in glutamate levels have been found in resected epileptogenic hippocampi (47). Glx-to-creatine ratios in human epilepsy depend on the epileptic syndrome investigated and the area of interest within the brain and are not without controversy. While for example increased Glx-to-creatine ratios have been reported in the thalamus in juvenile myoclonic epilepsy, decreased levels have been reported in the frontal cortex (48). Another study investigating the prefrontal cortex in juvenile myoclonus epilepsy found also increased Glx-to-creatine ratios in the thalamus but decreased ratios in the medial prefrontal cortex, an area anatomically very close to the frontal cortex (15). A decrease in Glx is also considered an early biomarker of neuronal degeneration (49), and reduced frontal cortex Glx ratios have been linked to deficient frontal lobe functions in juvenile myoclonus epilepsy (48). No consistent effects of different types of AEDT on Glx levels in humans have been reported (4). It is assumed that antiepileptic drugs do not alter the glutamate concentration directly, but instead decrease the sensitivity of the glutamate receptor. A negative modulation of the voltage-gated channels might then lead to decreased glutamate concentrations (4). So far, no studies have been conducted on the effect of

antiepileptic drugs on Glx concentrations in dogs, making it impossible to distinguish the effect of the drug from the effect of the disease on the Glx results in or study. Remarkably, potential contribution of AEDT associated lower glutamate levels to suboptimal cognitive functioning in patients with epilepsy has been stated (5). Cognitive decline has also been reported in canine IE, with dogs showing cluster seizures and higher seizure frequency having more severe cognitive decline (45). In our study, IE dogs with AEDT had more often history of cluster seizures than those without AEDT. Therefore, having a higher risk for cognitive declines. However, because cognitive function has not been systematically investigated in our study, we cannot draw definitive conclusion on this aspect, and it remains unclear whether lower Glx levels also reflect lower cognitive function in our study population. Glx is difficult to detect with long echo time MRS at 1.5 T, and therefore, the only study investigating canine IE so far has not reported Glx levels (9). Glutamate levels have been measured in the CSF of IE dogs, and CSF levels of this metabolite have been reported as increased in drug-naïve dogs with IE, as well as in dogs under AEDT (50). On the first sight, these results may look contradictory to our data. However, as highlighted before, with the MRS technique applied in this study, one is not able to measure glutamate separately from glutamine. Therefore, the decrease in Glx could rather reflect a reduction in glutamine than one glutamate. Moreover, MRS detects the intra- and extracellular Glx pool. As the intracellular concentration of glutamate greatly exceeds the extracellular glutamate concentration, small increases in the extracellular glutamate concentration can be easily overlaid by intracellular changes (51). In human medicine, impairment of the glutamate–glutamine cycle has been identified in epilepsy, but as we only measured the sum of both metabolites we cannot make conclusions on this aspect. In future, these limitations may be overcome using magnetic field strength higher than 3T or utilizing advanced MRS techniques, which allows an improved separate evaluation of glutamate and glutamine (11).

Beside NAA and Glx, the third major brain metabolite in epilepsy which can be detected by MRS is GABA. GABA is not only the major inhibitory neurotransmitter in the brain, but it is also directly connected to Glx *via* the glutamate–glutamine cycle (11). Moreover, dogs under AEDT received medications with a GABAergic mechanism of action. GABAergic antiepileptic drugs have been shown to increase the GABA concentration in MRS in humans, and GABA concentration has been positively associated with seizure control in humans (4). In our study, GABA was detected in our MRS spectra and we could not identify any significant difference between healthy controls and IE dogs with and without AEDT, respectively. However, a mean %CRLB of GABA of more than 50% clearly demonstrates the difficulty of measuring GABA with the setup used and precludes definitive conclusions. This limitation is partially due to the much lower GABA concentration in comparison with other brain metabolites and the GABA is obscured by signals from more abundant metabolites. Measurements at higher magnetic field strengths and dedicated sequences could help to overcome this technical issue (52).

In this study, we selected our VOI based on human studies in patients suffering from idiopathic generalized epilepsy, which showed significantly different metabolite concentrations primarily in the thalamus (38, 39, 53). In addition, in humans, the thalamus is one of the most important center associated with the loss of consciousness in epilepsy. This also makes the thalamus region interesting for canine IE, which is associated with the loss of consciousness (29). Furthermore, the planning of the VOI in the thalamus is straightforward and contaminations from other tissue types can be mostly avoided despite the small size of the dog brain (**Figure 4**). In epileptic dogs, the thalamus has not been an area of major interest so far. One perfusion study indicated differences in perfusion in the brain of IE dogs compared to healthy control dogs, among others, also in the thalamus (54). Additional support for thalamic involvement has recently been reported by Unger et al. that found bilateral rotary saturation effects in the thalamus using phase-cycled stimulus-induced rotary saturation sequence in an Old English Bulldog with generalized tonic–clonic seizures (55). Even peri-ictal MRI changes have been found in parts of the thalamus in dogs (56). Network analysis of peri-ictal lesions has shown high correlation with cingulate lesions. Combining MRS of the thalamus with MRS in the cingulate gyrus could therefore be one approach for further studies. Although the role of the thalamus in canine IE has still to be elucidated, our study is in line with previous studies and supports the involvement of this brain region in the pathogenesis of canine IE. However, the involvement of the thalamus does not allow conclusions about a possible epilepsy focus. To locate seizure-onset zones, combining MRS with electroencephalography or advanced imaging techniques, such as phase-cycled stimulus-induced rotary saturation could improve diagnostic sensitivity (2, 55).

The limitations of the study include a small sample size and inhomogeneity of the study population. In spite of finite access to cases and healthy controls, this limitation is in balance with expected number of accessible cases per year and in line with animal welfare aspects that reduce the number of experimental animals used. The study population was heterogenous in aspect of (1) IE dogs with and without AEDT, (2) different breeds of dogs, and (3) dogs not matched for age and sex. It would have been necessary to include drug-naïve dogs only, to eliminate a potential bias due to medical treatment on the MRS results. On the contrary, this would have been a preselection criterion influencing the population to newly diagnosed epileptic dogs or dogs with a milder course of the disease, which does not require AEDT. Another limitation is the fact that we evaluated the thalamus only on one side what excludes a side-by-side comparison and does not allow conclusions about possible lateralization. The choice to use the right thalamus was completely arbitrary and not supported by clinical signs. Nevertheless, we cannot exclude that measuring in the minority of the cases the left thalamus instead of the right could have influenced our results. Until now, a potential influence of the dog breed on MRS results has not been investigated. Different breeds have different skull conformations, and this also affects the shape and size of the area of interest in the brain. These anatomical differences could lead to different tissue sampling

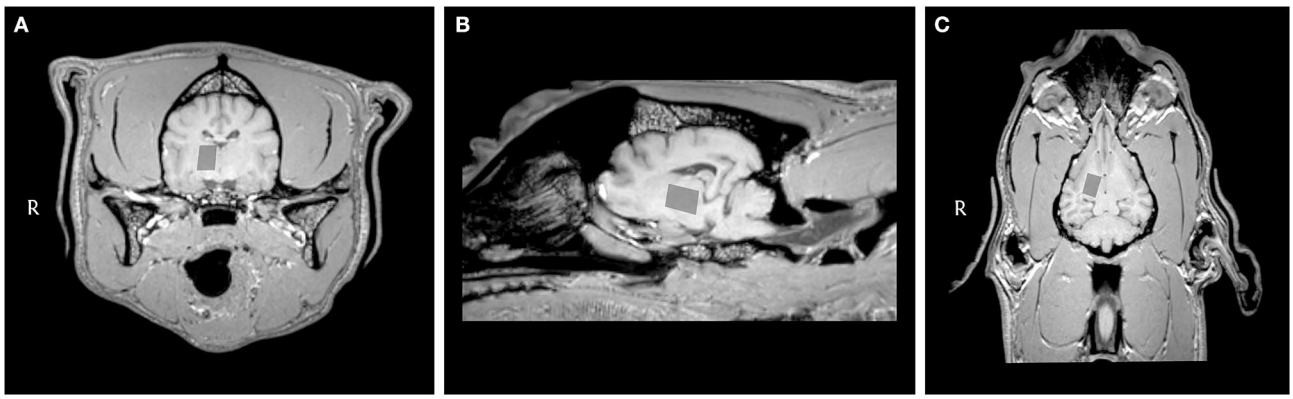

FIGURE 4 | Transverse **(A)**, sagittal **(B)**, and dorsal **(C)** T1-weighted MRI images of the brain of a dog with idiopathic epilepsy showing the location of the voxel of interest in the right part of the thalamus.

with a standardized VOI (e.g., different ratios of gray and white matter and even contamination of the VOI with CSF). Thus, we cannot fully exclude a potential bias based on this effect (33). As the study population was not matched for age and sex, we cannot rule out a potential effect on our measurements. Pairwise comparison between the treatment groups was not corrected for multiple comparison. Considering the set of statistical inferences, this seems to be balanced with the fact that comparing three groups is the lowest level of multiple comparisons possible. All these points probably contributed to the scatter of the results, and additional examinations on a larger and more homogeneous study population may help to get more precise answers.

In our study, MRS was used in research setting to assess advanced MRI techniques. However, it has been demonstrated that MRS, also in veterinary medicine, can be useful in a clinical approach (6–8, 57). Therefore, MRS should be considered as routine supplementary tool in selected patients together with conventional MRI to permit an improvement in diagnostic sensitivity. In human epilepsy research, it was suggested that future MRS studies should include MRS techniques that allow differentiation between glutamate and glutamine and to also measure GABA (11). Possibly, the availability of MRI scanners with higher field strength and the availability of new sequences will also allow this in canine epilepsy research. Furthermore, longitudinal studies with a larger and homogenous study population could improve and unfold the potential of MRS in the investigation of IE in veterinary medicine. This might help to develop biomarkers for drug response in (canine) IE and could strengthen the dog as model for the human epilepsy.

In 2015, the veterinary epilepsy task force has introduced MRI guidelines for canine IE, but these guidelines do not specify the use of MRS (1). Such consensus recommendation exists for MRS methods in humans and rodents (58, 59). By addressing MRS in canine epilepsy and applying human recommendations for reporting of methods (**Supplementary Table S1**) and results, we also aimed to improve standardization in MRS in veterinary medicine and we hope that in the next update of the veterinary epilepsy task force, imaging guidelines for MRS in IE will be included (60).

CONCLUSION

Using MRS, we have detected reduced NAA-to-creatine ratios in IE dogs with AEDT compared to healthy controls and IE dogs without AEDT, as well as reduced Glx-to-creatine ratios in IE dogs under AEDT compared to IE dogs without AEDT. MRS can be considered as an additional imaging technique to characterize disease severity and an additional tool for canine epilepsy research, but technical limitations have to be kept in mind when interpreting the results. Further studies are needed to improve and unfold the potential of MRS in the investigation of IE in veterinary medicine and possibly create a canine model for the study of epilepsy with MRS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Cantonal Authorities according to Swiss Law under Animal License Nos. ZH272/16 and ZH046/20. Written informed consent was obtained from the owners for the participation of their animals in this study.

CONTRIBUTION TO THE FIELD

Magnetic resonance spectroscopy (MRS) is an advanced neuroimaging technique, which allows non-invasive estimation of metabolite concentrations within a selected volume of interest. Although MRS is extensively investigated in human epilepsy and rodent epilepsy models, MRS information in canine epilepsy is

scarce. The aim of our study was to assess and compare thalamic MRS in healthy control dogs and in idiopathic epileptic dogs affected by generalized seizures, as well as to assess possible differences between idiopathic epileptic dogs with and without antiepileptic drug treatment. Significant differences, similar but not in complete accordance with MRS studies in human medicine, were detected between the investigated canine groups. In this article, we discuss these novel results and the possible reasons for the discrepancies between human and veterinary medicine. With our study, we encourage the use of MRS in canine epilepsy research and propose to add a standardized MRS protocol in the magnetic resonance imaging guidelines for canine idiopathic epilepsy, similar to the consensus recommendation approved for MRS application in humans and rodents. Further studies are needed to improve and unfold the potential of MRS in the investigation of idiopathic epilepsy in veterinary medicine, which may be used as canine model for the study of epilepsy with MRS.

AUTHOR CONTRIBUTIONS

KB, HR, and NZ designed the study. KB and FS collected clinical data. NM collected the MRS data and drafted the initial manuscript. NZ selected the MRS protocol and performed the LCModel and the statistical analysis. All authors edited the manuscript and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.885044/full#supplementary-material

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ORIGINAL RESEARCH
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Phenotype of Idiopathic Epilepsy in Great Swiss Mountain Dogs in Germany—A Retrospective Study

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Genetic predisposition of idiopathic epilepsy (IE) has been demonstrated in individual breeds. According to the responsible breeding association in Germany, the average incidence of registered Great Swiss Mountain Dogs (GSMDs) with seizures between the years 1999 and 2019 is 2.56%, a genetic predisposition in this breed is suspected. To describe the seizure phenotype and to examine seizure causes, a retrospective, questionnaire-based study was performed. In cooperation with the Swiss Mountain Dog Association of Germany e.V. (SSV e.V.), 114 questionnaires filled in by owners of GSMD displaying seizures and filled in by their respective veterinarians between the years 2005–2021 were evaluated. Seizure characteristics, clinical and further examinations, treatment, treatment responses, and pedigree information were collected. In this study, 94 (83.06%) dogs had IE (suspected genetic epilepsy) confirmed with confidence level TIER 1, 2, or 3. The remaining 20 dogs showed the signs of structural epilepsy, reactive seizures, or epilepsy of unknown cause and were therefore excluded from further analysis. The average age at seizure onset was 28.83 months. Male GSMDs were significantly more often affected by IE than females. The most common seizure type was focal evolving into generalized seizures (64.5%). Seizures often began with vomiting, retching, or salivation. Cluster seizures (CS) (48.9%) and status epilepticus (SE) (37.2%) were observed in a large proportion of dogs. During the observation time, a total of 49 animals (52.13%) died. Out of those, 19 dogs (20.21%) were euthanized in SE or during CS and 14 dogs (14.9%) died spontaneously during CS or SE. The median age at death was 4 years, and the median survival time for the time, when the dog was suffering from seizures, was found to be 18 months. Both occurrence of CS ($p = 0.0076$) and occurrence of SE ($p = 0.0859$) had an impact on survival time. In GSMD, idiopathic epilepsy presents with a severe phenotype with frequently occurring CS and SE. This study could serve as basis for further genetic evaluations as well as to provide individual treatment recommendations.

Keywords: idiopathic epilepsy, seizure, phenotype, dog, incidence, breed

INTRODUCTION

Epilepsy is one of the most common neurological chronic diseases in humans and dogs (1–3). The prevalence is generally reported to be 0.6–0.75% in the overall canine population and up to 18.3% in certain breeds (1, 4–6). Epilepsy is described as a disorder of the brain, characterized by a persistent predisposition to produce epileptic seizures (7, 8). The definition of seizure etiology has been standardized for veterinary medicine by the IVETF consensus: a distinction is made between structural epilepsy (StE) and idiopathic epilepsy (IE), which in turn both differ from reactive seizures (RS) (7).

Furthermore, according to its etiology, IE is divided into epilepsy of unknown cause, genetic epilepsy, and suspected genetic epilepsy (7), with this study referring to the latter. The basis of suspected genetic epilepsy is formed by familial accumulation, breed prevalence >2%, and/or genealogical analysis (7). In contrast to genetic epilepsy, the identification of a causative gene has not yet been determined (7). Some dog breeds, including a striking number of herding and working dog breeds, are already described to be predisposed to IE (2, 6, 9). It should be noted that a severe clinical manifestation of IE has already been confirmed in herding dogs such as the Australian Shepherd and the Border Collie (10, 11). The Swiss Mountain Dog Association of Germany e.V. (SSV e.V.) recorded 6,179 registered Great Swiss Mountain Dogs (GSMDs) in their database during 1999–2019, of which 158 dogs were registered to have seizures (12). However, not all dog owners provided data about their GSMD, an unknown number of additional dogs with seizures might be suspected. On average, this results in a rate of 2.56% GSMD with seizures and during the years 1999–2019, the frequencies vary from 0.93 to 4.41% (12). This is not equal to the rate of German Great Swiss Mountain Dogs with IE. Based on Sauer-Delhées et al.'s (13) study, the Swiss Kennel Club for Great Swiss Mountain Dogs reported a rate of 2% of dogs with epilepsy between 1999 and 2019 in Switzerland. In contrast to this study, in the Swiss questionnaire-based study, data of 12 GSMD were provided for analysis within less than a year (13). In addition, it was found that there are significant differences regarding heritability, clinical manifestation, seizure semiology, and treatment responses of IE between "geographically distinct populations of the same breed (6)".

The present questionnaire-based study is therefore dedicated to clarifying the hypothesis, which idiopathic epilepsy in German Great Swiss Mountain Dogs is characterized by a severe phenotype.

Abbreviations: ASD, Antiseizure drug; CS, Cluster seizure; CSF, Cerebrospinal fluid; CT, Computed tomography scans; EEG, Electroencephalography, GSMD, Great Swiss Mountain Dog; IE, Idiopathic epilepsy; IVETF, International Veterinary Epilepsy Task Force; µl, Microliter; mg, Milligram; ml, Milliliter; MRI, Magnetic resonance imaging; n, Number of dogs; RS, Reactive seizures; SE, Status epilepticus; StE, Structural epilepsy; SSV e.V., Swiss Mountain Dog Association of Germany e.V.; TIER 1-2, Confidence level for the diagnosis of IE.

MATERIALS AND METHODS

Study Population

This retrospective study was conducted in collaboration with the SSV e.V. This breeding club designed the questionnaire after contacting Diplomates of the European College of Veterinary Neurology and provided questionnaires of Great Swiss Mountain Dogs presenting seizures, collected from 2005 to 2021. Thus, several generations and litters in the population of German GSMD showing seizures, registered in the SSV e.V. and bred within or outside the SSV e.V., were identified, but the exact number of GSMDs with seizures is not known. The SSV e.V. was informed either by breeders or owners of the specific dogs displaying seizures and provided the questionnaires. As a further requirement, clinical and imaging examinations from veterinary practices and clinics were used. These included blood, urine, and cerebrospinal fluid (CSF) examinations, findings of ultrasonography, radiographs, and magnetic resonance imaging (MRI) or computed tomography scans (CT), as well as video recordings of the seizure events. Furthermore, a statement regarding the clinical assessment and evaluation of the further examinations was completed by the treating veterinarian or by the SSV e.V. consulting veterinarian. In addition, based on this information, expert opinions were written by Diplomates of the European College of Veterinary Neurology for the majority of the questionnaires, to determine whether the dogs were suspected or reasonably suspected of having idiopathic or structural epilepsy or reactive seizures, depending on the completeness of the available data. With the help of the patient management system ("easy vet," VetZGmbH, Isernhagen, Germany) of the Clinic for Small Animals, Foundation of the University of Veterinary Medicine Hannover, additional information on study participants who were patients there themselves could be obtained.

Idiopathic epilepsy was diagnosed according to the International Veterinary Epilepsy Task Force consensus proposal confidence levels (14). Dogs with IE are usually between 6 months and 6 years old at the age of their first seizure and show at least two unprovoked epileptic seizures 24 h apart (14). In addition, physical and neurological examination, minimum database blood test, and urinalysis are without specific findings in the interictal period, which corresponds to the TIER 1 confidence level of the IVETF (2, 14). Based on this, fasting and postprandial bile acids are carried out at the TIER 2 level, as well as MRI of the brain and CSF examination, which should also be unremarkable (14). If electroencephalography (EEG) is also performed, this is referred to as the TIER 3 level (14).

The questionnaire was divided into individual topics and included the following items: signalment of the dog, medical history of the dog, seizure history, age of the dogs at seizure onset, seizure frequency and duration, seizure characteristics, used antiseizure drugs (ASDs), and effect and side effects of the ASDs. Through further contact of the participants with the SSV e.V. or the Clinic for Small Animals of the University of Veterinary Medicine Hannover Foundation, time of death, cause of death, and possible response to therapy could be

additionally determined, if these had not already been noted in the questionnaire.

After the data review of the information collected on each animal, 20 dogs with confirmed or presumed structural brain disease, reactive seizures, syncope, or paroxysmal dyskinesia were excluded from the study (**Figure 1**).

Of these, 7 dogs had presumed reactive seizures caused by either hypoglycemia, liver disease, electrolyte disorders, or abnormal hematologic findings such as anemia (15). A total of 6 dogs were diagnosed with structural epilepsy based on MRI or CT findings or were suspected to have structural epilepsy based on age older than 6 years at the onset of the first seizure or abnormal findings on interictal neurologic examination without more in-depth diagnostic testing (14). Additionally, 5 dogs were diagnosed with idiopathic epilepsy of unknown cause. These dogs either had their first seizure at an age of <6 months and had no family history of seizures, or atypical seizure characteristics. Animals <6 months of age or >6 years of age at first seizure onset were not included because they did not have the most common age spectrum of seizure onset for IE as defined by the IVETF (7). Exceptions were dogs that have been subjected to further diagnostics in the sense of confidence level TIER 1 or 2 of the IVETF consensus statement and these diagnostics proceeded without any special findings (2 dogs) (14). Furthermore, syncope was suspected in one dog, paroxysmal dyskinesia was suspected in another, and thus, both were excluded from the study (14, 16). Dogs suspected of suffering from IE because of typical seizure characteristics, familial accumulation of the seizure disorder, and required age at first seizure onset between 6 months and 6 years were included (24 dogs; **Figure 1**).

After applying all inclusion and exclusion criteria, data from 94 study participants with suspected or diagnosed idiopathic epilepsy with suspected genetic origin (confidence levels TIER 1, 2, and 3) could be analyzed (**Figure 1**).

Statistical Analysis

The subsequent statistical analysis was performed using Microsoft® Excel 2021 and a commercial statistical software [Statistical Analysis System for Windows SAS®, version 9.4 using the SAS® Enterprise Guide® Version 7.15 Client (SAS Institute Inc., Cary, North Carolina, USA)]. The data to be evaluated were checked for normal distribution using the Shapiro–Wilk and Kolmogorov–Smirnov tests and by visual assessment of individual histograms. Descriptive statistics are provided; in case of a non-significant deviation from the normal distribution, mean values were taken. In contrast, when there was a significant deviation from the normal distribution, the median (m) and minimum and maximum (min–max) were described. Logistic regression was used to assemble correlations between qualitative characteristics, as well as between quantitative and qualitative variables. The Wilcoxon two-sample test was applied to non-normally distributed quantitative data and the Fisher's exact test to qualitative data. A p-value of < 0.05 was considered statistically significant and a p-value < 0.1 was considered statistically noticeable.

RESULTS

Study Population

The participating study population of the present work consists of a total of 94 Great Swiss Mountain Dogs with idiopathic epilepsy. IE was diagnosed or suspected in these dogs according to confidence level 1–3 due to typical seizure characteristics, familial accumulation, and required age at seizure onset between 6 months and 6 years (suspected IE: 24 dogs; TIER 1: 46 dogs; TIER 2: 23 dogs; TIER 3: 1 dog). Overall, MRI and CSF examination were performed for 23 (24.5%) of the dogs (TIER 2 confidence level).

Deviations from the basic population of 94 included dogs in the descriptive analysis result from incomplete questionnaires and were thus evaluated as missing and not interpreted further.

The gender distribution of GSMD is 37.2% female (n = 35) and 62.8% male (n = 59). Of these, 9 (9.6%) were spayed female, 26 (27.7%) were intact female, 6 (6.4%) were chemically neutered male, 9 (9.6%) were neutered male, and 44 (46.8%) were intact male (**Figure 2**). Male GSMDs were found to be significantly more likely to be affected than females in this study population ($p = 0.0133$).

The average weight of the animals is 50.29 kg (range 31–75 kg). The median shoulder height is 69.5 cm (range 52–76 cm). The age of the dogs at seizure onset averages 28.83 months (range 4–79 months). However, neither sex (p = 0.6613) nor reproduction status (p = 0.1179) showed a significant effect on age at seizure onset.

Patient History

A history of special events at birth was reported for 14 (15.2%) GSMD. This included information that the dog was the first-born puppy before a caesarian-section was necessary (n = 4; 4.4%), that delivery occurred by caesarian-section (n = 6; 6.5%), or that stillbirths occurred in the litter (n = 2; 2.2%). Pre-existing conditions were mentioned in 28 (30.1%) dogs, these included allergies/food intolerances (n = 11; 11.8%), gastrointestinal diseases (n = 14; 15.1%), cardiac diseases (n = 1; 1.1%), infection-related diseases (n = 15; 16.1%), and liver or kidney diseases (n = 2; 2.2%).

For 7 (7.5%) dogs, owners reported accidents in the form of a car accident (n = 2; 2.2%), contact with an electric fence (n = 1; 1.1%), or an unobserved event (n = 4; 4.3%).

In 49 (52.69%) dogs, owners were aware of relatives of their dog experiencing epileptic seizures.

About 2 weeks before their dog's first seizure, 5 (5.4%) owners reported an event in terms of anesthesia (n = 3; 3.3%), an infectious disease (n = 1; 1.1%), or both (n = 1; 1.1%) in their animal.

In addition, periods in which the dog presented episodic behavioral changes such as chewing/smacking (n = 10; 10.9%), excessive licking (n = 6; 5.5%), restlessness (n = 3; 3.3%), fly-snapping (n = 2; 2.2%), drowsiness (n = 2; 2.2%), clinginess (n = 1; 1.1%), aggression (n = 1; 1.1%), and hallucinations (n = 1; 1.1%) were recorded.

