UNIVERSITÀ DEGLI STUDI DI TORINO

Doctoral School in Life and Health Science

RESEARCH DOCTORATE IN VETERINARY SCIENCE FOR ANIMAL HEALTH AND FOOD SAFETY

Department of Veterinary Science

TITLE

Management and treatment of canine epileptic patients in acute phase

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ABBREVIATIONS

AED = antiepileptic drug
AMPA = α-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid
AUC = Area under the curve
ATP = Adenosine triphosphate
Bpm = Beat per minute
Ca^{2+}= Calcium
Cl^- = Chloride
C_{max} = Maximal concentration
CNS = Central nervous system
CPP = Cerebral perfusion pressure
CRI = Constant rate infusion
CS = Cluster seizures
CSF = Cerebrospinal fluid
EMSE = Epidemiology-based mortality score in status epilepticus
GABA = γ-aminobutyric acid
HPLC = high-performance liquid chromatography
ICP = Intracranial pressure
IM = Intramuscular
IV = Intravenous
IVETF = International Veterinary Epilepsy
Task Force
K^+ = Potassium
KBr = Potassium Bromide
LEV = Levetiracetam
MAP = Mean arterial pressure
MES = Maximal Electroshock Seizure
Mg^{2+} = Magnesium
MRI = Magnetic Resonance Imaging
mRS = Modified Rankin Scale
Na^+ = Sodium
NMDA = N-methyl-d-aspartate
NO = Nitric oxyde

PB = Phenobarbital

SE = Status epilepticus

STESS = Status Epilepticus Severity Score

SUDEP = Sudden Unexpected Death in Epilepsy

$T_{1/2}$ = Elimination half-life

T_{max} = Time at maximal concentration

V_d = Volume of distribution

VTH = Veterinary Teaching Hospital

Summary

ELECTROCHEMICAL BASIS OF NEURONAL FUNCTION

The cell membrane is a phospholipid lipid bilayer with the two hydrophilic, polar layers exposed to the extracellular matrix and the cytoplasm, respectively. In between, the hydrophobic non-polar layer is crossed by protein macromolecules, including ion channels, ligand receptors, and ionic pumps, that are therefore in contact with both the extracellular fluid and the cytoplasm (Figure 1) (Larsson, 2006).

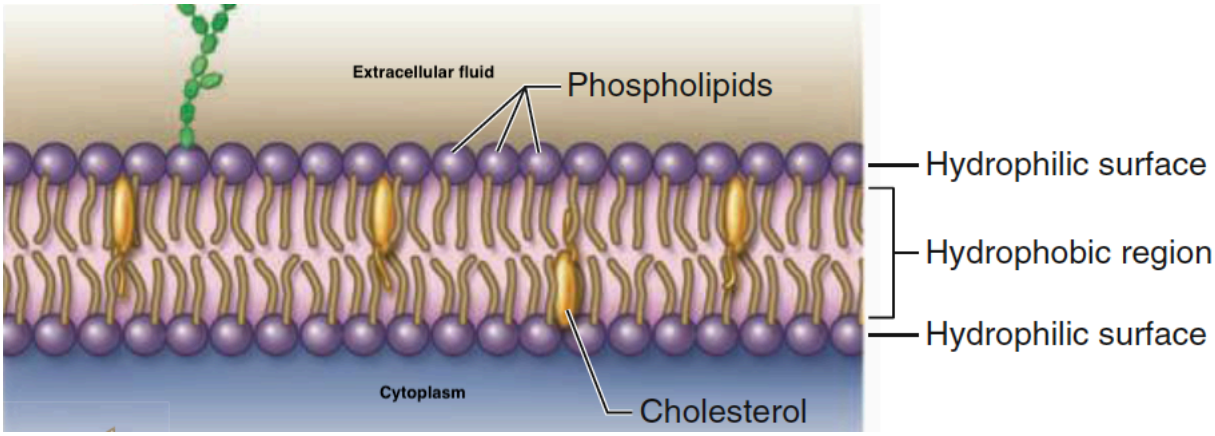

Figure 1: Cell membrane structure *(from: Junqueira's basic histology. Text and atlas, 14th ed., 2016)*

The cell membrane is characterized by a selective permeability: uncharged molecules pass through the neuronal cell membrane by passive transport according to their specific gradient concentration. Most polar and charged molecules, however, cannot pass freely through the neuronal cell membrane and their movements rely on electrochemical gradients. The cell membrane is therefore relatively impermeable to ions as Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) and Calcium (Ca^{2+}).
As a rule, anions and cations are electrostatically attracted to hydrogen and oxygen atoms of water, respectively. This mechanism prevents the passage of ions across the hydrophobic cell membrane and is the responsible for the particular distribution of inorganic ions across the neuronal membrane. Almost all cell membranes are characterized by an electric potential that usually is negative inside the cell compared to the extracellular compartment.
The transmembrane ion concentration is maintained by means of two different mechanisms (Figure 2):

- Passive transport: passive diffusion of ions across ion channels according to their concentration gradient
- Active transport: active, adenosine triphosphate (ATP)-dependent transport of ions against their concentration gradient, by means of ATP-dependent ion pumps.

(Mescher, 2016)

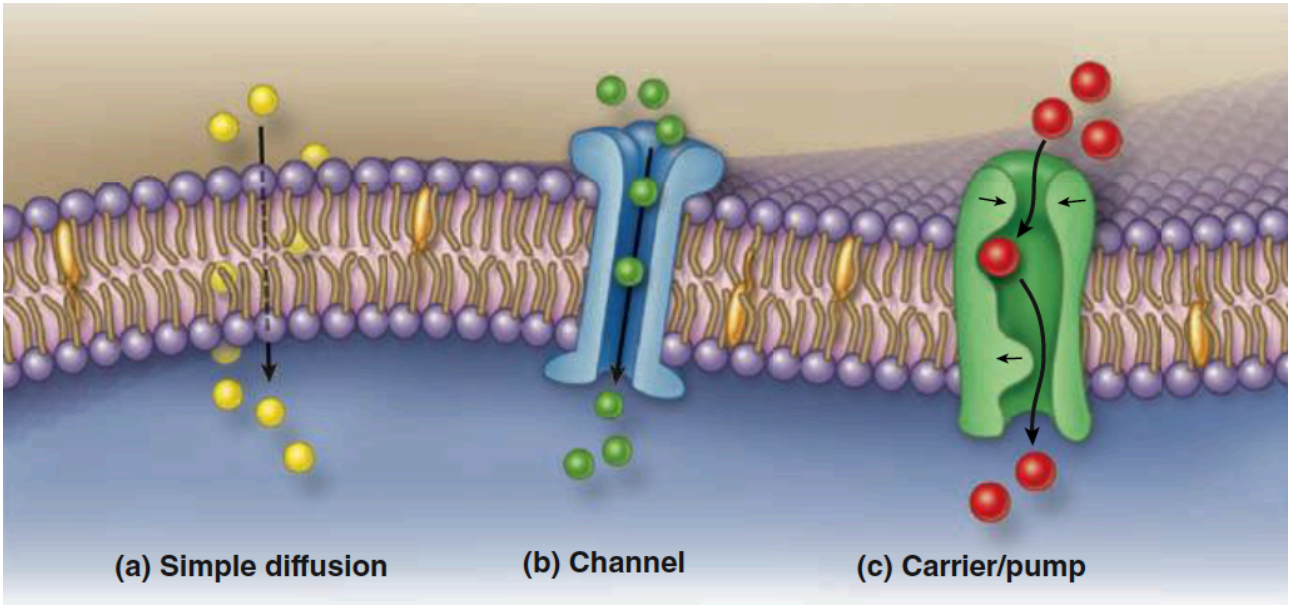

Figure 2: Major mechanisms by which molecules cross cell membrane *(from: Junqueira's basic histology. Text and atlas, 14th ed., 2016)*

Na^+, K^+ and Cl^- but not Ca^{2+} are capable of passive transport and their movement produces energy defined as *diffusion pressure.* In basal conditions, concentrations of Na^+, Cl^-, and Ca^{2+} are higher extracellularly, and the concentrations of K^+ and impermeable anions are higher intracellularly (Fig. 3). Therefore, K^+ ions tends to diffuse from the intracellular to the extracellular compartment, while Na^+ molecules assumes the contrary direction. Because of non-diffusible negatively charged intracellular molecules, a separation of charges develops, creating therefore an electric potential difference. This voltage determines an electrical pressure that prevent ions movement.

Ionic movement continues until an equilibrium between electrical and diffusion pressure is reached and no further net ionic movement occur. The electrical potential associated with this condition is defined as *equilibrium potential*. This is different for each ion and is defined as the voltage difference across the membrane that offsets the diffusion pressure of an ion to move down its concentration gradient. The equilibrium potential is therefore proportional to the difference between the intracellular and the extracellular concentration of ions. The summation of the different equilibrium potential generates the *resting membrane potential* of cells (Table 1) (Etsuro E. Uemura, 2015a).

Ion	Extracellular concentration (mmol/L)	Intracellular concentration (mmol/L)	Equilibrium potential (mV)
Na^+	150	15	+ 40
K^+	5	100	-100
Ca^{2+}	2	0.0002	+ 124
Cl^-	150	13	-75

Table 1. Intracellular and extracellular distribution of ions across the neuronal cell membrane and respective equilibrium potentials

Another variable involved in the development of the equilibrium potential is the permeability of the cell membrane to different ions, defined as the ease with which a molecule diffuses across the cell membrane. Therefore, an ion with high concentration gradient but low membrane permeability (e.g., Ca^{2+}) will not contribute substantially to the formation of the resting membrane potential.
Modifications of either ion concentrations or permeability will therefore affect the resting membrane potential of the cell. However, ion movements in basal conditions are not sufficient to generate significant changes in concentration and only consistent variations of permeability due to ion channel opening or closing can alter the membrane potential of a cell (Daube and Squire, 2009; Koester and Siegelbaum, 2013a; Etsuro E. Uemura, 2015a).

Ion channels

Ion channels are transmembrane proteins that allow ion transfer between the intracellular and the extracellular compartment, creating an aqueous pathway through the lipid membrane layers. Most of them open and close in response to specific electrical, mechanical or chemical stimuli and ion transfer do not necessitate of external sources of energy (passive transport).
Rapid changes in membrane potential that underlie the transmission of signals through the nervous system are mediated by ion channels.
According to their gating system, they can be divided in non-gated and gated channels. Gating involves a change of the channel's conformation in response to an extrinsic stimulation as follow (Figure 3):

- Non-gated ion channels: responsible for the ion flow across the cell membrane according to their gradient concentration, they contribute to the establishment of the resting membrane potential.
- Voltage-gated channels: open to changes in membrane potential and are therefore involved in the generation and conduction of the action potential and neurotransmitter release, neuronal excitability, spontaneous neuronal activity;
- Ligand-gated channels: open after binding of a specific chemical transmitter;
- Mechanically-gated channels: respond to particular physical stimuli such as pressure or stretch.

(Daube and Squire, 2009; Etsuro E. Uemura, 2015a)

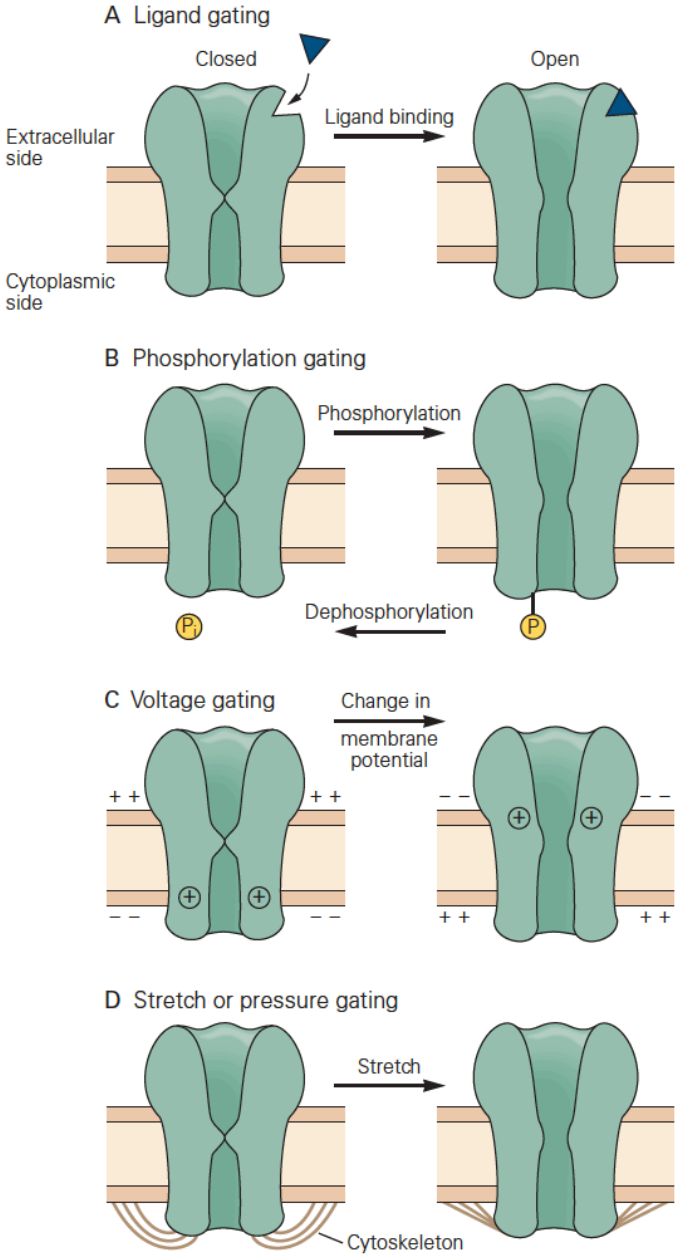

Figure 3: Mechanisms of opening and closing of ion channels *(from: Principles of Neural Science, 5th ed. Kandel et al., 2013)*

Ion channels can also be classified according to their selectivity for one or few types of ions. The exact mechanism of selectivity has not been established yet and many theories have been proposed over time. It is currently thought that ion channels selectivity is based on chemical interactions and molecular sieving based on pore diameter (Siegelbaum and Koester, 2013).

Ion pumps

In addition to ion channels, ion transporters/pumps represent another important means of transfer of ions across the cell membrane.

Ion channels and ion pumps, although displaying similar characteristics, differ in some crucial point:

- Ion pumps are not involved in rapid neuronal signaling like ion channels but play a crucial role in establishing and maintaining the concentration gradients of important ions across the cell membrane.
- While ions can flow from one side of the cell membrane to the other freely in open ion channels, an ion pump must undergo a series of conformational changes for an ion to move across the cell membrane and therefore, the speed of ion flow is considerably lower (100 – 100.000 times lower) compared to that found in the ion channels.
- A source of energy (usually ATP) is necessary for ion pumps involved in the maintenance of ion gradient, because for this task ions need to be transported against their chemical and electrical gradient. For this reason, ion movements provided by the ion pumps are defined as active transport.

The most important ion pump is the Na^+/K^+-ATPase pump. Although it is not directly involved in the generation of an action potential, the Na^+/K^+-ATPase pump is essential for the maintenance of the correct concentrations of Na^+ and K^+ ions across the cell membrane despite their constant leaking through the cell membrane. The ion pump mechanism moves 3 Na^+ ions outside and 2 K^+ ions inside the cell (Siegelbaum and Koester, 2013; Etsuro E. Uemura, 2015a).

NEURONAL EXCITABILITY

Neurons communicates between each other by means of electrical and chemical stimulations.
In basal conditions, cells are characterized by a relatively static membrane potential defined as *resting membrane potential*. This equilibrium is maintained through a selective ionic permeability of the cell membrane. When neurons receive excitatory or inhibitory signals, transient changes in the resting membrane potential occur, generating excitatory or inhibitory graded membrane potentials. When the electrical stimulus and the concurrent changes in the resting membrane potential are strong enough, the neuronal membrane undergoes dynamic reversal of membrane potential, called *action potential.*
The resting membrane potential of neurons is about -65mV and reflects the asymmetric distribution of ions (K^+, Na^+, Cl^-, fixed anions) across the neuronal membrane.
This asymmetric distribution is maintained by ion pumps, in particular the Na^+/K^+-ATPase pump that moves Na^+ out of the cell and K^+ inside the cell, against their electrochemical gradient.
At the levels of dendritic synapses, input from other neurons generates local membrane potentials referred as *graded potentials*, whose amplitude is directly proportional to the intensity of the stimulus received. Thousands of graded potentials occur at cell bodies and dendrites of neurons and travel to the axon hillock (also known as *trigger zone)* of the neuron. At this level, graded potentials are added to generate *action potentials.* The summation can take place by means of:

- Spatial summation: graded potentials from different synapses are added up in the post-synaptic neuron;
- Temporal summation: graded potentials obtained by successive stimulation of presynaptic terminals are summated in the post-synaptic neuron.

If the resultant action potentials are strong enough to reach and exceed the *threshold potential* (-55mV), the trigger zone stimulates the propagation of burst of action potentials along the axon. If the stimulus is too weak to overcome the threshold potential, an action potential is not generated and the graded potentials decay instead. This mechanism is defined as the *all-or-none phenomenon* that characterized the physiology of action potentials.
The threshold potential is reached by the opening of a sufficient number of voltage-gated Na^+ channels. Not all voltage-gated Na^+ channels open simultaneously. Some voltage-gated Na^+ channels start to open as the membrane starts to depolarize. When the graded potential reaches the threshold potential, more voltage-gated Na^+ channels open.
The function of grade potentials is therefore to shift the membrane potential. The term *depolarization* represents a decrease in the potential difference across the cell membrane because excitatory graded (or postsynaptic) potentials lead to more positive values of the membrane potential. *Hyperpolarization,* defined as an increased difference in potentials across the membrane, represents a shifting of the membrane potential towards more negative values caused by inhibitory graded (or

postsynaptic) potentials. The occurrence of depolarization or hyperpolarization is dependent on the transmitter involved in the synaptic transmission and the subsequent change in ion permeability that alters membrane excitability (Siegelbaum and Kandel, 2013; E.E. Uemura, 2015).

An action potential is defined as a brief reversal in membrane potential due to the increased membrane permeability of Na^+ and K^+ cause by the activation of voltage gated Na^+ and K^+ channels. The duration of the entire process is about 2 ms and can be divided in a *rising phase* and a *falling phase*. These two phases are directly correlated to the three states (resting, activated and inactivated) of the Na^+ ion channels, determined by the opening or closure of the activation and inactivation gates as follow (Figure 4):

- Resting state: activation gate closes the ion channel and the inactivation gate is open. As a consequence, no Na^+ ions flow into the neuronal cell.
- Activated state: during the rising phase of the action potential both activation and inactivation gates of the Na^+ ion channels are open, allowing Na^+ ions flow into the neuronal cell.
- Inactivated state: the inactivation gate closes the channel preventing the ion flux into the cell, while the activation gate is still open.
- De-inactivation: reset of the resting state after the inactivated state. Occurs only when the repolarizing membrane potential is sufficiently negative to go below the threshold voltage. A subsequent action potential cannot be generated without restoring the inactivated state of the Na^+ channels back to the resting state.

(Etsuro E. Uemura, 2015b)

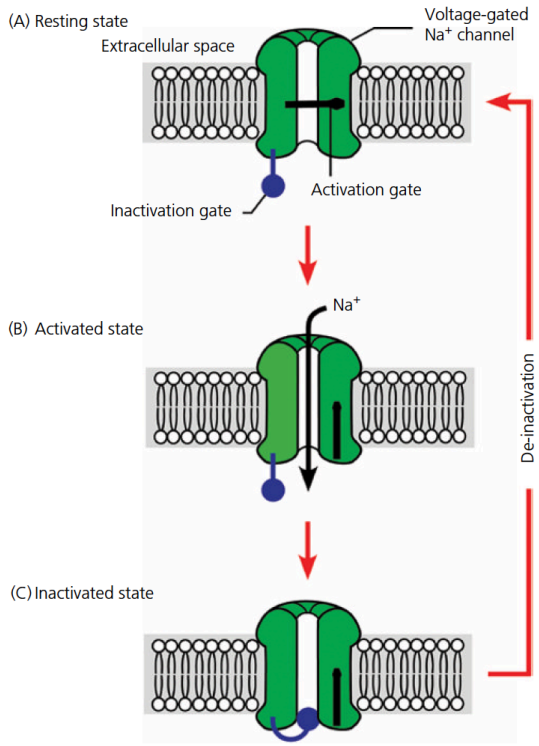

Figure 4: the three states of voltage-gated Na^+ channels *(from Duke's Physiology of domestic animals, 13th ed., 2015)*

During the rising phase, as a consequence of the opening of the Na^+ channels and the concurrent Na^+ ions flux into the neuronal cell, the neuronal membrane depolarizes towards the Na^+ equilibrium potential (+62mV). This part of the rising phase, when the inside of the neuronal cell is positive compared to the outside, is defined *overshoot.* Subsequently, during the falling phase, Na^+ channels close following the inactivated state and voltage-gated K^+ channels open, causing K^+ ion flux outside the cell and subsequent repolarization, approximately after 1 ms after membrane depolarization. At the end of the falling phase, the membrane potential is far more negative than the resting potential because K^+ efflux leads the membrane potential towards the K^+ equilibrium potential (-80mV). This part of the falling phase is referred as *hyperpolarization or undershoot* (Figure 5).

When voltage-gated K^+ channels close, the resting membrane potential is gradually restored (Koester and Siegelbaum, 2013b; Etsuro E. Uemura, 2015a).