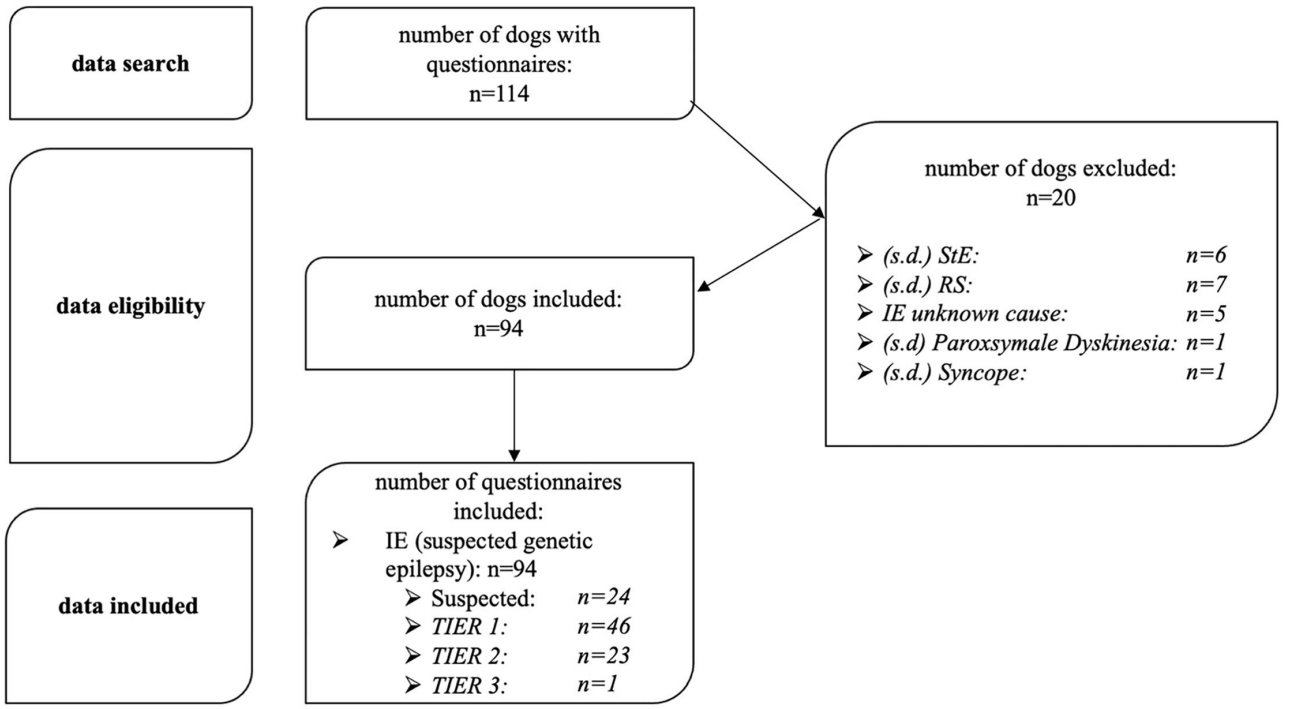

FIGURE 1 | Study protocol for data analysis, inclusion and exclusion criteria. n, number of dogs; StE, structural epilepsy; IE, idiopathic epilepsy; suspected, suspected idiopathic epilepsy; TIER, confidence level (TIER 1,2,3); RS, reactive seizures; s.d., suspected diagnosis.

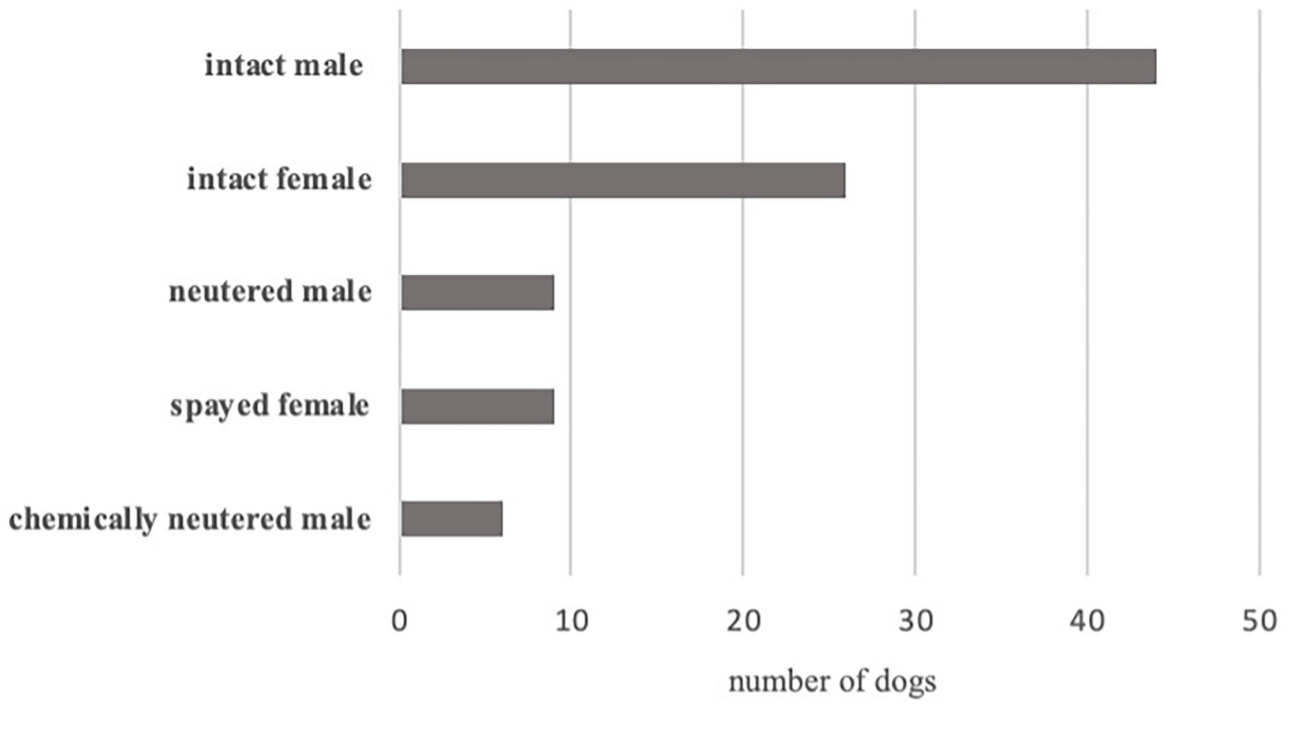

FIGURE 2 | Distribution of sex of Great Swiss Mountain Dogs with idiopathic epilepsy, whole study population.

Seizure Characteristics

In more than half of the dogs (n = 60; 64.5%), focal epileptic seizures evolving into generalized epileptic seizures were described (**Table 1**). Focal epileptic seizures started with vomiting (n = 39; 43.8%), retching (n = 21; 23.6%), hypersalivation (n = 19; 21.4%), or head shaking (n = 15; 16.9%) (**Table 1**). Generalized seizures were described in 31 (33.3%) dogs. A total of 5 (5.4%) dogs had a generalized seizure without observing the onset of the seizure. Single focal seizures were shown by 5 (5.5%) dogs (**Table 1**). Tonic-clonic seizures were described in the majority of GSMD (73 dogs; 90.1%) and tonic seizures in 8 (9.9%) dogs. In addition, autonomic signs during a seizure event were noted in 68 (82.9%) GSMD in the form of hypersalivation (n = 61; 74.4%), urination (n = 58; 70.7%), or defecation (n = 8; 9.8%).

Prodromal signs were observed in 24 (25.8%) GSMD and manifested in behavior changes such as restlessness (n = 18; 19.4%), clinginess to owner (n = 8; 8.6%), searching for quiet places (n = 4; 4.3%), disorientation (n = 2; 2.2%), or aggression (n = 1; 1.1%).

The postictal phase lasted for 1 h (median value; range 1–4,320 min) and was reported in 90 dogs. A total of 60 dog owners provided information on the postictal behavioral changes in their GSMD. Of these, 59 GSMD owners described them as follows: disorientation (n = 45; 75%), restlessness (n = 30; 50%), ataxia (n = 21; 35%), clinginess (n = 18; 30%), drowsiness (n = 15; 25%), polyphagia (n = 15; 25%), polydipsia (n = 11; 18.3%), aggression (n = 10; 16.7%), vocalization (n = 10; 16.7%), blindness/running into objects (n = 5; 8.3%), or inappetence (n = 1; 1.7%).

Seizure triggers of GSMD were reported by 12 (12.9%) patient owners: seizures occurred predominantly after stressful situations, excitement, exertion/intense play, and specific noises.

In most GSMD, seizures usually occurred early in the morning (n = 69; 77.5%), during sleep (n = 72; 78.26%), or during periods of rest (n = 84; 91.3%). In some dogs, seizures occurred during the day (n = 55; 61.8%), at night (n = 47; 52.8%), in the evening (n = 39; 43.8%), or during excitement (n = 15; 16.3%). No differences in the triggering situation or time points were described between the first seizure event and the following ones.

TABLE 1 | Seizure characteristics of Great Swiss Mountain Dogs with idiopathic epilepsy (n = 93/100%; *n = 91/100%; $n^{\bullet}$ = 89/100%).

Seizure type	Number of dogs	Percentage
Focal seizures*	5	5.49
Generalized seizures	31	33.33
Generalized seizures with unknown start	5	5.38
Focal seizures evolving into generalized seizures	60	64.52
- Hypersalivation•	19	21.35
- Vomiting•	39	43.82
- Retching•	21	23.60
- Head shaking•	15	16.85
Cluster seizure	46	48.94
Status epilepticus	35	37.23

n, number of dogs; n, number of dogs in the category focal seizures, n•, number of dogs in the category focal starts.*

Seizure Frequency, Cluster Seizures, and Status Epilepticus

The median interval between the first and second seizure in the GSMD study population was 30 days (range 1–79 days). The median number of seizures per 6 months was 4 (range 1–35 seizures/6 months). Most frequently, a seizure lasted 3 min (range 0.2–20 min) and was based on the subjective perception of the owner. The median number of seizures before initiation of ASD treatment was 3 seizures (range 1–7 seizures). The age at initiation of therapy with ASDs was 30 months (median value; range 5–82 months). At least one status epilepticus was observed in 35 (37.23%) GSMDs and 46 (48.94%) dogs showed cluster seizures (**Table 1**). Of these, 11 dogs developed status epilepticus only and 22 dogs displayed cluster seizures only, with 24 dogs experiencing both. A correlation between the occurrence of cluster seizures and status epilepticus was shown in the present GSMD study population (p = 0.0052).

However, evidence for a correlation between age at seizure onset (p = 0.9719), interval in days between first and second seizure (p = 0.9472), number of seizures per 6 months (p = 0.5688), and gender (p = 0.3710) and occurrence of status epilepticus could not be demonstrated. In addition, age at seizure onset (p = 0.4159) or the interval in days between the first and second seizures (p = 0.8245) did not significantly affect the occurrence of cluster seizures.

ASD Treatment

In the study population, continuous ASD treatment was administered to 72 dogs (76.6%). In those cases, 54 (57.4%) dogs were treated with 1 ASD, 17 (18.1%) with 2 ASDs, and one (1%) dog with 3 ASDs. Phenobarbital was administered to 60 (63.8%) GSMD. The last dosage the owners administered to their dogs of continuous phenobarbital application given was 5.8 mg/kg/d (median value; range 1.1–11.3 mg/kg/d) and was reported in 57 dogs. Phenobarbital serum levels were reported in 19 cases (median 20.12 μg/ml; range 5.5–38.7 μg/ml). Imepitoin was used at a median dose of 23.6 mg/kg/d in 17 dogs. Potassium bromide was used at a median dose of 23 mg/kg/d in 14 dogs. Levetiracetam was administered to 4 dogs: of these, 1 dog received pulse protocol, modified from Packer et al. (17), and another dog received levetiracetam after each seizure, but no protocol was specified. The third GSMD was treated with a dose of 95.2 mg/kg/d, and for the fourth dog, only the drug was mentioned. A total of 35 (37.2%) owners reported treating or having their dog treated with diazepam during seizures. In addition, medium chain triglyceride oil was administered in 3 dogs with no indication of the dosage or regimen of use.

The following ASD side effects were observed in 43/62 (69.4%) GSMD: drowsiness (n = 18; 29%), polyphagia (n = 25; 40.3%), polydipsia (n = 22; 35.5%), incoordination/unsteady gait (n = 11; 17.7%), or vomiting (n = 1; 1.6%). The

adverse reactions occurred in 36/57 (63.2%) dogs after phenobarbital administration, in 9/15 (60%) animals after imepitoin administration, and in 7/15 (46.7%) after potassium bromide administration.

Owners reported that 5 (9.4%) dogs became seizure free after ASD treatment. In another 5 (9.4%) dogs, owners could not give exact details about the treatment response. In 27 (50.9%) GSMDs, owners reported a reduction in seizure number, seizure length, or seizure intensity at the time of questionnaire submission. This information corresponds to the subjective feeling of the pet owner, as an exact seizure frequency could not be calculated based on the study design and questionnaire evaluation. According to the owners, 16 (30.2%) GSMDs did not respond to ASD treatment.

Statistically, the age of the GSMD at seizure onset showed no significant effect on the response to therapy ($p = 0.2617$).

Outcome

A total of 49 (52.13%) GSMDs died in the study population during the study period. Of these, 19 (20.2%) were euthanized in SE or CS, and 14 (14.9%) dogs died spontaneously during SE or CS. Another 3 (3.2%) dogs died, and 6 (6.4%) dogs were euthanized during this period with no indication of cause of death. In 7 (7.5%) dogs, only the time of death was noted, leaving the manner and cause of death unknown. Moreover, 11 of the dead dogs were diagnosed with IE TIER 2 and one dog with TIER 3. In addition, in 45 (47.9%) dogs, the survival status was not described (**Table 2**). Since there is no explicit question regarding this aspect, it can be assumed that these 45 dogs are still alive.

Dogs died with a median age of 48 months (range 10–207 months), with a median survival time, from first seizure to time of death, of 18 months (range 0.5–188 months). Neither age at seizure onset ($p = 0.2217$) nor gender ($p = 0.3419$), number of ASDs applied ($p = 0.6942$) or administration of phenobarbital ($p = 0.4125$) or administration of imepitoin ($p = 0.1791$) were able to significantly influence survival time. In addition, neither gender ($p = 0.9324$) nor reproduction status ($p = 0.3809$) had a significant effect on age at death. However, the occurrence of CS was found to have a significant impact on survival time ($p = 0.0076$), and the occurrence of SE was found to have a statistically noticeable impact on survival time ($p = 0.0859$).

TABLE 2 | Survival rate of the entire study population at the time of evaluation; SE, status epilepticus; CS, cluster seizure.

Outcome	Number of dogs	Percentage
Death, unknown reason	10	10.64
Death during SE or CS	14	14.89
Euthanasia because of SE or CS	19	20.21
Euthanasia, unknown reason	6	6.38
Probable alive	45	47.87

DISCUSSION

In this study, the phenotype of idiopathic epilepsy in GSMD is described.

In predisposed breeds, the prevalence of IE ranges from 1.25 to 18.3%, and over the years, more and more dog breeds have been added to the predisposition list for idiopathic epilepsy (4, 6, 18–22). The basis for a suspicion of genetic epilepsy is a familial accumulation, a breed prevalence of >2% and/or a genealogical analysis (7). From the mountain dogs, the Bernese Mountain Dog is already on this list (6, 21). The SSV e.V. also became aware of increased numbers of GSMD exhibiting seizures and reported a rate of 2.56% GSMD with seizures, with frequencies varying from 0.93 to 4.41% during the years 1999–2019 (12). More than half of the owners who participated in the present questionnaire-based study had knowledge of their dogs' relatives presenting epileptic seizures.

Based on these findings, a breed predisposition of idiopathic epilepsy is highly suspected in the Great Swiss Mountain Dog and the hypothesis of this study that GSMD predominantly present a severe phenotype of idiopathic epilepsy needs to be investigated.

The statistical analysis of the underlying study population indicates that male GSMDs are significantly more often affected by IE than females. This finding is supported by other studies that have previously demonstrated the same effect in some other breeds, such as the Bernese Mountain Dog (21), Australian Shepherd (11), Finnish Spitz (23), Irish Wolfhound (4), and Golden Retriever (24). The sex distribution of GSMD in the Swiss study by Sauer-Delhées et al. (13), which has a smaller number of participants, records 61.8% male affected animals, mirroring our results.

Another characteristic considered in our study is the age of GSMD at seizure onset, which is on average of 2.4 years. This is slightly lower than the Swiss GSMD of the Swiss Breeders' Association in which the onset of the disease is reported to be 3 years on average (13). However, the time of seizure onset in other herding dog breeds such as the Bernese Mountain Dog was reported to be 2.2 years (21) and for the Border Collie at 2.37 years (10). Those numbers are very similar to our findings and further support our analysis.

A differentiation between primary generalized seizures and focal seizures evolving into generalized seizures is elementary. Focal seizures have a regional origin and are characterized by motor signs, such as facial twitching or head shaking (25, 26). Possible signs can also be attributed to the autonomic nervous system, such as vomiting and salivation, or manifest in behavioral changes and are characterized by their short duration (seconds to minutes) and their frequent transition into a generalized epileptic seizure (7, 25). Primary generalization has been reported as the most frequently occurring seizure type in many dog breeds in the older literature (6, 21, 24). After the implementation of the described differentiation, focal seizures with secondary generalization are now referred to as the dominant seizure type in dogs with idiopathic epilepsy (7). Furthermore, the described seizure type is also reported in most dog breeds, such as the Poodle with 60% (27) and the Border Collie with 78% (10). In this study, focal seizures, which generalize secondarily, were

described in more than half of the dogs (64.5%) and presented as vomiting (43.8%), retching (23.6%), hypersalivation (21.4%), or head shaking (16.9%). Likewise, Sauer-Delhées et al. (13) recorded focal motor seizures with secondary generalization in more than 50% (7/12) of the cases in the Swiss GSMD population. However, in their study, characteristics such as vomiting shortly before a seizure were assigned to autonomic signs and not to the focal onset (13), which affects the comparability.

Administration of ASDs forms the basis of IE therapy (28). In a study by Berendt et al. (29), however, no statistically significant differences in age at death or number of years with epilepsy were found between drug-treated and untreated dogs. However, in a previously published manuscript, an increase in seizure frequency without long-term therapy was detected (30). Considering the treatment response to ASDs in our study, seizure freedom was achieved in only 5 (9.4%) dogs. Seizure freedom, respectively, a positive treatment response in studies, is defined as a seizure-free period equal to more than three times the length of the longest interictal period prior to initiation of therapy and lasting at least 3 months (31). Furthermore, 27 (50.9%) owners of German GSMD reported a reduction in seizure number, seizure length, or seizure intensity after the administration of ASDs at the time of questionnaire completion compared to the start of ASD administration. To determine the percentage of seizure frequency reduction and time frame of seizure freedom, the baseline seizure frequency should be considered, and the exact seizure frequency must be calculated (31). Such data were not sufficiently reported in the questionnaire of this study. However, data on seizure reduction and seizure freedom in canine epilepsy correspond to the subjective perception of the animal owners (10, 11, 13). According to owners in our GSMD study population, 16 (30.2%) dogs responded inadequately to ASD treatment. Considering subjective perception, the aspects of remission (9.4%) and seizure reduction (50.9%) of our German GSMD are reflected in similar studies (10, 11, 13). Compared to other breeds, according to Packer et al. (32), the Border Collie was the breed least likely to go into remission (0%) or achieve a reduction in seizure frequency of $\geq$50% (40%) (32). However, in Hülsmeyer et al. (10), the remission rate of Border Collies was 18%. In Australian Shepherds, a remission rate of 13% was reported (11). In the Swiss study of GSMD with IE, 33.3% of animals (4/12) showed a reduced seizure frequency with therapy; however, the remaining 66.7% (8/12) did not respond adequately to antiseizure drugs (13).

An older age at seizure onset seems to be associated with an increased likelihood of achieving remission or reduction in seizure frequency by $\geq$50% (32). In the study population of Australian Shepherds by Weissl et al. (11), a relationship between age at seizure onset and seizure control was also detected. However, in this study, age of GSMD at seizure onset did not reveal a statistically significant effect on response to treatment. This was in line with the results of evaluations of German Border Collies with IE (10). Consequently, the multifactorial influences on treatment response and drug resistance are presented and mirrored in this study, influences, which remain largely unknown despite years of research into the underlying mechanisms (33).

Complementarily, under the influencing risk factors for achieving remission, the occurrence of cluster seizures should be considered (32). In this study, 35 (37.23%) GSMDs suffered at least one status epilepticus, 46 (48.94%) dogs showed cluster seizures, and a correlation between the occurrence of cluster seizures and status epilepticus could be established. Similar results were also detected in the following studies: Sauer-Delhées et al. (13) determined the occurrence of status epilepticus in 41.6% of GSMD in Switzerland and 50% suffered cluster seizures. In a study of Belgian Shepherds with IE, serial seizures were reported in one-third of the dogs (34). Cluster seizures were described in 68% of Australian Shepherds with IE and status epilepticus in 60% (11). In the German study by Hülsmeyer et al. (10), a total of 94% of all included Border Collies experienced at least one episode of cluster seizures and 60% of all dogs experienced at least one episode of status epilepticus.

Furthermore, in our study, neither the influence of age at first seizure nor the time in days between the first and second seizure proved to be statistically significant on the occurrence of cluster seizures or status epilepticus. Similarly, no statistically significant relationship was found between sex and the occurrence of status epilepticus. The evaluation of the Australian Shepherd study revealed the same findings, and the severity of the clinical course, meaning the occurrence of serial seizures or a status epilepticus, was also independent of the age at seizure onset and of gender (11).

The occurrence of cluster seizures and status epilepticus in contrast to single, short seizures does not only have an impact on the severity of the clinical course, but also on the decision to euthanize the diseased dog: approximately two-thirds of dogs with epilepsy are euthanized due to status epilepticus or cluster seizures (35). Studies found that the occurrence of cluster seizures is associated with a shorter survival time (35), as well as a higher possibility of euthanasia (36). In addition, dogs that suffer from serial seizures have a lower chance of achieving seizure freedom (32). It should also be noticed that patients who did not receive therapy had a significantly increased risk of being euthanized (29). In a study of "Risk Factors for Survival of Dogs with Epilepsy", death or euthanasia was directly associated with epilepsy in 52% of dogs, with a significant proportion experiencing pre-existing cluster seizures, status epilepticus, or both (36).

Overall, in the study population presented here, 52.13% (49/94) of GSMDs died during the study time. About 35.1% (33/94) dogs were euthanized or died spontaneously in direct association with IE. Proportionately, 20.2% (19/94) were euthanized in status epilepticus or during cluster seizures. About 14.9% (14/94) of dogs died spontaneously during status epilepticus or cluster seizures.

In comparison, in the Swiss GSMD study, 25% (5/20) of the affected animals died due to seizures (13). This result can be attributed to the much shorter research time in contrast to the discussed study. In Irish Wolfhounds with IE, the lethality was reported to be 60.3% (76/126) (4). For comparability with our work and others, a mortality of 52% (76/146) can be calculated here from the total study population (4). In a Border Collie study,

47% (23/49) of the participating dogs had died at the time of evaluation: 74% (17/23) of the animals were euthanized due to IE and two dogs died in SE (10). Again, a mortality of 34.7% (17/49) can be calculated from the total study population of Border Collies (10). Regarding Petit Basset Griffon Vendéen with epilepsy, 45/471 dogs died in a study population, of which 13.3% (6/45) died from epilepsy itself (20). However, when converted to the total study population, consisting of 471 Petit Basset Griffon Vendéen, only 1.3% (6/471) died as a direct result of epilepsy (20). In the Australian Shepherd, death due to IE was reported in 28% (14/50) (11).

In our German GSMD with IE, the median age at death was 4 years with a median survival time from first seizure to time of death of 1.5 years. This outcome was also shown by Heske et al. (5) in a cohort study of epilepsy, in which the median survival time after diagnosis was reported to be 1.5 years. In another study on "Premature Death, Risk Factors, and Life Patterns in Dogs with Epilepsy", the median age of several breeds of dogs at death was found to be 7 years and the survival time was found to be 2.3 years (29). The median age of Australian Shepherds at death was 3.1 years; there was no calculation of survival time (11). German Border Collies with IE, on the other hand, were slightly older (5.17 years) than Australian Shepherds or GSMD at time of death; the median survival time (2.07 years) of Border Collies was similar to our results (10). Such a short survival time in GSMD with IE should be highly considered to develop further studies on new treatment schemes.

Statistically, no significant influence on survival time by age at first seizure, sex, number of antiseizure drugs, or administration of phenobarbital or imepitoin was found in the study discussed here. In contrast, Heske et al. (5) and Berendt et al. (29) found that females lived longer than males after a diagnosis of epilepsy, although this refers to a mixed dog breed population. Also, in the mixed population of dogs with IE, a higher age at diagnosis correlated with a shorter survival time (5). On the other hand, in a study on Border Collies, comparable to our study, there was no significant correlation between survival time and sex, reproduction status, or number of drugs administered (10).

In addition, in German GSMD, neither sex nor reproduction status had a significant effect on age at death. This was also confirmed by the study on German Border Collies (10). Regardless of this, this study was able to highlight that the occurrence of cluster seizures has a significant impact on survival time and the occurrence of status epilepticus has a statistically noticeable impact on survival time. Both cluster seizures and status epilepticus have been reported as risk factors for survival in dogs with epilepsy in previous studies (29, 35, 37), what has also been demonstrated in Border Collies (10). Treatment of these severe forms of seizures in GSMD such as a new concept of a pulse therapy could be considered (17).

Furthermore, a retrospective study of 407 dogs with epilepsy found an association between cluster seizure frequency and euthanasia, as significantly more dogs with frequently occurring CS were euthanized compared to dogs with less frequently occurring CS (35). Owners struggle with enduring the occurrence of cluster seizures or status epilepticus in their dog (29, 38). This not only significantly affects the quality of life of pet owners, but also the decision to euthanize their dog (36, 39). It can be suspected that owners of Great Swiss Mountain dogs with recurrent CS and SE have a similar reduced quality of life and fear that their pet is suffering, what leads to early decision for euthanasia.

In 52.69% (49/94) of GSMD relatives displayed seizures, strengthening the assumption of the genetic component already supported by the SSV e.V. surveys with a rate of 2.56% of dogs with seizures in 1999–2019. Sauer-Delhées et al. (13) stated that 28% (36/128) of all GSMD included in the study had a history of epilepsy in the family. Furthermore, in 45% (9/20) of the 20 dogs with seizures, seizures also occurred in family members (13). In Border Collies, a common ancestor could be identified in 29 affected dogs, "strengthening the suspicion of a genetic background of idiopathic epilepsy in Border Collies" (10). In the Australian Shepherd study, a pedigree of 42 IE-affected dogs was established, revealing a common ancestor of 29 affected Australian Shepherds in two subpopulations and an affinity for cluster seizures in littermates, full siblings, or half siblings (11). Pedigree analysis of Hungarian Magyar Vizsla revealed that all affected dogs in the study were traced to a common sire (40). Nevertheless, the identification of a causative gene has not been successful in the above-mentioned breeds (10, 11, 13, 21, 40, 41) and was not part of the objective in the discussed study.

A limitation of this study is its retrospective design. In some cases, not all data were available. In addition, the suspected diagnosis IE was based on the questionnaires and additional information from the treating veterinarians, without MRI and CSF examination (TIER 1). However, this influence was minimized by re-examining the dogs' data. In addition, the questionnaire contained standardized questions, and all free-text responses were compared with medical records.

In conclusion, the hypothesis that German Great Swiss Mountain Dogs show a severe clinical course of idiopathic epilepsy similar to other breeds such as the Australian Shepherd or Border Collie (6, 10, 11) can be confirmed in this study because of substantial percentages of dogs with status epilepticus and cluster seizures, as well as a high mortality rate. The current results can serve as a basis for genetic evaluations to identify a causative genetic defect, as well as to provide individual therapy recommendations, such as modified medication management during preictal phases, adding a second or third drug in case of an insufficient drug response and to raise awareness among GSMD owners, breeders, and the veterinary community.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was conducted in accordance with the Animal Welfare Act by following the ethical and data protection

guidelines of the University of Veterinary Medicine Hannover. The owners gave their informed consent to the anonymously evaluated scientific analysis of the data when filling out the questionnaire.

AUTHOR CONTRIBUTIONS

HU, NB, AT, and JN were responsible for the conception of the study. AT, AB-N, HU, NB, and CF performed the data acquisition. TO performed the statistical analysis, data analysis, and manuscript writing. AT supervised the data collection and manuscript editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neuroimaging in the Epileptic Baboon

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Characterization of baboon model of genetic generalized epilepsy (GGE) is driven both electroclinically and by successful adoption of neuroimaging platforms, such as magnetic resonance imaging (MRI) and positron emission tomography (PET). Based upon its phylogenetic proximity and similar brain anatomy to humans, the epileptic baboon provides an excellent translational model. Its relatively large brain size compared to smaller nonhuman primates or rodents, a gyrencephalic structure compared to lissencephalic organization of rodent brains, and the availability of a large pedigreed colony allows exploration of neuroimaging markers of diseases. Similar to human idiopathic generalized epilepsy (IGE), structural imaging in the baboon is usually normal in individual subjects, but gray matter volume/concentration (GMV/GMC) changes are reported by statistical parametric mapping (SPM) analyses. Functional neuroimaging has been effective for mapping the photoepileptic responses, the epileptic network, altered functional connectivity of physiological networks, and the effects of anti-seizure therapies. This review will provide insights into our current understanding the baboon model of GGE through functional and structural imaging.

Keywords: neuroimaging, genetic generalized epilepsy, baboon, PET, MRI

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INTRODUCTION

Epilepsy is a condition of recurrent seizures or a single seizure in the setting of an epileptogenic lesions on a brain magnetic resonance imaging (MRI) scan or epileptiform abnormalities on a scalp electroencephalography (EEG) (1). Clinically, seizures are characterized by stereotyped, episodic changes in behavior. As seizures are rarely recorded in brief scalp EEG samples, clinicians rely on the detection of interictal (between seizures) epileptic discharges (IEDs), which can serve as markers for the seizure type. Epilepsies are classified mainly as focal, i.e., seizures beginning in one region of the brain, or generalized, i.e., seizures are associated with a simultaneous activation of both cerebral hemispheres (2). Developments in structural neuroimaging, intracranial EEG recordings, and analysis of histopathological samples collected by resective surgery in people with medically refractory epilepsy have provided important insights into the pathophysiology underlying focal epilepsies. Knowledge with regards to the mechanisms underlying generalized epilepsies (IGEs), are less apparent; they are mainly idiopathic (without a known etiology), not associated with neuroimaging abnormalities, and not amenable for intracranial EEG sampling or resective surgeries. While IGEs are presumed to be genetic in etiology, monogenetic mutations, mainly affecting ion channels, are encountered in only 3% of the cases (2). There is still a large gap for understanding likely polygenic affects underlying epileptogenesis in idiopathic (IGE) or

genetic (GGE) generalized epilepsies, and animal models could provide new revelations with respect genetic and neurodevelopmental mechanisms.

Over fifty years ago, a prominent French neuroscientist, Robert Naquet, and his associates, Eva and Keith Killam et al. (3) first published their observations of photosensitivity, i.e., the predisposition of visual stimuli, such as flickering lights, to induce seizures. Photosensitivity of the baboon was quickly embraced for activation of seizures in a laboratory setting, allowing the recording of IEDs and seizures with scalp and intracranial EEG electrodes (4–6). The Senegalese *Papio hamadryas papio* (*P.h. papio*) appeared to be more photosensitive than other subspecies, including *P.h. anubis and cynocephalus* (7), remaining the preferred subspecies for electrophysiological evaluation and for testing the efficacy of known and novel anti-seizure medications. Photosensitivity was observed to be maximal in the morning hours and at intermittent photic stimulation (IPS) frequencies of 20–25 Hz. This model of photosensitivity was later adopted for testing known and novel anti-seizure medications (8, 9). However, technological improvements allowed electrophysiological investigations and anti-seizure medication testing in rodents and mice, decreasing interest for further developing the baboon model due to limitations related to cost and availability. Still, even after years of developing the baboon model, there is limited information regarding the underlying pathomechanisms and natural history.

Observation of spontaneous generalized tonic-clonic seizures (GTCS) in the largest captive baboon pedigree in the world, housed in the Southwest National Primate Research Center (SNPRC, Texas Biomedical Research Institute, San Antonio, Texas), reinvigorated research into this model. The pedigreed colony has 16,000 members over 6–8 generations, consisting mainly of *P.h. anubis*, *cynocephalus* and their hybrids; in contrast to the baboons studied in France, the founding members of this breeding colony originated from East Africa (10). On one hand, the pedigree presents a unique resource for evaluating the potential genetic effects of the epilepsy and led to the characterization of the epileptic phenotype. On the other hand, improved neuroimaging capabilities offers a new approach to evaluating underlying pathophysiology associated with this phenotype.

A retrospective case-detection survey of veterinary records between 1980 and 2007, demonstrated a prevalence of 26% for the expression GTCS or seizure-like behaviors in the pedigree (11). Forty-six spontaneous GTCS were recently semiologically characterized in 7 baboons; most of the seizures occurred in sleep or upon awakening (*12*). Preconvulsive semiologies were noted in 4 baboons, consisting of unilateral or bilateral rotatory behaviors and generalized or lateralized (myo)clonic activity; the ensuing convulsive portion of the seizures had a mean duration of 47 (+/-21) s. The total seizure duration was 54 (+/-21) s. Postictally, most of the baboons demonstrated myoclonus as they were recovering their upright posture following seizures (12). In addition to GTCS, generalized myoclonic, especially eyelid myoclonus, and absence seizures were recorded by on scalp video-EEG studies in 671 baboons (13). Generalized spike-and-wave discharges were noted in 324 (49%) baboons, and these were more commonly 4–6 Hz, but at times 2–3 Hz, frequency. Photoepileptic responses were recorded in 156 (23%) of the epileptic baboons. While these numbers may have been partially inflated with the use of low-dose ketamine (5–6 mg/kg) for sedation during scalp EEG studies; low-dose ketamine also activates generalized IEDs in asymptomatic animals predisposed to epilepsy. Nonetheless, these studies confirmed a genetic predisposition for epilepsy in this pedigree, and an electroclinical model that resembled juvenile myoclonic epilepsy (JME).

Only recently was a genetic etiology confirmed. Based upon whole genome sequencing in electroclinically well-characterized 42 epileptic and 19 controls, the RBFOX1 emerged as the only statistically significant association (14). RBFOX1 mutations have been identified in genetic focal and generalized epilepsies in humans and may act as a susceptibility gene in both humans and baboons (15, 16). RBFOX1 is an RNA-binding protein that regulates splicing of epilepsy candidate genes (e.g., *GABRG2*, *SYN1*, *KCNQ2*, *SCN8A*, *SLC12A5*) and plays a key role in neuronal excitation and may cerebral cortex development (17), changes to transcriptomic expression and splicing patterns of neuronal genes (18), and miRNA crosstalk that impacts homeostatic downscaling of excitatory synapses (19). As these gene interactions may be clinically relevant, larger samples of baboons will need to be studied in this pedigree. Furthermore, based upon a relatively consistent electroclinical phenotype within the pedigree, the epileptic baboon provides a suitable animal model for neuroimaging and evaluation of anti-seizure therapies.

This review will focus on the status of neuroimaging in the epileptic baboon. Based upon its phylogenetic proximity and similar brain anatomy to humans, the epileptic baboon provides an excellent translational model. Its relatively large brain size compared to smaller nonhuman primates or rodents, gyrencephalic structure compared to the lissencephalic brains of rodents and mice, and less variability in the cortical structures than humans, all contribute to the potential for identifying imaging biomarkers even in smaller cohorts than required for human studies. However, the studies described in this review were all performed in a single center, namely the SNPRC, and have not been validated by other centers. Nonetheless, because of the baboon's potential role in the neuroimaging of neurological disorders and treatment effects, several centers have developed PET and MRI templates. We will review the contribution of structural MRI as well as functional MRI and PET studies to our understanding of the baboon model, and more specifically the insights offered by functional imaging into the electrophysiological networks underlying photosensitivity, the epileptic network, and the effects of therapeutic interventions. We will also address gaps in the understanding of the structural and functional mechanisms, which will need to be addressed by prospective studies utilizing newly developed and/or complementary imaging techniques.

PRESENT STATE OF NEUROIMAGING IN THE EPILEPTIC BABOON

PET and MRI Brain Templates

While the advantages of neuroimaging the epileptic baboons are numerous (described above), there are also many challenges. Some disadvantages to humans include the need to sedate or anesthetize baboons due to their size and strength, interference by the large snout and air-filled sinuses which can distort the acquisitions, and their smaller brain size. Nonetheless, due to the potential for translatability, several centers have developed neuroimaging platforms and brain atlases to facilitate neuroanatomical analyses.

These centers all strived to develop high-resolution neuroanatomical atlases to allow the identification of regions of interest and cortical/subcortical landmarks, based upon anatomical or MR templates that be co-registered with PET studies, and normalized to a standard space for whole brain analyses and statistical parametric mapping. Early templates were limited to axial sampling, using the anatomical segmentation of a single baboon brain, and were not electronically accessible. Riche et al. (20) produced an anatomical atlas cut into 15 mm thick slices in the orbitomeatal plane to simulate the transaxial PET acquisition and planar resolution. Subsequently, radioligand PET to identify and validate anatomically defined cortical or subcortical structures. Black et al. (21) published the first electronically accessible baboon template based upon the Davis and Huffman anatomical atlas from a single baboon and limited to the subcortical structures (22). Greer et al. (23) developed an MR-based high-resolution atlas (centered at the mid-sagittal line, AC-PC orientation), averaging 6 datasets that were reformatted using a voxel size of 0.5 mm^3 to create representative MRI, that could be converted into PET space. A subsequent study aligned histological slices of baboon brain with anatomical MRI, first in two dimensions using block-face photographs of the brain slices, subsequently co-registering them in three dimensions with an *in-vivo* acquired MRI (24); this approach could link research relying on post-mortem microscopic material analysis with applications using *in-vivo* macroscopic imaging analysis. Further refinement of MRI templates was achieved by Love et al. (25), who created a template from 89 baboon brains of a heterogenous group of baboons based upon subspecies and sex to better represent their variability in brain morphometry. This template aimed to represent both hemispheres symmetrically to analyze side-to-side structural or functional differences and provided the first tissue probability maps, facilitating brain normalization or segmentation. Agaronyan et al. (26) created a fully segmented brain atlas, part semiautomatically, part manually, on an upscaled dataset using inter-slice interpolation. This atlas utilizes the earlier versions to guide segmentation, labeling and identification, hence achieving a higher resolution template to improve throughput analyses.

Structural MRI

While structural MRI scans are essentially normal in people with IGE, statistical parametric mapping first demonstrated increased regional gray matter concentrations (GMC) or volumes (GMV) in the frontoparietal cortices, but their cortical distribution varied among studies (27–29). While histopathological substrate underlying increases of GMV or GMC is unknown, but it is thought to be related to cortical developmental effects or increased synaptic connectivity, potentially due to increased synaptic density and/or anomalous synaptic pruning in adolescence (30). Decreases in GMV have been also reported, most consistently affecting the thalami bilaterally (31, 32), suggesting chronic, seizure-induced damage.

The potential for correlating structural MRI findings with histopathology could allow the baboon model to provide insight into potential underlying neurodevelopmental abnormalities; however, until now histopathological studies have not demonstrated evidence of abnormal cortical development or dysplasia (33). The only common structural abnormalities are deviations in ventricular sized or shape. Occipital horn variants, including unilateral or bilateral elongation or enlargement (colpocephaly), occurred in 25% of MRI scans (T1-weighted three-dimensionally acquired) in 208 baboons (**Figure 1B**). Historically, colpocephaly is associated with craniofacial trauma, which are typically due to convulsive seizures leading to falls from elevations [**Figure 1A**; (34)]. Temporal horn enlargement is less common and lacks any association with trauma or epilepsy.

Gyral and sulcal changes were compared between baboons with generalized interictal epileptic discharges (IEDs) on scalp EEG, some with witnessed seizures, others without, to those with normal scalp EEG and without witnessed seizures, using voxel-based morphometry [VBM, (35)]. Male brain volumes were ∼15% larger than those of females. While no significant differences were noted between the IED+ and IED- groups, there was a subtle increase in brain volume in the baboons with IEDs. This finding was unexpected, but an increased thickness of the cerebral cortices, especially of the postcentral gyrus and associated parietal regions responsible for generating absence seizures, were also noted in the GAERS model (36). However, post-hoc analyses demonstrated significant decreases in sulcal areas involving the central, intraparietal and cingulate sulci [**Figure 2C**; (35)], all of which are associated with brain regions giving rise to generalized ictal and interictal discharges in rodent and humans IGEs (36, 37), suggesting either a neurodevelopmental abnormality or seizure-induced changes.

GMC changes were also documented with VBM in epileptic baboons [**Figures 2A,B**; (38)]. GMC increases were noted in the frontopolar, orbitofrontal and anterolateral temporal cortices bilaterally. Decreases of GMC were noted in the primary visual cortices and thalamic nuclei, including reticular, anterior and medial dorsal nuclei. Increased GMC may reflect an underlying neurodevelopmental abnormality, either due increased cellularity or the absence of synaptic pruning (30); in adolescents with new onset JME, increased GMV is noted more diffusely, and decreases over time to a lesser extent than in age-matched healthy controls. As these baboons were mainly adults, it is possible that GMC increases were also more diffuse early on, but then normalized either as a function of cortical maturation or due to seizure-induced cell loss (38). The latter explanation is suggested by histopathological exams of two adult epileptic

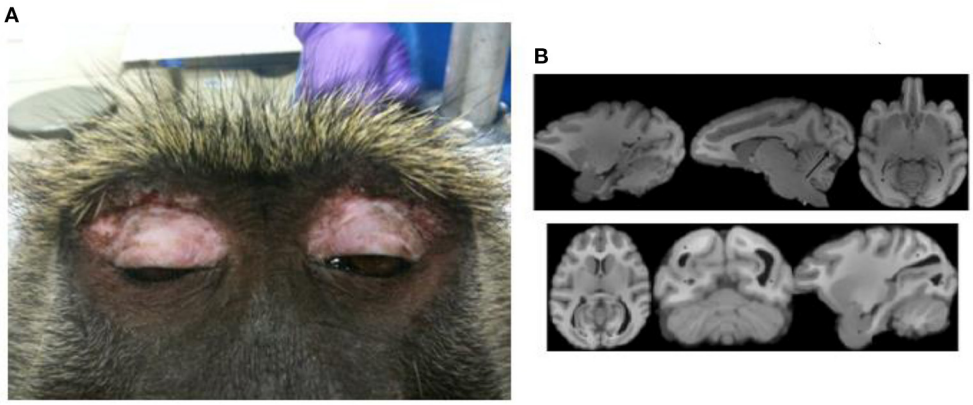

FIGURE 1 | Craniofacial trauma and colpocephaly. **(A)** Demonstrates periorbital scarring due to repeated craniofacial trauma due to seizures. **(B)** Shows normal ventricular configuration (upper series) and enlarged occipital horns (lower series). Adapted from *Comparative Medicine*, references (33, 34), respectively.

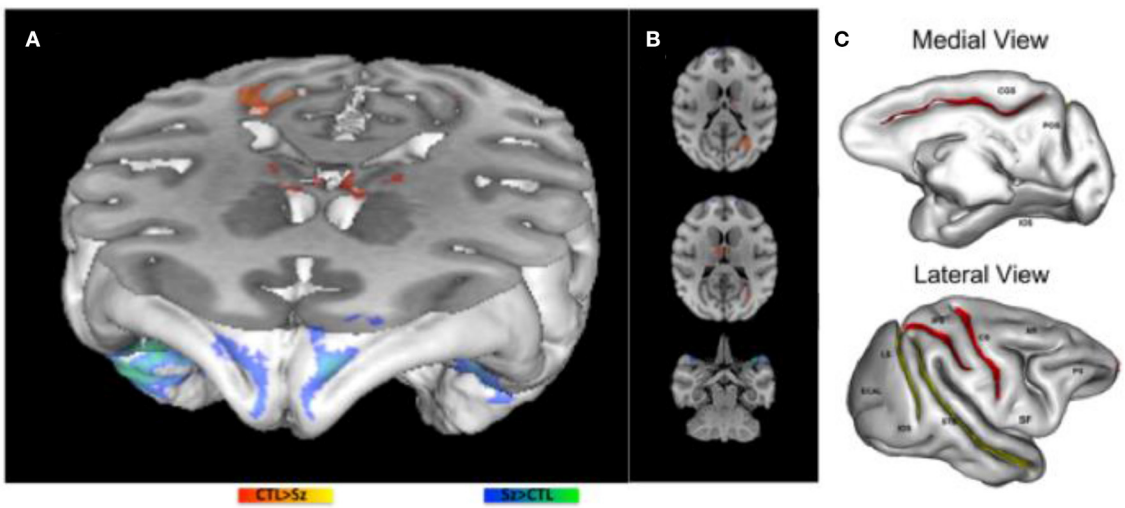

FIGURE 2 | Morphometric MRI Analyses in the Epileptic Baboon. **(A,B)** demonstrate increases (blue) and decreases (red) in gray matter concentration in epileptic baboons compared to controls. **(C)** Demonstrate significant decreases (red) and marginally significant decreases (yellow) in sulcal areas of baboons with IEDs on scalp EEG compared to healthy controls [adapted from *Epilepsy Research*, references (35, 38), respectively].

baboon brains, demonstrating evidence of decreased cortical cellularity, especially of neurons, in the frontoparietal cortices, though maximal in the sensorimotor region, while frontopolar, orbitofrontal and visual cortices were relatively spared (39). On the other hand, decreased GMC was encountered in the primary visual cortices of epileptic adult baboons (38), a brain region that is implicated in photosensitivity and participating as an integral part the baboon's epileptic network (40). Consistent with human studies (31, 32), thalamic GMC was also reduced in the baboons, and involve specifically the reticular, anterior and medial dorsal nuclei of the thalamus, all of which are involved in the generation of generalized spike-and-wave discharges (38). To determine whether the sulcal morphometric and GMC/GMV abnormalities are neurodevelopmental in etiology and/or evolve before and during epilepsy requires prospective, serial neuroimaging in larger samples of young epileptic baboons, ideally starting before the onset of epilepsy.

Microstructural differences had not been published in baboons so far, but could play an important role in determining connectivity differences that may pre-exist the onset of epilepsy and those that result from seizure-related injury. Fractional anisotropy (FA) appeared to be increased sensorimotor and premotor pathways in people with IGE, but decreased in the internal and external capsules, in one recent seed-based study human IGE, suggesting seizure related plasticity (41). Increased motor connectivity is also apparent in people with JME and their asymptomatic siblings, supporting genetic predisposition to connectivity (42, 43). Similar to human IGE, seed-based analyses in the genetic absence epilepsy in rats from Strasbourg (GAERS) model also showed decreases in FA in the corpus

callosum and internal capsule, but researchers were also able to demonstrate correlation of FA changes with seizure activity (44). Furthermore, treatment with ethosuximide appeared to prevent microstructural damage in these pathways and/or restore connectivity (44). These comparisons across species demonstrate the unique ability of animal models to illuminate mechanisms and treatment effects. Reanalyzing the baboon imaging data in a larger and better selected cohort may help validate these findings.

Functional PET and MRI

Anesthesia

The advantage of performing functional PET or MRI studies in treatement-naïve baboons is counterbalanced by the need for sedation and anesthesia. Gas inhalant anesthesia using 1% isoflurane is particularly challenging, as the blood oxygen level dependent (BOLD) signal responses are progressively attenuated above 1.0 MAC. Ketamine, used at lower doses for sedation of baboons during scalp EEG recordings, does not attenuate BOLD or EEG signal and increases cerebral blood flow [CBF, (45)]. With prior EEG recordings used as a screening tool, an ideal infusion rate could be identified which would avoid activation of seizures as well as EEG suppression, usually between 4-6 mg/kg/hr, for functional imaging studies (46).

Photosensitivity

$H_2^{15}O$-PET provides an excellent, minimally invasive method for studying cerebral blood flow (CBF) changes in association with visual stimulation in normal individuals (47–50). Similar to BOLD-fMRI, $H_2^{15}O$-PET takes advantage of neurovascular coupling to deliver visuospatial representations of brief electrophysiological events; the metabolic demand of activated or discharging neurons leads to localized changes in CBF and cerebral blood volume (47). $H_2^{15}O$-PET provides a more direct measure of CBF than BOLD-fMRI which relies on deoxygenation of venous blood but does not have the same spatial or temporal resolution of BOLD-fMRI.

$H_2^{15}O$-PET studies were performed in the morning, when the baboons are maximally photosensitive, utilizing IPS at 25 Hz, the frequency range most likely to activate photoepileptic responses (3–5). Six to eight injections are performed, about 10–12 min apart as the tracer's half-life is only 2 min in duration. During resting scans, CBF changes are recorded for 90-second radiotracer uptake period. For activation scans, IPS was typically started one minute prior to $H_2^{15}O$-injection, as occipital CBF reaches a steady state after one minute of continuous stimulation (51). The averaged resting scans are subtracted from activation scans, and these PET images are either co-registered with the baboon's own MRI, or with an average MRI for group analyses (46).

The photosensitive group exhibited more diffuse frontotemporal cortical and subcortical activations than control group (**Figure 3B**). Symmetrical cortical activations were mainly noted in the orbitofrontal cortices and anterior cingulate gyri, as well as the medial and anterolateral temporal cortices. Other activations were less symmetric, initially with a predominantly left hemispheric lateralization of the parietal and sensorimotor cortices when sampling CBF changes at IPS onset (**Figure 3A**), but shifting more to the right hemisphere once achieving steady state (**Figure 3B**). Activations were noted in both conditions in the right medial frontal regions including right anterior cingulate and orbitofrontal cortices, as well as the right mesiodorsal nucleus, though more bilaterally over time, reflecting effects of early and sustained activation of generalized IEDs recorded by scalp EEG. Similarly, the left posterior cingulate was also suppressed in both states, a brain region which is activated in the controls during IPS. There was diffuse deactivation of the basal ganglia and cortices early on, where deactivations in steady state pertained mainly to the caudal brainstem and cerebellum. Also subcortically, activations were noted in the left putamen, compared to the right putamen in controls. Another unexpected finding was the absence of occipital lobe activation in the photosensitive baboons; in photosensitive humans, EEG-fMRI demonstrates consistent occipital activation at the onset of IPS (54). While there was diffuse occipitoparietal activation noted in the control baboons, photosensitive baboons only showed minimal occipital activation early on during IPS, suggesting the possibility of an endogenous cortical inhibition induced by IPS. Nigral inhibitory pathways were also activated, reflected by regional CBF increases noted in the midbrain and globus pallidum, in baboons exhibiting myoclonic seizures during the tracer uptake period compared to baboons with only IEDs (55). This appears to be an important inhibitory pathway; even structural MRI demonstrated enlargement of the globus pallidum in epileptic baboons compared to controls (38).