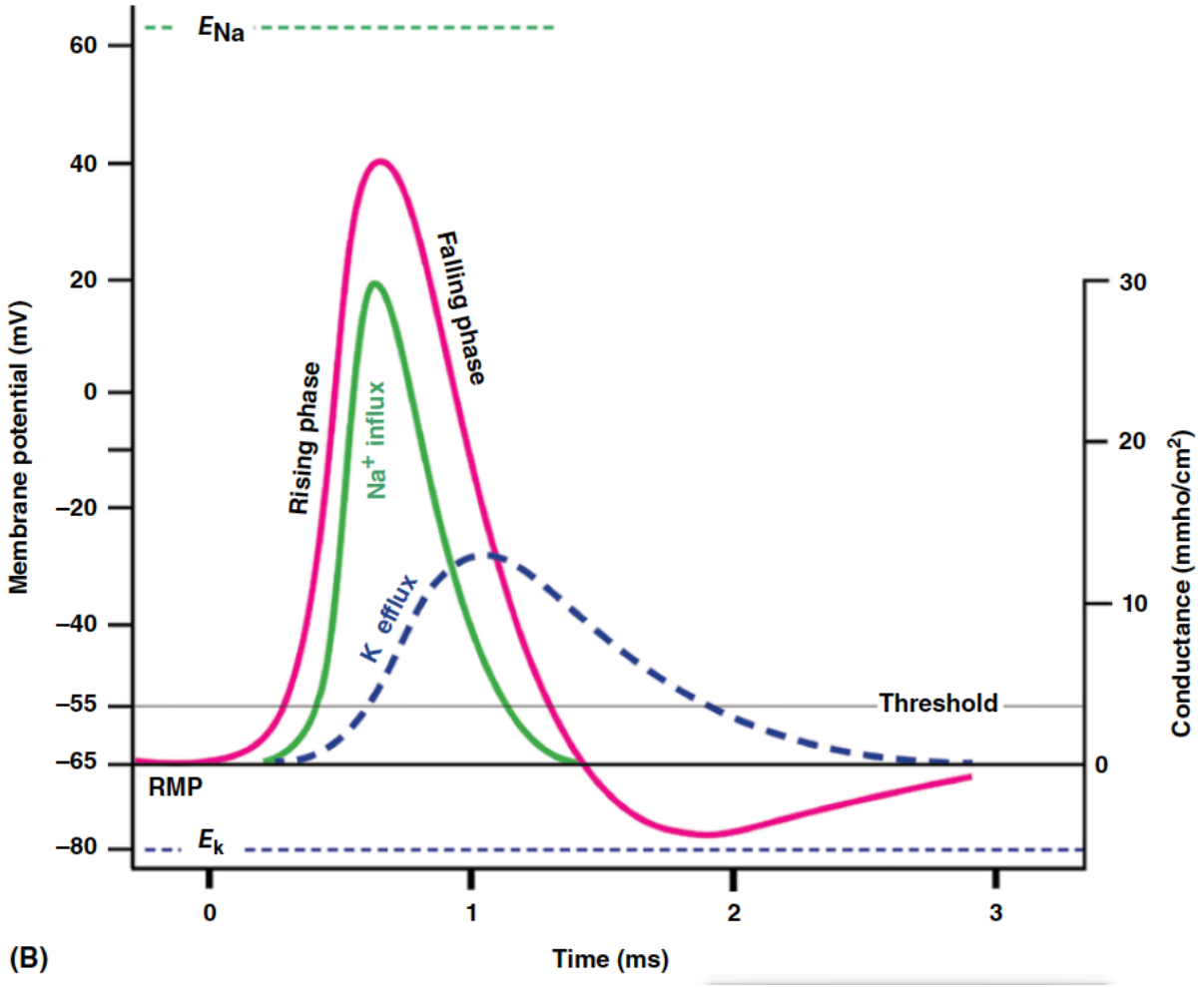

Figure 5. Time course of changes in membrane potential and in membrane permeability during an action potential. *The horizontal dashed lines represent the equilibrium potentials (E_k) for K^+ and Na^+ (from: Fundamentals of canine neuroanatomy and neurophysiology, Uemura E., 2015)*

After the generation of an action potential, a period of time is necessary for a cell membrane to become excitable again. This phase is defined *refractory period* and represents the time needed for the voltage-gated Na^+ channels to reset from the inactivated state to the resting state.
The refractory period is divided in:

- Absolute refractory period: after the initiation of an action potential, a second action potential cannot be triggered, regardless of the amplitude of the stimulus applied to the neuronal cell. This period corresponds nearly to the entire duration of the action potential and initiate after the inactivation of the Na^+ channels.
- Relative refractory period: it follows the absolute refractory period, starts during the repolarization phase and lasts until closure of the K^+ ion channels. During this phase a second action potential can be triggered only by very strong stimuli that exceed the threshold potential.

  This difficulty in generating action potentials is related to the necessity of de-inactivation of Na+ channels, which in turn requires membrane depolarization. Not all inactivated Na^+ channels undergo de-inactivation simultaneously and the generation an action potential requires a much stronger depolarizing potential to recruit a sufficient number of de-inactivated Na^+ channels. Furthermore, when membrane potential undergoes hyperpolarization, a much stronger current is necessary to shift the membrane potential to threshold.

(Koester and Siegelbaum, 2013b; Etsuro E. Uemura, 2015a).

Although action potentials can be generated with just two types of voltage-gated channels, it is now known that many types of voltage-gated Na^+ and K^+ channels, with different biophysical and functional properties, are expressed in different nerve and muscles cells. Also, the function of these different channels can even vary within a single neuronal cell. For example, many variants of voltage-gated K^+ channels that differ in modalities of activation, voltage-activation range and sensitivity to various ligand exist. Four of these variants are particularly important for the nervous system:

- Slowly activating K^+ channels: directly involved in the repolarization process as previously discussed;
- Calcium-activated K^+ channels: activated by an increase in intracellular Ca^{2+} concentration during depolarization;
- A-type K^+ channels: activated by depolarization, almost as rapidly as Na^+ channels and rapidly inactivated during prolonged depolarization;
- M-type K^+ channels: only small depolarizations are required for the opening, but the activation is very slow. They are closed by the neurotransmitter acetylcholine.

Similarly, at least five major types of voltage-gated Ca^{2+} channels and eight types of Na^{+} channels with different structural and functional variations are expressed within the nervous system. Furthermore, other gene families encode voltage-gated channels selective for Ca^{2+} that have mixed permeability also for Na^{+} and K^{+}. These differences have important consequences for membrane excitability. Most neurons contain voltage-gated Ca^{2+} channels that open after depolarization and some neurons have voltage-gated Cl- channels involved in membrane depolarization. Many neurons have also cation channels slowly activated by hyperpolarization, permeable to Na^{+} and K^{+}, that therefore give rise to an inward depolarizing current during hyperpolarization and repolarization.

This great variety of voltage-gated ion channels is essential for neurons that are involved in more complex information-processing modalities.

Excitability properties vary between regions of the neuron, because different regions have different types of ion channels that support specialized functions. For example, the trigger zone of the axon is characterized by a high density of voltage-gated Na^{+} channels that are sensitive to relatively small deviations from the resting potential. These characteristics explain the low threshold for action potential generation at this site.

Excitability properties also vary between types of neurons because through the expression of different ion channels, neuronal electrical properties can be modulated according to the information that has to be processed. How a neuron responds to a particular input is determined by the proportions of different types of voltage-gated channels in the cell's input and integrative region. For example, some cells respond with the generation of a single action potential to a constant excitatory input, while others with a train of action potentials at a constant rate of firing and still others with accelerating or decelerating train of action potentials. Some neurons with certain voltage-gated Ca^{2+} channels and hyperpolarization-activated cation channels are able to generate pacemaker currents that allow spontaneous firing of neurons without the need of any external stimulus.

Glial cells (in particular astrocytes) are involved in the maintenance of a correct neuronal excitability by means of controlling the extracellular concentration of K^{+}. With the mechanism called *spatial buffering,* when the extracellular concentration of K^{+} increase due to neuronal activity, astrocytes incorporate K^{+} and transfer it to one cell to another through their gap junctions preventing extracellular K^{+} accumulation.

(Koester and Siegelbaum, 2013b; Siegelbaum and Koester, 2013)

SYNAPTIC TRANSMISSION

Synapses are special site of contact where electrical and chemical impulses are transferred between different neurons.

Electrical synapses

In electrical synapses ion current flow passively through a gap junction between neurons. Here the separation between two neurons is minimal (4nm) and much less than the nonsynaptic space between neurons (20 nm) and the pore of the channel has a large diameter of 1.5 nm, which allows inorganic ions and small organic molecules to pass between 2 cells.

During excitatory synaptic transmission, voltage-gated ion channels in the presynaptic cell generate the current that depolarizes the postsynaptic cell. A large current is therefore necessary in order to produce a change in potential in the postsynaptic cell. To generate such a large current, the presynaptic terminal must have considerable size to contain many ion channels. Concurrently, the postsynaptic terminal must be relatively small, because according to the Ohm's law, a small cell has a higher input resistance than a large cell and undergoes a greater voltage change in response to a given presynaptic current.

In electric synapses, the change in potential of the postsynaptic membrane is directly related to the size of the change in potential of the presynaptic membrane, and even a minimal subthreshold depolarizing current is sufficient to create some current in the postsynaptic membrane and cause depolarization.

At electrical synapses, the synaptic delay (time between the presynaptic spike and the postsynaptic potential) is extremely short.

Most electrical synapses can transmit both depolarizing and hyperpolarizing currents and electrical transmission is also involved in orchestrating the actions of groups of neurons, because current crosses the membranes of all electrically coupled cells simultaneously. Furthermore, in addition to providing speed and synchrony in neuronal signaling, electrical synapses are also involved in transmission of metabolic signals between cells (Siegelbaum and Kandel, 2013; E.E. Uemura, 2015).

Chemical synapses

In contrast to electrical synapses, at chemical synapses there is no structural continuity between pre and postsynaptic neurons. In chemical synapses as a result of the depolarization of a presynaptic terminal, voltage-gated Ca^{2+} channels in the presynaptic membrane open and due to its electrochemical gradient Ca^{2+} enters the neuronal cell. Calcium entry determine the fusion of the synaptic vesicles with the active zones of the presynaptic membrane and the consequent release of neurotransmitters into the synaptic cleft (a narrow space 20 – 40 nm wide between the presynaptic

and postsynaptic membrane) by exocytosis. Here, neurotransmitters diffuse across the synaptic cleft and bind to their receptors on the postsynaptic membrane. This mechanism determines the opening or closing of specific ion channels on the postsynaptic membrane. The resulting flux of ions alters the membrane conductance and potential of the postsynaptic cell.

The synaptic delay of chemical synapses is therefore longer compared to the electrical synapses, because chemical transmission requires several biochemical steps and can last from 0.3 ms to several ms.

In chemical synapses the current in the presynaptic membrane must reach the threshold and create an action potential in order to release neurotransmitter and induce a response in the postsynaptic cell. Even though chemical transmission isn't fast as the electrical transmission, it has the important property of amplification, due to the fact that a single synaptic vesicle releases thousands of molecules of transmitters that can open thousands of ion channels in the postsynaptic cell. With this mechanism, a small presynaptic nerve terminal that generate only a weak electrical current can depolarize a big postsynaptic cell (Siegelbaum et al., 2013a; Siegelbaum and Kandel, 2013; E.E. Uemura, 2015).

The strength of both types of synaptic transmission can be enhanced or reduced by cellular activity due to their plasticity property.

Electrical synapses are primarily involved in the transmission of rapid and stereotyped depolarizing signals, while chemical synapses are capable of more variable signaling that can be either excitatory or inhibitory. Most synapses within the brain are chemical (Siegelbaum et al., 2013b).

Neurotransmitters

In both the peripheral and the central nervous system (CNS), a variety of chemical molecules act as neurotransmitters (Figure 6).

In order for a substance to be considered as neurotransmitter, it must meet four criteria (Schwartz and Javitch, 2013):

1) The molecule has to be synthetized in the presynaptic neuron;
2) It must be present in the presynaptic terminal and released in sufficient amount to cause a precise action on the postsynaptic neuron;
3) When administered exogenously, it must determine the same action as released endogenously;
4) The removal of the molecule from the synaptic cleft is mediated by specific mechanism.

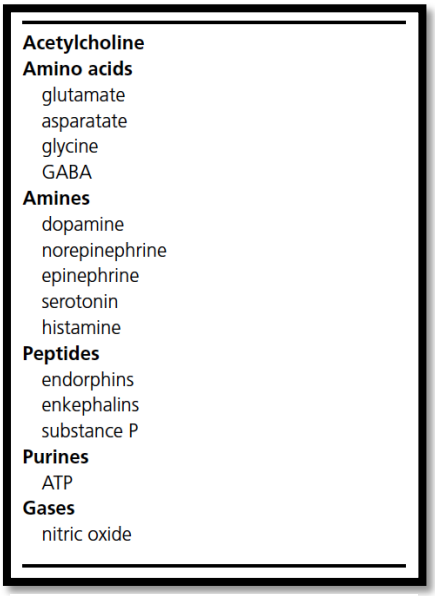

Figure 6. Major neurotransmitters of the CNS *(from Fundamentals of Canine Neuroanatomy and Neurophysiology, Uemura E., 2015).*

Two main categories of neurotransmitter can be found in the nervous system: small-molecule transmitters and neuroactive peptides (short polymers of amino acids). Both classes are contained in vesicles: neuropetides are stored in large vesicles which release their content by exocytosis, while small-molecule transmitters are packaged in small vesicles which release their content thorugh specific Ca^{2+} channels. While small vesicle can contain only small-molecule neurotransmitters, large vesicles can include both classes of transmitters. Both types of vesicles can be found in the majority of neurons, but in different proportions. Small vesicles are typical of neurons that use glutamate, γ-aminobutyric acid (GABA) and glycine as neurotransmitters, whereas large vesicles are characteristic of neurons that use catecholamines and serotonin (Schwartz and Javitch, 2013).

Although a variety of chemical molecules serve as neurotransmitters, the action depends on the properties of the postsynaptic receptors and not the chemical properties of the molecule itself. The interaction with receptors is typically transient, lasting from milliseconds to minutes but some neurotransmitters can cause long-term changes lasting for hours or even days. Furthermore, increasing evidence suggests that astrocytes play a role in synthetizing, storing and releasing neurotransmitters and expressing receptors involved in astrocyte function modulation (Schwartz and Javitch, 2013).

The first neurotransmitter discovered was *acetylcholine*. It plays a major role in the peripheral nervous system, where it is released by motor neurons and neurons of the autonomic nervous system, but it also plays an important role in the CNS in maintaining cognitive function. It is synthesized from choline and acetyl coenzyme A in axon terminals, and neurons that release acetylcholine are called cholinergic neurons

Glutamate is the primary excitatory transmitter of the CNS along with *aspartate*, while the major inhibitory transmitter is a glutamate derivative, the *GABA*. The other inhibitory neurotransmitter of the CNS is the amino acid called *glycine*, which is mainly found in the spinal cord. Glutamate and glycine are universal cellular constituents as well as neurotransmitters. Glutamate is derived from α-ketoglutarate, an intermediate in the tricarbocylic acid cycle, while glycine is synthetized from serine and it is also an allosteric modulator of the N-methyl-d-aspartate (NMDA) receptors. Its biosynthesis within neurons is not completely understood. (Etsuro E. Uemura, 2015c).

Many neuromodulators, such as *dopamine*, are monoamines. There are several dopamine pathways in the brain, and this neurotransmitter is involved in many functions, including motor control, reward and reinforcement, and motivation. *Noradrenaline (or norepinephrine*) is another monoamine and is the primary neurotransmitter in the sympathetic nervous system where it works on the activity of various organs in the body to control blood pressure, heart rate, liver function and many other functions. Dopamine, norepinephrine, and *epinephrine* are synthesized from tyrosine. Neurons that release norepinephrine or epinephrine are called adrenergic neurons. Neurons that use *serotonin* (another monoamine) are called serotoninergic and project to various parts of the nervous system. As a result, serotonin is involved in functions such as sleep, memory, appetite,

mood and others. *Histamine*, the last of the major monoamines, plays a role in metabolism, temperature control, regulating various hormones, and controlling the sleep-wake cycle, amongst other functions. Serotonin is derived from the amino acid tryptophan and histamine from histidine.
The *nitric oxide* (NO) is an unusual neurotransmitter that diffuse freely into the target neuron to bind to intracellular proteins and it is derived from oxygen and arginine (Etsuro E. Uemura, 2015c).

A neuron can produce and release different types of neurotransmitters. These are stored in particular intracellular organelles called synaptic vesicles. Their release is induced by the Ca^{2+} influx through the voltage-gated channels that open in response to an action potential in the presynaptic terminal. In response to this stimulus, the vesicle membrane fuses with the presynaptic membrane and neurotransmitters are released into the synaptic cleft by exocytosis. Usually, transmitters are stored in vesicles at synapses and released by exocytosis, but some molecules can be released into the synaptic cleft directly from the cytoplasm (E.E. Uemura, 2015).
The synaptic action of neurotransmitters can be terminated by different mechanisms. The most important mechanism of removal of neurotransmitters from the synaptic cleft are *enzymatic inactivation* within the synaptic cleft or *diffusion* away from the synaptic cleft. The enzymatic degradation is typical of cholinergic synapses. Following enzymatic inactivation, neurotransmitters constituents are re-uptaken by the presynaptic terminal for re-synthesis of neurotransmitters (this is the case of acetylcholine, hydrolyzed by the acetylcholinesterase and taken back up into cholinergic nerve as choline for new production of acetylcholine). The other mechanism of removal allows the diffusion of neurotransmitters in the circulation and proteolysis by extracellular peptidases or the transportation into neurons or astrocytes. Glutamate for example is transported back into the presynaptic terminal or into the astrocytes. In presynaptic terminals it is repackaged into synaptic vesicles. While in astrocytes glutamate is converted to glutamine and then transferred to the presynaptic terminal and repackaged in synaptic vesicles to be used as neurotransmitter (Schwartz and Javitch, 2013).

Receptors

Within the synaptic cleft, neurotransmitters released bind with specific receptors (special signal recognition proteins) on the postsynaptic membrane. This bond determines changes in the postsynaptic membrane permeability to selected ions through their ion channels, causing the distribution of these ions across the neuronal membrane according to their electrochemical gradient. When the neurotransmitter bond results directly in a conformational change of the ion channel that leads to the opening of the channel itself, ion channels are defined as *directly gated* and receptors associated with this type of channels are defined as *ionotropic* receptors. Neurotransmitters involved in this mechanism include acetylcholine, glutamate, glycine and GABA. When, in contrast, ion channels are separated from the binding site of the neurotransmitter, they are called *indirectly gated*

ion channels and the associated receptors are defined *metabotropic*. Binding of neurotransmitters to this type of receptors activates a protein, called G-protein, that in turns activates a second messenger system that either directly opens the ion channels or activates an enzyme that opens the channel by phosphorylating the channel portion. While activation of ionotropic receptors determines a very quick synaptic response (few milliseconds), metabotropic receptors lead to slow and long-lasting synaptic action. Neurotransmitters (except NO) can bind to different receptor types and each receptor type can have several subtypes which trigger different effect. Neurons can receive both excitatory and inhibitory inputs and the nature of a synaptic potential (whether it is excitatory or inhibitory) is determined not by the type of neurotransmitter but by the type of ion channels activated in the postsynaptic membrane by the neurotransmitter. Although some transmitters can produce both excitatory and inhibitory potentials by acting on different ionotropic receptors at different synapses, most neurotransmitters produce a single predominant type of synaptic response. Whether a synaptic terminal is predominantly excitatory or inhibitory can be predicted by its morphology. In particular, two morphological types are common within the brain: Gray type I and II. Most type I synapses are glutamatergic and therefore excitatory, while most type two synapses are GABAergic and inhibitory (Schwartz and Javitch, 2013; Siegelbaum et al., 2013b; Siegelbaum and Kandel, 2013).

Glutamate receptors

Glutamate receptors can be either ionotropic or metabotropic. Excitatory synaptic transmission is always mediated by ionotropic glutamate receptor-channels that are permeable to Na^+ and K^+, while metabotropic receptors can produce either excitation or inhibition.

Ionotropic glutamate receptors are integral membrane proteins composed of four homologous subunits forming a central ion channel pore, with each subunit containing two extracellular ligand binding regions. These two regions are historically referred to as S1 and S2 and they are formed by two extracellular stretches of aminoacids.

Three subtypes of glutamate ionotropic receptors are known:

- α-amino-3-hydroxy-5- methyl-4-isoxazole propionic acid (AMPA) receptors;
- kainite receptors;
- NMDA receptors.

Names reflect the agonist of the receptors. The AMPA and kainite receptors are sometimes referred to as non-NMDA receptors. The NMDA receptors are ligand gated-channels permeable not only to Na^+ and K^+ but also to Ca^{2+} and their opening requires extracellular glycine as a cofactor, which in physiological conditions is sufficiently concentrated to allow NMDA receptor activation. Furthermore, NMDA receptor opening not only relies on the type on neurotransmitter, but also on the membrane voltage: at resting membrane potential (-65 mV), magnesium (Mg^{2+}) block ion current through the ion channel by binding to a specific site within the channel pore. After membrane depolarization,

Mg^{2+} is removed by electrostatic repulsion, allowing Na^{+} and K^{+} to flow through the ion channel (Traynelis et al., 2010).

Central synapses that use glutamate as neurotransmitters contain both NMDA and AMPA receptors. Since at normal resting potential the majority of NMDA receptors are blocked by Mg^{2+}, as a result excitatory post-synaptic potential is predominantly determined by ion flow through the AMPA receptors, in which ion current are generated and interrupted very quickly. After depolarization, NMDA receptors are activated, and more charge flows through these channels. The time needed for the ion flow to rise and consequently decay is longer than the time needed for the AMPA receptor current and for this reason, NMDA receptors contribute only to a late, slow phase of the excitatory post-synaptic potential. Even though NMDA receptors are not directly involved in the generation of excitatory post-synaptic potentials, they have the essential function of translating electrical inputs in biochemical ones. They exert this function by uniquely conducting Ca^{2+} into the post-synaptic cell, causing rise in Ca^{2+} concentrations that in turns activate various Ca^{2+}-dependent signaling cascades. Some of these biochemical reactions are involved in the mechanism of synaptic plasticity, defined as the biological process by which specific patterns of synaptic activity result in changes in synaptic strength.

The conduction of Ca^{2+} ions inside the cell though the NMDA channels can also have negative effects on neural cells. Excessive high concentrations of glutamate are in fact thought to cause an overload of Ca^{2+} in the postsynaptic cell that can activate Ca^{2+}-dependent proteases and phospholipases that produce free radicals, toxic to cells (*glutamate excitotoxicity*).

For this reason, it is thought that glutamate toxicity can be involved in cell death after cluster seizures or status epilepticus, for example, and therefore, it has been postulated that agents selectively blocking NMDA receptors may represent a protection against these detrimental effects. Unfortunately, hallucinations related to NMDA receptor blockade have so far limited the clinical use of these compounds. Furthermore, it has to be considered that physiological levels of NMDA receptor activation are necessary for the protection of neurons from damage and cell death.

Glutamate receptors are usually clustered at post-synaptic sites in the membrane, opposed to glutamatergic pre-synaptic terminals. The majority of excitatory synapses in adult central nervous system contain both NMDA and AMPA receptors, while NMDA receptors prevail in young animals (Koester and Siegelbaum, 2013b; Siegelbaum et al., 2013b; Siegelbaum and Kandel, 2013).

GABA receptors

Although excitatory glutamatergic synapses represent the most abundant type of synapses within the brain, inhibitory synapses are essential for a correct function of the CNS by preventing excessive stimulation and coordinating activity among network of neurons. GABA is the most important inhibitor neurotransmitter of the brain and bind to two receptors, $GABA_A$ (ionotropic receptor that directly

opens Cl^- ion channels) and $GABA_B$ (metabotropic receptors that activates a second-messenger cascade, which often indirectly open a K^+ channel).

$GABA_A$ receptors consist of six subunits (namely, $\alpha1$, $\alpha2$, $\beta1$, $\beta2$, γ and δ). They are activated by the binding of two molecules of GABA in clefts formed between the two α and β subunits. To date, more than 800 different receptor subtypes have been discovered (Bowery et al., 2002; Olsen and Sieghart, 2008).

Typically, the resting membrane potential of a neuron s -65 mV that is slightly more positive than the Cl^- equilibrium potential (-70mV). At this value, the chemical gradient of the Cl^- that pushes the ion into the cell is slightly greater than the electrical force opposing Cl^- influx. Therefore, the opening of Cl^- channels leads to a positive current that correspond to an influx of ion into the cell following its electrochemical gradient, causing a membrane hyperpolarization (a net increase in the negative charge inside the membrane). However, some neurons within the CNS have a resting membrane potential that is approximately equal to the Cl^- equilibrium potential and in these cells an increase in Cl^- conductance does not cause hyperpolarization but inhibits the cell from firing an action potential in response to a near-simultaneous excitatory postsynaptic potential.

Hyperpolarization of neurons with metabotropic $GABA_B$ receptors is caused by the opening of K^+ channels. Because the equilibrium potential of this ion is even more negative that the one of Cl^- (-80mV), they produce more profound hyperpolarization, but while $GABA_A$ receptor activation causes fast and short responses, $GABA_B$ receptor activation is associated with slower and more persistent variations.

Paradoxically, $GABA_A$ receptors can cause excitation. This can happen because after intense periods of stimulation Cl^- influx into the cell can dramatically increase, causing the Cl^- equilibrium potential to be more positive than the resting membrane. At this stage, the opening of Cl^- channels leads to an efflux of ion and consequent depolarization of neurons. This condition is typical of newborn brain cells, where the intracellular Cl^- concentration is higher than normal, but can happen also in mature neurons where it is thought to contribute to epileptic discharge development (Siegelbaum et al., 2013a).

All four regions of a nerve cell (axon, terminals, cell body and dendrites) can be presynaptic or postsynaptic. Therefore, many types of contact between different part of different neural cells can be generated. The most common types are axodendritic, axosomatic and axoaxonic. The proximity of a synapse to the trigger zone influence its effectiveness: the more the synapse is near to the trigger zone, the greater the action potential will be, therefore axosomatic synapses are more effective than axodendritic contacts. In some neurons, this mechanism is compensated by the presence of higher concentrations of glutamate in distal synapses than in proximal ones, thereby minimizing the spatial effect in synaptic integration.

In contrast, axoaxonic synapses have no direct effect on the trigger zone of the postsynaptic neuron and their function is related to the control of the amount of neurotransmitter released in the synaptic cleft.

Excitatory synapses are mostly found on neuronal dendrites, whereas inhibitory synapses predominate on the cell body, where they can interrupt the excitatory inputs traveling down the cell's dendrite to the soma.

In conclusion, synaptic transmission in the brain rely on three major neurotransmitters, acting on five main classes of ionotropic receptors. The presence of a variety of receptor subtypes encoded by distinct but related genes ensure the enormous diversity in the postsynaptic properties of synapses in the brain (Siegelbaum et al., 2013b; E.E. Uemura, 2015).

PATHOPHYSIOLOGY OF SEIZURE ACTIVITY

An epileptic seizure is defined as the clinical manifestation of a temporary disruption of brain function, due to an excessive, synchronous and sustained discharge of a group of neurons in the brain (Fisher et al., 2017, 2014).

Epileptic seizures are therefore associated with change in the excitability of a group of neurons, and in particular with a persistent increase of neuronal excitability, due to a variety of causes. The excessive discharge alone is not sufficient to cause an epileptic seizure and a *synchronization* of a group of neurons is necessary.

Many ways of synchronization of neurons have been identified:

- Glutamatergic interconnections between pyramidal cells of the cerebral cortex;
- Gap junctions between cortical neurons that allow a low resistance current flow between neurons;
- Inhibition: many cortical pyramidal cells in a defined area are connected by GABAergic neurons. Therefore, discharge of a single interneuron can synchronously hyperpolarize an entire population of cortical cells. However, when the inhibition fades, voltage-dependent currents of pyramidal cells are activated simultaneously. These currents are relatively inactive at resting potential, but hyperpolarization causes activation and therefore synchronous depolarization of a group of pyramidal cells.

Furthermore, changes that promote further synchronization, such as growth of collateral excitatory glutamatergic neurons, occur over time in brain of patients affected by epilepsy.

Within an epileptic focus, each neuron is characterized by a stereotypic and synchronized electrical activity called paroxysmal depolarizing shift, a sudden, large (20 – 40 mV) and long-lasting (50 – 200 ms) intracellular depolarization, that triggers a train of action potentials and that is followed by an afterhyperpolarization The conductance of a paroxysmal depolarizing shift is depicted in Fig. 7. The depolarizing phase results primarily from activation of AMPA and NMDA glutamate receptors and voltage-gated Na^+ and Ca^{2+} channels. Afterwards, hyperpolarization occurs by activation of ionotropic and metabotropic GABA receptors and voltage-gated and Ca^{2+}-activated K^+ channels. (Westbrook, 2013).

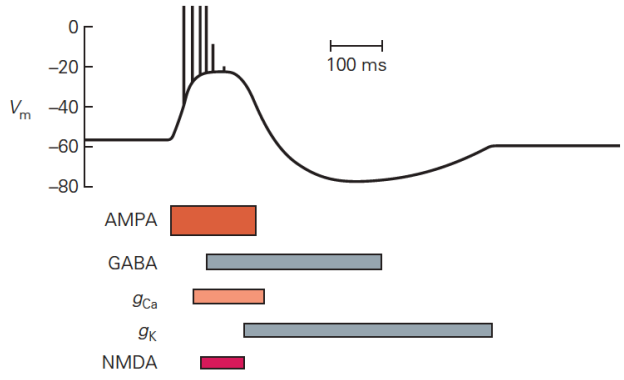

Figure 7. Conductance underlying the paroxysmal depolarizing shift of a neuron in a seizure focus. *g_{Ca} = voltage gated Na^+ and Ca^{2+} channels; g_K = voltage-gated and Ca^{2+}-activated K^+ channels (from: Principles of Neural Science, 5th ed, Kandel et al., 2013).*

The function of the afterhyperpolarization is to limit the duration of the paroxysmal depolarizing shift. When only a small group of neurons is affected by this abnormal electrical activity, no clinical manifestations are evaluated. The abnormal electrical activity is usually limited to the seizure focus by inhibitory effects of the excited region on the surrounding tissue by means of GABAergic inhibitory interneurons (Fig. 8A and 8B).

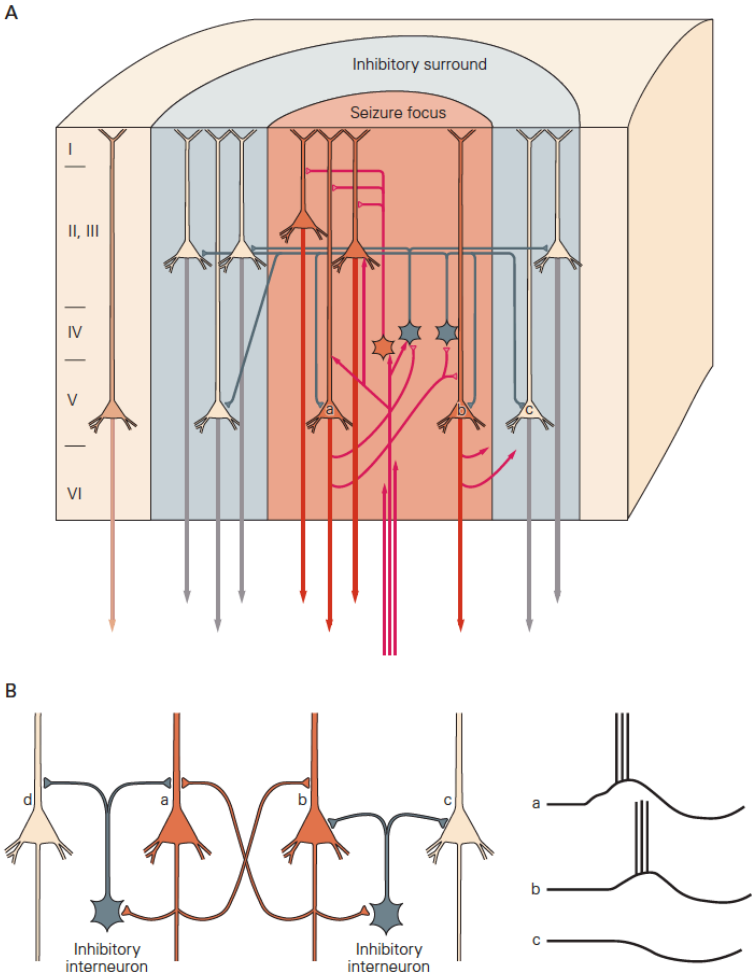

Figure 8. Spatial and temporal organization of a seizure focus.

A) The pyramidal cell "a" represents the typical neuron in a seizure focus. Its activity activates in turn another pyramidal cell "b", but also GABAergic inhibitory interneurons (gray). These interneurons can reduce the activity of cells in a seizure focus temporally and prevent the firing of these cells outside the epileptic focus ("c").

B) Synaptic connections and activity patterns for cells "a", "b" and "c".

(from: Principles of Neural Science, 5th ed., Kandel et al., 2013)

When the inhibitory surround is overcome and the afterhyperpolarization gradually disappears, neurons in the seizure focus become synchronously excited and the abnormal electrical activity spreads beyond the original focus, giving rise to a **focal seizure**, caused by continuous high-frequency train of action potentials.

Pyramidal neurons firing represents a key factor in the spread of a focal seizure. This results in a relative decrease activity of inhibitory GABAergic interneurons caused by a reduction in the release of GABA (presynaptic mechanism), a change of the Cl^- gradient responsible for the $GABA_A$ receptor-mediated ion flux or a reduction in GABA receptor activity (postsynaptic mechanism).

Other mechanism involved in the loss of inhibitory surround include changes in dendritic morphology, density of receptors and ion channels, or extracellular K^+ ion accumulation (S. Platt, 2014).

If the electrical activity within a seizure focus is sufficiently intense, a spread to other brain region can occur. This spread follows the normal cortical pathways and therefore thalamocortical (projection fibers), subcortical (association fibers) and interhemispheric (commissural fibers) pathways can all be involved in seizure spread. Prolonged firing also transmits action potentials to distant sites in the brain, which in turns may trigger trains of action potentials in neurons that project back to the seizure focus (backpropagation). Thalamocortical connections are extremely important in this regard. When both cerebral hemispheres become involved in the seizure spread, the seizure becomes **secondarily generalized**. The spread usually occurs within a few seconds but can also take many minutes. Rapid secondary generalization usually occurs if the original seizure focus is located in the neocortex, more than in the limbic system (in particular hippocampus and amygdala). An epileptic seizure can also be **primarily generalized**, when normal brain activity is disrupted in both cerebral hemispheres simultaneously (Figure 9) (Westbrook, 2013).

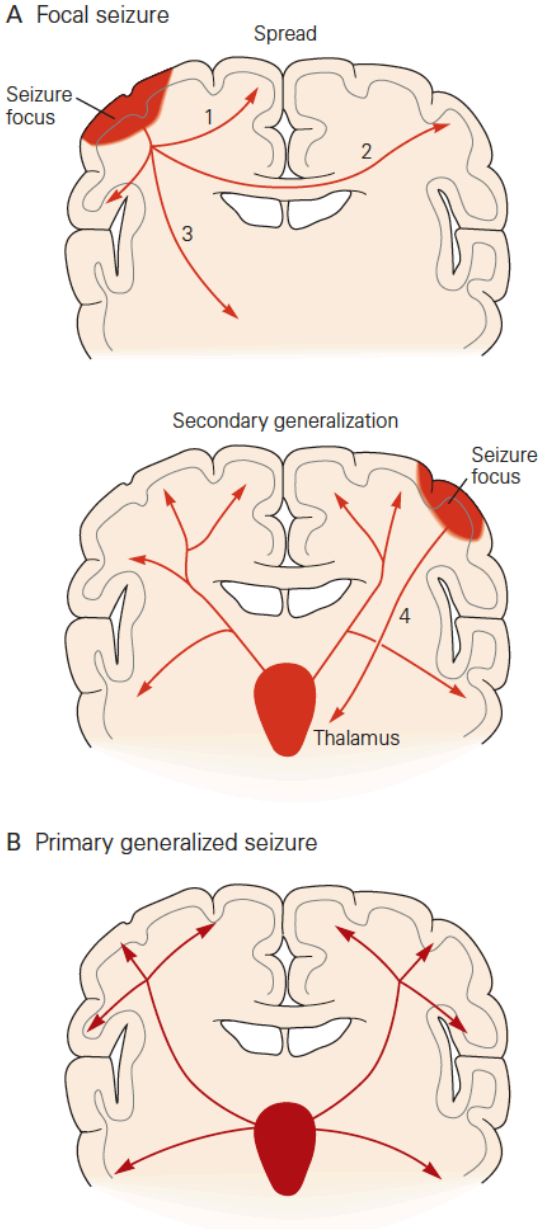

Figure 9. Seizure propagation pathways *(from: Principles of Neural Science, 5th ed., Kandel et al., 2013)*

A general feature of seizures is that the clinical manifestations depend on the location and extent of brain regions that are affected.

Seizures are followed by a rise in extracellular K^+ as a result of the excessive discharge. This can lead to a transient elevation in extracellular K^+, leading to a reduced K^+ equilibrium across the cell membrane and reduce outflow of K^+ ions. The net current will become inward, further predisposing neuron to depolarization, giving rise to a "vicious cycle" (S. Platt, 2014; Westbrook, 2013).

Not only functional changes (i.e.: concentrations of ions, changes in neurotransmitters levels) but also structural changes in both neurons and glia occur in epileptic foci. It has finally been demonstrated that neuronal microenvironment contributes substantially to the etiology of epilepsy. This microenvironment is largely influenced by glial cells. Proven mechanisms by which glial cells contribute to epileptogenesis are reported in Figure 10 (Patel et al., 2019).

Glial factor and/or pathway	Function	Biochemical changes in epilepsy	Mechanism of hyperexcitability
$K_{ir}4.1$	• Clearance of extracellular K^+ • Set resting membrane potential	• Downregulated in animal models and in human epileptic tissues • Prominent reduction in perivascular astrocytic end feet	• Increase in extracellular K^+ • Impaired clearance of extracellular glutamate
AQP4	• Transmembrane water transport • Colocalized in perivascular astrocyte end feet and functions in concert with $K_{ir}4.1$	• Differential regulation in epileptic tissue • Loss or mislocalization in perivascular astrocyte end feet	• Decreased clearance of extracellular K^+ • Impaired K^+ buffering and GJ coupling
Connexins (GJ proteins)	Ionic and biochemical coupling between CNS cells	• Differential regulation in epileptic tissue • Functional consequences of changes in astrocytic GJ coupling remain inconclusive	Impaired spatial redistribution of K^+ and glutamate
NKCC1	Moves Cl^- into the cell	Increased expression	Impaired GABAergic inhibition due to increased intracellular Cl^- and depolarizing shift in the reversal potential of $GABA_AR$
KCC2	Moves Cl^- into the extracellular space	Decreased expression	Impaired GABAergic inhibition due to increased intracellular Cl^- and depolarizing shift in the reversal potential of $GABA_AR$
GLT1 or EAAT2	Clearance of extracellular glutamate	Results of expression studies in human epileptic tissues inconsistent	Increased extracellular glutamate
GS	• Expressed in astrocytes • Key enzyme in the glutamate–glutamine cycle • Sequesters glutamate by condensing it with ammonia to form glutamine	• Reduced expression and enzymatic activity in hippocampus of individuals with MTLE • Downregulated in gliotic tissue in mice	• Decreased neuronal GABA synthesis due to disruption in astrocytic supply of glutamine • Impaired GABAergic inhibition
Adenosine kinase	Phosphorylates adenosine (endogenous anticonvulsant) and terminates its action	Increased expression	Decreased level of adenosine
Gliotransmitters	Regulation of neurotransmission and synaptic plasticity	Increased release of glutamate and ATP	Increased activation of glutamatergic and purinergic signalling
BBB	Astrocyte end feet support the maintenance of BBB integrity	• Extravasation of albumin and peripheral immune cells into the brain following BBB disruptions • Increased albumin–TGFβ signalling in astrocytes	• Decreased expression of $K_{ir}4.1$, glutamate transporters and GJ proteins • Impaired K^+ and glutamate buffering • Increased neuroinflammation

Figure 10. Glial mechanisms of epileptogenesis. *AQP4, aquaporin 4; BBB, blood–brain barrier ; EAAT2, excitatory amino acid transporter 2; $GABA_AR$, GABA type A receptor ; GJ, gap junction; GLT1, glutamate transporter 1; GS, glutamine synthetase; KCC2, K^+–Cl^- cotransporter ; Kir4.1, inwardly rectifying potassium channel 4.1; NA , not available; NKCC1, Na^+–K^+–Cl^- cotransporter 1; MTLE, mesial temporal lobe epilepsy ; TGFβ, transforming growth factor- β (from Neuron-glia interactions in the pathophysiology of epilepsy. Patel et al., 2019)*

However, many important questions on glial contribution to epileptogenesis remain unanswered yet. Gliosis is a histological feature of epileptic brain, but the causal relationship between gliosis and epileptogenesis still has to be defined. Recent studies have raised the suspicion that functional changes that occur in gliotic tissue can also alter distant brain region and that glial cells not only contribute to structural epilepsy, but that a general glial dysfunction can be a feature of all forms of epilepsy. Furthermore, it is well known how glial cells are involved in recovery of neuropathological lesions following CNS insult, but uncontrolled and exaggerated gliosis may contribute to the development of neuropathological lesions associated with epilepsy. Glial cells also produce molecules that modify extracellular matrix and therefore influence neuronal activity, but the extent of this influence and its possible role in epileptogenesis still has to be evaluated (Patel et al., 2019).

Mechanisms involved in interictal-ictal transition can be divided in nonsynaptic and synaptic.
Nonsynaptic mechanisms involve:

- Alteration of ionic microenvironment: increase of extracellular K^+ due to repetitive ictal and interictal activity causing increased neuronal excitability.
- Active ion transport: hypoxia or ischaemia may cause Na^+-K^+ pump failure and subsequent interictal-ictal transition due to abnormal neuronal excitability, while disfunction of Cl^--K^+ co-transport mechanism (that control the Cl^- concentration and gradient across the cell membrane) can cause decreased function of GABAergic inhibition leading to increase neuronal excitability.
- Pre-synaptic terminal bursting: bursts of action potentials in thalamocortical relay cells can cause synchronization of many neurons.
- Ephaptic interaction: when the extracellular space between cells is reduced, reciprocal excitation of adjacent neurons can occur in the absence of synaptic connections.

Synaptic mechanisms include instead all the alterations that cause decrease synaptic inhibition of increased synaptic excitation. As already reported, GABA is the primary inhibitory neurotransmitter of the CNS, therefore involved in the generation of inhibitory postsynaptic potentials. During repetitive stimulation of cortical circuits, the amplitude of the inhibitory postsynaptic potentials gradually decreases. This reduction is thought to be caused by decrease in GABA release, desensitization of GABA receptors or alterations in the ionic gradient because of intracellular Cl^- accumulation. For this reason, GABA analogues molecules, GABA metabolism blockers or molecules that facilitate the effect of GABA can have all anticonvulsant effect.
Conversely, glutamate represents the most important excitatory neurotransmitter of the CNS and its synaptic release is directly involved in the initiation and spread of the seizure activity. Both AMPA and NMDA receptors antagonists are therefore potent anticonvulsant and an altered function of these receptors is thought to be involved in seizure generation. In particular, it has been hypothesized that NMDA receptors may change after neuronal damage and that after an insult, new

receptors are generated with either less sensitivity to Mg^{2+} or more sensitivity to glycine. Furthermore, NMDA receptors are susceptible to many endogenous agents (including glycine, polyamines, steroids, neuropetides, pH, redox state and NO) that may cause changes in excitability. In addition to receptors abnormalities, glial glutamate transporter abnormality, causing a reduction of glutamate clearance from the extracellular fluid have been implicated in epilepsy.
In addition, catecholamines have been reported to have proconvulsant effect in several genetic models of epilepsy. In particular, dopamine was found to be decreased in the CNS of affected animals, while noradrenaline was increased. It has been proven that decreased dopamine lowers the seizure threshold facilitating seizures generation. Opioids peptides can have both proconvulsant and anticonvulsant properties: kappa-agonists suppress electrical discharge, while μ-agonist can cause inhibition of inhibitory interneurons in experimental models (Westbrook, 2013).

Inflammation within the brain has been reported as one of the pathophysiological mechanism of seizures and epilepsy. Evidences of this causative role of inflammation have been demonstrated by the identification of the anticonvulsant effect of steroids and anti-inflammatory treatments in case of drug-resistant epilepsies, by the identification of increased levels pro-inflammatory agents in case of febrile seizures in humans and by the identification of an immune system activation in some patients affected by epilepsy. However, recently it has been postulated that inflammation might be a consequence as well as a cause of epilepsy. Seizure activity may in fact induce brain inflammation and seizure-associated cell death can contribute to this. Both innate and adaptative immunity have been implicated in epilepsy (S. Platt, 2014).

To date, the mechanism that determine the termination of an epileptic seizure has not been completely understood. The only certainty is that termination is not caused by metabolic exhaustion (Westbrook, 2013).
Intrinsic mechanisms of seizure termination acting on single neurons include:

- Intracellular ion-activated K^+ current: increased Ca^{2+} influx during a paroxysmal depolarizing shift of consequent to glutamate release activates Ca^{2+}-dependent membrane K^+ current causing K^+ efflux and hyperpolarization (cessation of firing). Also, Na^+ entry may activate Na^+-dependent K^+ current causing reduced neuronal excitability.
- Transmembrane ion gradients: as previously described, during seizure activity a progressive increase in extracellular K^+ concentration occur causing depolarization of neurons and subsequent firing increase. With further accumulation, the membrane potential becomes more depolarized than the firing threshold, Na^+ channels inactivate, ceasing neuronal firing. The increased extracellular K^+ concentrations may also lead to an increased intracellular Cl^- concentration through the action of the K^+-Cl^- co-transporter. In this setting, the action of GABA to open Cl^- channels could enhance membrane depolarization to the point of becoming

refractory to further firing of action potentials. Seizure activity also results in a decreased extracellular Ca^{2+} concentrations that may inhibit synaptic transmission.