The Epileptic Network

With the help of EEG-fMRI, the epileptic network underlying absence seizures has been well-defined in humans and rodents (44, 56). The onset and resolution of paroxysmal generalized spike-and-wave complexes in people with absence and myoclonic seizures are associated with transient activation of the medial frontothalamic network (37, 56). BOLD changes precede the paroxysmal EEG discharge by about 15 s, with initial rises in BOLD signal occipitally, parietally and orbitofrontally (56, 57); nonetheless, the order latency of activations and deactivations can vary widely between subjects (57). The generalized spike-and-wave is followed by sustained decreases in BOLD signal across the frontoparietal cortices. This is one reason for overall decreases in frontoparietal BOLD signal during random sampling in IGE patients.

But even without EEG-fMRI, functional PET was able to demonstrate activation of the corticothalamic network during IPS (46). Furthermore, correlation analyses of CBF changes with resting IED rates also resulted in the activation of the posterior aspects epileptic network (**Figure 3C**; 56), which included symmetrical activations of the primary visual cortices, occipito- and temporo-parietal association cortices, insula, and sensorimotor cortices, all brain region that were active both ictally and interictally during intracranial EEG recordings (40). However, other brain regions that were activated by IPS and were electrophysiologically active on EEG, namely the anterior cingulate, orbitofrontal cortices, even the thalamus, were either deactivated or not activated. As suggested by

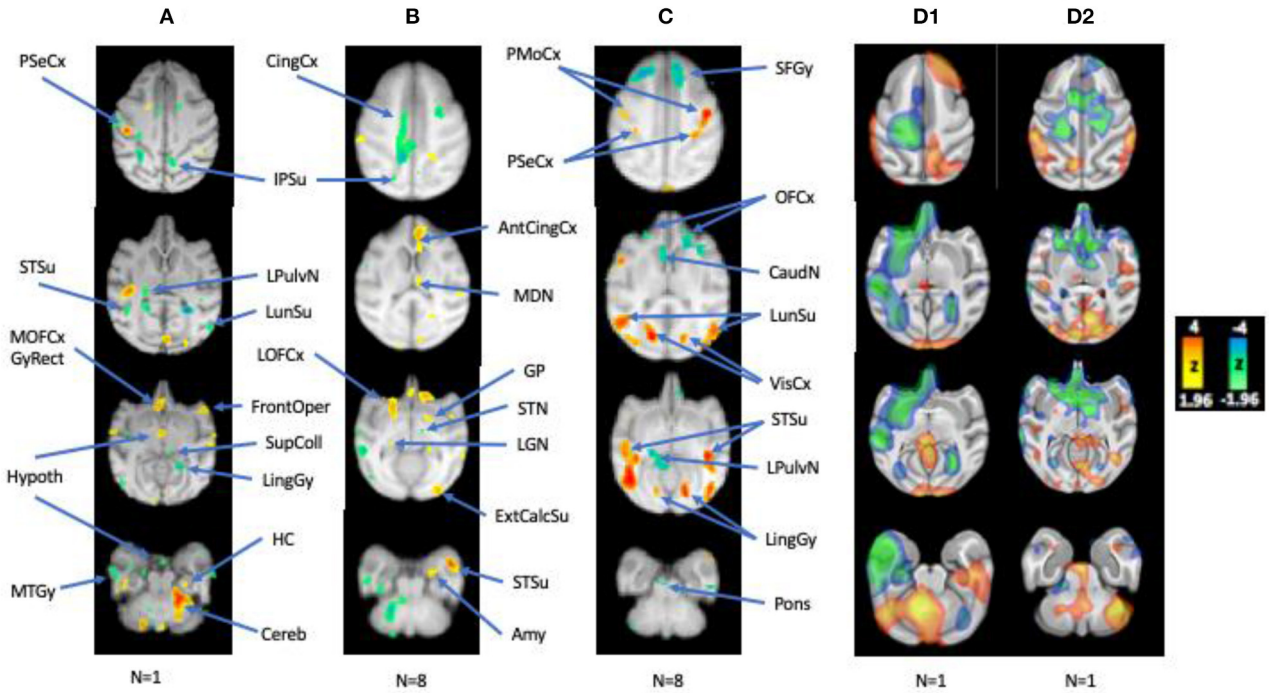

FIGURE 3 | Regional CBF Changes with IPS, Correlated with IEDs, and during HF-VNS therapy. **(A,B)** Early and late CBF changes with IPS, **(C)** CBF changes correlated with interictal epileptic discharges, **(D1,D2)** CBF changes in a photosensitive baboon and a non-photosensitive animal related to HF microburst VNS Therapy at rest (compared to the resting-state average from nine epileptic baboons). L hemisphere is on the left. Adapted from references (46, 52, 53).

EEG-fMRI recordings of absence seizures in humans, near-infrared recordings of oxyhemoglobin, deoxyhemoglobin and total hemoglobin in the GAERS cortex, deoxygenation and CBV are decreased before the onset and after the end of the generalized spike-and-wave discharge (58), which may account for the paradoxical deactivation is these brain regions. Subcortical CBF changes were mainly represented by deactivations in the left caudate and lateral pulvinar nucleus, which were activated during IPS, in the pontine nucle and cerebellum.

This network was also supported by FC analyses comparing 10 epileptic and 10 control baboons, matched for gender, age, and weight (59). Independent component analyses (ICA) identified 14 unique components/networks, and FC maps were generated for each group's brain networks (**Figure 4**). The epileptic group demonstrated network-specific differences in FC when compared to the control animals: sensitivity and specificity of the two groups' functional connectivity maps differed significantly in the visual, motor, amygdalar, insular, and default mode networks. Significant increases of FC were found in visual cortices of the epilepsy group's maps for the default mode, cingulate, anterior parietal, motor, visual, amygdalar, and thalamic networks. More importantly, **Figure 4** demonstrates that the connectivity maps of the visual and parietal networks reconstitute the entire epileptic network, including for the corticothalamic pathways that express generalized ictal and interictal epileptic discharges.

Treatment Effects

Functional neuroimaging can also be used to evaluate acute or chronic treatment effects for anti-seizure medications or devices. We compared FC changes after intravenous injection of valproic acid (VPA 20 mg/kg) and following 1-week of orally administered VPA (20–80 mg/kg/day) between epileptic and control baboons (60). Similar to study above (59), FC was increased in most cortical networks of the epilepsy group, but less so for the subcortical networks (60). After intravenous VPA, FC was increased for the basal ganglia network in the epileptic baboons with respect to the medial frontal cortices, but decreased for longer cortico-cortical pathways. Increased basal ganglia connectivity may reflect an acute upregulation of cortical inhibition by subcortical networks, while decreasing cortico-cortical connectivity is likely to reflect decreased activation of the larger epileptic network. Cortical and basal ganglia connectivity was also increased with pontine nuclei and cerebellum, supporting potential activation of another pathway modulating cortical excitability. After oral VPA therapy, FC in the epileptic baboons approached control values in the amygdalar, precuneus, left parieto-occipital, parietal, orbitofrontal, and secondary visual areas. Increases of FC were noted for the medial frontal/(peri)cingulate and pontine networks, which may reflect brainstem-mediated neuromodulation *via* activation of biogenic amine secretion and neurotransmission.

In a more recent study, $H_2^{15}O$-PET changes were compared between high-frequency (HF; 300 Hz) microburst, and standard,

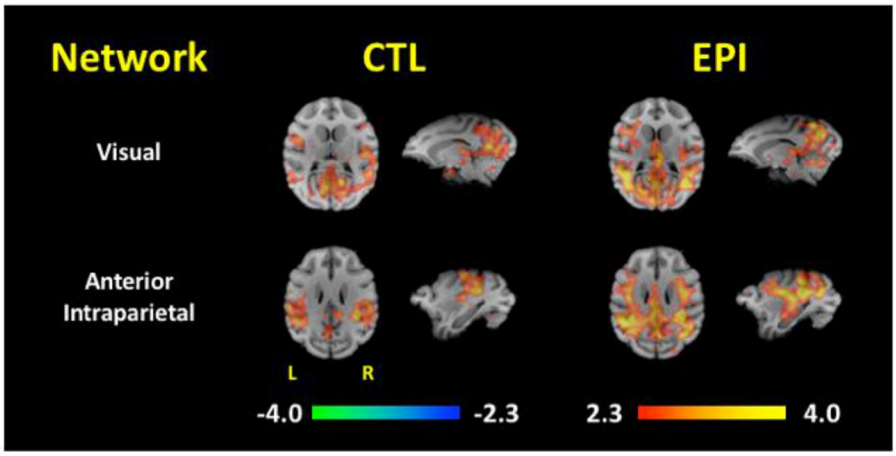

FIGURE 4 | Functional Connectivity Maps for the Visual and Anterior Parietal Networks. Functional connectivity maps are compared between asymptomatic healthy control (CTL) and epileptic (EPI) baboons. Note the increased connectivity of both visual and anterior partial networks to the medial thalamus and frontocentral cortices [Adapted from *Epilepsia*, reference (59)].

low-frequency (LF; 30 Hz) Vagal Nerve Stimulation (VNS) Therapy in two baboons with GGE, including one with photosensitivity (53). The baboons were selected based on video recordings and scalp EEG studies. Both were implanted with Sentiva™ M1000 devices capable of stimulating at LF- and HF-frequencies. CBF changes were compared for both modes of stimulation and resting scans in the first study. While spontaneous scalp IEDs were reduced in both baboons by HF- and LF-therapies, HF-VNS Therapy completely suppressed IEDs in one baboon (D2). Regional CBF changes were overall consistent between the two modes of therapy in both baboons with respect to the activation of the superior colliculus and cerebellum (**Figures 3D1,D2**). IED suppression by HF-VNS Therapy in one baboon was associated with bilateral deactivations of the frontal and temporal cortices, anterior cingulate and striatum, and of the medial thalamus, while the pons and cerebellum were both activated (**Figure 3D2**). Some therapeutic targets for both LF- and HF-VNS Therapy appeared to be subcortical, including the superior colliculus, brainstem nuclei, as well as the cerebellum, all structures that were either deactivated or inactive during IPS or spontaneous IEDs.

FUTURE DIRECTIONS

Structural and functional neuroimaging has contributed to our understanding of brain networks and pathophysiology underlying GGEs in humans and baboons alike, and the potential for translation is extraordinary. As such, different modalities may be used to characterize brain developmental effects which may contribute to the expression of seizures as well as seizure-induced cortical and subcortical damage. Serial neuroimaging could lead to a new perspective on the natural history of GGE in the baboon and identify neuroimaging biomarkers for epileptogenesis and sudden unexpected death in epilepsy (SUDEP) (61). As demonstrated by the studies above, GMC/GMV measurements and FC connectivity changes could track the evolution of epileptic networks. Quantitative trait analyses could map genetic effects on brain development and connectivity (10, 62). Several newer PET-ligands could contribute to our understanding of neurodevelopmental mechanisms associated with epileptogenesis. As SV2A modulates the exocytosis of synaptic vesicles, SV2A-PET can quantify synapses and synaptic connectivity in the cerebral cortex (63, 64). Translocator protein or TSPO-PET, on the other hand, can evaluate inflammatory changes that may also contribute to epileptogenesis (65). Both can also be used to monitor seizure-related loss of cortical and brainstem neurons. Ultimately, the epileptic baboon model may be used to validate of neuroimaging markers by electrophysiological testing and the availability of brain tissue.

AUTHOR CONTRIBUTIONS

CS and FS designed the studies and performed the research and analysis. CS wrote the review. FS provided critical input. All authors contributed to the article and approved the submitted version.

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GLOSSARY

Cortical regions of interest	Subcortical structures
(M/L)OFCx, medial/lateral orbitofrontal cortex	CaudN, Caudate nucleus
FrontOper, frontal operculum	LPulN, lateral pulvinar nucleus
SFG, superior frontal gyrus	LGN, lateral geniculate nucleus
GyRect, gyrus rectus	SupColl, superior colliculus
PMoCx, primary motor cortex	Cereb, cerebellum
PSeCx, primary sensory cortex	GP, globus pallidum
VisCx, visual cortex	STN, subthalamic nucleus
LingGy, lingual gyrus	Hypoth, hypothalamus
(Ant)CingCx, (anterior) cingulate cortex	MDN, mediodorsal nucleus thalami
MTGy, middle temporal gyrus	HC, hippocampus
STSu, superior temporal sulcus	AMY, amygdala
ExtCalcSu, external calcarine sulcus	
IPSu, intraparietal sulcus	
LunSu, lunate sulcus	

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The effect of phenobarbital treatment on behavioral comorbidities and on the composition and function of the fecal microbiome in dogs with idiopathic epilepsy

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Phenobarbital (PB) is one of the most important antiseizure drugs (ASDs) to treat canine idiopathic epilepsy (IE). The effect of PB on the taxonomic changes in gastrointestinal microbiota (GIM) and their functions is less known, which may explain parts of its pharmacokinetic and pharmacodynamic properties, especially its antiseizure effect and drug responsiveness or drug resistance as well as its effect on behavioral comorbidities. Fecal samples of 12 dogs with IE were collected prior to the initiation of PB treatment and 90 days after oral PB treatment. The fecal samples were analyzed using shallow DNA shotgun sequencing, real-time polymerase chain reaction (qPCR)-based dysbiosis index (DI), and quantification of short-chain fatty acids (SCFAs). Behavioral comorbidities were evaluated using standardized online questionnaires, namely, a canine behavioral assessment and research questionnaire (cBARQ), canine cognitive dysfunction rating scale (CCDR), and an attention deficit hyperactivity disorder (ADHD) questionnaire. The results revealed no significant changes in alpha and beta diversity or in the DI, whereas only the abundance of Clostridiales was significantly decreased after PB treatment. Fecal SCFA measurement showed a significant increase in total fecal SCFA concentration and the concentrations of propionate and butyrate, while acetate concentrations revealed an upward trend after 90 days of treatment. In addition, the PB-Responder (PB-R) group had significantly higher butyrate levels compared to the PB-Non-Responder (PB-NR) group. Metagenomics of functional pathway genes demonstrated a significant increase in genes in trehalose biosynthesis, ribosomal synthesis, and gluconeogenesis, but

a decrease in V-ATPase-related oxidative phosphorylation. For behavioral assessment, cBARQ analysis showed improvement in stranger-directed fear, non-social fear, and trainability, while there were no differences in ADHD-like behavior and canine cognitive dysfunction (CCD) scores after 90 days of PB treatment. While only very minor shifts in bacterial taxonomy were detected, the higher SCFA concentrations after PB treatment could be one of the key differences between PB-R and PB-NR. These results suggest functional changes in GIM in canine IE treatment.

KEYWORDS

canine idiopathic epilepsy, phenobarbital, gastrointestinal microbiota, short-chain fatty acids, butyrate, behavioral comorbidities

Introduction

Idiopathic epilepsy (IE) is a common neurological disease in dogs, with an unknown underlying cause and no identifiable structural brain abnormalities (1). IE is typically treated lifelong with antiseizure drugs (ASDs) (2). Phenobarbital (PB) is an ASD that is used as the primary treatment of choice for canine IE due to its widespread availability, tolerance, and affordability (2–4). It mainly acts on the allosteric site of gamma-aminobutyric acid subtype A ($GABA_A$) receptors, causing the prolongation of its receptor channel opening, which enhances response to inhibitory neurotransmitter gamma-aminobutyric acid (GABA) or in higher concentrations, open the GABA channel alone, causing neuronal hyperpolarization (5). However, some dogs with IE continue to have a seizure despite PB treatment, which is referred to as "PB resistance" (6). The role of the microbiota–gut–brain axis (MGBA) in PB or drug-resistant epilepsy has not yet been fully explored.

MGBA is a bidirectional communication system between the enteric microbiota and the brain, which mainly consists of the central nervous system (CNS), enteric nervous system (ENS), and gastrointestinal microbiota (GIM) (7, 8). Communication is exerted through many pathways, namely, neural tracts, immunological, inflammatory, and neuroendocrine pathways (9). In the last decade, scientific evidence has increased substantially, highlighting the relation between GIM and neuropsychiatric and neurological disorders, namely, epilepsy (10–12). The first milestone in epilepsy was made by a preclinical study in mice by Olson and colleagues, which showed specific GIM to be responsible for increasing seizure threshold (13). This study led to an increased interest in MGBA relating to epilepsy and its role in treatment response.

In human medicine, patients with epilepsy differ in their GIM from healthy controls (14). Moreover, a difference in the GIM between human patients with drug-sensitive and drug-resistant epilepsy was also noted. A study found that the GIM composition of drug-sensitive epilepsy was similar to that of healthy people, while the GIM of patients with drug-resistant epilepsy showed an increased abundance of rare bacteria (11). In veterinary medicine, a study showed a significant reduction in GABA and short-chain fatty acids (SCFAs) producing bacteria in dogs with epilepsy compared to healthy controls (15). Another study in canine IE showed a change in microbiota composition when treating dogs with a medium-chain triglyceride-enriched diet, a diet used to improve seizure control (16).

Normally, GIM diversity and their functions can be affected to varying degrees by several factors, such as diet, environment, disease, and medication (17, 18). One of the main metabolites of GIM is SCFAs. SCFAs are fatty acids with fewer than six carbons produced by bacteria, such as Bifidobacterium, Clostridium, and Lactobacillus during polysaccharide fermentation in the colon (19, 20). The main SCFAs produced by GIM are acetate, propionate, and butyrate, which are absorbed through the intestine before being circulated to the liver and other organs, namely, the brain (9, 20). SCFAs play a major role in maintaining a healthy intestine as they are the main energy source of enterocytes and promote intestinal barrier integrity (21, 22). Furthermore, SCFAs work *via* MGBA, providing their anti-inflammatory, immunomodulatory, and neuroprotective abilities (23–25).

Like in people, fear- and anxiety-related behaviors, as well as cognitive dysfunctions, have been reported in treated and untreated dogs with IE (26–32). To date, it has not yet been completely elucidated whether these epilepsy comorbidities are a risk factor for the development of epilepsy, part of the epilepsy phenotype, a side effect of some ASDs, or a combination of all three factors. Therefore, the aim of the current study was to further investigate the effect of PB on behavior and on the composition and function of GIM as well as their association to PB response in dogs with IE. The results of this study could lead to a better understanding of the association of GIM and their function in canine IE and epilepsy comorbidities.

Materials and methods

Dogs

Owners of drug-naïve dogs with IE [Tier II confidence level (33)] were recruited and first informed about the current standard of care for IE management (2). Then, only dogs were included in the study for which the owners had given formal consent to initiate PB treatment. Only one dog per household was allowed to be enrolled. Diets were recorded and not modified, and the owners were told not to change the diet throughout the study period.

Phenobarbital treatment

The initial PB dose given was based on the International Veterinary Epilepsy Task Force (IVETF) consensus statement: 2.5 mg/kg orally every 12 h (34). The dose of PB was not changed during the 90-day study period. On days 30 and 90, the PB serum concentrations were measured.

Fecal samples

The fecal samples were collected prior to PB treatment (D0) and 90 days after continuous PB treatment (D90). The samples were stored in a plastic tube (5 ml, 57 × 15.3 mm, polypropylene; Sarstedt AG & Co. KG, Nümbrecht, Germany) at −80°C before being shipped with dried ice to the Gastrointestinal Laboratory of Texas A&M University, College Station, Texas, USA, for qPCR-based dysbiosis index (DI) and SCFA analysis, and further delivered to Diversigen, Inc., Houston, TX, USA for metagenomics by shallow DNA shotgun sequencing.

Shallow DNA shotgun sequencing (metagenomics)

The microbial DNA from fecal samples was extracted and quantified using the MoBio PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) and the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), respectively. For sequencing libraries preparation, the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) was used before the libraries were pooled. After this step, SPRI bead purification and concentration were processed using SpeedBeads Magnetic Carboxylate Modified Particles (Cytiva Life Sciences, Marlborough, MA, USA). The resulting pooled libraries were denatured by NaOH before being diluted and spiked by 2% PhiX. The metagenomic sequencing was performed on an Illumina NextSeq 500 System using NextSeq 500/550 High Output 150 cycle kit (1 x 145 bp reads), followed by being multiplexed on the sequencer before converting to FASTQ files and filtering for low quality (Q-score <30) and length (<50). Adapter sequences were trimmed, and all sequences were trimmed to a maximum length of 100 bp prior to alignment. The raw sequences were made using NCBI Sequence Read Archive before analysis with established pipelines. In terms of taxonomic classification, FASTA sequences were aligned making a curated database, which contained all representative genomes in the NCBI RefSeq representative genome collection for prokaryotes (release 86) with additional manually curated strains for bacteria (35). Alignments were made at 97% identity and compared to reference genomes. The input sequences were listed for taxonomy assignment as the lowest common ancestor, which was compatible with not <80% of the reference sequences. OTUs accounting for <one million of all species-level markers and OTUs with <0.01% of their unique genome regions matching as well as <0.1% of the whole genome were discarded. For downstream analysis, normalized and filtered tables were used in QIIME2.

Alpha diversity was evaluated by the number of species, Shannon–Wiener index, Pielou's index, and observed operational taxonomic units (OTUs) using a rarefied OTU table. Beta diversity was evaluated by weighted and unweighted UniFrac distance measures, and principal coordinate analysis (PCoA) plots using the Bray–Curtis dissimilarity.

The differences between each GIM community were investigated by Bray–Curtis distance metric analysis and beta diversity with QIIME2. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) groups were observed with alignment at 97% identity against a gene database derived from the NCBI RefSeq representative genome collection for prokaryotes with additional manually curated strains for bacteria mentioned above for functionally annotated genes (36, 37). The directly observed KO counts reported as relative abundance within each sample were expressed in a KO table and downstream tables. KOs were then collapsed to level-2 and level-3 KEGG pathways and KEGG modules.

Fecal dysbiosis index and quantitative real-time PCR

All fecal samples were analyzed, and DNA was extracted from 100 mg of each fecal sample using a MoBio PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). The total bacteria and specific bacterial taxa (i.e., *Faecalibacterium*, *Turicibacter*, *Streptococcus*, *Escherichia coli*, *Blautia*, *Fusobacterium*, *Clostridium hiranonis*, and *Bifidobacterium*) were analyzed using Quantitative PCR assays (qPCR). The PCR conditions were performed in the following order: at 95°C maintained for 2 min, 40 cycles at 95°C for

5 s, and then annealing for 10 s at the optimized temperature using 10 μL of SYBR-based reaction mixtures (5 μL of SsoFast™ EvaGreen® supermix [Bio-Rad Laboratories GmbH, Düsseldorf, Germany]), 1.6 μL of high-quality PCR water, 0.4 μL of each primer (final concentration: 400 nM), 1 μL of 1% BSA (final concentration: 0.1%), and 2 μL of DNA (1:10 or 1:100 dilution). The qPCR results were reported in log amount of microbial DNA (fg) for each taxon per 10 ng of isolated total DNA. The DI was calculated combined with qPCR assay results. The DI at 0 was used as a cut-off for normobiosis (DI <0) (38).

SCFAs analysis

The concentration of SCFAs, namely, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate was analyzed by stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay [modified from (39)]. The fecal samples from each time point were thawed at room temperature and then vortexed for 30 min before being centrifuged for 20 min at 2,100 g at 4°C. The fecal supernatants from each sample were separated using serum filters (Fisherbrand serum filter system, Fisher Scientific Inc., Pittsburgh, PA, USA). The amount of 500 μL of each supernatant sample was mixed with 10 μL of internal standard (200 mM heptadeuterated butyric acid). A C18 solid-phase extraction column (Sep-Pak C18 1 cc Vac Cartridge, Waters Corporation, Milford, MA, USA) was then used to extract the mixtures. The samples were derivatized by N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) for 60 min at room temperature. The chromatographic separation and quantification of the processed samples were performed using a GC (Agilent 6890N, Agilent Technologies Inc., Santa Clara, CA, USA) connected to an electron ionizing MS (Agilent 5975C, Agilent Technologies Inc.). A DB-1 ms capillary column (Agilent Technologies Inc.) was used for separation. The separation was performed under the following temperature program: 40°C maintained for 0.1 min, increased by 5°C/min to 70°C, then maintained for 3.5 min, increased by 20°C/min to 160°C, and lastly increased by 35°C/min to 280°C and maintained for 3 min. This process lasted for 20.53 min. The MS in electron impact positive-ion mode was used with selective ion monitoring at mass-to-charge ratios (M/Z) of 117 (acetate), 131 (propionate), 145 (butyrate), and 152 (heptadeuterated butyrate; internal standard). In terms of quantification, the ratio was calculated using the area under curve of the internal standard and each SCFA. The minimum detection limits of acetate, propionate, and butyrate concentrations were 1.33, 0.43, and 0.12 μmol/g, respectively. If the SCFA concentrations were lower than the minimum detection limits of acetate, propionate, and butyrate concentrations, the concentrations were adjusted to 1.32, 0.42, and 0.11 μmol/g, respectively. Due to the different water contents of the individual fecal samples, the fecal SCFA concentrations were reported as μmol/g of fecal dry matter.

Questionnaires

The online questionnaires were sent to each owner at the beginning and the end of the study. These contained questions about seizure semiology, nutrition, behavior, and cognition based on the formerly validated cBARQ (40), ADHD (41), and CCDR (42) questionnaires.