- Energy failure: sustained neuronal activity markedly increase the energy demand, namely ATP. Some neurons are provided with ATP-gated K^+ channels that have the function to reduce neuronal activity when ATP levels start to decrease. Therefore, when ATP levels falls because of seizure activity, K^+ channels open and hyperpolarize the cells.
  Furthermore, *in vitro* evaluations demonstrate that decreasing extracellular glucose concentration terminates electrographic seizure-like activity.

Seizure termination is also promoted by mechanisms acting on networks of neurons:

- Glutamate depletion: a mechanism limiting synaptic transmission during prolonged seizure activity is the depletion of synaptic vesicles containing neurotransmitters. As the glutamate reservoir is replenished continuously, however, additional control mechanisms are necessary to prevent re-initiation of seizure activity.
- Intra and extracellular environment: sustained seizure activity causes increased CO_2 levels and anaerobic metabolism determining extracellular acidosis or intracellular acidosis with extracellular alkalosis. In vitro experiments have proven that acidification of the intracellular space can terminate seizure-like activity probably because of a decreased NMDA receptors function and loss of synaptic long-term potentiation. For this reason, carbonic anhydrases inhibitors (such as acetazolamide) may have some anticonvulsant effect as reported in human medicine. Furthermore, it has been hypothesized that intracellular acidification can reduce neuronal excitability by reducing gap-junction function.
- Glial buffering of glutamate: as already mentioned, glutamate can be removed from the synaptic cleft by glial cells, preventing accumulation and therefore excessive neuronal excitation. Failure of this mechanism may therefore predispose to prolonged seizure activity. In some epileptic brain tissue, evidence of a release of glutamate by glial cells has been proven, confirming the role of glial cells in seizure activity.
- Increased GABA-ergic inhibition: as previously reported, inhibitory interneurons are activated around the seizure focus, preventing the propagation of the seizure. Synaptic inhibition is mediated by GABA release that bind with GABA receptors on the postsynaptic membrane. The role of $GABA_B$ receptors in seizure termination is difficult to determine, whereas it is well known that a sustained seizure activity cause a desensitization of $GABA_A$ receptors that likely contributes to the failure of seizure termination. This mechanism is also the basis of loss of efficacy of benzodiazepines medications and it is due to increased internalization of receptors and change in subunit composition. Non-synaptic $GABA_A$ receptors are not involved in this process and maintain inhibitory effect.

(S. Platt, 2014)

During epileptic seizures, after a prolonged depolarization, neurons begin to repolarize and the afterhyperpolarization occurs. The cycle of depolarization and hyperpolarization is the mechanism responsible of the common tonic-clonic manifestation of epileptic seizures.
The period that follows an epileptic seizure, called post-ictal phase, is usually characterized by a decreased electrical activity and may be accompanied by behavioural and neurological signs such as obtunded mental state, blindness, disorientation and other neurological deficits (Westbrook, 2013)

An epileptic seizure implies an increased metabolic demand. During a focal seizure normal ATP levels are maintained within the brain, but in case of a generalized seizure, consequently to a transient apnea phase, a decrease in blood oxygen concentration occurs and this results in a decrease in ATP concentration and an increase in anaerobic metabolism. After a single generalized epileptic seizure, the oxygen levels are quickly restored without complications, but after repetitive or prolonged seizure activity (i.e.: cluster seizures or status epilepticus), permanent brain damages due to persistent oxygen deficit can occur.
In fact, during a generalized seizure, stimulation of the hypothalamus determines a massive activation of the sympathetic nervous system, with subsequent increased systemic blood pressure and serum glucose levels that initially compensate for the increase metabolic demand. However, these homeostatic mechanisms fail during prolonged seizure activity resulting in hypoxia, hypotension, hypoglycemia and acidemia, but also more severe systemic complications such as cardiac arrythmias, pulmonary edema, hyperthermia and rhabdomyolysis.
Brain damages following prolonged or repetitive seizure activity is also due to the release of glutamate during excessive stimulation of neurons (*excitotoxicity*). This process is responsible for cell death due to activation of cell-destructive enzymes such proteases, lipases, phosphatases and lipid peroxydases responsible for the production of detrimental free radicals. In fact, these are example of Ca^{2+}-dependent enzymes, and it is well known that overactivation of glutamate receptors leads to an increase in intracellular Ca^{2+} concentration (S. Platt, 2014; Westbrook, 2013).

CANINE EPILEPSY DEFINITIONS, CLASSIFICATION AND TERMINOLOGY

Until recent years, classifications, definitions, terminology, criteria of diagnosis and measures of evaluation of therapeutic outcome have differed between studies. Many of the definitions and criteria used in veterinary medicine in the past have reflected the proposals of the human International League Against Epilepsy. In 2014, a group of Veterinary Neurology Specialists and non-specialists founded the International Veterinary Epilepsy Task Force (IVETF) with the aim of creating a consensus statement on canine epilepsy (Volk, 2015).

According to this recent consensus statement, the term seizure defines any sudden, short lasting and transient event and does not imply that the event is epileptic (Berendt et al., 2015). An epileptic seizure is instead the manifestation of excessive, synchronous and usually self-limiting epileptic activity of neurons within the brain, while the term epilepsy defines a brain disease causing two or more unprovoked epileptic seizures more than 24 hours apart (Fisher et al., 2014). Epileptic seizures do not comprise seizures caused by metabolic or toxic disturbances. In these circumstances the term “reactive seizures” is used, and defines a seizure occurring as a response of a normal brain to a transient disturbance in its function. Epileptic seizures can be classified by their etiology or according to their semiology.

Aethiological classification

- Idiopathic epilepsy:
    - Genetic epilepsy: a genetic cause of the disease has been identified
    - Suspected genetic epilepsy: a breed prevalence greater than 2%, pedigree analysis and/or familial predisposition of epilepsy support a genetic cause of the disease, but the causative gene still has not been identified.
    - Epilepsy of unknown cause: the underlying cause has yet to be identified in patients with no evidence of structural epilepsy.
- Structural epilepsy:
  In this category, epileptic seizures are provoked by an intracranial disease identified with diagnostic investigations.

Semiological classification

- Focal epileptic seizures: as previously described, focal seizures are the clinical manifestation of an abnormal neuronal electrical activity that spreads beyond the original focus but remained limited to one cerebral hemisphere. The clinical signs depend on the cerebral areas involved and their function and for this reason focal seizures can display motor signs (e.g. facial twitches, repeated rhythmic jerks of one extremity, …), autonomic signs (e.g. dilated pupils, hypersalivation or vomiting), behavioural signs (change in behavior such as anxiousness, restlessness, fear, aggressiveness).

- Generalized epileptic seizures: they represent the clinical manifestation of a bilateral cerebral involvement that may occur alone or as an evolution of a focal seizure. Usually the patient is unconscious, and salivation, defecation and urination can occur. Generalized epileptic seizures can be subdivided in convulsive when the patient displays motor activity (tonic-clonic, tonic, clonic, myoclonic) or non-convulsive, characterized by a sudden and generalized loss of muscle tone causing a collapse (atonic).
- Focal epileptic seizures evolving into generalized epileptic seizures: when a focal epileptic seizure spreads from the initial regional cerebral involvement to bilateral cerebral involvement, gives rise to the clinical manifestation of a generalized epileptic seizure after a usually brief (seconds to minutes) focal manifestation. Due to its brief nature, the focal onset is usually difficult to identify.

Regardless of the etiology and semiology, an epileptic seizure may be associated with different phases. The seizure per se defined the ictus (seizure activity). After its clinical manifestation, the epileptic seizure is followed by a period of time necessary for the brain correct functioning to be restored called post-ictal phase. This period can last from minutes to hours or days and typical clinical signs of these phase include disorientation, behavioural changes including aggression and compulsive locomotion, blindness, exhaustion and increased hunger and thirst.

In some patients a prodrome may also occur. This can last from hours to days and during this phase patients experience a change in disposition characterized especially by restlessness, anxiety, attention-seeking behavior. These clinical signs need to be differentiated from focal seizures. As a rule, prodromal signs are defined by their long-lasting nature whereas focal seizures can last only from seconds to minutes. When present, this window of time can represent the target for therapeutic options known as pulse treatment (Berendt et al., 2015; De Risio, 2014a).

EPIDEMIOLOGY OF EPILEPTIC SEIZURES IN DOGS

Epileptic seizures have been reported as one of the most common neurological disorders in dogs (Podell et al., 1995), with an estimated prevalence of 0.6-0.75 % in the general dog population (Heske et al., 2014; Kearsley-Fleet et al., 2013). The true prevalence is unknown, with rates that may differ substantially when evaluating different hospital populations, with rates ranging from 0.5 to 5% in non-referral population and from 1 – 2.6% in referral hospital population (Berendt et al., 2015).

In particular, according to Bellumori et al., only <1% of dogs examined at a veterinary teaching hospital was affected by confirmed epilepsy and when only dogs affected by a genetic condition were took into exam, epilepsy prevalence was 2.6% (Bellumori et al., 2013). When a population of neurological referral cases was evaluated, the forebrain appeared to be the most frequent neurological localization affecting 20% of canine patients referred and idiopathic epilepsy accounted for 41% of patients diagnosed with a forebrain disease (Fluehmann et al., 2006). According to a study based on veterinary care claims in Sweden, the incidence rate of epilepsy (including both idiopathic and so-defined “symptomatic” cases), was estimated to be 18 per dogs-years at risk and 0.75% of the entire population (666.249 dogs) studied that had at least one veterinary claim for epilepsy (Heske et al., 2014). When taking into account epilepsy of unknown origin in 92 UK first opinion practices, Kearsley-Fleet and colleagues reported a prevalence of 0.62% (Kearsley-Fleet et al., 2013). When focusing always in the UK first opinion practice, a recent study by Erlen and colleagues reported a 1-year period prevalence of 0.82% for dogs having at least one seizure (Erlen et al., 2018). In Japan, the prevalence of epilepsy has been recently estimated to be approximately 1.9% of all dogs referred to a referral teaching hospital (Hamamoto et al., 2016).

This data suggests that differences in countries (and therefore canine breeds) are not associated with prevalence of epilepsy. Prevalence varies instead when different types of epilepsy both from a semiological or an aethiological point of view are analyzed.

Epilepsy has been associated with a shortened life expectancy, increased risk of developing neuro-behavioral comorbidities and reduced quality of life of both the patients and the owners (Berendt et al., 2015).

CLUSTER SEIZURES (CS) AND STATUS EPILEPTICUS (SE)

Cluster seizures

Definition

According to the consensus statement of the IVETF, the term “cluster” in epilepsy defines an incidence of epileptic seizures within a given period (usually one or a few days) which exceeds the average of incidence over a longer period for the patient. Clinically, CS can be defined as two or more epileptic seizures within a 24-h period (Berendt et al., 2015). Before this consensus statement, definitions of CS have been inconsistent between publications (Patterson, 2014; S. R. Platt, 2014a). In the past, clustering was for example considered as an increase over the patient’s typical seizure frequency, or a typical pattern of distribution of epileptic seizures and therefore a deviation of randomness (S. R. Platt, 2014a).

Pathophysiology

The mechanism of seizure clustering has not been completely understood but of course implies either impaired termination or increased excitation, possibly due to secondary alterations from an initial seizure that promote a second attack, or an excess of seizure promoting factors (Taubøll et al., 1991). Furthermore, seizures occurring within 8 hours of a prior epileptic seizure have been reported to be significantly more likely to arise from the same epileptic focus than seizure separated by a longer period of time (Haut et al., 1997). These data supported in the past the establishment of the concept of CS.

Clinical features

Clinical patterns of CS are the same reported for single epileptic seizures.

Occurrence and prevalence

The prevalence of CS occurrence during epilepsy varies widely between studies and comparisons are difficult because of the different definitions used in the past. Data available in veterinary medicine for canine patients are limited. In a study including 407 dogs with idiopathic epilepsy, 41% experienced CS at least once during their epilepsy history, regardless of the specific breed (Monteiro et al., 2012). A more recent study reported a prevalence of CS of 49.1% among 384 patients included in the publications (Packer et al., 2016). In a recent prospective investigation of newly diagnosed epileptic dogs, the evaluation of the course of the disease highlighted the occurrence of CS in 30% of patients affected by idiopathic epilepsy and 95% of patients affected by structural epilepsy (Fredsø et al., 2017).

While in human medicine a significant association between occurrence of CS and SE has been detected (Haut et al., 2005), this correspondence has not been evaluated in canine patients (Monteiro et al., 2012; Miyoko Saito et al., 2001). Instead, German Shepherds and boxers were found to be significantly more likely to suffer from CS (Monteiro et al., 2012).

In people, previous history of head trauma, degree of seizure control and hormonal asset have been reported to be associated with the occurrence of CS (Haut et al., 2005; Scharfman and MacLusky,

2006; Velíšková and DeSantis, 2013). In canine patients none of these variables have been specifically associated with the risk of CS or studied in depth (Friedenberg et al., 2012; Van Meervenne et al., 2015), but still a certain degree of hormonal influence has been reported in female dogs (Van Meervenne et al., 2015). To date, only gender and neutered status have been reported to associated to CS occurrence, with intact dogs being 1.4 times more likely than neutered dogs to develop CS and intact male dogs being twice as likely as neutered males to suffer from CS (Monteiro et al., 2012).

When looking at specific breeds affected by idiopathic epilepsy, CS occurrence varies considerably. Border Collies, Australian Shepherds and Dalmatians appear to be the canine breeds more prone to develop CS (and SE) during epilepsy, with percentage of 49%, 48% and 63.6%, respectively (Hülsmeyer et al., 2010; Licht et al., 2002; Weissl et al., 2012). Other specific breeds affected by epilepsy that are reported to develop CS (sometimes in association with SE) in variable percentage are Belgian Sheperd, English Springer Spaniel, Finnish Spitz, Standard poodle, Italian Spinone and Rottweiller (L. De Risio et al., 2015; Gulløv et al., 2012; Heske et al., 2015; Licht et al., 2002; Patterson et al., 2005; Seppälä et al., 2012; Viitmaa et al., 2013).

Therapeutic considerations

In human medicine the use of benzodiazepines, either rectally, orally, intranasally or intravenously and intramuscularly represent the approved mainstay of CS emergency treatment (Haut, 2015; Jafarpour et al., 2018).

Because of the lack of consistent definitions of CS for companion animals, and lack of published information, there are no clear-cut recommendations that can be unequivocally made for monitoring and treatment of CS (Patterson, 2014). To date, according to the latest guidelines on epilepsy treatment in veterinary patients, along with the establishment of an emergency treatment, a long term anti-epileptic treatment should be started in case of CS occurrence (Podell et al., 2016).

Emergency treatment basically consist of benzodiazepines administration because fast-acting and effective (Patterson, 2014; S. R. Platt, 2014a). Rectal or intranasal routes of administration are usually preferred to intravenous/intramuscular injections when given in a domestic environment or in a in-hospital setting when a venous access has not been already established during an emergency situation (S. R. Platt, 2014a). To date, a single study has been focused on the out-of-hospital treatment of single epileptic seizures in dogs and this was based on the rectal administration of diazepam (Podell, 1995).

Status epilepticus

Definition

SE has historically been defined as a seizure activity lasting for at least 30 minutes (time needed to cause a permanent brain damage) (Patterson, 2014). Since this definition is clinically unacceptable, the IVETF has defined the SE as an epileptic seizure which shows no clinical signs of arresting after a duration encompassing the great majority of seizures of that type in most patients or recurrent epileptic seizures without resumption of baseline CNS function interictally. Clinically, it has a double definition: either SE represents a continuous seizure activity greater than 5 minutes or, in case of generalized epileptic seizures, two or more epileptic seizures between which there is an only a partial recovery of the state of consciousness (Berendt et al., 2015).

Pathophysiology

As for CS, the basic pathophysiological mechanisms of SE involve failure of seizure termination processes due to excessive excitation or insufficient inhibition. Recently, it has been postulated that the failure of inhibition may be caused by a shift in the functional properties of GABA receptors after prolonged seizure activity (Goodkin et al., 2005). Furthermore, repetitive and continuous neuronal firing increase the metabolic demand, which is exacerbated by glutamate-mediated excitotoxicity and decrease GABA inhibitory neurotransmission. Chronic seizures can alter neuronal and glial expression of glutamate receptors and uptake transporters, further contributing to epileptogenesis During SE, many molecular triggers activate neuronal membrane receptors. The NMDA receptors are one of those and in several animal models, NMDA receptors antagonists have been shown to contrast seizure activity. Excessive concentrations of glutamate can activate NMDA receptors, causing the opening of Ca^{2+} ion channels and therefore a cascade of intracellular chemical events that eventually terminate in cell death (Barker-Haliski and Steve White, 2015; Cho, 2013).

Pyramidal cells of the hippocampus and the amygdala are particularly sensitive to neuronal necrosis during SE, as shown in experimental studies. Both of these regions are rich in GABA and therefore their destruction can predispose to further seizure activity.

The increased metabolic demand of the brain cells during seizure activity can be initially satisfied by compensatory factors, but after 30 minutes of continuous seizure activity these compensatory factors cannot be enough and brain damage occur. Hyperthermia, hypoxia and hypotension related to SE can further worsen the neuronal damage as well.

Clinical features

As for single epileptic seizures, CS and SE may exhibit different clinical patterns by being convulsive or non-convulsive, as previously described. Non convulsive status epilepticus, defined as a prolonged seizure activity seen only on electroencephalography without evident clinical signs, may be difficult to recognize in veterinary patients and to date only few retrospective case series have been performed to further characterize this particular clinical condition (Granum et al., 2019; Raith et al., 2010). Recently, the prevalence of electrographic SE, defined as seizure activity detectable

by electroencephalography lasting more than 10 minutes, has been reported in 12% of patients during a six-year period. According to this retrospective study, the mortality rate was higher in patients affected by non-convulsive SE, compared to non-affected patients (50% vs 19%) and risk factors for electrographic SE included young age, seizure activity within 8 hours before the electroencephalographic monitoring and history of CS (Granum et al., 2019).

Furthermore, if during SE, recurrent convulsions persist without or with inadequate treatment, a progressive decrease of convulsive seizure activity may be seen, and motor manifestations of SE become increasingly subtle and difficult to evaluate. At this point, patients may appear stuporous or even comatose, exhibiting only subtle twitches of the body (usually the extremities) (S. R. Platt, 2014b).

Along with the manifestation of seizure activity, severe systemic changes occur during SE. These life-threatening alterations include hyperthermia, hypertension, tachycardia and cardiac arrythmias, hypoglycemia and acidosis. The initial physiologic response during SE is a massive release of catecholamines resulting in increased blood pressure, heart rate, plasma glucose concentration and increase incidence of cardiac arrythmias. These changes result also in a consistent increased cerebral blood flow (200% to 700%) that has the function to supply to the increased metabolic demand of neuronal cells. With persistent (usually more than 30 minutes) seizure activity though the brain metabolic rate remains high while compensatory mechanisms are no more able to maintain increase cerebral blood flow due to exhaustion leading to neuronal cell death. This leads to increased intracranial pressure that can potentially cause cerebral herniation. Ventilation is usually impaired during seizure activity leading to hypoventilation. Also, autonomic dysfunction can also occur causing hypersalivation and increase in tracheobronchial secretions. All these abnormalities can lead to hypoxia that further worsen the condition of neuronal cells causing cerebral lactic acidosis and hypotension. The continuous muscle contraction caused by seizure can also cause increase in body temperature and subsequent rhabdomyolysis and myoglobinuria that can further compromise renal function along with hypotension and acidosis (Hawkes and Hocker, 2018; Walton, 1993).

Occurrence and prevalence

Epidemiologic studies on SE in dogs are limited. A 1999 retrospective study revealed a 0.44% prevalence of SE in a referral hospital. The cause of seizures was found to be primary or genetic epilepsy in 26.8% of cases, secondary epilepsy in 35.1% of cases, reactive in 6.7%, undetermined in 25.8%, and related to low concentrations of antiepileptic drugs (AEDs) in 5.7% (Bateman and Parent, 1999). According to the results of the study performed by Saito and colleagues, approximately 59% of epileptic dogs would experience one or more episodes of SE during their lifetime (Saito et al., 2001). SE can represent a clinical feature of any etiological type of epilepsy. Another retrospective study found that SE was more frequently associated with structural-metabolic epilepsy than with genetic/unknown epilepsy (Platt and Haag, 2002). This association was confirmed by the retrospective study of Zimmermann et al. in 2009 in which a higher risk for SE was found in

dogs that experienced seizures secondary to toxin exposure. Structural brain disease was a common cause of SE in dogs older than 5 years, and these dogs had a lower probability of survival compared to dogs with genetic epilepsy and metabolic epileptic seizures (Zimmermann et al., 2009). A 2012 retrospective study of dogs with idiopathic epilepsy showed a high prevalence of cluster seizures, but a low prevalence of SE (Monteiro et al., 2012). This finding has not been confirmed in another study, were canine patients with idiopathic epilepsy showed a high prevalence of SE (M Saito et al., 2001). The occurrence of SE during idiopathic epilepsy is also influenced by the specific canine breed: in a study focusing of idiopathic epilepsy in Australian Shepherd, SE has been reported in 12% of cases, while SE occurred only in 4% of patients in a study evaluating idiopathic epilepsy in Border Collies (Hülsmeyer et al., 2010; Weissl et al., 2012). No gender prevalence has been documented in dogs with SE.

The mortality rate associated with canine SE varies between studies and has been reported to be 23 – 38% (Bateman and Parent, 1999; M Saito et al., 2001; Zimmermann et al., 2009). These rates represent the mortality of SE associated with euthanasia. True mortality rate due to spontaneous death are lower considerably lower (2 – 5%) (Patterson, 2014).

SE following intoxication have a good prognosis (up to 85% chances of survival) and usually no seizures are recorder during long term follow up (Brauer et al., 2011; Jull et al., 2011; Zimmermann et al., 2009). Conversely, dogs with idiopathic epilepsy experiencing SE tend to have a worse prognosis compare to other idiopathic epileptic patients (Arrol et al., 2012).

A poor prognosis has also been reported in case of SE during structural epilepsy and after 6 hours of continuous seizure activity.

TREATMENT OF CS AND SE

CS and especially SE represent two important neurological emergencies and treatment has to begin even before a diagnostic evaluation of the epileptic patient. The initial management of these conditions should be based on basic principles of life support and AEDs administration to stop seizures. Medical and neurological examinations should be performed serially and recorded to detect signs of improvement or deterioration of patients (Haley and Platt, 2012).

Systemic stabilization

As in any emergency situation, the *"ABC principle*" (airway, breathing and circulation) should be assessed first. An intravenous catheter should be placed as soon as possible in epileptic patients for fluid therapy and AEDs administration. Appropriate oxygenation and ventilation should be ensured. Hypoxia and respiratory acidosis (detected by arterial blood gas analysis) may emerge from hypoventilation and should be addressed and treated immediately because may precipitate SE. Normal blood oxygenation help preventing intracranial hypertension since hypoxemia (PaO_2 < 50 mm Hg) causes increased cerebral blood flow and increased intracranial pressure (ICP). Airway management and respiratory monitoring may be difficult before termination of seizure activity, but if

possible, the airway patency should be checked while the patient is unconscious in order to avoid hypoxia. The administration of 100% oxygen by means of a mask or a nasal tube is recommended in these patients. If spontaneous breathing is not detected or ventilation is not adequate, then intubation and mechanical ventilation are necessary. Respiratory acidosis should also be differentiated from metabolic acidosis, that usually resolves once the seizure activity stops and treatment is based only on adequate fluid therapy administration.

Hypoglycemia may be detected in patients affected by SE and may represent either a cause or a consequence of it. For this reason, low glucose serum concentration should be promptly corrected with the administration of dextrose that can be added to intravenous fluids (to make a 2.5% or 5% solution based on patients need).

Hypertension is typical of early phases of SE due to seizure activity and therefore treatment is directed at cessation of it, while hypotension usually occurs as SE progresses and can be worsened by AEDs administration.

Hypotension and hypovolemia are usually treated with fluid (crystalloids) boluses. Hypotension should be especially avoided because since cerebral perfusion pressure (CPP) depends on both mean systemic arterial pressure (MAP) and ICP (CPP = MAP – ICP), increased ICP or reduced MAP can impair it. Through autoregulation, the brain is able to maintain normal cerebral blood flow with CPP ranging from 50 to 150 mm Hg; however, during SE autoregulation may be impaired, potentially compromising CPP if either systemic blood pressure is reduced. Hypovolemia should be avoided since it contributes to hypotension and hypoperfusion. Low-volume resuscitation may be beneficial in patients that require resuscitation and have increased ICP and cerebral edema.

Systemic hypertension can also cause increase in ICP. Since CPP = MAP – ICP, patients with increased ICP develop increased MAP to maintain CPP. Reflex bradychardia is usually associated with this hypertensive condition and this complex of clinical signs is usually known as *Cushing's reflex.*

In case of clinical signs suggestive of increased ICP, hyperosmolar therapy should be instituted. Mannitol is an osmotic diuretic that increases the osmotic gradient between intravascular and extravascular fluid compartments, causing fluid to move out of the tissues into the blood for a mean period of 3.5 hours and up to 8 hours. The increase in blood volume also contributes to the decrease ICP due to the dilutional effects and resultant decrease in blood viscosity. Mannitol may also act as free radical scavenger, minimizing the oxidative injury to the brain. It has to be administered at a dose of 0.5 – 2 g/kg intravenously over 20 – 30 minutes and effect appear within 30 minutes from the administration.

Hyperthermia can occur frequently during SE and can be life-threatening if exceeds 40°C. In this case, passive cooling should be promptly performed.

Indwelling urinary collection system should be placed for hygienic reasons and to allow precise urine production.

(Blades Golubovic and Rossmeisl, 2017a; Haley and Platt, 2012; Patterson, 2014; S. R. Platt, 2014b)

Management of patients affected by CS and SE

Furthermore, a patient affected by CS and especially SE, due to the conditions themselves or to AEDs received, can be recumbent and necessitate a specific management, beyond the monitoring of vital parameters. Such patients must in fact be kept in a well-padded cage, kept dry and clean by means of bends, slings or hammocks and mattresses. They should be turned every 4-6 hours to prevent pressure sores at the level of bony protuberances. If detected, these should be clipped, cleaned and dried and padded with doughnut bandages to prevent worsening. In order to prevent corneal ulceration, eyes should be lubricated with artificial tears every 4 hours. The positioning of an indwelling urinary catheter can help monitoring urine production and therefore renal function but can also help keeping clean and dry the patient during the hospitalization.

When epileptic patients are unable to eat and drink spontaneously, a naso-oesophagenal or naso-gastric feeding tube should be placed to assure adequate nutrition and hydration. If conscious and able to swallow properly, they should be offered food and water while in sternal recumbency and kept in the same position for at least 5 minutes after eating and drinking to avoid aspiration.

Body temperature should be checked frequently, and heat support or cooling should be provided depending on needs.

(Haley and Platt, 2012; S. R. Platt, 2014b)

Cessation of seizure activity

Together with the systemic stabilization of the patient, seizure activity should be address and stopped as soon as possible after presentation. The goal of an anti-epileptic therapy in these patients is to stop seizure activity (ideally both clinical and electrical) and prevent recurrences. Several AEDs have been proved to be effective in the treatment of CS and SE, but no single AED is ideal. Usually, the choice is made taking into account the timing and ease of administration, the time of onset and duration of the effect and related side-effects (especially cardio-respiratory depression) (S. R. Platt, 2014b).

To date, current treatment recommendations for CS and SE in canine patients are based on clinical experience and guidelines taken from human medicine (Patterson, 2014). Several algorithms for the antiepileptic treatment in case of CS and SE have been proposed. One of the most recent, proposed by De Risio and Platt is reported in Figure 11.

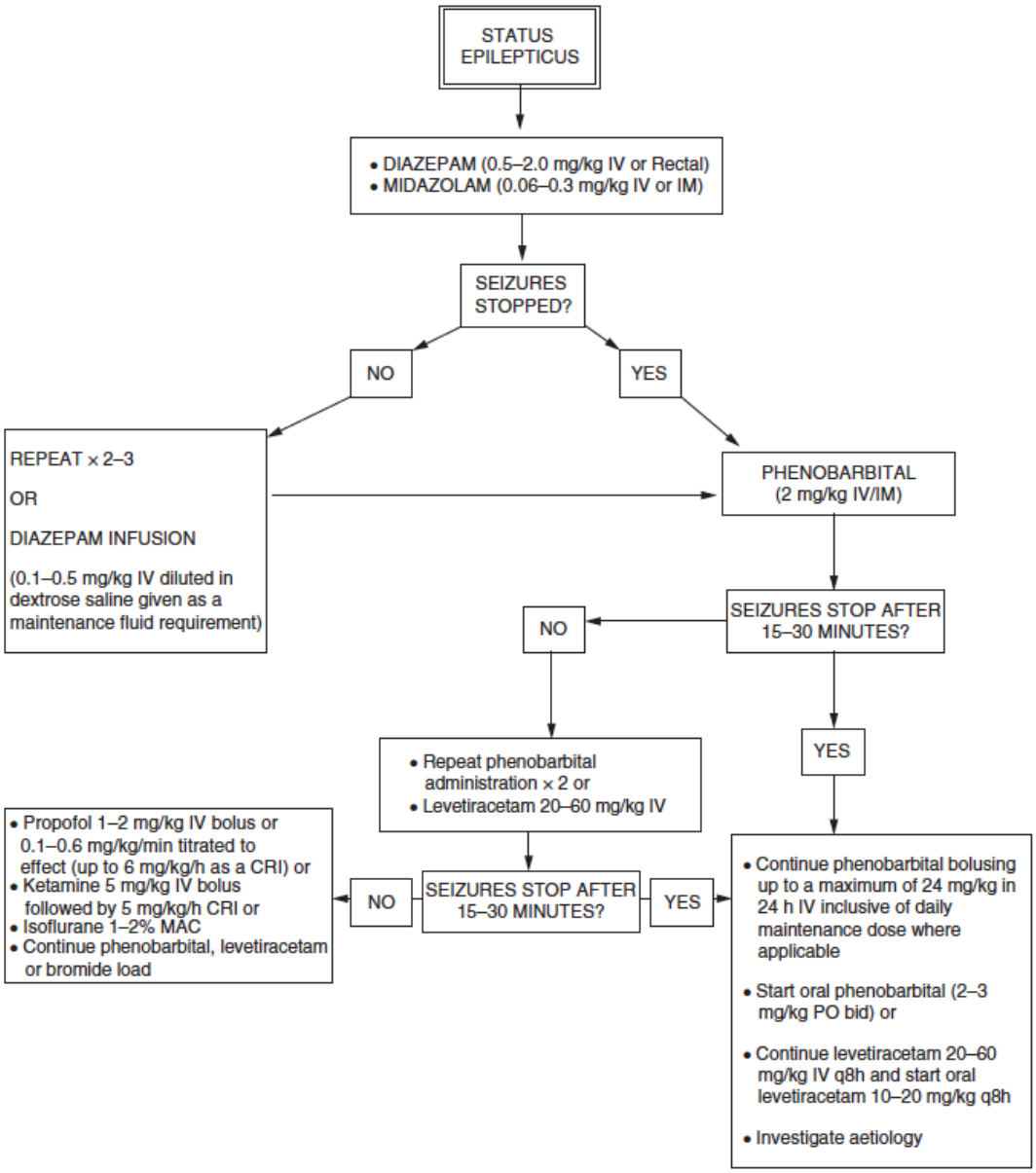

Figure 11. Proposed algorithm for the antiepileptic treatment of SE in canine patients *(from: Canine and Feline Epilepsy, De Risio and Platt, 2014)*

PHARMACOLOGICAL TREATMENT OF CS AND SE

Benzodiazepines

Benzodiazepines are a class of psychoactive medications classified as minor tranquilizers with sedative-hypnotic properties.

The first molecule to be discovered was chlordiazepoxide in 1955, made available a few years later in 1960 by Hoffmann-La Roche as diazepam, a simplified version of chlordiazepoxide. The basic chemical structure of benzodiazepines is formed from the fusion of a benzene ring and a seven-membered diazepine ring (Papich, 2018).

The lipophilicity of these compounds determines their rapid penetration across the blood-brain barrier following intravenous (IV) administration.

Benzodiazepines bind to the benzodiazepine receptor binding site on the gamma subunit of the $GABA_A$ receptors. The activation of the benzodiazepine receptors enhances the effect of GABA on the $GABA_A$ receptors. This GABA-agonism leads to a neuronal influx of Cl^- due to an increased frequency of Cl^- ion channels opening, causing hyperpolarization of postsynaptic neurons. Benzodiazepines therefore do not alter synthesis, release or metabolism of GABA, but they only potentiate its action at the receptor sites. At higher doses, benzodiazepines also limit sustained repetitive neuronal firing, preventing the spread of seizure activity rather than suppressing the epileptic focus. A heterogeneity in $GABA_A$ subunits has been detected, causing different clinical actions and pharmacokinetics of benzodiazepines. Thus, some benzodiazepines are more effective than others as anticonvulsants. The highest concentration of $GABA_A$ receptors is found in the cerebral cortex, with very few receptor sites outside the CNS, hence the minimal cardiopulmonary effects of benzodiazepines. $GABA_A$ is a large macromolecule, which also contains a number of binding sites for other sedative drug classes such as barbiturates. This explains the synergistic effect of these drugs on $GABA_A$-mediated inhibition of the CNS (Papich, 2018; S. R. Platt, 2014c).

The two most commonly benzodiazepines used as AEDs in veterinary neurology are diazepam and midazolam.

<u>Diazepam</u>

It is the first drug of choice for the emergency treatment of CS and SE in dogs and cats, except in case of concomitant hepatic disfunction. Usually the intravenous or rectal administration is preferred during these circumstances. Therapeutic concentrations of diazepam are reported to be between 150 and 300 ng/ml. Reported dosages are 0.5 – 2 mg/kg (up to 20 mg as maximum dose) (Blades Golubovic and Rossmeisl, 2017a; S. R. Platt, 2014c). Mean peak plasma concentration are reached rapidly in less than 2 minutes when given IV. Rectal administration is a good alternative to the IV route of administration with an adequate absorption and peak plasma concentration reached within 15 minutes. Based on a pharmacokinetic study performed in seizuring dogs untreated with other medications, the administration of 1 mg/kg rectally resulted in a mean time to peak plasma

concentration of 14.3 min and peak diazepam concentration of 474 mcg/l (Podell, 1996). Recently, the administration of diazepam per rectum via compounded suppositories at a dosage of 2 mg/kg was evaluated and did not resulted in therapeutic plasma concentrations in a clinically useful interval (Probst et al., 2013). Reasons for the variable clinical efficacy reported after rectal diazepam administration include: unsuccessful delivery because of presence of fecal material, expulsion of drug, unpredictable absorption and extensive first-pass hepatic metabolism (S. R. Platt, 2014c).

Also, intranasal administration of diazepam has been investigated. Following a dosage of 0.5 mg/kg, mean peak plasma concentration was 448 $\pm$ 41 ng/ml at 4.5 min, compared with 1316 $\pm$ 216 at 3 min obtain with the IV route of administration. Maximum concentrations (C_{max}) were achieved in 10 min after nasal administration and exceeded the recommended therapeutic concentration (Platt et al., 2000). Following studies confirmed these findings (Musulin et al., 2011). However, even if intranasal administration can represent a valid therapeutic option in seizuring dogs, the absence of a commercially available high concentration preparation limits its use in practice.

It is metabolized in the liver by hepatic microsomal system in nordiazepam and oxazepam. Other metabolites with unknown antiepileptic properties are 3-hydroxydiazepam and temazepam. In particular, diazepam is metabolized in nordiazepam by cytochrome P2B11 and in oxazepam by cytochrome P3A12. These major metabolites have 33% of the activity compared to diazepam. For this reason, since phenobarbital (PB) is a potent inducer of cytochrome P450 system, patients under long-term seizure control with this molecule should receive a higher dosage of diazepam (2 mg/kg) in case of seizures (Wagner et al., 1998).

In dogs the elimination half-life ($T_{1/2}$) of diazepam and its metabolites following IV administration is short (0.25 hours and 2.5 to 5.2h, respectively) and for this reason it is not suitable for long-term seizure control in epileptic patients.

To date, human trials support diazepam as a first-choice AED for managing CS and SE. No such data are available in veterinary medicine, but diazepam still represents an established first-line treatment for these neurological emergencies. In 1995 Podell firstly evaluated the use of rectal diazepam at a dosage of 0.5 mg/kg for the at home management of CS in 11 dogs affected by idiopathic epilepsy over a 16-month period. Patients included were all under long-term seizure control with PB and one of them with PB and KBr (potassium bromide) in combination. Results demonstrated a significant decrease in the total number of single seizures and cluster seizures and in the average number of seizures per CS and the total number of single seizure events occurred before and after the administration of rectal diazepam (Podell, 1995).

Boluses of diazepam can be repeated 2 – 3 times (not exceeding 20 mg as a total dose). If boluses are not enough to control seizure activity, then an IV constant rate infusion (CRI) at a rate of 0.1 – 0.5 mg/kg/h should be considered. Care should be taken in order to protect the administration set from light and change it every 2 hours because of denaturation of diazepam in plastic containers.

Since in human medicine the administration of single injections of IV diazepam have been associated with seizure relapse within 2 hours, it has been hypothesized that multiple injections or CRI can result in better seizure control. However, this can lead to drug accumulation and more profound respiratory depression, sedation, hypotension and development of tolerance for infusions lasting more than 24 h (S. R. Platt, 2014c).

Midazolam

Midazolam acts in the same manner as other benzodiazepines and it is a more recently developed water-soluble benzodiazepine. At physiologic pH it becomes extremely lipophilic, determining a rapid onset of action. Reported dosages varies from 0.07 to 0.2 mg/kg given IV or intramuscularly (IM). Minimum therapeutic level reported in human medicine is 40 ng/ml.

Peak plasma concentrations after IM administration are reached within 15 minutes. Unlike diazepam, midazolam rectal administration resulted in erratic systemic availability with low plasma concentrations in one study. In another study, recta administration of parenteral midazolam solution achieved reported human therapeutic concentrations but with prolonged time to C_{max} (39 ± 14.49 mins). For these reasons, to date rectal diazepam is considered superior to rectal midazolam in seizuring patients (S. R. Platt, 2014c).

The intranasal administration of midazolam has also been investigated and reported to achieve the minimum therapeutic range within 15 mins, however the dosage given was not reported (Lui et al., 1991). the pharmacokinetics of midazolam gel (4% hydroxypropyl methylcellulose) given intranasally has also been investigated, showing a rapid absorption and a superior profile compared to the parenteral formulation (Eagleson et al., 2012). More recently, the pharmacokinetics of a midazolam gel formulation was also evaluated after oral administration and results indicated that buccal administration of gel formulation might be a viable alternative for midazolam administration in dogs (Aldawsari et al., 2018).

Midazolam undergoes hepatic metabolism by microsomal oxidation followed by glucuronide conjugation like other benzodiazepines. Alpha-hydroxy-midazolam represents the primary metabolite and has sedative properties being pharmacologically active.

In contrast to humans, in dogs the predominates the extrarenal excretion of this drug, probably through the bile. The elimination half-life following IV administration in dogs has been reported between 53 and 77 mins (Papich, 2018).

Clinical experience with midazolam for treating CS and SE in veterinary medicine is limited. In several human clinical trials, the efficacy of intranasal midazolam was reported to be similar to or better than that of IV or rectal diazepam. Only recently, a study comparing the clinical efficacy of intranasal midazolam versus rectal diazepam in 20 canine patients affected by SE was published, reporting a superior efficacy of midazolam compared to diazepam in terminating SE. In particular, intranasal midazolam terminated SE in 70% of patients, while rectal diazepam was successful in

only 20% of cases (Charalambous et al., 2017). More recently, the efficacy of intranasal and IV midazolam administration in dogs affected by SE was evaluated. Results of the study reported both route of administration as quick, safe and effective in controlling SE, however, the intranasal route demonstrated superiority when the time needed to place an IV catheter was taken into account (Charalambous et al., 2019)

The higher efficacy of intranasal midazolam can be due to the rapid achievement of therapeutic cerebral concentration by this route of administration because of the highly vascular nature of the turbinates and the avoidance of the first-pass metabolism associated with the intravenous administration (Haley and Platt, 2012).

Adverse effects

Common adverse effects of benzodiazepines include respiratory depression, hypotension and impaired consciousness (Papich, 2018). Paradoxical excitement/hyperactivity has been sometimes associated with benzodiazepines administration in both dogs and cats (S. R. Platt, 2014c). Diazepam has also been specifically associated with hepatotoxicity, causing hepatic necrosis, when given orally in cats. The toxicity has been suggested to be idiosyncratic as it is not experimentally reproducible and not associated with dose and duration of administration (Center et al., 1996).

The intranasal route of administration has been associated with transient irritation of the nasal mucosa.

No controlled trials evaluating adverse events associated with different benzodiazepines have been published in veterinary medicine. It is thought that diazepam may have a greater effect on respiratory depression compared to midazolam, even though the incidence of respiratory depression following benzodiazepines administration is considered low because of low density of binding sites in the brainstem (S. R. Platt, 2014c).

Both in human and veterinary medicine a “withdrawal syndrome” associated with physical dependence to benzodiazepines has been reported. In dogs the syndrome included generalized tonic-clonic seizures 24h after the discontinuation of benzodiazepines administration (increasing doses every 8h for 5 – 6 weeks) and the intensity increased proportionately with the dose and plasma and brain concentrations of diazepam (Sloan et al., 1993).

Tolerance

One of the main therapeutic concern associated with benzodiazepines administration is the development of tolerance, causing concurrent progressive increase in the number and severity of seizures. This issue represents one of the main reasons why benzodiazepines are generally considered unsuitable for long-term seizure control.

Despite decades of research, the understanding of how benzodiazepines tend to lose their efficacy over time is at least incomplete. Furthermore, there is a considerable variance in the published data

due to the application of different methodologies, species, treatment regimens, and benzodiazepines that makes comparisons difficult.

Neuroadaptive mechanisms underlying benzodiazepine tolerance are an example of the result of neuronal plasticity. $GABA_A$ receptors are composed by different subunits, with the most common receptor subtype being composed of two α, two β, and one γ subunit. In situ hybridization and immunohistochemical studies have shown that $GABA_A$ receptor subunits display a distinct CNS distribution with a differential cellular localization pattern, suggesting that $GABA_A$ receptor subunits have a specialized function. Of the GABAergic subunits, α1, β1, β2, β3, and γ2 subunits are found throughout the brain and the exact binding site of benzodiazepines at the $GABA_A$ receptor is located between the α and γ subunit.

Several mechanisms underlying benzodiazepines tolerance have been proposed over the last decades. The general assumption is that chronic benzodiazepine use leads to compensating changes in the CNS. This way, the $GABA_A$ receptor may become less responsive to the continuing acute effects of benzodiazepines, either as a result of adaptations in the $GABA_A$ receptor itself, intracellular mechanisms, or changes in other neurotransmitter systems, such as the glutamatergic system:

$GABA_A$ System Hypotheses

- $GABA_A$ receptor uncoupling: the $GABA_A$ receptor contains two GABA-binding sites and one benzodiazepine-binding site that are allosterically coupled, that is, binding to the benzodiazepine-binding site potentiates binding of GABA to the GABA-binding site. Benzodiazepines are generally referred to as positive allosteric modulators because their binding alters the $GABA_A$ receptor conformation with an increased capacity to bind GABA, leading to increased channel opening frequency, increased chloride influx, and, consequently, to hyperpolarization. $GABA_A$ receptor uncoupling is defined as a decreased ability of benzodiazepines to enhance GABA-induced inhibitory post-synaptic potentials at the $GABA_A$ receptor. It has been therefore hypothesized that chronic treatment affects the benzodiazepines' capacity to pharmacologically enhance the GABA response (i.e., tolerance leads to uncoupling). A decreased coupling may develop as a result of changed $GABA_A$ receptor subunit composition, alterations to the $GABA_A$ receptor itself or its second messenger ligands, or any process affecting the conformational state of the $GABA_A$ receptor.
- Alterations in $GABA_A$ receptor subunit expression: caused by a general downregulation of $GABA_A$ receptors throughout the brain.

Glutamate System Hypotheses

Chronic and/or increased activation of the GABAergic system during benzodiazepine treatment may pertubate glutamatergic transmission. The basis of benzodiazepine tolerance could then lie in sensitization of the glutamatergic system, a putative process that could account for the withdrawal symptoms after chronic benzodiazepine discontinuation.

In rodents, the development of tolerance to the effects of the benzodiazepines diazepam and chlordiazepoxide was prevented by coadministration of the NMDA receptor antagonists. These data suggest therefore that NMDA-dependent mechanisms contribute to the development of benzodiazepine tolerance. However, the evidence does not support a universal and replicable glutamatergic component, also because molecular data are diverse and sometimes inconsistent. There are, however, indications that NMDA receptor blockade can prevent tolerance to at least some benzodiazepine effects (S. R. Platt, 2014c).