Statistics and data analysis

The data were collated and analyzed using Prism® Version 9.3.1 (GraphPad Software, San Diego, CA, USA). The level of significance was set at a *P*-value of <0.05. A one-tailed Wilcoxon matched-paired signed-ranks test was used to compare each alpha diversity parameter, taxonomic levels, genes related to metabolic pathways, qPCR-based DI, SCFA concentrations, and behavioral questionnaires between two-time points. To compare the difference between PB-R and PB-NR groups at D90, a one-tailed Mann–Whitney U test was used for analysis. The same method was also used to compare the results at D0 between both groups in order to rule out any potential biases. Multivariate statistical analysis for beta diversity (analysis of similarity, ANOSIM) was performed using Primer 7 (Plymouth Routines in Multivariate Ecological Research Statistical Software, v7.0.13) (43). Univariate statistics were then performed on alpha diversity and bacterial taxa for all taxonomic levels using JMP Pro 12 (Cary, NC, USA). *P*-values were adjusted for multiple comparisons with Benjamin & Hochberg FDR at a *P*-value of <0.05.

Results

Dogs

Twelve dogs with IE Tier II confidence levels (33) were included in the study (Table 1). The signalment of the dogs is summarized in Table 1. The mean age (± standard deviation) of the dogs at the beginning of the study and their age at IE onset were 3.5 ± 2.2 and 2.7 ± 1.7 years, respectively. Of the 12 dogs, 10 dogs were male and two dogs were female. The dog breeds varied, with no breed being overrepresented. The administered dose of PB was 2.3 ± 0.4 mg/kg every 12 h. The PB serum concentrations at D30 and D90 were 19.8 ± 5.3 and 18.1 ± 3.7 mg/L, respectively. After the start of PB treatment, seven dogs were seizure-free, while five dogs had at least one seizure within the 90-day study period. In this study, the dogs experiencing seizure freedom since starting PB were grouped as PB-Responder (PB-R), and the dogs not responding to the initial dose were grouped as PB-Non-Responder (PB-NR).

TABLE 1 Signalment of dogs included in the study, age of IE onset, diets, treats, experience of cluster seizures (CS) or status epilepticus (SE) in the last 90 days before starting phenobarbital (PB), PB concentrations (conc.) 30 days post-treatment (D30), and 90 days post-treatment (D90), responses to PB, seizure frequency (SF) per month during the study, dysbiosis index (DI) at pre-treatment (D0), and D90 of each dog.

Case number	Breed	Gender	Age (year)	Age of IE onset (year)	Diets	Treats	CS/SE before PB	PB conc. (mg/L)		PB response	SF	DI	
								D30	D90			D0	D90
1	American Bully	male	2.2	2.2	CDF from chicken	Fish and chicken treats, F, V, M	–	14.8	25.1	PB-NR	3	−2.2	−3.2
2	Labrador Retriever	male	4.7	4.5	CDF+CCF from duck	Sausages from poultry	CS	18.5	22.1	PB-R	0	−1.5	−5.0
3	Peruvian Hairless Dog	male	0.7	0.7	CDF from chicken	Pork, chicken, and rabbit treats, F, V, M	–	19.3	14.7	PB-R	0	0.1	−4.6
4	Poodle	male	6.9	6.2	CDF from beef	F, V	SE	24	24	PB-NR	0.33	−2.8	−4.8
5	Rhodesian Ridgeback	female	3.6	3.6	CDF from duck, lamb, and horse	Pork, beef, and horse treats	CS	33.1	17.6	PB-R	0	5.2	−2.2
6	Australian Shepherd	male	2.8	2.8	CDF from ostrich	V	–	16.8	17	PB-R	0	−0.9	−5.5
7	Labrador Retriever	male	4.9	4	CDF from chicken, pork, lamb, and fish	Beef and chicken treats, eggs, F, V	–	24.5	16.6	PB-R	0	−6.9	−4.8
8	Crossbreed	male	7.8	0.7	CDF from fish	Beef and duck treats, F, M	CS	21	17.3	PB-NR	0.33	−0.3	−5.7
9	Rottweiler	male	3.5	3.5	CDF from duck	–	CS	18	15.4	PB-R	0	−4.3	−3.1
10	Crossbreed	male	1.2	1.2	CDF from lamb	–	–	17.3	17.2	PB-NR	1	−4.9	−5.1
11	French Bulldog	male	3.3	1.3	CDF from chicken	–	–	19.2	13	PB-NR	0.33	−5.4	4.7
12	Dachshund	female	0.7	0.5	CDF+CCF from duck	Beef treats, M	CS	10.9	17.5	PB-R	0	−3.8	−3.5

CDF, commercial dry food; CCF, commercial canned food; F, fruits; V, vegetables; M, milk products such as cheese and yogurt; PB-R, PB-Responder; PB-NR, PB-Non-Responder.

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Alpha diversity

No differences in alpha diversity parameters, namely, species richness, Shannon–Wiener index, Pielou's evenness, and OTU rarefaction, were found within the samples when comparing the two-time points (D0 and D90) (Figure 1).

Beta diversity

No significant microbiome clustering between D0 and D90 or between the PB-R and PB-NR group at D90 was observed.

Also, when comparing feces from the PB-R group and PB-NR group sampled at D0, no significant microbiome clustering was detected. There was also no significant difference in beta diversity in the PB-NR group at D90 compared to D0, which was similar to the PB-R group. The beta diversity is shown in Figure 2.

Taxonomic difference

The metagenomics by shotgun gene sequencing showed no significant change in fecal bacterial taxa at phylum, family, genus, and species level. There was only a significant decrease in the order Clostridiales at D90 compared to D0 ($P = 0.04$) (Figures 3, 4). The sequence data are deposited in the NCBI Short Read Archive (SRA) database (Accession Number: PRJNA849257).

Functional genes analysis

The comparisons of the functional genes concentrations measured by metagenomics analysis between D0 and D90 demonstrated a significant increase in the number of microbial ribosome genes (KEGG module M00178, $P = 0.02$), genes associated with the conversion from oxaloacetate to fructose 6-phosphate in the gluconeogenesis pathway (KEGG module M00003, $P = 0.04$), and D-glucose 1-phosphate to trehalose in the trehalose biosynthesis pathway (KEGG module M00565, $P = 0.04$). Additionally, a significant decrease in V-type ATPase genes as part of the oxidative phosphorylation pathway (KEGG module M00159, $P = 0.01$) was evident. The sequence data are deposited in the NCBI SRA database (Accession Number: PRJNA849257).

Dysbiosis index

In parallel to the sequencing data, the qPCR-based DI comparing D0 and D90 did not show any significant changes (Table 1). At D0 and D90, 10/12 (83%) and 11/12 (92%) dogs had a DI < 0, respectively.

Short-chain fatty acids

The total fecal SCFA concentrations and the concentrations of propionate and butyrate increased significantly from D0 to D90 ($P = 0.04$, 0.03, 0.03, respectively), while acetate concentrations showed a marginal trend but did not reach statistical significance ($P = 0.05$). There were no significant changes in the levels of isobutyrate ($P = 0.36$), isovalerate ($P = 0.31$), and valerate ($P = 0.16$) between both time points. The comparison between the PB-R group and the PB-NR group revealed that the PB-R group had significantly higher concentrations of butyrate ($P = 0.03$). Respective graphs are shown in Figure 5.

Behavioral analysis

The cBARQ, ADHD, and CCDR values were compared between D0 and D90. The cBARQ results showed a significant decrease in stranger-directed fear (0.20 ± 0.32 [D0] vs. 0.08 ± 0.19 [D90], $P = 0.03$), non-social fear (0.49 ± 0.41 [D0] vs. 0.34 ± 0.26 [D90], $P = 0.04$), and an increase in trainability (0.85 ± 0.44 [D0] vs. 1.01 ± 0.39 [D90], $P = 0.02$). The results showed that stranger-directed fear, non-social fear, and trainability improved after PB treatment. Neither ADHD nor CCDR scores changed between D0 and D90. All CCDR values at D0 (34.91 ± 3.75) and D90 (35.75 ± 6.73) were under the score of 50, below the canine cognitive dysfunction (CCD) score (32). Comparing each parameter between the PB-R group and PB-NR group revealed no significant differences.

Discussion

PB as the primary drug of choice has a long history in canine IE treatment. However, its role in behavior and GIM has not been fully elucidated. PB improved stranger-directed fear, non-social fear, and trainability. Apart from the abundance of the bacterial order Clostridiales being reduced after PB treatment, no other GIM changes could be demonstrated. The fecal DI indicated that 92% of the dogs were within the reference interval established for healthy dogs. The functional metagenomics analysis mainly demonstrated changes in protein synthesis and glucose metabolism pathways, whereas there was an increase in the total SCFAs, butyrate, and propionate.

Similar to the current study, another study in nine dogs showed no changes in the alpha and beta diversity of GIM from fecal samples of drug-naïve IE dogs compared to 30 days after PB- or imepitoin treatment (15). However,

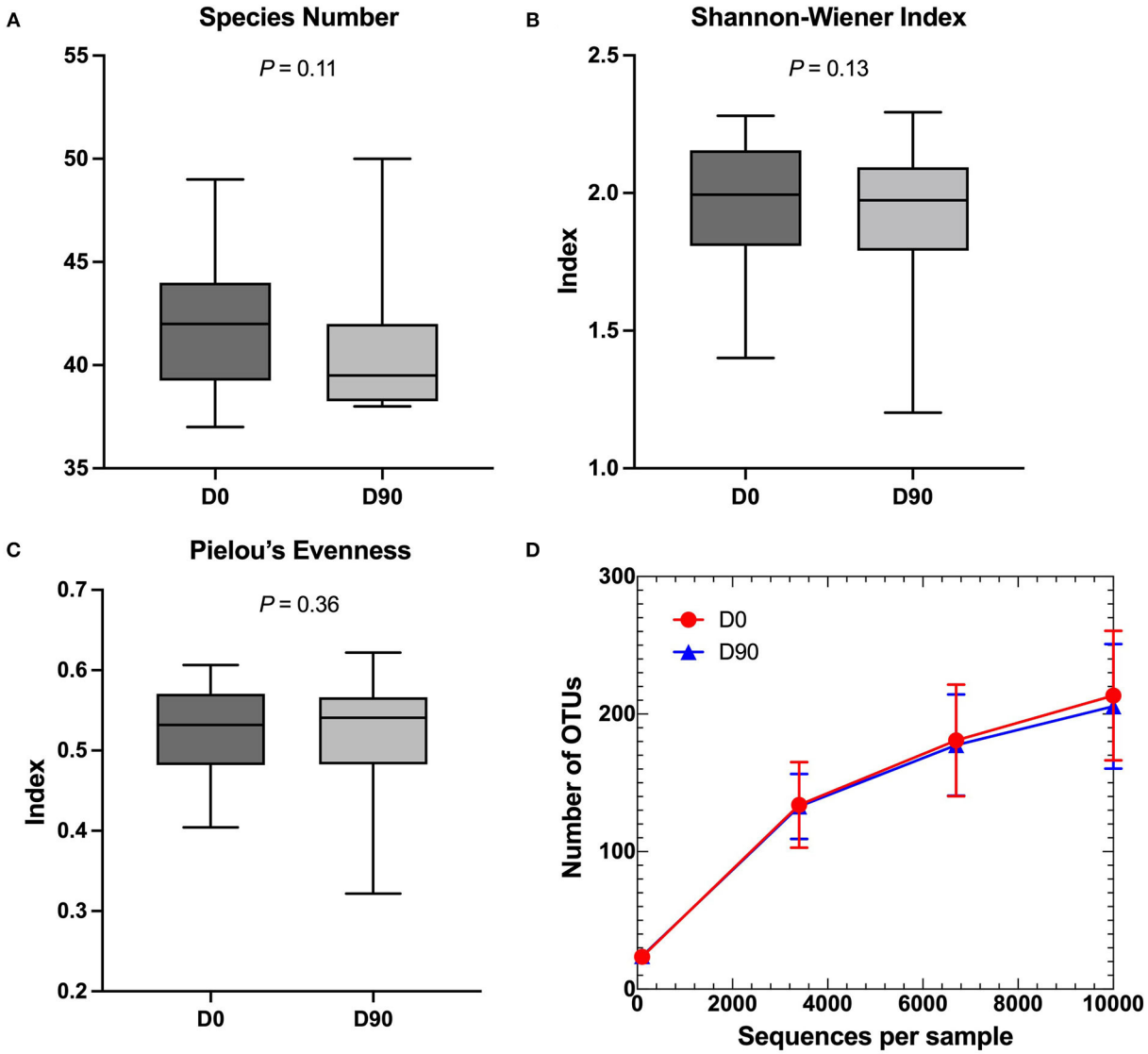

FIGURE 1
(A–C) Comparison of alpha diversity parameters in box and whiskers plots. **(A)** Species number, **(B)** Shannon–Wiener index, and **(C)** Pielou's evenness demonstrated no significant changes between pre-phenobarbital treatment (D0) and 90-day post-treatment (D90). Lines in the boxes represent the median of each group and the boxes represent the interquartile range, whereas the whiskers represent the minimum and maximum data. **(D)** Rarefaction curve based on the number of observed operational taxonomic units (OTUs). The red circles and blue triangles represent the mean of sequences per sample at D0 and D90, respectively. The error bars represent standard deviations.

the dosage of PB might play a role here. In a preclinical study, the effects of different PB concentrations on several bacteria, namely, Bifidobacterium, Bacteroides, Enterococcus, Eubacterium, Clostridium, and Staphylococcus were tested *in vitro*. The study found that generation time and lag time during the growth of some bacteria were affected depending on PB concentrations, while the lag time was influenced only by a high concentration of PB. Additionally, the metabolite alpha-ethyl-benzeneacetamide was found as a by-product of Bifidobacterium in both low and high PB doses (44). One of the metabolite synonyms is 2-phenylbutyramide, which was shown in several studies in mice as having a promising antiseizure effect by inhibiting neuronal acetylcholine receptors (45, 46). In the present study, a starting dosage of PB was used (2, 3). Higher PB concentrations might have yielded a different effect and produced a similar effect *in vivo* as seen *in vitro* in terms of how bacteria are affected.

In the study by Olson and colleagues in mice, *Akkermansia* and *Parabacteroides* bacteria were considered important for seizure control by increasing the seizure threshold (13). In contrast, no genus *Akkermansia* was detected in the current study by metagenomics analysis, while *Parabacteroides*

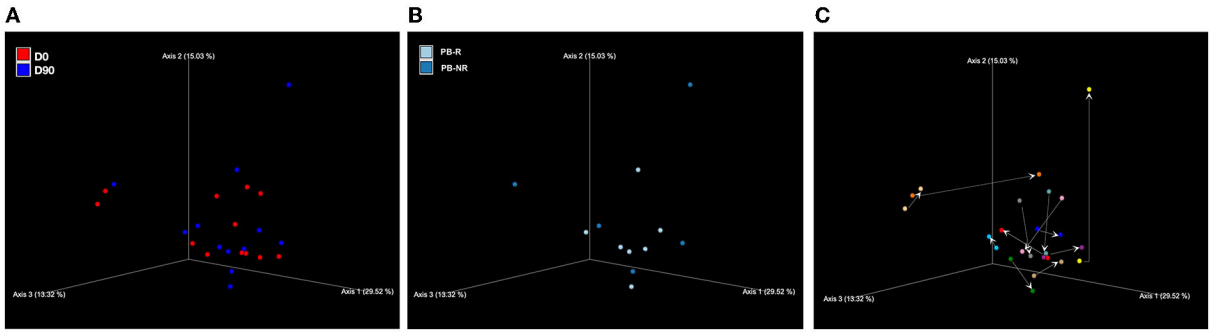

FIGURE 2
Principal coordinates analysis (PCoA) of weighted UniFrac distances of taxa diagrams demonstrating beta diversity **(A)** between the day prior to phenobarbital (PB) treatment (D0; red) and 90 days post-PB treatment (D90; blue), and **(B)** between PB-R group (light blue) and PB-NR group (dark blue) at D90. There was no significant microbiome clustering. **(C)** Changes in PCoA weighted UniFrac distances of taxa after PB treatment. The arrows show the direction from D0 (arrow tail) to D90 (arrow head) of each dog.

accounted for only 0.1% of sequencing reads in this study. It is worth noting that *Akkermansia* populates mainly the enteric crypts in dogs, so they might not shed easily into feces (47). To investigate this hypothesis, an intestinal biopsy could provide more information. In another canine study, *Bacteroidaceae* species within genus 5-7N15 were more abundant in dogs fed with a medium-chain triglyceride diet (16). These bacteria have previously been suggested to prevent aggressive behavior and occupy a similar niche in humans as *Akkermansia*.

In our study, there was a significant decrease in Clostridiales after PB treatment, which inversely correlates with the SCFA concentration results. Clostridiales are a complex bacterial order, namely, SCFA producers, pathogens, and many others. It is most likely that there were some changes in another Clostridiales member in lower taxa, other than SCFA-producing bacteria, which caused an overall decrease in this order abundance. In the aforementioned study by García-Belenguer and colleagues, the reduction in SCFA-producing bacteria at the family level was reported after 30 days of PB- or imepitoin treatment. Nevertheless, the SCFA concentration was not measured in that study (15).

The concentration of total SCFAs, propionate, butyrate, and also to some extent, acetate, did increase with PB treatment in our study. This is supported by the results of a study by Xie and colleagues (48). The researchers investigated the effects of oral GABA administration on different concentrations of SCFAs in mice and found that GABA could increase the production of total SCFAs, acetate, propionate, and butyrate as well as increase acidity in the intestine. Both PB and GABA work on the same receptor, which is the $GABA_A$ receptor (48). However, the reason why PB increases the SCFA production of GIM is unknown. SCFAs are essential for gut and brain health. Due to their being small molecules of fatty acids, they can be rapidly absorbed and transported *via* circulation to the liver and other organs, namely, the brain, easily crossing the blood–brain barrier (BBB) (49). In another study, serum saturated fatty acid concentrations in PB-treated dogs were evaluated and were significantly higher than in controls (50). The beneficial functions of SCFAs are well studied in the intestine but only to a lesser degree in CNS. SCFAs have several positive effects that could potentially support brain functions and palliate epilepsy through MGBA. One of the well-known functions of SCFAs, particularly butyrate, is their anti-inflammatory effect (23, 51, 52). Inflammation plays an important role, especially in drug-resistant epilepsy (53, 54). Nonetheless, whether butyrate can alter brain inflammation in epilepsy requires further studies. However, a study in mice showed that intestinal inflammation could increase convulsant activity and reduce ASD efficiency (55). Furthermore, epilepsy is associated with a change in glucose metabolism (56). Butyrate can provide an alternative energy resource for the brain (57), resulting therefore in a potential improvement in epilepsy. This could be similar to a ketogenic diet in humans and dietary medium-chain triglyceride in canine IE management, where ketones provide an alternative source of energy (56). Butyrate also has neuroprotective effects, which can be explained in several dimensions, such as the ability of neurogenesis stimulation (58), prevention of neuronal apoptosis (59), suppressed demyelination, and promoted remyelination (60), BBB permeability restoration (61), and antioxidation (25). A couple of preclinical studies in rodent models also found that butyrate may have direct antiseizure effects through the histone deacetylase pathway (62, 63). In our study, the butyrate level after PB treatment was significantly higher in the PB-R group compared to the PB-NR group. Nevertheless, it should be taken into account that this study had a small sample size in both groups, which might influence the results. A future study with a larger sample size would be needed to confirm these results. Further studies might also consider adding butyrate to a dog's diet, either

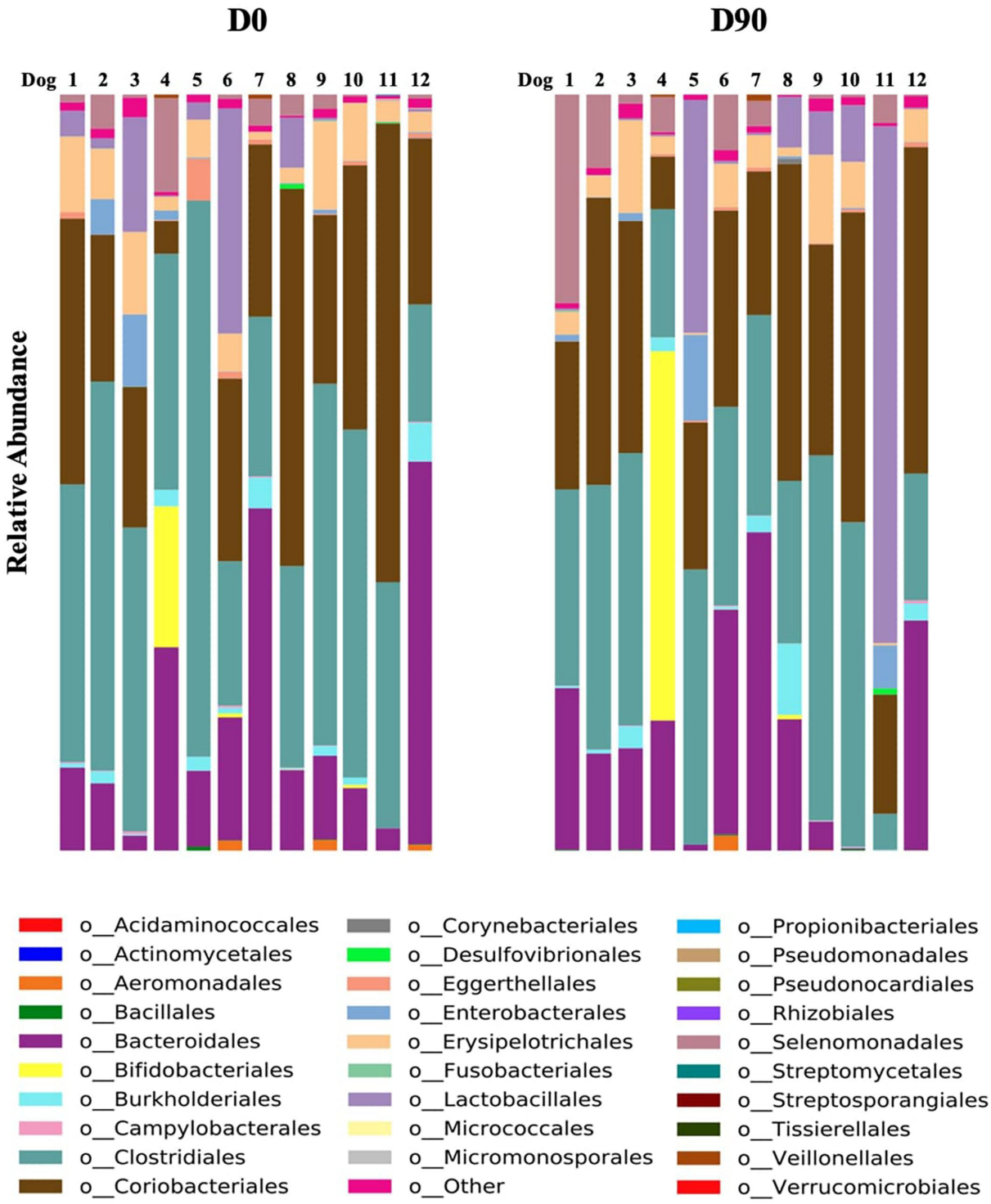

FIGURE 3
Relative abundance of each order prior to phenobarbital treatment (D0) and 90 days post-treatment (D90) in fecal samples of each dog ($n = 12$).

directly or indirectly by modifying the microbiome (probiotic or high fiber diet) (57, 64), to see whether this can improve epilepsy management.

Regarding the functional aspect of GIM, we also found that PB treatment could increase the number of genes associated with protein synthesis and carbohydrate metabolism. However,

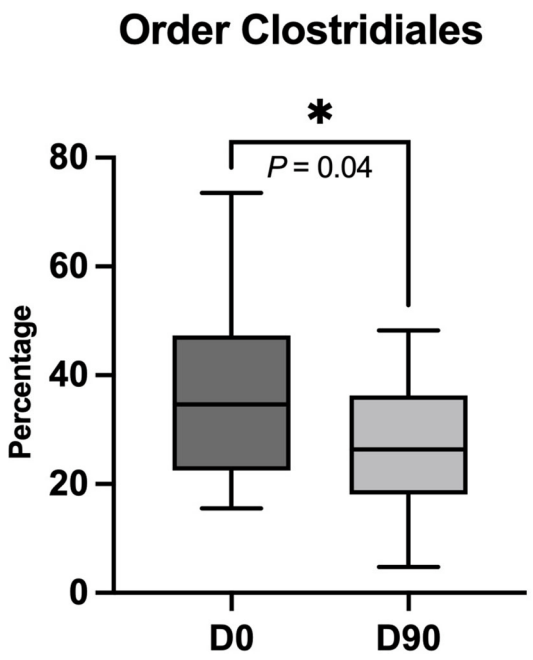

FIGURE 4
Significant decrease in order Clostridiales in percentage after 90 days of phenobarbital treatment (D90) compared to pre-treatment (D0). Lines in dark and light gray boxes represent the median at D0 and D90, respectively. The box limits indicate the interquartile range, while the whiskers represent the minimum and maximum data. *$P < 0.05$ (one-tailed Wilcoxon matched-paired signed-ranks test).

it appears that the GIM under PB treatment was under stress conditions. There are several conditions that could be stressful for GIM, such as osmotic stress, oxidative stress, and acid stress from their SCFA fermentation (65–67). To survive under these conditions, GIM have stress defense mechanisms, well-known ones such as trehalose biosynthesis and transcriptional regulation (65). Interestingly, we found that there was an increased abundance of genes associated with the trehalose biosynthesis pathway during metabolism from D-glucose 1-phosphate to trehalose (KEGG module M00565). Bacterial trehalose biosynthesis typically occurs under abiotic stress in order to increase trehalose concentration (66, 68). On the one hand, the elevation of trehalose concentration nourishes the GIM themselves (66). On the other hand, the accumulation of trehalose results in bacterial rehydration without cellular function deterioration by reducing water activity, cell volume restoration, and stabilizing protein as well as cellular turgor pressure (65). Other than surviving stress conditions by trehalose biosynthesis, an increased number of ribosomes are also essential. In the present study, the abundance of genes related to microbial ribosomal production (KEGG module M00178) was significantly increased, which potentially resulted in an increased number of microbial ribosomes. As mentioned above, transcriptional regulation is also necessary for GIM to alleviate stress conditions. The defense strategies of bacteria are generally "programmed" in specific genes. The responses depend on the type of stress the GIM are facing and the transcription is regulated to react appropriately to each condition. However, the success of transcriptional regulation is determined by the subsequent ribosomal translation, the number and speed of the ribosomes playing a crucial role in this process (69, 70). Since ribosomes are an important factor in bacterial protein synthesis, increasing the ribosome number is not only important for stress responses but also for viability and their protein-related productivity (71). Other elevated gene numbers detected in this study were genes associated with pathways in gluconeogenesis during the metabolism of oxaloacetate to fructose 6-phosphate (KEGG module M00003). GIM normally require glucose as the main carbon source for their metabolism, which they normally uptake from the surrounding environment. When glucose is insufficient, the GIM use gluconeogenesis as an alternative method to synthesize glucose from non-sugar substrates, namely, amino acids and intermediates from the tricarboxylic acid cycle (72). In contrast, V-type ATPase genes as part of oxidative phosphorylation decreased in this study. The V-type ATPase gene is located in the bacterial cytoplasmic membrane and functions as an ATP hydrolysis-driven ion pump, which relates to several GIM processes, namely, intracellular pH regulation and neurotransmitters release (73). However, it is still unclear how the reduction in the V-type ATPase gene might affect this situation. Furthermore, the alterations of the four genes associated with the KEGG modules (M00003, M00159, M00178, and M00565) showed no relation to SCFA production. In our study, we compared the results before and after PB treatment. Each dog received the same diet throughout the study period. Since the diet is one of the important factors affecting GIM (18) as is the dog breed (74), it is worth noting that the lack of dietary and breed standardization in this study might have affected the results. In addition, an age, breed, and diet matched healthy control group could have also added value to the interpretation of the study results.

Fear- and anxiety-related behaviors as well as cognitive dysfunction are well-known epilepsy comorbidities in dogs (26, 27, 32). To date, it has not yet been completely elucidated whether these epilepsy comorbidities are a risk factor for the development of epilepsy, part of the epilepsy phenotype, a side effect of some ASDs, or a combination of all. Our study is the first prospective study focusing on the effect of PB on behavior in dogs with IE. For behavioral assessment in this study, the positive effects of PB were observed by improving stranger-directed and non-social fear as well as trainability. There was no significant difference between the PB-R group and the PB-NR group. These results might appear surprising at first sight, as the opposite is more likely to have been predicted. However, prior to our study, there had been no behavioral longitudinal studies in either animals or humans with epilepsy treated with

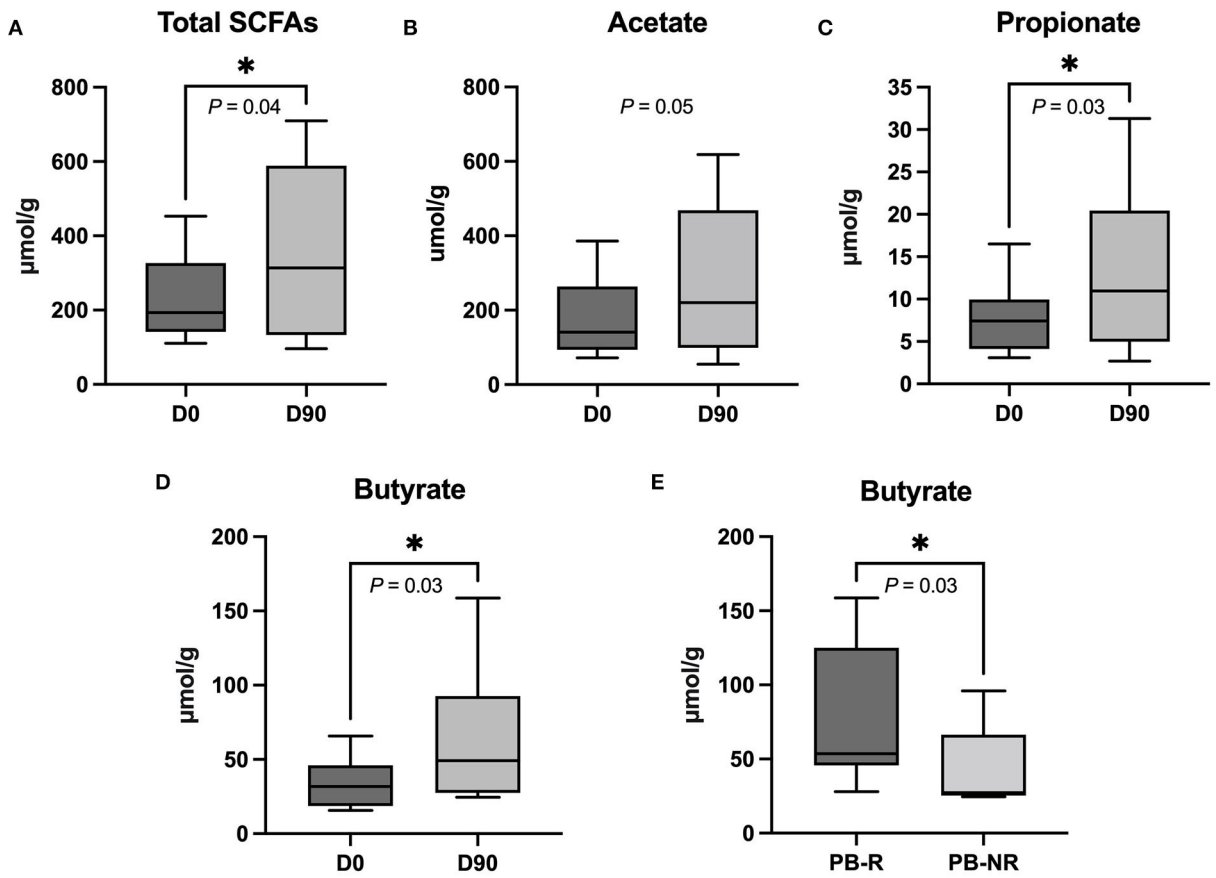

FIGURE 5
(A) The total short-chain fatty acids (SCFAs), **(C)** propionate, and **(D)** butyrate levels in fecal samples increased significantly when comparing 90 days after phenobarbital (PB) treatment (D90) to the day before PB treatment (D0). **(B)** The graph shows an upward trend of acetate at D90 compared to D0. *$P < 0.05$ (one-tailed Wilcoxon matched-paired signed-ranks test). **(E)** Butyrate level at D90 differed between PB-R and PB-NR. *$P < 0.05$ (one-tailed Mann–Whitney *U*-test). The lines in the boxes indicate a median value and the box limits represent the interquartile range. The whiskers indicate minimum and maximum data.

PB as monotherapy (26). In humans, PB has been used not only to treat seizures but also to treat anxiety (75). Nonetheless, in a small cross-sectional study, it was shown that people with epilepsy treated with primidone, which gets metabolized to PB, were more likely to show anxiety (76). Similar single reports on anxiety-related behavioral changes seen in dogs with IE exist for primidone, but not for PB (77). The influence of PB on cognition remains unclear, with original studies in humans even reporting a potential improvement (75). In dogs with IE, cross-sectional studies did not reveal an improvement or a deterioration in fear/anxiety or cognitive impairment with PB monotherapy (27, 78, 79). Potential improvement in terms of behavior, but also deterioration in individual cases after treatment with individual drugs have been shown for levetiracetam (80) and imepitoin (77). For other drugs, however, this link is not as clear. Nonetheless, aggression as a side effect, which is often idiosyncratic in nature, as seen in individual cases treated with PB, potassium bromide, levetiracetam, and/or zonisamide, has been reported in the literature (77, 81). In addition, polypharmacy and drug resistance have also been associated with cognitive impairments and fear/anxiety, especially in dogs with IE treated *via* monotherapy (27, 32, 79). Interestingly, in one of these studies, pharmacoresistance was an influencing factor, but an association with seizure frequency could not be found (79). This further highlights that the severity of epilepsy is not only reflected by its seizure frequency but also by the severity of its comorbidities. With regard to the influence of ASDs on cognition, Packer and colleagues reported that the trainability of adult dogs with IE was significantly worse compared to controls when dogs received potassium bromide, zonisamide, or polypharmacy (32). The current evidence in canines rather suggests that epilepsy is one of the main factors triggering the development of fear/anxiety and cognitive deficits (27, 29, 32, 78, 79). It is worth mentioning that the diagnostic methods for anxiety and cognitive dysfunction are complex. In humans, the gold standard of anxiety disorder diagnosis is

a clinical interview by a specialist (82), while mild cognitive impairment is normally assessed using the Montreal Cognitive Assessment and Mini-Mental State Examination (83). To date, there is a paucity of data showing that self-reported or symptom-based questionnaires could replace the gold standard (82). In dogs, diagnoses of fear- and anxiety-related behaviors and cognitive dysfunction are as complicated as in humans and the gold standards have not been established. Therefore, history taking and completion of validated questionnaires by dog owners as well as the performance of dog behavioral tests or observations and behavioral development assessments are used in combination as diagnostic procedures (84, 85). In the present study, only validated online questionnaires were used to evaluate the behavioral comorbidities. This should be kept in mind, when considering the results, as it is a limitation of the current study.

It is possible that the beneficial effects on stranger-directed and non-social fear-related behavior and trainability after PB treatment may be due to elevated SCFAs, particularly the butyrate level (57). Such effects were also shown in human cases and mice models receiving a high fiber diet, modulating the GIM to produce these SCFAs (86, 87). An improvement in anxiety-like behavior and cognitive impairment was shown in a number of studies on sodium butyrate, a salt form of butyric acid in rodent models (88–90). Sodium butyrate is also a histone deacetylase inhibitor and was reported to be a potential anti-depressant, inducing short-lasting histone hyperacetylation in the hippocampus and frontal cortex as well as temporarily increasing brain-derived neurotrophic factor (BDNF) expression in mice (91). Not only in preclinical studies was the inverse correlation between butyrate concentration and the degree of anxiety observed but also in humans with anorexia nervosa (92), which agrees with our findings. In our study, improvement in trainability without alleviating cognitive ability was observed, which could be explained by its multifactorial correlation. In dogs, trainability is a combination of motivation and willingness to follow the commands of their owners, difficulty in being distracted, and positive reaction to correction as well as cognitive skills (40, 93). Therefore, the improvement in fear- and anxiety behaviors could also play a positive role in trainability.

Conclusion

PB treatment in canine IE could affect GIM taxonomically and functionally including an increase in SCFAs level as well as altering glucose and protein metabolism of GIM by increasing the abundance of their functional genes. In addition, the level of butyrate among PB-R was significantly higher than that of PB-NR, showing that butyrate could be essential and beneficial for seizure control. This could be explained by providing an alternative efficient energy source and by the anti-inflammatory and neuroprotective effects of butyrate. Moreover, PB treatment may have the potential to alleviate fear- and anxiety-related behaviors as well as trainability in dogs with IE. Future large-scale studies including consulting a behavior specialist are needed to confirm these results.

These findings could be an important first milestone showing that the difference in microbial functional reactions could be the key difference between the effective and ineffective treatment of dogs with epilepsy. More studies on GIM functions and their role in epilepsy management, as well as behavioral comorbidities, are needed to better our understanding and to consider the development of alternative effective treatment strategies in canine IE.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA data: PRJNA849257/Temporary Submission ID: SUB11612854.

Ethics statement

Ethical review and approval was not required for the animal study because all dogs in this study received the standard of care, therapeutic and diagnostic work-up. The fecal samples were collected after voiding. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AW planned and performed this study as well as interpreted the results and drafted the manuscript as a part of her Ph.D. thesis. SM and HV planned, co-supervised, and reviewed the study. JS, MK, and RP performed the laboratory part and supervised and reviewed the study. SL, LB, and AB-N were responsible for some dogs from their own institutes and reviewed the study. GM-W supervised and reviewed the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author HV served as a paid consultant in the field of epilepsy for Boehringer Ingelheim, CEVA Animal Health, Nestle Purina, and as a contract researcher for Nestle Purina, Desitin Pharma, and Boehringer Ingelheim. Author JS is an employee of the Gastrointestinal Laboratory at Texas A&M University that provides microbiome testing on a fee-for-service basis and is on the scientific advisory board for Nestle Purina and received speaker honoraria from Royal Canin, Nutramax Laboratories, ExeGi Pharma, LLC, and Hill's Pet Nutrition, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metabolic fingerprinting of dogs with idiopathic epilepsy receiving a ketogenic medium-chain triglyceride (MCT) oil

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Consumption of medium-chain triglycerides (MCT) has been shown to improve seizure control, reduce behavioural comorbidities and improve cognitive function in epileptic dogs. However, the exact metabolic pathways affected by dietary MCT remain poorly understood. In this study, we aimed to identify changes in the metabolome and neurotransmitters levels relevant to epilepsy and behavioural comorbidities associated with the consuming of an MCT supplement (MCT-DS) in dogs with idiopathic epilepsy (IE). Metabolic alterations induced by a commercial MCT-DS in a population of 28 dogs with IE were evaluated in a 6-month multi-centre, prospective, randomised, double-blinded, controlled cross-over trial design. A metabolic energy requirement-based amount of 9% MCT or control oil was supplemented to the dogs' stable base diet for 3 months, followed by the alternative oil for another 3 months. A validated, quantitative nuclear magnetic resonance (NMR) spectroscopy platform was applied to pre- and postprandially collected serum samples to compare the metabolic profile between both DS and baseline. Furthermore, alterations in urinary neurotransmitter levels were explored. Five dogs (30%) had an overall reduction in seizure frequency of $\geq$50%, and were classified as MCT-responders, while 23 dogs showed a $\leq$50% reduction, and were defined as MCT non-responders. Amino-acid metabolism was significantly influenced by MCT consumption compared to the control oil. While the serum concentrations of total fatty acids appeared similar during both supplements, the relative concentrations of individual fatty acids differed. During MCT supplementation, the concentrations of polyunsaturated fatty acids and arachidonic acid were significantly higher

than under the control oil. β-Hydroxybutyric acid levels were significantly higher under MCT supplementation. In total, four out of nine neurotransmitters were significantly altered: a significantly increased γ-aminobutyric acid (GABA) concentration was detected during the MCT-phase accompanied by a significant shift of the GABA-glutamate balance. MCT-Responders had significantly lowered urinary concentrations of histamine, glutamate, and serotonin under MCT consumption. In conclusion, these novel data highlight metabolic changes in lipid, amino-acid and ketone metabolism due to MCT supplementation. Understanding the metabolic response to MCT provides new avenues to develop better nutritional management with improved anti-seizure and neuroprotective effects for dogs with epilepsy, and other behavioural disorders.

KEYWORDS

metabolome, neurotransmitter, epilepsy, biomarker, medium-chain triglyceride, canine

Introduction

Epilepsy is the most common neurological disorder in dogs affecting an estimated 0.6–0.8% (1–3) of the population in first opinion practise. Epilepsy is defined by its recurrent seizure activity (4), but is often associated with behavioural and cognitive comorbidities (5), such as anxiety (6–10), ADHD-like behaviour (11), deficits in general trainability (8, 12), spatial memory (13, 14) or earlier signs of canine cognitive dysfunction (15). Although there is active international research in this field, therapeutic approaches to canine epilepsy focus on drugs for seizure suppression (16, 17), instead of treating epileptogenesis or the disease itself, including the comorbidities mentioned above (18). The cellular, pathophysiological mechanisms and the biological manifestation of seizures leading to epilepsy are still unclear. However, chronic administration of anti-seizure drugs (ASD), often in tandem as polytherapy, comes with a delicate balance between the desired seizure-suppressive effects and drug-related adverse effects (19). Despite appropriately managed polypharmacotherapy, around one-third of dogs with idiopathic epilepsy continue to have difficulty controlling seizures (20–23). Seizure activity and drug related adverse effects contribute to the reduction in quality of life (QoL) for both dogs and their owners (5, 24–28) emphasising the need for new treatment strategies.

Dietary interventions, such as ketogenic diets (KD) have been explored as a therapeutic option to prevent and treat seizures in humans with epilepsy since the early 1920s. Originally, such diets were designed to mimic the metabolic state with its biochemical changes of fasting, as this has been shown to evoke anticonvulsant properties (29). Since then, dietary modification in seizure control has been extensively studied in human medicine (30–32) and some rodent epilepsy models (33–37), showing varying levels of anti-seizure effects.

In veterinary medicine, dietary manipulations have attracted increasing consideration as an alternative approach to managing seizure activity and behaviour in dogs with idiopathic epilepsy (IE) (11, 38, 39). In 2015, Law et al. compared an MCT kibble diet (test formula contained 5.5 % MCT; 10% of the total formula calories from C8, C10, C12) to a standardised placebo diet on its seizure-controlling effects in 21 chronically non-responsive ASD-treated dogs with IE in a crossover trial design (39). Anti-seizure properties and a significant rise in blood β-hydroxybutyrate (BHB) concentrations were found. In 2020, another study showed that the supplementation of a commercial MCT-oil as 9% of the metabolic energy (ME) rate on a various base diet had proven similar positive anti-seizure effects and resulted in an increase in BHB serum concentrations (40). The in average daily feed ration consisted of at least 50% carbohydrates in both studies. This supports the hypothesis, that medium-chain fatty acids (MCFA), such as caprylic, capric, and lauric fatty acids, as the main constituent within the MCT, may possess direct mechanistic effects in the anticonvulsive properties and come with specific metabolic adaptions (41–46).

Abbreviations: ASD, Anti-Seizure Drug; AA, Arachidonic Acid; BHB, Beta Hydroxybutyric Acid; C10, Decanoic Acid = Capric Acid; C8, Octanoic Acid = Caprylic Acid; DS, Dietary Supplement; FA, Fatty Acids; GABA, γ-Aminobutyric Acid; GPR, G-Protein Coupled Receptor; IE, Idiopathic Epilepsy; IVETF, International Veterinary Epilepsy Task Force; KBr, Potassium Bromide; Kcal, Kilocalories; KD, Ketogenic Diet; KD-R, Ketogenic Diet Responders; LEV, Levetiracetam; MCT, Medium Chain Triacylglyceride; ME, Metabolic Energy; NR, Non-Responders; PB, Phenobarbital; PC, Pyruvate Decarboxylase; PDH, Pryruvat Dehydrogenase; PG, Prostaglandin; PUFA, Polyunsaturated Fatty Acids; R, Responders, RVC, Royal Veterinary College; TCA, Tricarboxylic Acid; TAG, Triacylglyceride; QoL, Quality of Life.

Recently, we have shown that a 9% ME MCT supplemented diet has also a positively affects anxious behaviour and cognitive abilities in some dogs with IE (40, 47). However, the knowledge about how MCT alters the metabolism and may lead to these brain effects in dogs is limited. In the past, MCT containing diets has been shown to confer neuroprotective effects in humans (48). The same suggestion has been made in dogs after 3 months of MCT consumption, which led to significant global changes in the lipid metabolism. A considerable increase of the saturated C17:0 fatty acyl moieties (lysophosphatidylcholine, phosphatidylcholine) was highlighted and discussed as associated with the neuroprotective, downstream metabolites triheptanoin (49). Triheptanoin is a triacylglyceride (TAG) consisting of three heptanoate (C7:0) fatty acids (FA). Especially, C17:0 fatty acyl moieties can be metabolised to C7:0 moieties after five cycles of β-oxidation. If it is hypothesised that triheptanoin may influence anaplerotic mechanisms in the brain by delivering tricarboxylic acid cycle substrates and supporting mitochondrial metabolic pathways (37, 50, 51).

Overall, most of the beneficial effects of MCFA have been attributed to ketone body formation. More recent results has raised the question, whether MCFA *per se* and its derived downstream metabolites may play a much more relevant role in the treatment of epilepsy.

This study aimed to explore the metabolomic effects behind an oral MCT oil supplementation in epileptic dogs governing, as previously reported, anti-epileptic and pro-cognitive properties. Thus, appropriate physicochemical methods were chosen to identify, and measure altered metabolites in serum and urine putting them into a physiological context.

Materials and methods

This clinical study was conducted following the guidelines of the International Cooperation of Harmonization of Technical Requirements for Registration of Veterinary Products (VICH) GL9 Good Clinical Practices (GCP) and the European Agency for the evaluation of Medical Products (EMEA). This study was approved by the Royal Veterinary College Clinical Research Ethical Review Board (CRERB) (URN 2016 1558).

The clinical trial

A 6-month multicenter, prospective, randomised, double-blinded, controlled, dietary crossover trial was conducted to investigate the short-term effects of an MCT oil supplement to a "control" dietary oil supplement (DS) for the management of canine epilepsy and their correlation with metabolic responses caused by this dietary modification (Figure 1). The clinical study design has been published separately in detail (52), as were the results of the effect of the supplement on seizure frequency (40) and cognition (47). Briefly, dogs diagnosed with IE (International Veterinary Epilepsy Task Force tier II level diagnosis) and not responding to at least one appropriate chosen and administered ASD were recruited at five different study centres in three countries for this dietary trial. Each dog was randomly assigned a first DS-1, either the MCT- or control-DS, instructed to supplement alongside their normal diet and fed initially for 3 months (day 1 to day 90 ± 2), followed by a respective switch to the second DS-2 for another 3 months (day 90 to day 187 ± 2). The first 7 days of dietary crossover, DS period 2 (day 90 to day 97 ± 2) were considered as wash-out period and allowed metabolic adaption.

Dietary supplementation and test products

A total of 9% of the daily metabolic energy requirement was translated into the individual oil volume prescribed per day and added on the top of their usual energy amount. Throughout the study, all dogs were fed twice daily with their regular diet receiving half amount of the total daily oil requirement. ASD treatment was not withheld at any point nor changed in this study. Concomitant changes in ASD medication or diet during participation led to study exclusion.

Both used oils were commercially available dietary supplements suitable for human consumption. While the test oil was an MCT-DS purified from palm and rapeseed oil containing high levels of two medium-chain fatty acids (MCFA) 50–65% caprylic acid (C8) and 30–50% capric acid (C10) (End GmbH, Germany, Batch No. L 16 M12), the control oil was colourless, extra-virgin olive oil with 8 kcal per ml containing 11% saturated, 11% polyunsaturated, and 78% g monounsaturated fat (Filippo Berio, Italy, Batch No LE194M04). To ensure a throughout blinded study design, both oils were dispensed in brown bottles and all owners were instructed on how to store and supplement. The base diet was recorded, but not individually assessed.

Sample collection and preparation

Serum and urine samples were collected based on a standard operating procedure for different metabolomic approaches to interrogate the changes in metabolic profile seen in epileptic dogs receiving a daily ketogenic DS onto a stable base diet and anticonvulsive medication regime (Figure 1).

Urine samples

On the morning of each scheduled study visit (V1–V3), time-matched urine samples were collected from each

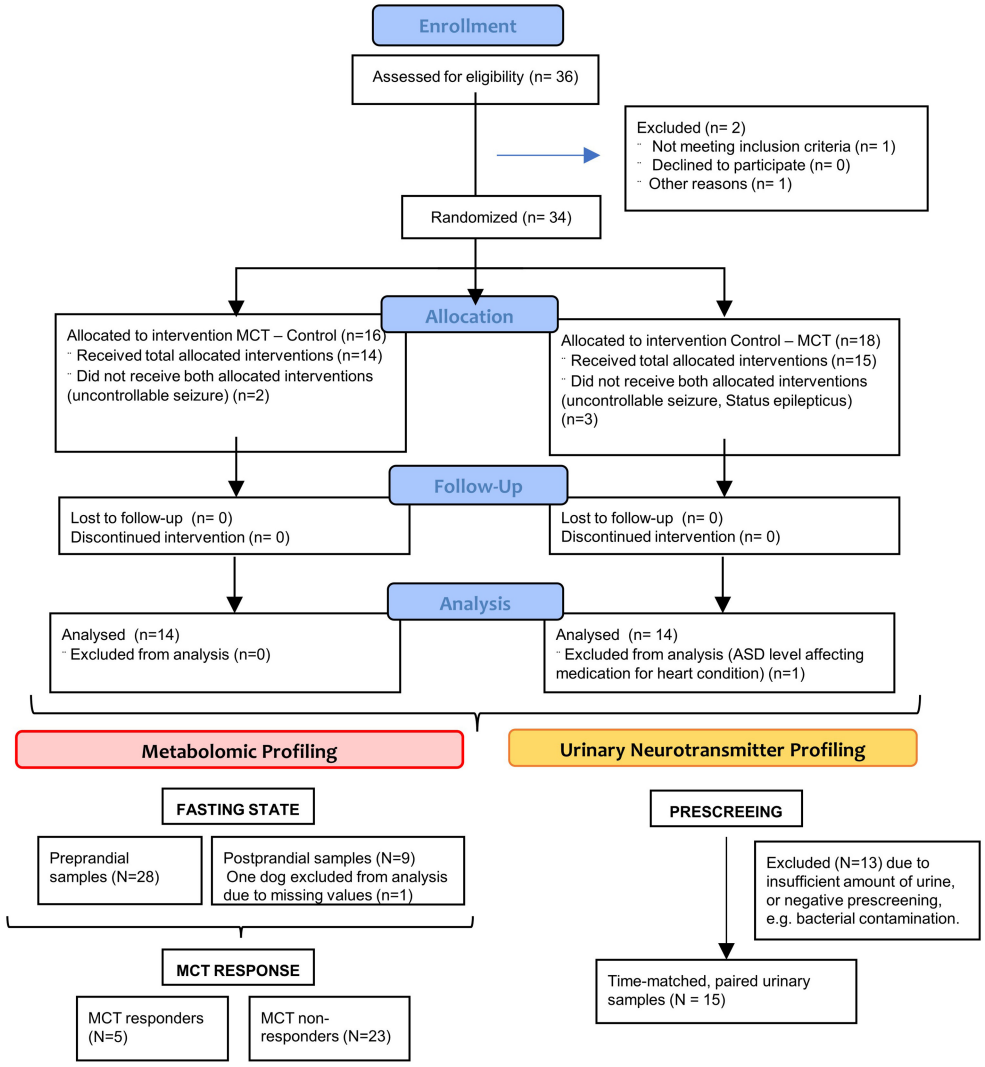

FIGURE 1
CONSORT flow diagram—Flow diagram of the progress through the phases of this randomized clinical trial of two groups (that is, as shown, enrolment, intervention allocation, follow-up, and data analysis).

dog using the free catch method, and transferred into sterile urine tubes. The samples were identified as baseline, control DS phase or MCT-DS phase samples. As quality pre-screening standard, part of the urine was subsequently analysed for urine specific gravity, pH, nitrite, protein, ketones, bilirubin, bacteria, crystals, and blood (erythrocytes, leukocytes) at two local laboratories (Diagnostic Laboratory Services, Royal Veterinary College, United Kingdom; IDEXX Laboratories, Inc., Ludwigsburg, Germany). Only dogs with unremarkable urine analysis at all study visits were included into further analysis. Aliquots with 500 ul of urine were prepared and stored in cryovials at −80°C up to trial completion.