Other proposed mechanisms involved in the development of benzodiazepines tolerance include the role of transcriptional and neurotrophic factors, serotonin dopamine and acetylcholine systems and neurosteroids:

- Neurotrophic factors: neurotrophic factors that have discovered so far include brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4, and nerve growth factor (NGF). Since they act as potent factors in regulating fast synaptic inhibition, adaptations leading to tolerance following chronic benzodiazepine treatment could in part be mediated via these neurotrophic factors. In support, brain-derived neurotrophic factor and neurotrophin-4 were found to acutely reduce postsynaptic $GABA_A$ receptor immunoreactivity This reduced immunoreactivity was hypothesized to be caused by a reduction in $GABA_A$ receptor surface expression.
- Serotonin, dopamine and acethylcoline systems: even if studies on this particular topic are scant, there is evidence that these receptors systems can modulate the $GABA_A$ receptor functionality.
- Neurosteroids: acute or chronic neurosteroid treatment may change $GABA_A$ receptor subunit expression. In light of the plasticity-inducing actions of neurosteroids on inhibitory signaling, long-term enhancement of the GABA system with benzodiazepines may in turn evoke changes in the neurosteroids system such as changes in neurosteroid synthesis and metabolism, although classical benzodiazepines may differ in their potency to cause such changes.

Furthermore, clinical experience accumulated over the course of decades suggests that prolonged seizures and SE are more difficult to control than brief seizure episodes and that patients experiencing SE are more likely to develop resistance to first line antiseizure drugs (i.e. benzodiazepines) and require second- and third-line interventions. The more accounted explanation for this reduced benzodiazepine efficacy has been reported to be a reduction in the number of benzodiazepine receptors. These elements account then for a role of the duration of seizure activity in the development of benzodiazepines tolerance (Deeb et al., 2012).

In conclusion, a considerable variance in the published data on benzodiazepines tolerance mechanisms exist. Altogether, it appears that none of the proposed putative mechanisms can sufficiently explain tolerance development. Thus, multiple mechanisms may (synergistically) coexist, or an additional yet undiscovered mechanism may be present.
(Löscher et al., 1996; Loscher and Schmidt, 2006; Rundfeldt et al., 1995; Vinkers and Olivier, 2012)

Phenobarbital (PB)

Phenobarbital (5-ethyl-5-phenylbarbituric acid) is a phenyl barbiturate which has the longest history as AED for seizure control. It can be administered orally, IV or IM It is currently considered the first-choice AED both in dogs and cats, due to its pharmacokinetic profile, relative safety, affordable cost, efficacy and greater data from veterinary studies compared to other AEDs (De Risio, 2014b).

Mechanism of action

Phenobarbital act by binding the $GABA_A$ receptors, both directly activating GABA receptor-gated Cl^- channels and increasing the affinity of GABA for its own receptor by allosteric effect. These mechanism result in a prolonged opening of Cl^- channels and an increased influx of Cl^- into the cell, causing hyperpolarization and thereby increasing seizure threshold and decreasing the spread of discharge to surrounding neurons by creating an inhibitory postsynaptic potential. PB also interacts with glutamate AMPA receptors decreasing neuronal excitatory postsynaptic currents and decreases the conductance of Ca^{2+} across the cell membrane by inhibiting the voltage-gated Ca^{2+} channel. This molecule has not a specific action of neurons of epileptic foci, but act on all neural cells of the brain, affecting normal synaptic transmission. In conclusion, PB increases the seizure threshold and decreases the spread of discharge to surrounding neurons (Papich, 2018).

PB represents the mainstay of treatment for convulsive SE both in human and veterinary medicine. Due to its relatively slow distribution to the CNS, which may take 20 – 30 minutes due to its decreased lipophilicity, PB is not suitable as a first-line AED to immediately stop the seizure activity. The recommended loading dose in naïve dogs is 12 -24 mg/kg IV in order to achieve therapeutic concentrations rapidly, but these dosages have been associated with profound hypotension and cardio-respiratory depression. In order to minimize these severe side effects, the total dose can be divided in several daily dosages to a maximum total 24h dose of 24 mg/kg. In this regard, several protocols have been proposed. Also, the intramuscular or oral administration of the loading dose has been proposed in order to reduce the severity of the reported side effects.

Pharmacokinetics

Phenobarbital bioavailability ranges between 86 – 97% and it is absorbed within 2 hours after oral administration from the gastro-intestinal tract. For this reason, disturbances of the correct functioning of this tract or presence of food may cause delay in the absorption.

The percentage of protein bound is around 45% and the peak concentration after oral assumption is reached in 4 hours. The volume of distribution (V_d) after a single administration is 0.7 ± 0.15 l/kg in dogs and 0.77 ± 0.02 l/kg in cats. The steady state of this drug is achieved in 10 - 20 days in dogs. Phenobarbital has a hepatic metabolism mediated primarily by hepatic microsomal enzymes (of which it is a potent inducer), and only 25% of the drug is excreted unchanged with urines. The elimination half-life ($T_{1/2}$) after a single oral dosage of 5 mg/kg has been reported as 46.3 ± 11.3 h or 88.7 ± 19.6 h in dogs.

In dogs, PB induces the hepatic microsomal cytochrome P450 enzymes (subfamilies 1A, 2B, 2C and 3A), causing an increased clearance of hepatically metabolized medications in epileptic patients chronically treated with PB. As a result, in most dogs treated with PB, the clearance of the drug increases and elimination half-life decreases progressively. This can lead to a therapeutic failure, due to progressive reduction of PB serum concentrations and significant changes in peak and though concentrations during a dosing interval.

PB metabolism can also be affected by diet and urine pH. In particular, low-protein and low-fat diets lead to a more rapid elimination of PB and urine alkalinization increases renal excretion of PB.

Pharmacokinetics interaction

The two main mechanisms of pharmacokinetics interaction between PB and other medications or endogenous substances are the induction of the cytochrome P450 enzymes (influencing medications metabolized by the same enzymes) and the increase in the concentration of the plasma α1-acid glycoprotein (a plasma protein involved in binding and transport several molecules), that affects the unbound fraction of other concurrently administered medications.

In particular, PB can interact with other specific AEDs:

- Benzodiazepines: the increased clearance and decreased plasma concentrations during concurrent administration of PB are attributed to PB induction of cytochrome P450 enzymes.
- Levetiracetam: the increased disposition of levetiracetam is caused by PB induction of exidative enzymes.
- Zonisamide: both induction of cytochrome P450 enzymes and plasma α1-acid glycoprotein concentration play a role in the in the reduction of maximum serum concentration, apparent elimination half-life and bioavailability and in the increased clearance of the drug.

Conversely, the concurrent administration of cytochrome P450 enzymes inhibitors and PB can cause inhibition of PB metabolism, resulting in increased serum concentration and possibly toxicity.

Common molecules included in this category are: cimetidine, omeprazole, lansoprazole, chloramphenicol, trimethoprim, fluoroquinolones, tetracycline, ketoconazole, fluconazole, itraconazole, fluoxetine, felbamate and topiramate.

Other common molecules that can be affected by a decreased therapeutic effect because of an alteration of the pharmacokinetics during concurrent administration of PB are corticosteroids, cyclosporine, metronidazole, digoxin, phenylbutazone and some anesthetic agents such as thiopental.

Side effects

PB associated side effects can be divide in transient and dose dependent and idiosyncratic.

Dose-dependent side effects include sedation, ataxia, polyphagia, polydipsia/polyuria. These side effects are usually evident during the first two weeks of treatment and subside in the subsequent 1 – 3 weeks due to the development of tolerance.

Hyperexcitability, restlessness and aggression have been occasionally reported in dogs during the first weeks of PB treatment but considering that these behavioural changes can represent also comorbidities in epileptic patients (especially those affected by idiopathic epilepsy) the real cause of these abnormalities is difficult to establish.

Idiosyncrasic side effects are rare and include hepatotoxicity, haematologic changes, superficial necrolytic dermatitis, pancreatitis, dyskinesia and anxiousness, hypoalbuminaemia. Although a direct cause-effect relationship has not been proven for these adverse effects, they are classified as idiosyncrasic because they resolve after discontinuation of PB.

Other barbiturates

A potential but still unproven cerebral protective effect during SE has been described for thiopentone (or thiopental sodium, an ultrashort-acting thiobarbiturate) and pentobarbitone (a short-acting oxybarbiturate).

As short-acting barbiturates, they are generally used as anaesthetic agents, however, they possess properties that are favourable to cerebral physiology: they decrease cerebral blood flow, cerebral oxygen consumption and ICP and cause dose-dependent depression of the EEG.

Both molecules can control at least the physical manifestations of seizures as general anaesthetics, and pentobarbitone has also the ability to scavenge oxygen radicals and decrease cerebral oxygen demand (Blades Golubovic and Rossmeisl, 2017a).

Both molecules are rapidly metabolized in the liver by the CYP450 system and eliminated by kidneys. They should be given IV to effect because a high individual variability has been reported. Hypotension and cardiac toxicity are the most common adverse effects associated with the administration (Papich, 2018).

Propofol

Propofol (2,6 diidropropylphenol) is an alkylphenol commonly used as intravenous anaesthetic for sedation, induction or maintenance of general anaesthesia. It is water insoluble but highly lipid soluble.

It is a $GABA_A$ receptor agonist that acts at different sites from those targeted by benzodiazepines and barbiturates. Its mechanism of action includes the increase opening of Cl^- channels, increased Ca^{2+} conductance and reversible inhibition of NMDA receptors, resulting in hyperpolarization of postsynaptic cell membranes and causing anesthesia and amnesia. However, the mechanism of action as anticonvulsant is still unknown.

Due to its very short elimination half-life, propofol is generally administered as CRI. Recommended protocols report a loading dose of 1 – 2 mg/kg to 2 -6 mg/kg followed by a CRI of 0.1 – 0.6 mg/kg/min or 0.15 – 0.4 mg/kg/min titrated to effect up to 6 mg/kg/h. Duration of CRI varies between specialists, even though it has been recommended to continue the CRI for at least 6 hours, then 24h in case of further seizure activity.

Propofol administration produces a favourable neurological status by the decrease of ICP (in patients with both normal and elevated ICP), the decrease of CPP and the decrease cerebral metabolic oxygen consumption (Blades Golubovic and Rossmeisl, 2017a).

Pharmacokinetics

Propofol is rapidly absorbed and distributed to the CNS. Due to its lipophilic nature it results in a large volume of distribution (17.9 l/kg in mixedbreed dogs), which has been reported to be higher in Greyhounds compared to other breeds. Furthermore, dogs older than 8.5 years have been reported to have a slower clearance compared to younger. For these reasons, in these categories of patients, recovery can be potentially slower.

The body clearance of propofol is rapid and presumed to be extrahepatic, even if the site is unclear and significant species differences have been postulated. Propofol is excreted as inactive metabolites with urines (Papich, 2018).

Side effects

Propofol administration has been associated with apnoea in a rate of administration-dependent fashion (i.e. the faster the administration, the more likely the incidence of apnoea). Respiratory depression caused by propofol may result in mild hypoxemia, hypercapnia and acidosis.

Propofol administration has been associated with myoclonic movements of unknown origin in both humans and dogs. Furthermore, a propofol-infusion syndrome associated with high dosages (> 4 mg/kg/h) or prolonged administration (> 48h) has been reported in human medicine but has not been evaluated in veterinary patients yet. This syndrome is due to impairment of mitochondrial activity and use of free fatty acids, with resulting mismatch between energy needs and use. Clinically, this syndrome has been described as metabolic acidosis, rhabdomyolysis, hyperkalemia, lipaemia, renal failure, hepatomegaly and cardiovascular collapse (Rossetti and Lowenstein, 2011).

Ketamine

Ketamine is a phencyclidine derivative with dissociative anaesthetic properties. It is a noncompetitive NMDA receptor antagonist and as such prevents the binding of glutamate at the receptors, avoiding the conduction of Na^+, K^+ and Ca^{2+} and inhibiting the thalamocortical and limbic system and depressing the reticular formation nuclei. Ketamine do not interact with GABA receptors. There is evidence of some anticonvulsive effect of ketamine and the use of ketamine as a treatment option for SE is controversial. In fact, NMDA receptors activation has been reported in later phases of SE causing a perpetuation of seizure activity. For this reason, the use of NMDA antagonist during refractory and prolonged SE have been postulated, even though to date only one case report describing the management of a refractory SE in a dog has been published and no proper clinical studies focused on the effectiveness and the safety of ketamine CRI in these patients have been published (Serrano et al., 2006).

However, due to its effect on cerebral blood flow (increase) due to CNS vasodilation and increased blood pressure and therefore increased ICP, ketamine should be avoided in neurologic patients with suspected increased ICP.

Ketamine has stimulatory effect on the cardiovascular system and only mild depressant effect on the respiratory system.

(Höfler and Trinka, 2018; Rossetti and Lowenstein, 2011)

Inhalant anaesthetics (isoflurane, sevoflurane, halotane)

Inhalant anaesthetic are considered both in human and veterinary medicine the last chance during refractory SE. They enhance the inhibitory $GABA_A$ receptor-mediated currents therefore decreasing thalamic neuronal membrane excitability. They also have a positive effect on the cerebral blood flow and ICP (increase), while they decrease cerebral oxygen consumption (Blades Golubovic and Rossmeisl, 2017a; S. R. Platt, 2014b).

Veterinary studies regarding the use of inhalant anaesthetic agents in SE are lacking.

LEVETIRACETAM (LEV)

Levetiracetam is the (S)-enantiomer of alfa-ethyl-2-oxo-1-pyrrolidine acetamide, an ethyl analog of piracetam, classified as nootropic agent considered “pharmacologically safe”. It is structurally unrelated to any other AED and has a novel mechanism of action that has still not been completely understood (Papich, 2018).

This molecule was approved in the United States in 1998 as an oral treatment of partial onset seizures in adult human patients. Almost 10 years later, in 2006, a parenteral formulation was approved for bridge therapy, as an alternative for treatment of partial seizures when oral administration is not feasible (De Risio, 2014c).

Mechanism of action

The postulated mechanism of action of LEV is thought to be associated with the binding to the synaptic vescicle 2A (SV2A) protein on the presynaptic membrane and the modulation of the synaptic vesicle fusion and subsequent neurotransmitters release by exocytosis (Lynch et al., 2004). Other reported mechanisms that may be involved in the anti-epileptic effect of LEV include the alteration of ion flow through Na+-dependent Cl-/HCO3- channels by inhibition, modulation of K+ and N-type high-voltage activated Ca2+ channels, reduction of glutamate release by modulation of the presynaptic P/Q-type voltage-dependent Ca2+ channels, opposition of allosteric inhibition of GABA and glycine-gated current and antagonism of neuronal hypersynchronization. In addition, a recent study suggests that LEV may reduce the spread of excitation elicited by seizures within the astroglial functional syncytium, with stabilizing consequences for neuronal–glial interactions (Crepeau and Treiman, 2010; Stienen et al., 2011).

Pharmacokinetics

One of the main reasons for the increasing use of LEV in both human and veterinary medicine is the high safety profile, associated with favourable pharmacokinetics. In fact, LEV pharmacokinetics comes especially close to fulfilling the desirable pharmacokinetic characteristics for an AED:

- Bioavailability: LEV has a high oral bioavailability and the co-administration of food slows the rate but not the extent of absorption.
- Plasma protein bound: LEV is not significantly bound to plasma proteins (less than 10%).
- Metabolism and elimination: LEV is primarily (89% in dogs) eliminated with urines, with a renal clearance of 0.69 $\pm$ 0.16 ml/min/kg (similar in humans and dogs) that of course progressively reduces in case of renal dysfunction. Approximately 50-62% is excreted unchanged, while the remaining fraction is metabolized through hydrolysis by hydrolases, amidases and β-esterases in the blood, liver and other tissues. Only a small proportion of LEV undergoes oxidation.

The metabolism of LEV is not mediated by the cytochrome P450 pathway within the liver.

- Parmacokinetics model: LEV has linear and time-independent kinetics.

- Drug interations: LEV is not vulnerable to important drug interactions, nor does it cause clinically significant alterations in the kinetics of concomitantly administered drugs

(Perucca and Johannessen, 2003; Strolin Benedetti et al., 2004).

Several pharmacokinetics studies have been performed over the years in healthy dogs, evaluating pharmacokinetics parameters of LEV adiministered by different routes. Table 2 reports the results of the studies (Dewey et al., 2008; Isoherranen et al., 2001; Moore et al., 2010; Patterson et al., 2008; Peters et al., 2014; Strolin Benedetti et al., 2004).

Reference	Dose	F(%)	Vd (l/kg)	Cl (ml/kg/min)	AUC (h*μg/ml)	C_{max} (μg/ml)	T_{max} (h)	$T_{1/2}$ (h)	MRT (h)
Isoherranen et al., 2001	20 mg/kg IV once	NA	0.5 ± 0.1	1.5 ± 0.3	NA	NA	NA	3.6 ± 0.8	5.0 ± 1.2
Strolin Benedetti et al, 2004	54 mg/kg PO once	NA	NA	NA	425.4 (M) 320.0 (F)	55.1 ± 12.9 (M) 53.7 ± 7.5 (F)	2.5 ± 2.4 (M) 1.4 ± 0.5 (F)	3.1 (M) 2.3 (F)	NA
Dewey et al., 2008	60 mg/kg IV once	NA	0.48 ± 0.08	1.4 ± 0.28	768 ± 179	254 ± 81	NA	4.0 ± 0.8	6.0 ± 0.9
Patterson et al., 2008	20 mg/kg IV once	NA	0.55	2.1 ±0.3	166 ± 27	37 ± 5	NA	3 ± 0.3	NA
	20 mg/kg IM	113	0.6	2.3 ± 0.3	176 ± 20	30 ± 3	0.7 ± 0.3	3 ± 0.4	NA
	20 mg/kg PO once	100	0.59	2.1 ± 0.3	167 ± 23	30 ± 4	1.4 ± 0.5	3 ± 0.3	NA
Moore et al., 2010	20 mg/kg PO once	NA	NA	NA	268.52 ± 56.33	59.91 ± 11.54	0.62	2.9 ± 0.2	NA
	20 mg/kg PO q8h for 7d	NA	NA	NA	289.31 ± 51.68	52.41 ±10.08	1	3.6 ± 0.8	NA
Peters et al., 2014	40 mg/kg rectally	NA	NA	NA	222 ± 72	36.0 ± 10.7	1.7 ± 0.5	NA	NA

Table 2. LEV pharmacokinetic parameters in healthy dogs. *NA: not available; d=days; F=bioavailability, Vd=volume of distribution; Cl= clearance; AUC= area under the curve; C_{max} = maximum concentration; T_{max} = time to maximum concentration; $T_{1/2}$ = elimination half-life; MRT= mean residence time*

With the advent of extended release oral formulation of LEV, several studies have been focused on the evaluation of the pharmacokinetics of these products.

In particular, Beasley and Boothe in 2015 evaluated the pharmacokinetics of an extended release formulation of LEV in 16 dogs given at a dosage of 30 mg/kg IV and orally, with and without food. Pharmacokinetic parameters for fasted versus fed animals, respectively, were (mean ± SEM): C_{max} = 26.6 ± 2.38 and 30.7 ± 2.88 μg/ml, T_{max} = 204.3 ± 18.9 and 393.8 ± 36.6 min, $t_{1/2}$ = 4.95 ± 0.55 and 4.48 ± 0.48 h, MRT = 9.8 ± 0.72 and 10 ± 0.64 h, MAT = 4.7 ± 0.38 and 5.6 ± 0.67 h, and F = 1.04 ± 0.04 and 1.26 ± 0.07%. Significant differences were limited to T_{max} (longer) and F (greater) in fed compared to fasted animals. Serum LEV concentration remained above 5 μg/ml for approximately 20 hours in both fasted and fed animals (Beasley and Boothe, 2015).

Boozer and colleagues aimed instead not only to evaluate pharmacokinetics parameters of extended release oral formulation of LEV but also to compare them with those of an immediate release formulation. The study was performed on 6 neurologically normal dogs with a crossover study design. Two different 500 mg oral formulations of extended release LEV and one 500 mg immediate release oral formulation were used. All extended release formulations of LEV had similar pharmacokinetic properties, with some exceptions. In particular, the generic extended release formulation 1 had a significantly lower AUC compared to other extended release formulations and Vd and Cl were significantly lower for brand and generic 2 extended release formulations compared with the generic 1 extended release formulation, as reported in Figure 12 (Boozer et al., 2015).

Variable	Brand-IR	Brand-ER	Generic-ER1	Generic-ER2
Tmax (h)	2.29 ± 1.42	6.00 ± 2.19†	5.33 ± 2.07	8.00 ± 2.83†
Cmax (μg/mL)	34.9 ± 6.40	29.4 ± 4.1‡	21.9 ± 3.4†	27.8 ± 4.0†
β (1/h)	0.173 ± 0.037	0.157 ± 0.017	0.159 ± 0.021	0.161 ± 0.030
$t_{1/2}$ (h)	4.17 ± 0.92	4.47 ± 0.50	4.44 ± 0.68	4.42 ± 0.81
AUC (h•μg/mL)	313 ± 80	361 ± 71‡	280 ± 62	380 ± 30‡
Vd (mL)	9,780 ± 2000	9,020 ± 1,110‡	11,800 ± 32,60	8,440 ± 2,110‡
Cl (mL/h)	1,680 ± 435	1,420 ± 270‡	1,850 ± 443	1,310 ± 94.7‡

*Formulations of levetiracetam were evaluated individually in a randomized crossover design, and each evaluation was separated by a minimum washout period of 7 days. Data from 6 dogs are represented for brand-IR, brand-ER, and generic-ER1; data from 5 dogs are represented for generic-ER2. †Value differs significantly ($P \leq 0.05$) from that for brand-IR. ‡Value differs significantly ($P \leq 0.05$) from that for generic-ER1.

β = Terminal rate constant. $t_{1/2}$ = Half-life.

Figure 12. Pharmacokinetics parameters of extended release oral formulation of LEV compared to immediate release. *IR: immediate release; ER: extended release (from. Boozer et al., 2015)*

Pharmacokinetic interactions

To date, it is well known how concurrent administration of other AEDs can affect serum concentration of LEV by increasing its clearance in both healthy and epileptic dogs.