Blood samples

At each study visit (V1–V3), two blood samples were collected from each dog. A pre-prandial sample was taken after 12 h of overnight fasting and the second in the post-prandial state 2 h after feeding the dog with its usual diet supplemented with the prescribed volume of DS. Routine anti-seizure medication was also given during feeding. Both samples were taken into both clotting activator dipotassium EDTA-containing (with serum-separation gel) polypropylene blood-collection tubes and plain polypropylene blood-collection tubes (International Scientific Supplies Ltd). Serum tubes were left to coagulate on ice for 15 min prior to centrifugation. The samples were centrifuged for 10 min at 2,000 × g at 2–5°C, and

the resulting supernatant (=serum) was separated. Serum was stored in cryovials at −80°C up to study completion. Only dogs with a total amount of 300 ul per cryovial for each study visit were finally studied using a targeted metabolomics approach.

Metabolomic exploration methods

Two analytical methods [nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC)] were used to acquire metabolic data in serum and urine (66).

Neurotransmitter profiles in urine

Urine samples (∼2 mL) were sent to Doctors Data Inc. (St. Charles, IL) where they were stored at −80°C until analysis. The urine specimens were diluted according to their creatinine level. The diluted urine samples were mixed with internal standards and derivatized to ethoxycarbonyl-ethyl-ester derivatives with ethyl-chloroformate and ethanol-pyridine at room temperature. The derivatives of these neuro-biogenic amines were then extracted by polymeric reversed phase SPE cartridge to remove protein and water-soluble impurities. The analytes were analysed by Agilent 6460 liquid chromatography–mass spectrometry (LC-MS/MS) with Jet Stream (AJS) Electrospray Ionisation. Multiple reaction monitoring (MRM) transition ions were detected by MS/MS technique for each analyte of interest while a precursor ion is further broken down to a fragment ion for greatly improved selectivity. The chromatography separation was achieved on a Poroshell 120 EC-C8, 2.1 × 150 mm, 2.7 μM (Agilent Technologies, Palo Alto, CA). The urine-based concentration of the following neurotransmitters relevant for epilepsy and behavioural comorbidities were then quantified for statistical assessment: serotonine, dopamine, epinephrine, norephinephrine, glutamate, GABA, histamine, glycine, phenethylamine.

Metabolic profiles in serum

Both the pre- and post-prandial samples were measured on each visit day. While pre-prandial samples were used to elucidate the global shifts in metabolism, post-prandial samples were used for elucidating the immediate and short-term metabolic responses associated with DS consumption. Sample analysis was conducted using a validated, canine-specific proton nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabolomics platform quantifying 123 measurands (53). Sample preparation with buffer allocation was performed by a PerkinElmer 8-tip JANUS automated liquid handler (PerkinElmer Inc., Waltham, Massachusetts, United States) and nuclear magnetic resonance (NMR) measurement utilising a Bruker AVANCE III HD 500 spectrometer with a 5 mm triple-channel (1H, 13C, 15N) z-gradient Prodigy probe head (Bruker Biospin, Rheinstetten, Germany) with principles outlined in Soininen and colleagues (53, 54).

Ketone body measurements in serum

Pre- and post-prandial β-Hydroxybutyric acid (BHB) concentrations were measured on each visit day from the collected pre- and post-prandial serum samples. Th serum samples were shipped for subsequent analysis for BHB concentrations at one of the local laboratories (Diagnostic Laboratory Services, Royal Veterinary College, United Kingdom; IDEXX Laboratories, Inc., Ludwigsburg, Germany).

Statistical analysis

For all above-mentioned neurotransmitters in urine and metabolites in serum, (a) concentrations, (b) the percentage change relative to baseline and (c) specific ratios were statistically compared. Comparisons were made between the following two subgroups: (i) baseline and/ or type of DS; (ii) fasting state: pre- vs. post-prandial state; (iii) MCT response rate, based on reduction in seizure frequency defined as the number of seizures per month [subclassified as "R" (R) ≥ 50% reduction, "NR" (NR) ≤50% reduction], as reported previously (40).

Baseline and DS concentrations were compared using a two-sided, match-paired Student's *t*-test for parametric data and Wilcoxon matched-pairs signed-rank test for non-parametric data. When comparing MCT R with NR, unpaired two-sided *t*-tests for parametric data and Mann-Whitney U-test for non-parametric data were chosen (GraphPad Prism®; STATCON GmbH; Schulstr. 2; 37213 Witzenhausen, Germany). To account for multiple comparisons. *P*-value correction was conducted using false discovery rate (FDR) (55). The relationships between selected pairs of continuous variables were analysed using Pearson's correlation coefficient analysis. Non-parametric data are presented as median (25th−75th percentile), and parametric data are represented as mean values and standard deviations.

Results

Animal population

As formerly reported (40), the clinical trial was completed by 28 of the 36 recruited dogs, which included 18 different breeds at five different study sides in three different countries [UK ($N = 16$), Germany ($N = 14$); Finland ($N = 1$)]. The study population consisted of 16 males, of which were 12 neutered and 4 were intact, and 12 females, of which 10 were neutered and 2 intact. The population of dogs were on average 5.46 ± 2.61 years of age and weighed 25.6 ± 13.4 kilogrammes at the start of the trial. Twenty-five (89%) of the 28 dogs

received phenobarbital (PB) as ASD, while 32% received a second and 61% ($N = 17$) a third ASD as combination therapy. Predominantly, potassium bromide or levetiracetam was given as add-on medication (40%). During MCT supplementation a reduction in seizure frequency was observed in over 60% of dogs ($N = 17$). However, only thirty percent of dogs ($N = 5$) had an overall reduction in seizure frequency ≥50% and were from there classified as MCT responders (R). Thus, 23 dogs were grouped to MCT non-responders (NR). Twelve dogs showed ≤50% (21%; 6–42%) reduction, while three dogs had no change and eight dogs showed an overall increase in seizure frequency per month (8%; 2–33%), as reported previously (40).

Urine samples of 15 dogs (53%) were available in sufficient quantity and were sent for neurotransmitter level quantification. This subpopulation consisted dominantly of male-neutered (40%), pure-breeds (80%) receiving PB in 87% as ASD. The mean age was 5.75 ± 2.14 years of age and the mean weight was 26.1 ± 15.12 kilogrammes at the start of the trial. All five MCT-R were included (30%, $N = 5$), while the rest were classified as NR (70%, $N = 10$).

Feed ration

Most dogs (82%, $N = 19$) received a commercial dry food as a single diet (46%) or in combination with wet food (11%), raw feed (14%) or home-cooked add-on (11%). A variable self-cooked food or raw feed ration was given to the rest ($N = 8$). Fourteen out of 28 dogs were supplemented first with the MCT-DS, while 15 got the control-DS first, subsequently followed by the dietary switch to the other DS type.

Urinary neurotransmitter profile

Significant differences in four out of nine neurotransmitters were observed in dogs consuming the control-DS and MCT-DS after 90 days of DS consumption (Supplementary Table 1). In all 15 dogs, a significant increased γ-aminobutyric acid (GABA) concentration ($p = 0.044$) was detected during the MCT-phase (Figure 2A). This increase was accompanied by a significant shift in the balance between GABA and glutamate (Figure 2B). Supplementation of MCT has resulted in a relative increase of 50% on the GABAergic side ($p = 0.025$) (Figures 2B,C). Moreover, significant differences were observed between R and NR during the MCT-DS phase. MCT-R were also found to have significantly lowered urinary concentrations of histamine ($p = 0.006$), glutamate ($p = 0.046$) and serotonin ($p = 0.012$) during the MCT-DS phase, but not during the control-DS phase [histamine ($p = 0.345$); glutamate ($p = 0.323$); serotonin ($p = 0.269$)]. The same was found for all three metabolites when compared to baseline.

Serum metabolite concentrations

Similarly, to the urinary neurotransmitters, significant differences in metabolite profiles were observed both between the MCT-DS and control-DS supplemented dogs after 90 days of supplementation, and between R and NR during dietary supplementation with MCT-DS (Supplementary Tables 2–4, in SI units).

Consumption of MCT-DS

Changes in lipid, amino-acid and ketone metabolism were observed during MCT consumption, as several metabolite concentrations and ratios were significantly altered (Figure 3A).

In pre-prandial state, seven metabolites and six ratios were altered with statistical significance between Control DS and MCT DS phase (Supplementary Table 2). While two amino acid ratios were shown to be decreased related to baseline [ratio of alanine to total branched-chain amino acids (Ala/BCAA), ratio of alanine to valine (Ala/Val)], a significantly increased concentration of Arachidonic acid (AA) ($p = 0.0464$) was found. Concerning to the total fatty acids amount, proportional (%) concentrations of AA ($p = 0.0081$) and PUFA ($p = 0.0162$) were significantly elevated, while saturated fatty acids (SFA, $p = 0.0450$) were lowered in comparison to the Control DS. BHB serum concentrations were significantly increased by MCT oil ($p = 0.033$). Blood glucose ($p = 0.01$) and leucine levels ($p = 0.001$) were significantly lowered under MCT compared to baseline. For both oils, the serum concentration of acetate was higher (MCT, $p = 0.0232$; control, $p = 0.0044$), but lower for albumin (MCT, $p = 0.0059$; control, $p = 0.0006$) and stearic acid (MCT, $p = 0.004$; control, $p = 0.0061$) compared to baseline. HDL particle size decreased significantly under both oils (MCT, $p = 0.002$; control, $p = 0.002$).

In post-prandial state, two metabolites and four ratios were altered with statistical significance between Control DS and MCT DS phase (Supplementary Table 3). As in the pre-prandial state, Ala/BCAA ratio was decreased in the MCT DS phase. In proportion to the total fatty acids amount (%), the concentration of AA ($p = 0.0357$) was elevated, while oleic acid ($p = 0.0182$) and PUFA ($p = 0.0184$) were significantly lowered. MCT intake led to a thirty percent higher BHB serum concentration than Control-DS ($p = 0.008$). LDL particle size was significantly larger under MCT than to Control-DS ($p = 0.0391$). Under both oils, the triglyceride content of large HDL particles' triacylglyceride content was higher compared to baseline (MCT, $p = 0.046$; control, $p = 0.047$).

MCT response rate

Significant differences were found in the metabolic signature between MCT R ($N = 5$) to NR ($N = 23$). Four out of 123 measurands were significantly altered (Figure 3B,

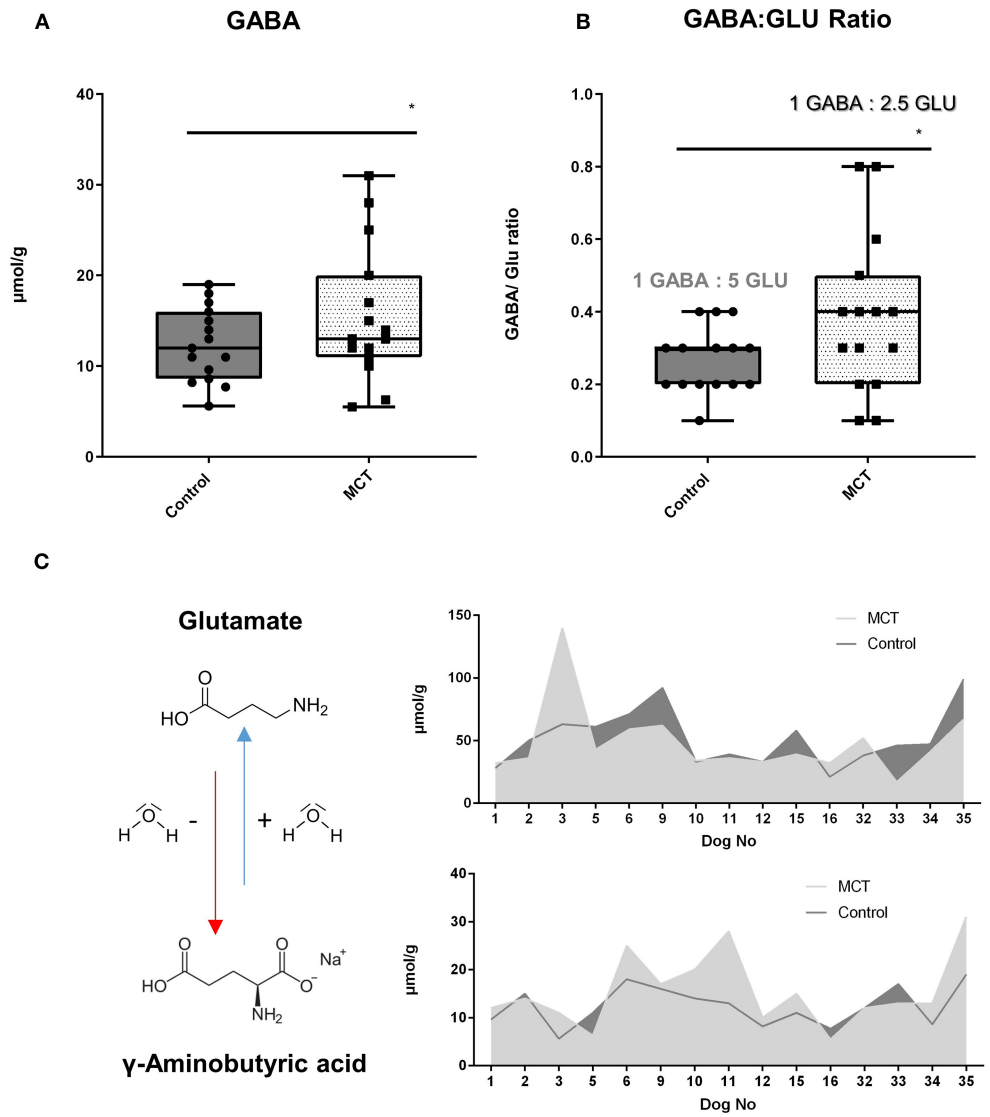

FIGURE 2
Urinary neurotransmitter profile: Effect of the medium-chain TAG dietary supplement (MCT-DS, light pointed) on the γ-Aminobutyric acid (GABA)—glutamate balance in urine compared with the control dietary supplement (Control-DS, dark-grey). Significant **(A)** increased γ-aminobutyric acid (GABA) concentrations ($P = 0.0044$) and a shift in the GABA/glutamate ratio **(B)** during the MCT phase was found ($P = 0.0025$). A predominant shift to the GABAeric side in the GABA/glutamate ratio **(C)** was observed under MCT-DS consumption (light grey) compared to the Control DS (dark-grey). Data are shown as box-and-whisker plots (central lines of the box represent the median, lower and upper limits of the box represent the 25th and 75th percentiles and whiskers represent the minimum and maximum). Two-sided Wilcoxon's matched-pairs rank tests were used to compare control and MCT-DS groups. *$P < 0.05$.

Supplementary Table 4). MCT-R showed higher relative (%) concentrations compared to the total fatty acid amount of AA ($p = 0.0283$) and overall Ω6 fatty acids ($p = 0.0171$), in their serum. Serum concentrations of pyruvate (MCT: 0.013 mmol/l vs. Control: 0.023 mmol/l; $p = 0.0368$) and TAG (MCT: 0.579 mmol/l vs. Control: 1.149 mmol/l; $p = 0.0034$) were significantly decreased in abundance, when compared to NR (Figure 3C). In 19 dogs, the dietary composition data could reconstructed (R = 4, NR = 15, Supplementary Table 5). On dry matter basis, dietary composition differed not significantly between MCT-R and MCT-NR ($p = 0.579$).

BHB-TAG ratio—The ketogenic BHB yield per fat

A ratio of BHB to TAG was calculated by dividing the serum concentration of BHB through TAG. The beneficial effects of MCFA on the brain are in humans mainly attributed to the

A

Metabolite	PREPRANDIAL		POSTPRANDIAL	
	MCT	Control	MCT	Control
Ala/BCAA	-	+	-	+
Ala/Val	-	+	-	-
Albumin	-	-	X	X
AA	+	-	X	X
AA%	+	=	+	-
BHB	+	-	+	=
LDL-Diameter	X	X	-	-
OleA%	X	X	-	+
PUFA%	+	=	-	-
SFA%	=	-	X	X

* Significant p-value (MCT vs. Control) after FDR.

B

Metabolite	PREPRANDIAL	
	MCT-R	MCT-NR
AA%	+	-
Omega6%	+	-
TAG	-	+
Pyruvate	-	+

C

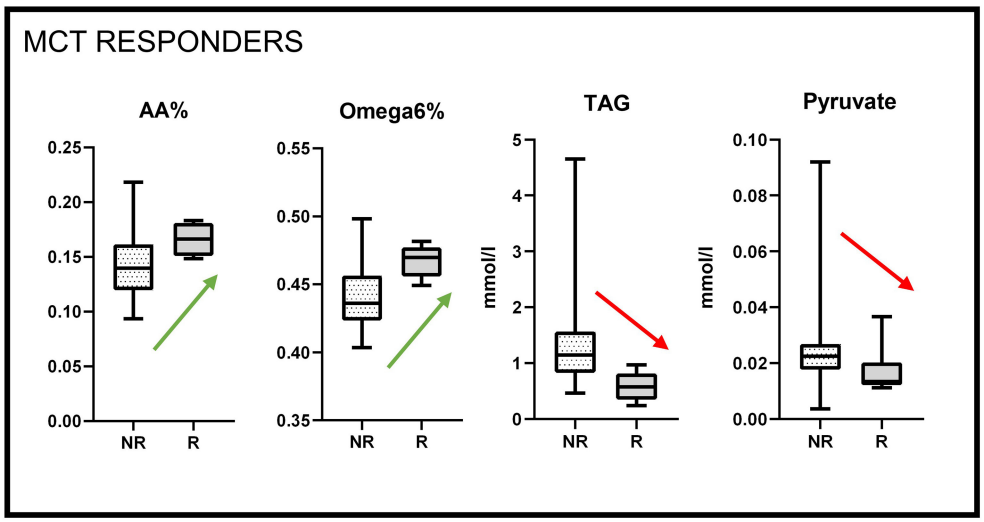

FIGURE 3
Serum metabolite profile: Effects of the medium-chain TAG dietary supplement (MCT-DS, light-grey) on serum metabolites compared with the control dietary supplement (Control-DS, dark-grey) in **(A)** pre-prandial (light-orange) and post-prandial (dark-orange) state ($N = 28$). If significant between both dietary supplements **(A)**, altered parameters are labelled according to their change related to baseline (red = decreased, blue = stable, green = increased, x = not significant). MCT-R **(B)** showed significant abundances in four different metabolites. MCT-R **(C)** show a shift in their fatty acid profiles to increased serum levels of arachidonic acid ($P = 0.0283$) and Ω 6 fatty acids ($P = 0.0171$), but decreased levels of triglycerides ($P = 0.0034$) and pyruvate ($P = 0.0386$). Data are shown as box-and-whisker plots (central lines of the box represent the median, lower and upper limits of the box represent the 25th and 75th percentiles and whiskers represent the minimum and maximum). Two-sided Wilcoxon's matched-pairs rank tests were used to compare control and MCT-DS groups. *$P < 0.05$.

ability of these fatty acids to provide energy sources in the form of ketone bodies, such as BHB. As BHB production depends on the overall fatty acid metabolism and not only MCFA, BHB/TGA ratio was calculated and presented.

The clinical relevance was then evaluated by correlating the documented seizure control and relative change after the dietary switch to MCT-DS (Supplementary Table 6). Significant association was found between BHB-TAG ratio and seizure frequency per months ($N = 28$, $p = 0.0150$) (Figure 4A). The relative change in seizure frequency under MCT consumption correlated negatively with BHB-TAG ratio ($N = 28$, r: −0.455, $p = 0.015$) (Figure 4B). The lower the BHB-TAG ratio was, the higher the seizure frequency per month, or lower the change in seizure frequency under MCT.

A dietary supplementation independent analysis ($N = 84$, MCT, Control, baseline) had shown again another significant negative correlation between the occurring seizure frequency per month and the BHB-TAG ratio (r: −0.387; $p = 0.0016$) (Figure 4C). Thus, the higher the BHB concentration is in relation to the TAG concentration, the better was the seizure control in our study population.

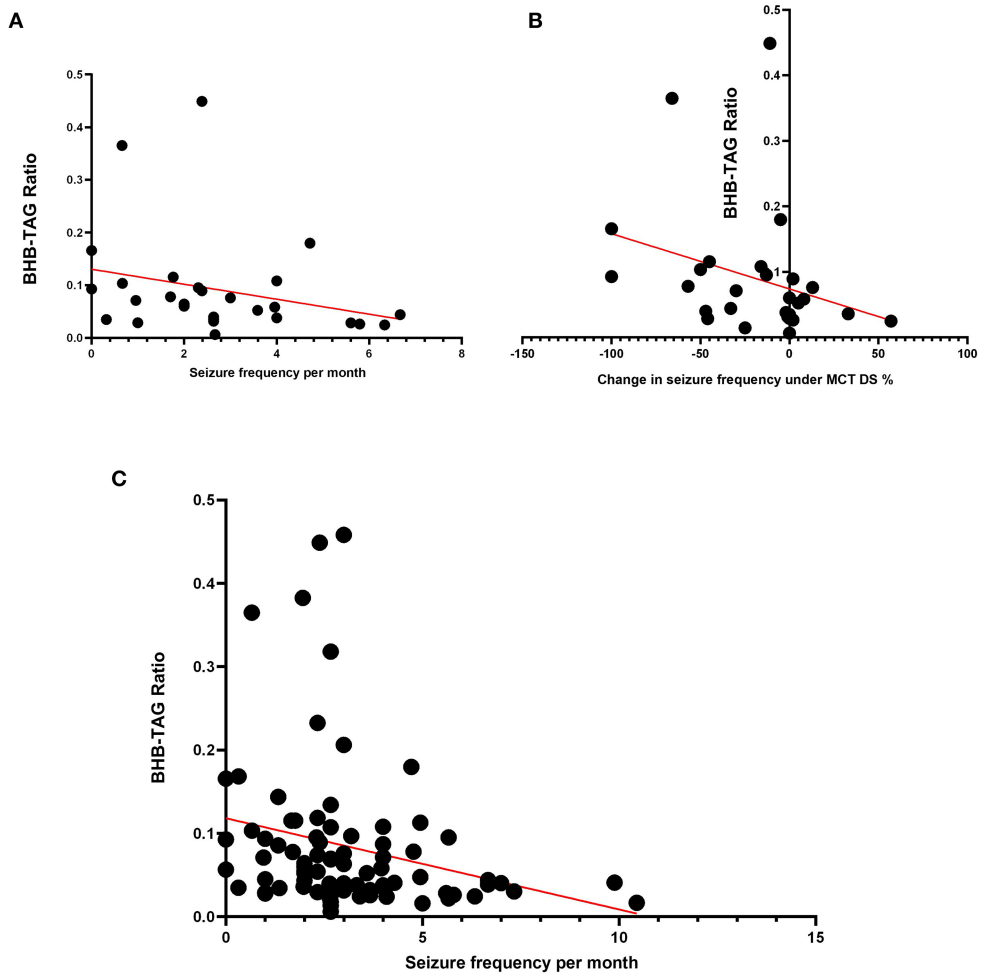

FIGURE 4
BHB-TAG Ratio: The BHB-TAG ratio was evaluated on its clinical relevance as a monitoring tool. **(A)** When MCT was consumed, a significant association between BHB-TAG ratio and seizure frequency per month ($N = 28$, $p = 0.0150$) was found. **(B)** The relative change in seizure frequency correlated negatively with BHB-TAG ratio ($N = 28$, r: −0.455, $p = 0.015$) under MCT intake. The lower the BHB-TAG ratio was, the higher the seizure frequency per month, or lower the change in seizure frequency under MCT. The higher the reduction in seizures (−%), the higher the BHB-TAG ratio. **(C)** Independent of diet or dietary supplementation, a significant negative correlation was found between the occurring seizure frequency per month and the BHB-TAG ratio ($N = 84$; r: −0.387; $p = 0.0016$).

Discussion

The metabolic and peripheral neurotransmitter changes induced through dietary supplementation of MCT oil were characterised in urine and serum in a canine population with idiopathic epilepsy (Figure 5). Although over 60% of the dogs experienced a reduction in seizure frequency, only 30% of the dogs had a reduction of 50% or more in seizure frequency (= MCT-R). The major finding of the current study was, that these responders had a different metabolic response to MCT oil than those dogs who showed no or less improvement. In summary, MCT consumption influenced significantly the amino-acid metabolism (serum alanine/BCAA and alanine/valine decreased compared to the control oil) and fat metabolism. While the serum amount of total fatty acids appeared similar during supplementation with the MCT-DS and control oil, the relative amounts of individual fatty acids differed. MCT consumption led to an increase in the relative concentrations of PUFA and AA compared to the control oil. BHB serum concentrations were significantly higher under MCT-DS than with the control oil and to baseline. The serum metabolic signature of MCT-R differed significantly to NR by higher relative concentrations of AA and overall Ω6 fatty acids as well as significantly decreased concentrations of pyruvate and TAG. Four out of nine neurotransmitters were altered considerably during the MCT phase: increased GABA concentration, accompanied by a significant GABAergic shift in the GABA/glutamate balance. Significantly lowered urinary concentrations of histamine,

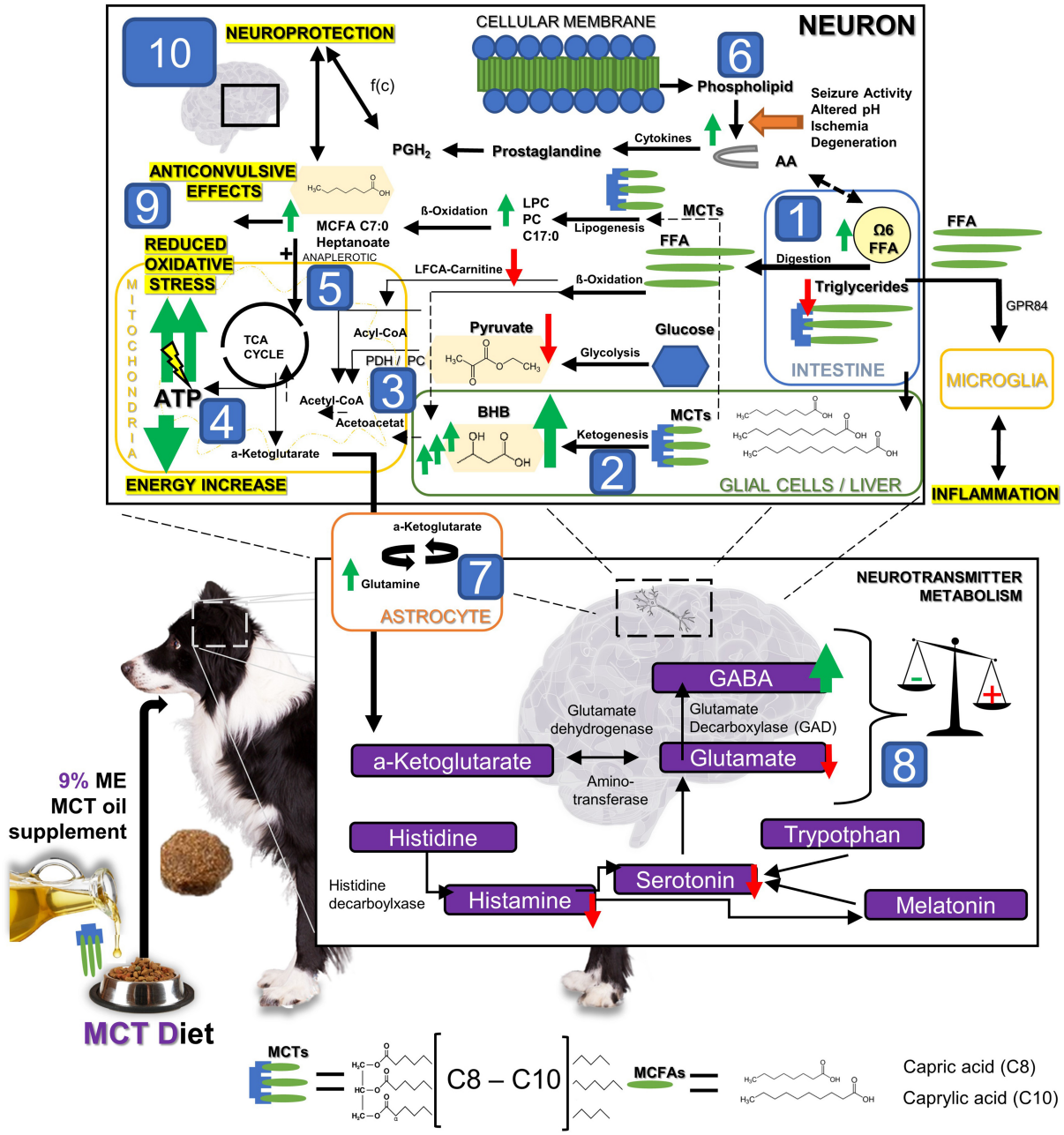

FIGURE 5
The hypothetical metabolic response to MCT oil supplementation: The dietary supplementation of MCT oil induces significant metabolic changes. Substantial changes in the lipid, energy and neurotransmitter metabolism have been detected in dogs with idiopathic epilepsy after 3 months under MCT DS as a nutritional, therapeutic approach. In brief, after oral intake, [1] MCT can be degraded in the intestinal lumen by lipases into MCFAs. MCFAs can be directly absorbed, transported into the liver and metabolized into C4 ketone bodies, such as beta-hydroxybutyrate (BHB). BHB levels [2] were significantly increased in all dogs during MCT consumption compared to the control oil phase. Based on these findings, MCT might lead to a significantly increased anaplerotic influx into the TCA cycle [3] and thereby evokes an additional production of adenosine triphosphatase (ATP) via the mitochondrial respiratory chain resulting in a compensation of epilepsy associated energy deficits [4]. Moreover, Pyruvate was found being especially decreased in MCT responders [3]. The compensation of energy deficits may thus also be given via (a) an increase in the enzymatic activity of the pyruvate dehydrogenase (PDH) activity, or/and (b) the promoted entry of oxaloacetate into the TCA by increased pyruvate carboxylase activity (PC). PDH and PC may be here both the key metabolic enzymes relevant for overcoming energy shortage under MCT consumption via the use of pyruvate. Furthermore, an additional *de-novo* lipogenesis from MCT into other MCFA via ß-oxidation leading to anaplerotic and neuroprotective effects has been hypothesized in the past [5]. Some prostaglandins deriving from arachidonic acid (AA) play significant roles in the processes of neurodegeneration and neuroinflammation, but also neuroprotection and regeneration. Prostaglandine can induce both depending on its concentration ratios of different types of prostaglandin [6].
(Continued)

FIGURE 5 (Continued)
The energy and neurotransmitter metabolism in neurons and astrocytes is tightly coupled [7]. Astrocytes take up GABA and glutamate from the synapse, and in turn, provide neurons with glutamine, an essential substrate for the re-synthesis of glutamate and GABA in the neurons. Based on our findings and previous research findings, we speculated that astrocytes are the main cellular compartment of MCFA metabolism and result in promoted astrocyte glutamine synthesis. The promoted glutamine supply from astrocyte metabolism of C8 and C10 MCFAs may lead then to elevated neuronal GABA synthesis [8], and thus aid in maintaining the inhibitory tone of the brain and provide another anticonvulsant mechanism of MCT oil supplementation. In summary, anticonvulsive effects may thus be provoked by I. compensating the energy shortage [2–5], II. influence on GABA/ glutamate balance via astrocyte metabolism [7–8], III. Reduction of antioxidative stress [9] via ATP increase and neuroprotective effects on the brain by MCT influencing metabolic pathways [10].

glutamate and serotonin were found during the MCT-DS phase in MCT-R.

In human medicine, urinary neurotransmitter excretion has been shown to effectively correlate not only with diverse neurological conditions (56–59), but also therapeutic effect of both dietary (60) and pharmacological interventions (61). Our urinary neurotransmitter analysis showed that both MCT-R and MCT-NR exhibited an increase in the GABA concentration and, an increase in the GABA/glutamate ratio compared to the control phase. Interestingly, a more significant reduction in glutamate was shown in MCT-R than in MCT-NR. GABA and glutamate are the primary inhibitory and excitatory neurotransmitters of the brain. The energy and neurotransmitter metabolism in neurons and astrocytes is tightly coupled, whereby astrocytes take up GABA and glutamate from the synapse, and in turn, provide neurons with glutamine, an essential substrate for the re-synthesis of glutamate and GABA in the neurons (62). Insufficient astrocyte glutamine synthesis is speculated to be closely linked to central nervous system (CNS) disease, with inhibition of glutamine synthesis resulting in seizures (63). Andersen and colleagues have recently demonstrated that astrocytes are the main cellular compartment of medium-chain fatty acid (MCFA) metabolism and result in promoted astrocyte glutamine synthesis (64, 65). The resultant increase in glutamine supply from astrocyte metabolism (66) of C8 and C10 MCFAs, may lead to elevated neuronal GABA synthesis, and thus aid in maintaining the inhibitory tone of the brain and provide an anticonvulsant mechanism of MCT oil supplementation. Given that GABA is formed through transamination of α-ketoglutarate to glutamate, which then undergoes decarboxylation by glutamic acid decarboxylase (GAD) to form GABA, our observed increase in GABA and decrease in glutamate could be therefrom consistent with previous studies (67). In addition, most recently dogs diagnosed with idiopathic epilepsy under anti-seizure pharmacotherapy were shown to have significantly higher GABA concentration in urine increased to a similar level as measured in healthy companions (68). However, even if plausible, this remains highly speculative, the measurements in urine do not let one conclude that the same changes can be found in the brain or reflect brain metabolism of MCFA being connected to neurotransmitter recycling in astrocytes. Based on our and previous *in-vitro* findings, we hypothesise that MCT may enhance anti-seizure drug-induced GABAgeric properties and thus leads to further seizure frequency reduction (65, 69–73). Future studies are necessary to directly measure metabolic processes within the brain tissue.

Our understanding of the pathophysiology of epilepsy is predominantly confined to inhibitory GABAergic and excitatory glutamatergic neurotransmission. However, a complex interplay exists between GABA, glutamate, histamine and serotonin. Monoamines such as histamine and serotonin are major modulators of the CNS and are thought to halt seizure activity (74). Elevated histamine levels suppress seizure activity and confer neuroprotection, whereas low levels are associated with seizures mediated through histamine H_1 receptors (74). MCT administration resulted in decreased urine histamine levels in MCT-R compared to MCT-NR. This might be explained by the MCT-NR group having higher seizure frequency than MCT-R. In former studies, we have found a significant positive correlation between seizure frequency and histamine serum concentration (personal communication, 2022). Another explanation is that the MCT-NR and MCT-R differ in their degree of neuroinflammation. There is increasing evidence that supports a pathogenic role of neuroinflammation in various CNS diseases, including epilepsy (75). Histamine is known to modulate inflammatory processes critically and is a regulator of innate and adaptive immune responses. Previous studies have shown that histamine can stimulate microglia, the resident immune cells in the brain, resulting in activation and subsequent production of proinflammatory factors tumour necrosis factor (TNF)-alpha and interleukin-6 (IL-6) resulting in neuroinflammation (76). *In vitro*, decanoic acid at a very low concentration was able to activated the inflammatory receptor G protein-coupled receptor 84 (GPR84). As a receptor for MCFA with carbon length of C9 to C14, it is normally expressed at very low levels mainly in monocytes, neutrophils and microglia in the brain, but can be induced via lipopolysaccharide (77–79). Although knowledge about the role of proinflammatory receptors regarding anticonvulsant or other effects is still unclear, an altered neuroinflammation might be one explanation for some dogs with idiopathic epilepsy not responding to therapy (80). Therefrom, it might be possible that MCT can

influence neuroinflammatory processes in some epileptic dogs via modulation of microglia activity in the brain. Although no adverse effects have been seen in the dogs under MCT consumption so far, more research about MCT effects on inflammation is needed.

Much like our observed changes in histamine, our results also showed a more significant reduction in urinary serotonin levels in MCT-R compared to MCT-NR. Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoaminergic neurotransmitter involved in fundamental brain functions such as the stress response, emotion and cognition. Accumulating evidence indicates that serotonergic neurotransmission modulates seizure susceptibility, and agents that generally elevate extracellular 5-HT levels, such as 5-hydroxytryptophan, inhibit both focal (limbic) and generalised seizures (81). At the same time depletion of brain 5-HT levels lowers the threshold for seizures (82). Our results are contradictory to what has been presented in the literature. However, there appears to be a disparity between the central (hippocampal) and peripheral (plasma) processes in regulating of 5-HT concentration. Maciejak et al. found that following intragastric administration of C8 and C10 fatty acids, there was an increased turnover of 5-HT in the hippocampus, detected through an increase in the concentration of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of 5-HT, and 5-HIAA/5-HT ratios, but a decrease in plasma serotonin levels (83). This is thought to be due to the alteration of the tryptophan pathway activity, whereby the MCFA displace tryptophan from its binding sites on plasma albumins. This results in the free fraction of tryptophan being metabolised peripherally by hepatic tryptophan 2,3-dioxygenase while at the same time surplus tryptophan passes into the CNS. Given that tryptophan is the sole precursor of 5-HT, it is thought that once in the brain, tryptophan is converted into several different neurotransmitters, including 5-HT. It is therefore likely, that the observed changes of peripheral 5-HT in plasma are similar to the changes that we observed in urinary output. The fact, that epileptic dogs had significantly increased serotonin levels in urine (68), the hypothesis that MCT might have serotonergic modulating effects seems feasible. How central and peripheral serotonin levels in body are affected by dietary MCT, should be another focus in future clinical studies on the nutritional management of canine epilepsy.

The metabolic signature also differed significantly between MCT-R and MCT-NR. Supplementation of MCT-DS, when compared to the control oil, resulted in significant differences in four serum metabolites playing crucial roles in lipid and energy metabolism. In human medicine, available data suggest that brain energy metabolism is impaired in people with epilepsy (84–87). Accordingly, deficits in glucose metabolism of canine epileptic brains are also likely (37, 88, 89). Issues in brain energy supply might occur from (i) impaired glycolysis from plasma-derived glucose, (ii) decreased TCA activity and (iii) decreased production of amino-acid or lipid precursors (90).

Our results have shown that serum pyruvate concentrations were significantly decreased in MCT-R. Once pyruvate enters the mitochondria, it is typically integrated into the tricarboxylic acid (TCA) cycle via conversion through the enzyme pyruvate dehydrogenase (PDH) into acetyl-CoA. A shortage of glucose-derived intermediates and metabolites entering the TCA cycle has been found in several rodent epilepsy models (91, 92). Given that a reduced PDH activity was proposed being relevant in spontaneous seizure activity (92, 93), MCT might increase enzymatic activity (71). As capric acid has been proposed to be able to upregulate pyruvate dehydrogenase kinase 4 expression in skeletal muscle of mice (94), single MCFA in the DS might also be able to overcome the energy shortage in the brain permanently by genetic upregulation of the PDH expression and by surpassing the necessity of PDH by providing an alternative and direct energy source for TCA cycle (37). In brief, after oral intake, MCT can be degraded in the intestinal lumen by lipases into MCFAs. MCFAs can be directly absorbed, transported into the liver and metabolised into C4 ketone bodies, such as BHB. BHB levels were significantly increased in all dogs during MCT consumption compared to the control oil phase (40). Based on that, MCT leads to a significantly anaplerotic influx into the TCA cycle and thereby evokes an additional production of adenosine triphosphatase (ATP) via the mitochondrial respiratory chain resulting in a compensation of energy deficits (95). Another alternative pathway explaining a decrease in pyruvate may be the promoted entry of oxaloacetate into the TCA by increased pyruvate carboxylase activity (PC). In summary, both, PDH and PC, may be the key metabolic enzymes relevant for overcoming energy shortage under MCT consumption (37, 96).

Broken down to both MCFAs C8 and C10, previous *in-vitro* research showed an upregulated mitochondrial respiration (64), which could depend on the cell type, dose or serum concentrations (71, 97, 98). C8 was shown to increase the anaplerotic influx into the TCA in the brain of rats. The measured anaplerotic influx in the brain into the TCA cycle was found to be significantly associated with glutamine production (72), which would also support our previous hypothesis about the linkage to astrocytic metabolism ADDIN EN.CITE (72, 99). Most recent research showed as well, indication of active MCFA metabolism in astrocytes, while βHB is metabolised in a different cellular compartment (73).

Based on those findings, it is likely, that MCT may lead directly via ketogenesis and indirectly via anaplerotic influx to compensation for epilepsy-associated glucose hypometabolism. Future *in-vivo* and *in-vitro* research should focus on gaining further insight into MCT derived energy supply and its influence on the anaplerotic influx within the neuronal tissue.

When considering global shifts in lipid metabolism, due to MCT-diet consumption, the first findings were made in 2015 by Law and colleagues (49). Under an MCT containing kibble diet, dogs showed significantly increased abundances of C17:0 long fatty acids. In this study, we have shown that

TAG concentrations are highly reduced in MCT-R serum. Triglycerides are the neutral storage form of diverse FA and occur primarily in hepatocytes. After eating, dietary lipids are hydrolyzed within the intestine. FA are re-esterified into TAG upon intestinal absorption, then packed into chylomicrons and delivered to the muscle and adipose tissue. The remaining TAG is then transported to the liver and processed into FA within the hepatocytes. In order to be metabolised, FAs are activated into acyl-CoA, which can then undergo oxidation or reconstituted into other lipids (100). Under normal conditions, the liver processes large quantities of FA and stores only small amounts as TAG. Upon fasting, TAG stored intracellularly are mobilised to release FA products, such as C4 ketone acetoacetic acid and BHB (101). Our results show changes in lipid metabolism, but how TAG metabolism is influenced long-term, over the year, by dietary added MCT requires future research.

In addition, the ratio of BHB to TAG was calculated, as tool to assess the ketogenic yield per fat depending on the established feeding regime (baseline without oil, with DS-1 or DS-2 oil). The clinical relevance was then investigated by analysing the association between the documented seizure frequency per months and the BHB-TAG ratio. To the authors knowledge, this has not been investigated in any species before. Independent from dietary supplementation ($N = 84$, MCT, Control, baseline) a significant negative correlation between the occurring seizure frequency per month and the BHB-TAG was found. This means, that the higher the BHB concentration was relative to the TAG concentration, the better was the seizure control in our study population. Exploring both DS separately, the same negative correlation was found under MCT, but not in the control oil feeding phase. As the baseline diet was documented to be stable over the entire study period, the fat profile was the only factor that was also altered, beside the type of oil. While the MCT oil contained C8 and C10, so MCFA, the used control oil was olive oil containing especially monounsaturated long chain fatty acids. Based on previous research, the type of fat may not play the only and significant role in the success of a ketogenic-orientated nutritional, therapeutic interventions (102–104), but also the fat profile and composition in relation to the patients complete diet (105–107). Future studies could consider therefrom BHB to TAG ratios and characterise the fat profile in the diet.

In this study, an increase in the relative concentrations of the Ω6 fatty acid AA and overall Ω6 fatty acids were detected in the serum of MCT-R. In humans, changes in AA metabolism have been extensively mapped in seizure states, wherefrom a correlation between AA concentrations and seizure control was often postulated (108). However, the investigations are not clear and quite conflicting. While some studies showed that KD-R (ketogenic diet responders) experienced a significant decrease in serum AA concentration compared to NR (109, 110), another has detected a 1.6- to 2.9-fold rise in AA levels in KD-R (111). The same controversy can also be discovered in rodent epilepsy models. While one study found that rats treated with KD experienced a decrease of serum AA concentration (112), another study in mice showed the same after oral Ω3 treatment, but with no change in seizure thresholds (113). From another perspective, AA can be enzymatically synthesised into diverse prostaglandins (PG) via membrane-associated cyclooxygenases (114). Some PGs play significant roles in the processes of neurodegeneration and neuroinflammation. In people with epilepsy, PGs are often found in higher concentrations in serum (115, 116). Depending on receptor subtype, cell population and receptor gene expression, PG can induce both neuroprotective and neuro-toxic effects in the brain (117). Therefrom, MCT could provide neuroprotection via influencing inflammatory stimuli from ongoing or past seizure activity. As a result, further elucidation is needed to evaluate how the MCT diet may be related to a serum AA increase and that could lead to improved seizure control.

Overall, the differences we have presented in serum metabolites and urinary neurotransmitters might be related to each other via the astrocyte metabolism. One critical function of astrocytes is the regulation of neurotransmitter homeostasis. Synaptically-released neurotransmitters, such as glutamate, GABA and glycine are taken up, metabolised and released as precursors back to the neurons (118). Moreover, MCFA may modulate astrocyte metabolism via activation of shuttle systems that provide fuel to neighbouring neurons in the form of lactate and ketone bodies (97, 119). Based on our finding and previous reported data from *in-vitro* research, we hypothesise that astrocytes may have a linking role between the neurotransmitter and energy homeostasis under MCT supplementation (72, 99). Further research is necessary, to explore this postulated linkage.

A few limitations of the present finding on metabolic and peripheral neurotransmitter changes induced through dietary supplementation of MCT oil should be considered. Due to the2 multicenter study design (52), variability and storage despite of standard operation procedures may impact our results. Furthermore, the number of dogs categorised as MCT-R was small ($N = 5$); thereby findings referring to this population must be interpreted cautiously. Another relevant limitation is the aspect, that the direct correlation between intracerebral and extracerebral metabolites (blood, urine) has been shown to a more or less extent in previous research. This has been discussed in detail in a recently published article from our group (68). Although the baseline diet was kept stable, urine collection was standardised and fasted serum samples were used to interrogate the global shifts in metabolism associated with diet consumption. Numerous factors can influence both body fluids.

Urinary neurotransmitter levels could arise from an additional synthesis outside the CNS. Besides the production in the PNS (120) or other organs, such the adrenal glands and kidneys (121), also the intestinal microbiome is capable of synthesising neuromodulating molecules (122–124). Although sex appears to influence urinary neurotransmitter profile in dogs (68), this should not be relevant in this study, as all samples

have been evaluated in a paired analysis. Diverse factors might be able to affect metabolic profiling. The seasonal and diurnal variance of diverse metabolites have been identified in humans (125, 126). Although no clear evidence for that problem was found in dogs (127), it can be relevant for some metabolites. Another impact on our study results could come from the individual's metabolism (age, breed, sex, diet) (128) and in a subpopulation of epileptic dogs also the type of ASD medication (129). However, as the dietary supplement was the only changing factor in the study, and comparison was performed pairwise, significant impacts should not be present.

Metabolic perturbations in urine and serum reflect the response to MCT dietary supplementation and therefore help in understanding potential physiological mechanisms. In conclusion, this study provides further evidence about MCT-derived effects on the lipid and energy metabolism in dogs with IE. In addition, it highlights the first candidate biomarkers (BHB, AA%, Ω6 FA%, pyruvate, TAG; BHB/TAG ratio) being of potential use in a clinical setting for individual treatment monitoring and customised adjustments of nutritional therapeutic interventions. Overall, the results presented by this study provide further evidence of the value of MCT as dietary management for both human and canine drug-resistant epilepsy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Royal Veterinary College Clinical Research Ethical Review Board (CRERB) (URN 2016 1558). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BB planned the conduction of the study, carried out the main practical work, did the patients recruitment and the sample acquisition at each study centre, carried out statistical analysis, and interpreted the results. AW, AB-N, TJ, AK, and AT supported study conduction with recruitment and sample acquisition of dogs with idiopathic epilepsy at their study location. HV and RP designed the study. CO supported the sample acquisition and conducted the metabolomic analysis with physicochemical methods. HV reviewed and edited the manuscript. All authors contributed to the interpretation of the results, the manuscript revision, and approved the final manuscript.

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Conflict of interest

Author BB was CEO of BrainCheck.Pet®, a label and veterinary practise specialised into epilepsy and paroxysmal disorders in cats and dogs. Authors CO was an employee and HL the Chairman of Board of PetBiomics Ltd., a company providing NMR metabolomics testing for dogs.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.935430/full#supplementary-material

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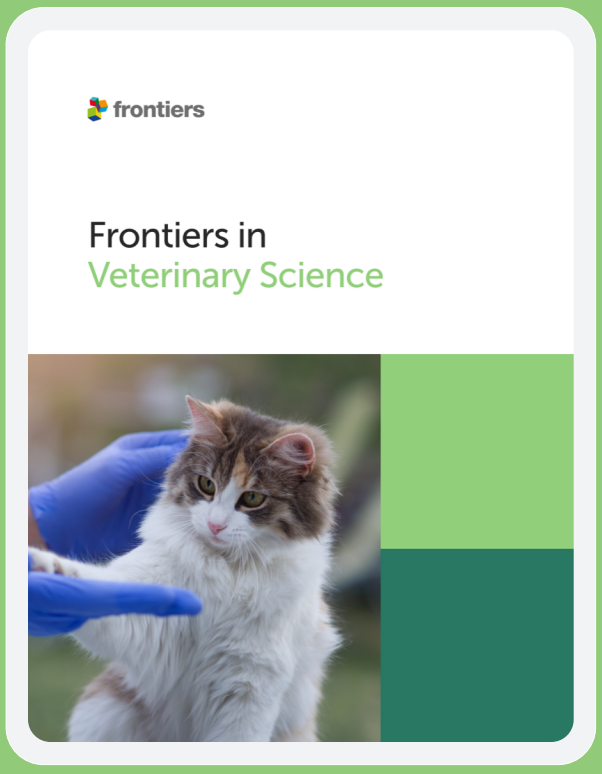

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