In healthy dogs, PB administration at a dosage of 2 – 3.3 mg/kg q12h for 21 days significantly altered the pharmacokinetics of LEV, causing a decrease in LEV peak concentrations (C_{max} from 32.39 ±

6.76 to 18.22 ± 8.97 μg/ml), a decrease in elimination half-life ($T_{1/2}$ from 3.43 ± 0.47 to 1.73 ± 0.22 h), and an increase in oral clearance (from 124.93 ± 26.93 to 252.99 ± 135.43 ml/h/kg). It has been postulated that the increased metabolism and clearance of LEV is caused by the induction of oxidative enzymes by PB, causing the need of higher LEV oral dosages in dogs concurrently administered with PB (S. A. Moore et al., 2011).

Subsequently in 2015, a prospective pharmacokinetic study was performed in order to evaluate the pharmacokinetics of LEV in dogs with epilepsy concurrently administered with conventional AEDs (PB and KBr). The results of the study performed on 18 client-owned dogs on maintenance treatment with LEV and phenobarbital (PB group, n = 6), LEV and KBr (KBr group, n = 6) or LEV, PB and KBr (PB- KBr group = 6) highlighted that the concurrent administration of PB alone or in combination with KBR increases LEV clearance in epileptic dogs compared to concurrent administration of bromide alone. In fact, compared to the PB and PB–KBr groups, the KBr group had significantly higher peak concentration (C_{max} 73.4 ± 24.0 versus 37.5 ± 13.7 and 26.5 ± 8.96 μg/ml, respectively, P < .001) and AUC (329 ± 114 versus 140 ± 64.7 and 98.7 ± 42.2 h*lg/mL, respectively, P < .001), and significantly lower clearance (CL/F) (71.8 ± 22.1 versus 187 ± 81.9 and 269 ± 127 mL/h/kg, respectively, P = .028) (Muñana et al., 2015).

Recently, also the population pharmacokinetics of extended-release formulation of LEV in epileptic dogs when administered alone or in combination with other AEDs (PB and zonisamide) has been investigated. Patients treated with LEV and PB had a lower C_{MAX} (13.38 μg/mL) compared to patients treated only with LEV (33.01 μg/mL) and patients treated with LEV and zonisamide (34.13 μg/mL), lower AUC (134.86 versus 352.95 and 452.76 hours*μg/mL, respectively), and higher CL/F (0.17 versus 0.08 and 0.07 L/kg/hr, respectively). The half-life was similar to values previously reported for healthy dogs. It was concluded that considerable variation exists in the pharmacokinetics of extended release formulations of LEV in dogs with epilepsy being treated with a common dose regimen. It was also reported that concurrent administration of PB contributed significantly to the variation, while other factors evaluated, including co-administration of zonisamide, were not shown to contribute to the variability (Muñana et al., 2018).

In regard of human medicine, the effect of the concurrent administration of other AEDs other than PB has also been evaluated. The results of the study proved that the concurrent administration of carbamazepine and phenytoin increase LEV clearance, causing significant reduction of plasma LEV concentrations (Sourbron et al., 2018a).

Due to the negligible rate of protein binding, hepatic metabolism and induction or inhibition of metabolizing hepatic enzymes, LEV has always been considered as a molecule with low potential for clinically relevant pharmacokinetic interactions with other medications (Isoherranen et al., 2001; Patsalos, 2004; Strolin Benedetti et al., 2004). It has been proved that LEV does not alter the the steady-state serum concentrations of other AEDs in dogs, namely PB and KBr (Muñana et al., 2012).

A synergic effect on seizure protection of LEV used in combination with benzodiazepines has also been proposed. This effect is not related to modification of plasma AEDs concentrations, nor associated with more pronounced side-effects or pharmacokinetics interactions. To date, clinical evidence on how and when to combine AEDs is still very limited and only preclinical studies are available (Kaminski et al., 2009). Experimental studies conducted on audiogenic seizures model in mice highlighted that the anticonvulsant potency of drugs enhancing GABAergic inhibition (i.e., benzodiazepines, PB, chlordiazepoxide, and valproate) was most effectively enhanced by more than 16-fold by LEV. The potency of other drugs augmenting GABAergic neurotransmission was also considerably increased, up to 11-fold increase. Likewise, the protective effects of glutamate receptor antagonists (NMDA and AMPA receptor antagonists) were also markedly enhanced in combination with LEV. In contrast, the potency of several Na^+ channels inhibitors (i.e., carbamazepine or phenytoin), Ca^{2+} channel inhibitors and β adrenergic receptor blockers were enhanced to much lesser degree (Matagne et al., 2001). Another experimental study performed on audiogenic seizure model reported synergic interactions of LEV with felbamate, gabapentin and topiramate, and less enhancing effect in combination with Na^+ channels inhibitors (Donato Di Paola et al., 2007). In these studies, the therapeutic index, defined as the ratio between toxic dose of 50% and dose of AED required to protect 50% animals against seizure, was dramatically increased in combination of LEV with valproate, clonazepam, PB.

Other experimental studies proved that LEV is not effective in Maximal Electroshock Seizure (MES) model, but only raises the seizure threshold (Löscher and Hönack, 1993). However, interactions between LEV and several other AEDs have been studied in the MES model with the use of isobolography, which is a method of studying drug interactions, by administering them at several fixed dose ratios. This method appears to have more predictive validity than traditional subthreshold methods involving dose-response analysis. This analysis reported that LEV administered at several fixed dose ratios in combination with topiramate, carbamazepine or oxcarbamazepine had a synergic effect, while the association with phenytoin, lamotrigine, PB or valproate produced additive effects. (Luszczki et al., 2007, 2006). Similarly to MES model, LEV resulted ineffective against seizures induced by supramaximal doses of pentylenetetrazol (Klitgaard et al., 1998). However, protection against seizure was obtained in a pentylenetetrazol model when LEV was administered in association with topiramate, even if topiramate alone was not able to protect mice against seizures (Sills et al., 2004).

Conversely, LEV showed very good efficacy in the rat amygdala kindling model. In particular, the coadministration of LEV increased up to four-fold the potency of other AEDs displaying dose-dependent efficacy (PB, valproate, clonazepam, carbamazepine) (Kaminski et al., 2009).

In the rat model of self-sustained status epilepticus, LEV resulted more effective than diazepam in suppression seizure activity and potentiated diazepam's anticonvulsant effects when used in combination (Mazarati et al., 2004).
Of course, these preclinical data need to be further investigated in order to evaluate as to what extent these AEDs combinations can provide a therapeutic benefit in patients with epilepsy.

Dosages and monitoring

LEV dosage recommendations in dogs are deducted from the results of pharmacokinetic studies and reference ranges reported in human medicine (Patsalos et al., 2008).
To date, two different treatment protocols have been reported in veterinary medicine, defined as "maintenance" and "pulse".
The recommended dose for the maintenance (long-term) treatment protocol is 20 mg/kg q8h or q6h given either parenterally or orally.
However, Volk and colleagues reported the development of tolerance in some dogs, when LEV was used chronically, similarly to what is reported in human medicine for several AEDs, including LEV. This phenomenon has been called "the honeymoon effect" and it has been reported for LEV and zonisamide regarding canine patients (BOGGS and JG, 2000; Volk et al., 2008).
For this reason, the introduction of the "pulse" treatment protocol was developed in order to avoid the induction of LEV tolerance. This protocol is based on the administration of an initial dose of 60 mg/kg either orally or parenterally after the occurrence of a first epileptic seizure or as soon as preictal signs are recognized, followed by 20 mg/kg q8h until the patient is seizure free for 48h. This protocol allows the rapid achievement of LEV therapeutic concentrations. This treatment protocol has been proposed also for the acute management of CS and SE (Packer et al., 2015; S. R. Platt, 2014a, 2014b).
As suggested by one author, LEV dose can be increased by 20 mg/kg increments until either efficacy is achieved, side effects become apparent or the drug becomes cost-prohibitive (Dewey, 2006).
Due to its metabolism, LEV dosages should be reduced in case of impaired renal function, while LEV dosages should be increased in case of concurrent administration of PB, as previously reported.
Regarding the extended-release oral formulation of LEV, pharmacokinetics after oral administration of 30 mg/kg q12h and 17 to 25 mg/kg in healthy dogs have been evaluated and reported to be safe (Beasley and Boothe, 2015; Boozer et al., 2015). Recently, in a study evaluating the population pharmacokinetics of extended-release LEV formulations in epileptic dogs, the mean study dose was 29.4 mg/kg (Muñana et al., 2018).
In human medicine the LEV therapeutic concentration monitoring is currently not common in clinical practice due to the wide therapeutic range and the low occurrence of side-effects (Sourbron et al., 2018b). In fact, a high individual variability has been noticed, and a specific therapeutic range has not been definitively established in human medicine, with reported reference ranges ranging from 12 – 46 μg/ml, 13 – 42 μg/ml, 5 – 30 μg/ml and 5 – 45μg/ml (De Risio, 2014c). In both human and

veterinary medicine, the evaluation of serum LEV concentrations can be valuable to evaluate compliance but also to individualize treatment in single patients by comparing the serum concentration with the rate of seizure control (De Risio, 2014c; Sourbron et al., 2018b). To date, the association between response to treatment and serum LEV concentrations have been investigated in only 2 studies and results failed to identify any association between the two variables in exam (Fredsø et al., 2016; Muñana et al., 2012)

Serum or plasma should be immediately separated from whole blood after sampling because LEV undergoes hydrolysis in blood collection tubes (Patsalos et al., 2008).

Due to LEV relatively short half-life, the sampling time in relation to the administration is extremely important and the collection of a through sample allows the assessment of the lowest concentration in the dosing interval, as for other AEDs. Furthermore, differences in plasma LEV concentrations following oral administration have been evaluated during the day. In particular, plasma LEV concentrations resulted higher in the morning compared to midday in one study. Authors hypothesized to refer these oscillations to the different rate of renal clearance during the day (Moore et al., 2010).

Adverse effects and laboratory alterations

Levetiracetam is well tolerated and generally safe in dos. It has been in fact associated with only mild and infrequent side effects. Most frequently reported in dogs are sedation, ataxia, decreased appetite and vomiting (Bhatti et al., 2015; Charalambous et al., 2016; Muñana et al., 2012; Volk et al., 2008). Other infrequent reported side effects include: polyphagia, polydipsia, diarrhea, aggression and restlessness (Packer et al., 2015). Recently, the administration of an intravenous dose of undiluted LEV (60 mg/kg) has been associated with a presumed anaphylactic idiosyncratic reaction in an 8-year-old female spayed Chihuahua affected by seizures. The patient died of respiratory failure and cardiac arrest after developing tachycardia, hyperglycemia, hypotension, and a dull mentation (Biddick et al., 2018).

According to the results of recent cross-sectional study, LEV administration in human patients was associated with a lower blood platelet count compared to control, while no changes were observed in hemoglobin concentration or white blood cell count (Bachmann et al., 2011). Conversely, no significant alterations of lipid profile or thyroid function were noted during LEV monotherapy does not cause any alteration on routine laboratory parameters both in both humans and dogs (Nishiyama et al., 2019). In dogs, LEV administration has not been associated with any relevant effects on routine laboratory parameters (Muñana et al., 2012). However, the effect of LEV on lipid profile and thyroid function to date has not been investigated in dogs.

Evidence of clinical efficacy

The majority of scientific studies performed in epileptic dogs have been focused on the efficacy of LEV administered as add-on to other AEDs.

Preliminary information on LEV efficacy in idiopathic dogs have been reported in 2004. Fifteen dogs affected by idiopathic epilepsy resistant to PB and KBr were enrolled in an open-label study and administered with LEV at a dosage of 7.1 – 23.8 mg/kg q8h in association with the other AEDs. This add-on treatment resulted in a temporary reduction in seizure frequency of 54% during the initial 3 months (Steinberg and Faissler, 2004). Subsequently, in 2008 Volk and colleagues reported a favourable response during the initial 2 – 3 months compared to baseline when pharmacoresistant idiopathic epileptic dogs were administered with LEV as add-on. In this study, 14 dogs diagnosed with idiopathic epilepsy resistant to PB and KBr were initially administered with 10 mg/kg of LEV q8h orally for 2 months. Nine out of 14 dogs were reported as responders, experiencing a decrease in seizure frequency of > 50%. Patients that experienced a decrease in seizure frequency of < 50% from baseline received then a higher dosage of 20 mg/kg of LEV q8h orally for 2 months and 1 further dog responded to LEV treatment after the increase dosage. Overall, the results of the study highlighted a significant decrease in seizure frequency of 77% and a decrease in seizure days per month of 68% in LEV responders. However, 67% of responders experienced an increase in seizure frequency after 4 – 8 months from the beginning of LEV treatment. This finding was explained by authors as a development of tolerance (*honeymoon effect*) as reported in human medicine (Volk et al., 2008).

However, the efficacy of LEV in idiopathic pharmacoresistant epileptic dog was not confirmed afterwards. The efficacy of LEV as add-on treatment was evaluated in 34 dogs affected by idiopathic epilepsy refractory to PB and KBr in a randomized, double-blinded, placebo-controlled cross-over study. After a prospective baseline period of 8 weeks, patients were randomly assigned to receive either oral LEV at a dosage of 20 mg/kg q8h or placebo for 16 weeks. After a 4-week washout period, patients were then crossed over to the alternate treatment for 16 weeks. Only 22 dogs completed the study, and as a consequence of the high dropout rate, comparisons were only made between dogs receiving LEV (n = 18) and placebo (n = 10) during the first treatment. Results of the study failed to demonstrate the efficacy of LEV compared to placebo when administered as add-on therapy in dogs with refractory epilepsy. There was no statistical difference in the number of dogs classified as responders with LEV administration (56%) compared to placebo (30%). A statistically significant decrease in weekly seizure frequency compared to baseline was reported in dogs receiving LEV, but the reduction in seizures with LEV was not significant when compared to placebo (Muñana et al., 2012). A placebo response during epilepsy trials has been reported both in human and veterinary medicine and it has been mainly attributed to regression to mean or the natural course of the epileptic disease. Furthermore, individual improvement can also be attributed to more careful attention provided during study participation and the potential for improved adherence to antiepileptic treatment regimen during study participation (Muñana et al., 2010).

A retrospective evaluation of LEV efficacy in epileptic dogs reported that LEV treatment resulted in 69% of ≤ 50% reduction of seizures with 15% of patients being seizure free (mean follow up time of

1.2 and 1.4 years, respectively). When response rate between different treatment protocols (LEV maintenance vs pulse treatment) was evaluated, no significant differences were found. In this retrospective study, 90% of dogs received treatment with other AEDs prior to LEV, but no associations were found between AED-use prior to LEV and treatment success (Packer et al., 2015). Concerning the evaluation of LEV efficacy as monotherapy, only two scientific studies have been published to date. In the first study, LEV monotherapy was evaluated in dogs affected by idiopathic epilepsy with a prospective single-blinded parallel group study design. Twelve dogs were included in the study and randomly assigned to receive either oral LEV (30 mg/kg/day or 60 mg/kg/day divided into 3 daily dosages) or oral PB (2 mg/kg q12h). Only six patients (1 in the LEV group and 5 in the PB group) completed the 1-year study period. No significant difference in the monthly number of seizures was evaluated in dogs treated with LEV before and after the initiation of treatment., whereas in the PB group, the initiation of treatment resulted in a significant decrease in seizure frequency. In conclusion, LEV resulted to be not effective as monotherapy compared to PB (Fredsø et al., 2016). Subsequently, LEV efficacy has been evaluated in dogs affected by structural epilepsy in a retrospective case series of 19 dogs. Seizure control was considered good if no seizures occurred within 3 months after the initiation of treatment or poor if seizure recurrence was evaluated within 1 month. LEV was administered orally at a mean dosage of 20 mg/kg. Ten out of 19 dogs were considered to have a good seizure control, while 9 dogs had a poor treatment outcome and a statistically significant reduction in CS was evaluated. Patients received other medications according to the specific diagnosis of structural epilepsy, when needed (Kelly et al., 2017).

Finally, the efficacy of IV LEV has been also evaluated in case of CS and SE. In a double-masked, placebo-controlled study,19 dogs affected with SE or CS were randomized to receive IV LEV (30 mg/kg in 5 patients and 60 mg/kg in 4 patients) or placebo in addition to a standard treatment protocol. Patients were enrolled in the study if they had another seizure after the hospitalization or were actively seizuring on presentation and LEV/placebo was administered within 30 minutes from the seizure activity. Patients were then monitored for at least 24 hours after admission for further seizure activity. The responder rate of LEV group was 56% compared to 10% for the placebo group, but the difference resulted not statistically significant, even if a trend to significance was evaluated and dogs in the placebo group received significantly more IV boluses of diazepam than the dogs in the LEV group. Similarly, no statistically significant differences were found when comparing the responder rate for the 2 different dosages of LEV. Based on the results of the study, the authors concluded that IV LEV could be a safe and potentially effective AED for the treatment of CS and SE, even if further studies are required to confirm this hypothesis (Hardy et al., 2012).

Due to its predominant renal metabolism, LEV has also been investigated as prophylactic AED in dogs undergoing extrahepatic porto-systemic shunt surgery. In a retrospective case review of 126 dogs, LEV was administered orally at a dosage of 20 mg/kg q8h for at least 24 hours before surgery (attenuation with ameroid ring constrictors) in 33% of patients and none of them experienced

postoperative seizures, whereas 5% of dogs not receiving prophylactic LEV resulted affected with postoperative seizure activity. Furthermore, none of the patients that experienced postoperative seizures survived to discharge from the hospital (Fryer et al., 2011). However, the results of the study performed by Fryer et al, have not been confirmed by another recent multi-institutional retrospective study of 940 dogs undergoing surgical attenuation of extrahepatic porto-systemic shunt. According to the results, the overall incidence of postoperative seizures was reported as low (8%) and prophylactic treatment with LEV was not associated with a reduced incidence of seizures (Mullins et al., 2019).

SUDDEN UNEXPECTED DEATH IN EPILEPSY (SUDEP)

Definition and epidemiology

In recent years, there has been much focus on sudden unexpected death in epilepsy (SUDEP), referring to the occurrence of an unexpected death of an otherwise healthy individual with epilepsy, usually in relation to a tonic-clonic seizure, for whom no cause of death can be identified.

SUDEP has been defined as the sudden, unexpected, witnessed or unwitnessed, non-traumatic, and non-drowning death in patients with epilepsy, with or without evidence for a seizure, with exclusion of documented status epilepticus, and when post-mortem examination does not reveal a structural or toxicological cause for death (Nashef et al., 2012). Cases confirmed with post-mortem examination excluding all other possible causes of death are usually referred as “definite SUDEP”, while non-confirmed cases are reported as “probable SUDEP” (Devinsky et al., 2016).

In human patients with chronic refractory epilepsy, SUDEP has been reported as the leading cause of premature death, accounting for 10–50% of all deaths (Shorvon and Tomson, 2011).

SUDEP incidence is low in young children, higher in adolescence, peaks in younger adults, and is reduced substantially thereafter. However, SUDEP might be underdiagnosed in older adults in whom sudden death might be attributed to cardiac events without careful investigation of alternative causes. (Shorvon and Tomson, 2011; Sveinsson et al., 2018).

Patophysiology

Discussions on the pathophysiology of SUDEP have focused on the final mechanisms by which a seizure could lead to cardio-respiratory arrest and death. Most research, performed on anesthetized sheep, has focused on the possibility that the primary physiological event is seizure-induced hypoventilation (either central obstructive or both) or cardiac dysrhythmia (Devinsky et al., 2016).

However, it is now accepted that SUDEP is a result from different mechanisms in different individuals, and that there might be a combination of mechanisms in every individual. Furthermore, most people with frequent seizures do not die in SUDEP; therefore, an individual susceptibility exists. In most cases, even the predisposed individuals will usually have had many non-fatal seizures before the final fatal one, which suggests that there are modulating factors that establish whether the course of the seizure will be benign or will lead up to SUDEP. Various aspects have been studied but a

satisfactory unitary explanation of the mechanisms of SUDEP remains elusive (Sveinsson et al., 2018).

Interest has also focused on prolonged QT syndromes, characterized by a life-threatening cardiac arrhythmia syndrome which represents a leading cause of sudden death especially in young people (Schwartz et al., 2012). These genetic disorders are channelopathies, as are some of the epilepsies. Some channelopathies might underpin epilepsy and prolong QT, which might contribute to SUDEP. Furthermore, seizures themselves can prolong the QT interval directly. Genetic long QT syndromes are associated with sudden death because of the risk of arrhythmia (Devinsky et al., 2016).

Sympathetic activation consequent to generalized tonic-clonic seizure and serotonin dysfunction have been associated as well with increased risk of SUDEP. In particular, serotonergic neurons are central chemoreceptors that stimulate breathing and cause arousal from sleep in response to hypercapnia. Medullary serotonergic neurons are inhibited by seizures, which might contribute to ictal apnoea and prolonged postictal hypoventilation (Shorvon and Tomson, 2011).

Pathology

Pathological changes in SUDEP do not explain cause of death but might provide clues regarding pathophysiology. Cardiac and pulmonary abnormalities are common in cases of SUDEP at autopsy. Neuropathological changes associated with SUDEP include causes of epilepsy and consequences of seizures but no specific lesion type or location was associated with an increased risk of SUDEP (Devinsky et al., 2016).

Risk factors

The risk of SUDEP has been reported to be higher in in male patients, in those with onset of epilepsy before the age of 16 years and in those who had had epilepsy for longer than 15 years.

The most important risk factor that has been reported is frequency of primary or secondary generalized tonic-clonic seizures.

Antiepileptic drug polytherapy was not a SUDEP risk factor when the analysis was controlled for generalized tonic-clonic seizure frequency. However, the association between AEDs and SUDEP is still complex and opinions are contradictory. Attempts have been made to use post-mortem AED blood concentrations to assess the level of compliance in SUDEP cases, but results are still conflicting.

Because SUDEP has been usually associated with the occurrence of a high frequency of generalized tonic-clonic seizures, the assumption that effective drug treatment would reduce the incidence of SUDEP is reasonable. However, polytherapy with AEDs had an associated increased risk of SUDEP in several studies, but this association may not be necessarily causal, and polytherapy might simply be a marker of severe epilepsy.

Although effective AED treatment is likely to be protective, AEDs in some circumstances might increase the risk of SUDEP. Some epilepsy drugs may have in fact potential effects on cardiac conduction through their membrane stabilizing effects and their effects on autonomic function. This

has been particularly pointed out for the Na^+ channel blocking drugs, such as carbamazepine and lamotrigine (Sveinsson et al., 2018).

Furthermore, epileptic encephalopathies (severe epilepsies beginning in early life in which frequent epileptiform activity and seizures contribute to developmental delays) have been found to carry a high risk of SUDEP.

Genetic mutations are linked to SUDEP in humans and to postictal death in animal models. Some have been labelled “SUDEP genes” and their association with cardiac disease has led to conclusions about the mechanism of death. It is unclear whether any of the epilepsies associated with genetic disorders confer an increased SUDEP risk independent of seizure severity and frequency (Shorvon and Tomson, 2011).

CLINICAL SCORING SYSTEMS

Scoring systems have been introduced in the 1980’s and subsequently developed and used in all diagnostic areas of medicine. Several parameters are evaluated and rated with points according to their value in order to simplify a complex clinical situation with a score. The application ranges from the classification of disease severity through determining the number of staff for the intensive care unit to the evaluation of new therapies under study conditions. In human medicine, scores have been also used to assist appropriate triage of patient groups (Fleig et al., 2011; Hayes et al., 2010).

In particular, an illness score is a number assigned to a patient that correlates with a probability that a specific outcome will follow. The use of an illness score to manage individual patients or establish a prognosis has several limitations, but illness severity scores are still a valuable and currently underutilized research tool. However, illness severity scores are not designed to be used in isolation to predict outcome for individuals, and predictions should be applied on a population basis (that is: where a dichotomous outcome, e.g. survival or death, is being predicted a score of 70% cannot predict a 70% chance of mortality for any 1 individual, but in a group of 100 similar patients, 70% may be expected to die, or 30% will survive).

Although several diagnosis-specific and diagnosis-independent scores have been proposed in recent years and are being used clinically, scoring systems in general have achieved limited adoption in the veterinary clinical research setting (Hayes et al., 2010).

Diagnosis-specific scores assess particular facets of a patient’s primary problem, while diagnosis-independent scores provide an objective assessment of a patient’s global illness status, typically derived from variables.

Severity scores can provide an objective tool for baseline assessment at admission or some other defined time point or can be calculated daily for patient trending and therefore represent an adjunctive tool for patient assessment, together with a traditional clinical assessment.

Variables used to determine outcome probabilities vary widely depending on the outcome being evaluated. They can be categorized into patient factors or factors relating to treatments and other

processes of care; variables can be weighted with respect to their relative contribution to outcome prediction and finally, they can be selected on the basis of expert opinion, by multivariable logistic regression analysis of patient data in a population of patients where the outcome is known, or by a combination of the two. In an example of the former, the canine modified Glasgow coma score for predicting outcome after head trauma was devised on neurological markers selected by expert opinion as analogous to the human Glasgow coma scale (Platt et al., 2001).

Ideally, selected variables should be independent of treatment or care process, as these factors are likely to vary among groups of clinicians and institutions. Inclusion of variables of this nature may decrease external validity and thus limit application of the model to the wider population. Furthermore, selection of variables that are infrequently measured or expensive to measure, however predictive, also may limit the wider applicability of the model.

A relevant issue in the development of models based on mortality outcome in veterinary medicine is the degree to which the frequency of euthanasia outweighs ‘‘natural’’ death in veterinary patients. The performance and timing of euthanasia reflects in fact multiple factors, including severity of patient illness, owner financial and emotional status, diagnosis of a disease anticipated to be terminal at some future point, subjective assessments of degree of suffering, and individual clinician perspective. If all euthanized patients are excluded from the model development data set, available patient data may be limited and biased. If all patients are included regardless of euthanasia status, the significance of a particular variable as a risk factor for death may be masked by patients euthanized for financial reasons. Handling of euthanized patients in veterinary models varies from complete exclusion to exclusion of some subsets to complete inclusion (Hayes et al., 2010).

Epilepsy-related clinical scoring systems

Status epilepticus has been defined as a severe neurological emergency in human medicine, with reported mortality rates ranging from 7 and 39%. To date, no consensus on the best treatment strategy has been reached and the risk benefit ratio of various treatment protocols is still unclear. The medical management of SE varies widely ranging from IV benzodiazepines in combination or not with other IV AEDs to induced coma with anaestethic agents. The ultimate goal is to find a compromise between the danger related to untreated SE and the damage induced by possibly unnecessary aggressive treatments (Leitinger et al., 2015b).

Accordingly, several scoring systems to predict the outcome of patients affected by SE at the onset of episodes, before the results of diagnostic procedures, have been developed and validated over the course of the years (Yuan et al., 2018).

Status Epilepticus Severity Score (STESS)

This was the first clinical score developed in 2006 for the assessment of short-term outcome in adult patients affected by SE. It was designed as a tool to predict the risk of death in these patients.

Variables taken into consideration for the development of the clinical score represented previously identified risk factors for short-term mortality in SE adult patients and included: age, history of previous seizures, consciousness impairment and seizure type. Variables were used to formulate three possible scores, by varying relative impact of age and consciousness impairment while the other two variables were kept constant. These models were applied retrospectively on 127 episodes of SE occurring in 107 patients. SE caused by cerebral anoxic events were excluded as this condition had been associated with a poor outcome, despite specific treatment. After assessing the receiver operating characteristic curves and the negative predictive value, the best score was subsequently validated on 34 consecutive adult patients with SE prospectively. The results of the study highlighted that none of the patients with a score below the identified cut-off were affected by a poor outcome (Rossetti et al., 2006).

Subsequently, in 2008, the potential utility of the STESS in the decision of SE treatment strategy was assessed by comparing outcomes of a cohort of 134 patients with and without come induction, stratified according to their STESS. The results of the study demonstrated that STESS is an excellent predictor of outcome: a favourable (low) STESS score (0-2) was in fact consistently related to both survival and likelihood to return to baseline clinical condition in surviving patients.

Conversely, STESS score was found to have a poorly efficient positive predictive value for death, therefore it was concluded that STESS score should not be used to justify medical support withdrawal. Furthermore, the survival was not found to be different depending on coma induction, regardless of the STESS score (Rossetti et al., 2008).

Over the years STESS score utility has then been assessed and confirmed by several other studies, with a negative predictive value of 96 – 100% and a positive predictive value of 25 – 39% (Goyal et al., 2015; Kang et al., 2016; Pacha et al., 2016).

Modified STESS (mSTESS)

Because of the low predictive value for bad outcomes and the ceiling effect in patients older than 65 years without pre-existing epilepsy, the association of the STESS score with the modified Rankin Scale (mRS) for the assessment of SE patients has been investigated. The mRS is an objective measure of disability or dependence in daily activities of a patient and it is commonly used in the evaluation of patients presented with neurological emergencies, such as SE, as a tool to measure their functional impairment, usually influenced by different comorbidities. The authors hypothesized that the inclusion of the baseline functional condition of the patient in the STESS could improve the prediction of mortality. The results of this retrospective study performed on 136 patients highlighted that the baseline functional condition defined by the mRS was independently associated with mortality in patients with SE and that by adding this variable to the STESS, the prediction of mortality improved and a correct classification of patients with a low risk of death (STESS 0 - 2) was obtained. Furthermore, the ceiling effect of the STESS resulted decrease and a higher value of positive

predictive value allowed a better identification of patients with a very high risk of mortality (González-Cuevas et al., 2016).

Epidemiology-based mortality score in status epilepticus (EMSE)

Considering the low positive predictive value of the STESS, in 2015 Leitinger and colleagues developed a new scoring system from, with the aim to increase the diagnostic accuracy. The EMSE was based on epidemiological data (i.e. published mortality rates) of aetiology, age, comorbidity, electroencephalography, duration of convulsive activity and level of consciousness. Published mortality rates for these potentially predictive parameters were transformed into points and the new scoring system was then compared with the STESS score as gold standard in a retrospective cohort of 92 SE patients. Sensitivity, specificity, positive and negative predictive values and number of correctly classified patients were calculated for both EMSE and STESS and EMSE performed better to predict patients with poor but also and good outcome. EMSE negative predictive value was 100%, positive predictive value was 69% with 89% of correctly classified patients.

The advantage of this scoring system relies on the opportunity to prognosticate outcome based on real data world, which can be adapted to regions in the world with different aetiology and comorbidity pattern, if epidemiological data are provided, and it can be adapted as new data emerge (Leitinger et al., 2015a).

END-IT score

Both STESS and EMSE have been developed and primarily used to predict survival versus death in the hospital setting. However, none of them were developed for the purpose of predicting the functional outcome of patients with SE after discharged.

In 2016 Gao and collegues performed a retrospective cohort study on 132 patients, with the aim of, firstly, identifying independent prognostic factors associated with the functional outcome of patients three months after discharge from the hospital setting and secondly, of establishing a prognostic score by incorporating these variables according to their determined relative contributions to the resulting functional outcome.

The several predictor variables with a possible association with the recovery of SE were chosen for statistical analysis including age, gender, history of epilepsy, Glasgow coma scale score, presence of the pupillary light reflex, SE etiology (encephalitis vs non encephalitis, as the most common etiology of SE), resistance to diazepam, SE duration, drug induced coma, use of 3 or more types of IV AEDs, complication by non-convulsive SE, need for tracheal intubation, presence of comorbidity, brain images (both CT and MRI). The mRS was used to measure the disability of the patients.

The statistical analysis showed that encephalitis, non-convulsive SE, diazepam resistance, image abnormalities and tracheal intubation were significant independent predictors of functional outcome at three months post discharge from the hospital. Using these predictors and their regression coefficients in the model, a simple sixpoint score was developed in order to predict the probability of

an unfavorable outcome in SE. The END-IT score resulted to have a high sensitivity of 83.9% with a positive predictive value of 70.3% and a specificity of 68.6% with a negative predictive value of 82.8% for the prediction of functional outcomes. The prognostic score achieved a predictive accuracy of 76.22% (Gao et al., 2016).

Several studies, both retrospective and prospective, have been focus on the comparison of the different scoring systems over the years. No substantial differences were found between STESS and EMSE among most studies (Kang et al., 2016; Pacha et al., 2016; Zhang et al., 2018). According to Lin and colleagues, the EMSE resulted to be the most specific, while the STESS was the most sensitive scoring system (Lin et al., 2019). In 2018, Reindl and colleagues reported a slightly higher diagnostic accuracy of the EMSE compared to STESS (Reindl et al., 2018). According to results of the prospective study performed by Giovannini and colleagues, the EMSE reported superior to STESS in the evaluation of outcome, but both scoring system were unreliable in predicting response of patients to antiepileptic treatment (Giovannini et al., 2017).

Scoring system have been developed also in the field of neonatal epilepsy. To date, 4 scoring system have been proposed:

Ellison's scoring system

It represents the first scoring system developed in 1981 for the assessment of newborn affected by seizures and the prediction of seizure disorders, mental retardation and motor dysfunction. It was performed on 96 newborn infants with seizures and variables taken into consideration included abnormality of electroencephalography, neurologic examination, etiology of seizures, length of seizure, type of seizure, and birth weight under or over 1500 grams. Patients included were then re-evaluated at 3 and 10 months. Chi square analysis documented that the scoring system was an accurate predictor of those infants with seizure disorders, mental retardation, and motor dysfunction and authors concluded that the score could assist the clinician in making decisions in regard to anticonvulsant therapy drug at initial hospitalization or at age of 3 months (Ellison et al., 1981).

Garfinkle's score system

The aim of the study was to identify independent prognostic indicators and design a predictive scoring system for neurodevelopmental outcome for term infants who experienced clinical neonatal seizures. Statistical analysis performed retrospectively on 120 term infants with neonatal seizures identified as independent prognostic indicators of adverse outcome (defined as death, cerebral palsy, global developmental delay and/or epilepsy) the following variables: method of delivery, time of seizure onset, seizure type, EEG background findings, and etiology. A five-point

scoring system was consequently devised using these predictors with a sensitivity of 81.1% and a specificity of 84.0% (Garfinkle and Shevell, 2011).

Scoring system for early prognostic assessment after neonatal seizures

Pisani and colleagues performed a retrospective study on 106 preterm newborns affected by seizures in order to devise a clinically viable risk scoring system for the early prediction of adverse neurodevelopmental outcome. The best outcome predictors on multiple logistic regression were birth weight, Apgar score at 1 minute, preictal neurologic examination and ultrasound at seizure onset, efficacy of the anticonvulsant therapy and presence of SE. These variables were used to devise 2 different scoring systems in which the variable background electroencephalographic activity was added only to the second.

The first scoring system resulted highly accurate, with a sensitivity of 85.7%, a specificity of 80.6%, and a positive predictive value of 89.6%. When background electroencephalographic activity was included, the score had a comparable accuracy with a sensitivity of 81.4%, a specificity of 83.3% and a positive predictive value of 90.5% (Pisani et al., 2009).

Scoring System as a Prognostic Tool for Epilepsy After Neonatal Seizures

Another retrospective study published in 2015 was instead focused on the evaluation of risk factors for the development of epilepsy in newborns with neonatal seizures and the subsequent development of a scoring system as a prognostic tool. Also, a validation of the Garfinkle's score system was carried out. The five independent predictors from Garfinkle's study and other known predictors were entered into hierarchical binary logistic regression models and analyzed through four steps to identify independent predictors of epilepsy. Of five potential predictors from Garfinkle's score, electroencephalograph background findings and etiology were predictive. Etiologies, gestation, mode of delivery, duration of seizures, and other risk factors at birth were found to be independent predictors. Duration of seizures has a different effect on prognosis depending on the gestational age (Soltirovska-Salamon et al., 2014).

EXPERIMENTAL SECTION

PROJECT 1

PHARMACOKINETICS OF RECTAL LEVETIRACETAM AS ADD-ON TREATMENT IN DOGS AFFECTED BY CLUSTER SEIZURES AND STATUS EPILEPTICUS

Background

Canine epilepsy is among the most common neurological diseases in dogs (Podell et al., 1995). CS are defined as the occurrence of two or more seizures within a 24-h period, with complete recovery of the state of consciousness in between; SE refers to seizure activity lasting for 5 min or longer or when there's no complete recovery of the state of consciousness between two seizure events (Berendt et al., 2015). CS and SE are potentially life-threatening neurological emergencies and are considered risk factors for spontaneous death or euthanasia of dogs affected by epilepsy (Arrol et al., 2012; Fredsø et al., 2014; Monteiro et al., 2012; Packer et al., 2014; M Saito et al., 2001). As such, these conditions are a frequent reason for presentation to emergency veterinary services (Berendt et al., 2007; Zimmermann et al., 2009). To date, first line therapy is intravenous or rectal administration of diazepam during the seizure event (S. R. Platt, 2014a, 2014b; Podell, 1995). Unfortunately, not all dogs will respond to benzodiazepines and can experience refractory SE. Moreover, prolonged seizure activity is known to decrease the effectiveness of benzodiazepines in human medicine (Deeb et al., 2012). LEV, a pyrrolidone derivative, is a novel AED that was approved in the United States in 1999 for the oral treatment of partial onset seizures in humans (Patsalos, 2004). Its mechanism of action is not fully understood, but it seems to differ completely from other AEDs. LEV is thought to act by binding the synaptic vesicle protein 2A on the presynaptic terminal, thus modulating exocytosis of neurotransmitters (Lynch et al., 2004). Due to its favorable therapeutic profile, LEV has been increasingly used for seizure control either alone or in combination with other first line AEDs in veterinary medicine (Packer et al., 2015). In their study published in 2014, Peters and colleagues found a rapid rise in serum LEV concentrations associated with maintenance of values above the targeted minimum concentration up to 9 h after rectal administration of a LEV formulation in healthy dogs(Peters et al., 2014).

Based on these premises, the aim of this pilot study was to determine the pharmacokinetics of LEV administered per rectum in dogs presented for CS or SE and possibly already in treatment with other long-term AEDs. We hypothesized that LEV administered per rectum would achieve the targeted minimum plasma drug concentration in patients affected by CS and SE. Furthermore, we report the response to treatment as preliminary information on the potential association of LEV administered per rectum as an adjunct to standard treatment in patients referred for CS and SE.

Materials and methods

Animals

The study was approved by the Bioethics Committee of the University of Turin (protocol #9834 dated 25/02/2016). The owners gave their written, informed consent to their dog's enrollment in the study. Client-owned dogs (minimum weight 20 kg) presented with CS or SE to the Veterinary Teaching Hospital (VTH), Department of Veterinary Science of Turin, between October 2016 and April 2017 were eligible for inclusion. SE was defined as a seizure event lasting more than 5 min or two or more seizures without complete recovery of consciousness in between. CS were defined as two or more seizures occurring within a 24-h period. Dogs were excluded if they were already in treatment with LEV for long-term seizure control or if further diagnostic tests indicated reactive seizures.

Study design

At the time of presentation to the VTH, seizure activity was immediately controlled by standard care comprising rectal administration of diazepam (at a dosage of 1–2 mg/kg if the dog was seizuring at presentation) followed by IV administration of phenobarbital (4–5 mg/kg q8h). As soon as possible after hospitalization, and always within 2 h from the presentation, LEV suspension (at a dosage of 40 mg/kg) was administered per rectum. The dosage was based on the results of a previous study (Peters et al., 2014). A rigid, sterile, male dog urinary catheter (BUSTER Disposable Dog Catheter, Buster, Kruuse, Germany) was cut to 5 cm length and inserted approximately 3 to 4 cm into the rectum. A syringe was then connected to inject the drug. The catheter was flushed with air immediately after the injection to ensure the administration of the remaining portion of LEV in the catheter. After removal of the catheter from the rectum, the anus was held closed for 5 min to prevent drug expulsion. The procedure was performed by the same investigator (G.C.) in all patients.

Venous blood samples were obtained immediately before drug administration (T0), and at 30 (T1), 60 (T2), 90 (T3), 120 (T4), 180 (T5), 240 (T6), 360 (T7), 720 (T8), and 1440 (T9) min thereafter. Blood samples were collected in ethylenediaminetetraacetic acid tubes, and plasma was separated immediately after sampling by centrifugation at room temperature (3500 × *g*, 5 min) and then frozen at − 20 °C until analysis. Patients were assessed for signs of adverse reactions specifically attributable to LEV administration (decreased appetite and vomiting) by the same investigator (G.C.) at each time point and between the experimental time points by the intensive care unit veterinarians. For the assessment of treatment efficacy, dogs were defined as "responders" if no further epileptic seizures occurred during the 24-h observation period between hospital admission and discharge; "non-responders" were dogs that experienced an additional epileptic seizure despite LEV administration in addition to the above-mentioned protocol in the 24-h period.

LEV suspension

Pure LEV powder (Levetiracetam European Pharmacopoeia Reference Standard, Sigma-Aldrich, Saint Louis, MO, USA) was purchased and mixed with sterile water to make a suspension with a LEV concentration of 200 mg/ml. This was done to reduce the volume of solution for rectal

administration and minimize the risk of accidental evacuation of the drug. The suspension was formulated and replaced every month. LEV suspension was stored at room temperature away from direct light and always vigorously shaken to suspend the powder before administration.

Determination of plasma LEV concentrations

LEV powder and all other reagents were purchased from Sigma-Aldrich. LEV was analyzed on a high-performance liquid chromatography (HPLC) system (Dionex Thermo Fischer Scientific, Sunnyvale, CA, USA) and separation was performed on a C18, 5 µm, chromatography column (Dionex Thermo Fischer Scientific) protected by a security guard precolumn. Chromatographic run was carried out at 35 °C for 20 min with a step gradient starting at 0 min with 95% solvent A (H_3PO_4 0.423% in water) and reaching 100% solvent B (acetonitrile) at 12 min. Detection was performed at λ = 210 nm. The limit of detection was 1 µg /ml. For LEV extraction, 500 µl of plasma were mixed with 10 µl of $HClO_4$ and 500 µl of methanol. The samples were then vortexed for 2 min and centrifuged at 17,000 × *g* for 5 min. Forty microliters of supernatant were then analyzed by HPLC. The unknown concentrations of LEV in samples were quantified by comparing the signal to standard calibration curve ($R^2 = 0.9947$). The recovery percentage was 99.2 ± 4.9%.

Data analysis

Continuous variables, including patient age and weight at inclusion, were reported as median (minimum – maximum) [min – max]. Pharmacokinetic parameters were estimated by plotting LEV concentrations versus time. Data were analyzed using a Chromelion 6 Chromatography data system (Chromelion 6 Chromatography data system, Thermo Fischer Scientific), and statistical analysis was performed using GraphPad InStat 3.0 (GraphPad InStat 3.0, GraphPad Software, La Jolla CA, USA). Parameters were area under the curve (AUC), maximum concentration (C_{max}), time to maximum concentration (T_{max}), and half-life (t ½). Non-compartmental analysis was performed with AUC calculated using the linear trapezoidal method. The Shapiro-Wilk test showed normal distribution of the dataset; data were reported as mean ± standard deviation (SD).

Results

A total of 36 dogs were presented for CS or SE to the VTH between September 2016 and April 2017. Eight dogs met the inclusion criteria and were included in the study. The other 28 patients were excluded because: body weight less than 20 kg (16/28), no consent given by the owners for inclusion in the study (7/28), long-term oral LEV administration for seizure control (3/28), and diagnosis of reactive seizures (2/28).

Among the eight dogs included in the study, five were intact females and three were males (two intact and one neutered); the median age and weight at presentation were 75 months (range, 43–126) and 34 kg (range, 24–52), respectively. Detailed information on signalment and history are reported in an additional file (see Additional file 1). Blood work comprising complete hematology and biochemistry panel, bile acid stimulation test, and blood ammonia concentration resulted within

normal limits. Four dogs were diagnosed with suspected idiopathic epilepsy based on signalment, history, and normal interictal neurological examination. Magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis were available for only one patient and were unremarkable. Signalment, history, and abnormal interictal neurological examination aroused suspicion of structural epilepsy in the four other patients. In two of these cases a neoplastic lesion (suspected glioma) was confirmed by MRI investigation. A space-occupying lesion was suspected in the other two patients based on signalment and the findings of neurological examination. The neurological examination was performed by a board-certified neurologist or a neurology resident under supervision of the board-certified neurologist.

At the time of inclusion in the study, four out of eight dogs had been receiving phenobarbital (PB) therapy for long-term seizure control; two were concurrently receiving potassium bromide (KBr) and one patient was on treatment with Imepitoin. The remaining three dogs had not received any previous AED therapy (see Additional file 1). The patients receiving PB alone or in combination with KBr had been in treatment for longer than the period needed to achieve steady state of the drugs (14 days and 1–3 months, respectively). PB dosage varied from 2.6 to 6.3 mg/kg q12h, (median, 3.6 mg/kg q12h); the KBr dosage was 40 and 27 mg/kg q24h in each of the two dogs, respectively. Imepitoin was administered at a dosage of 15.7 mg/kg q12h.

Plasma LEV concentrations at the nine time points are shown in Figure 13. At the first experimental time point (T1) the mean concentration was 28.2 ± 15.5 µg/ml ($n = 8$). At this time point (T1), plasma LEV concentrations reached the minimum target concentration of 5 µg/ml in all but one patient, in which it was slightly lower than the target (4.7 µg/ml). Plasma LEV concentrations remained above the minimum target range in all patients until T6 and in 7/8 (88%) patients at T7.

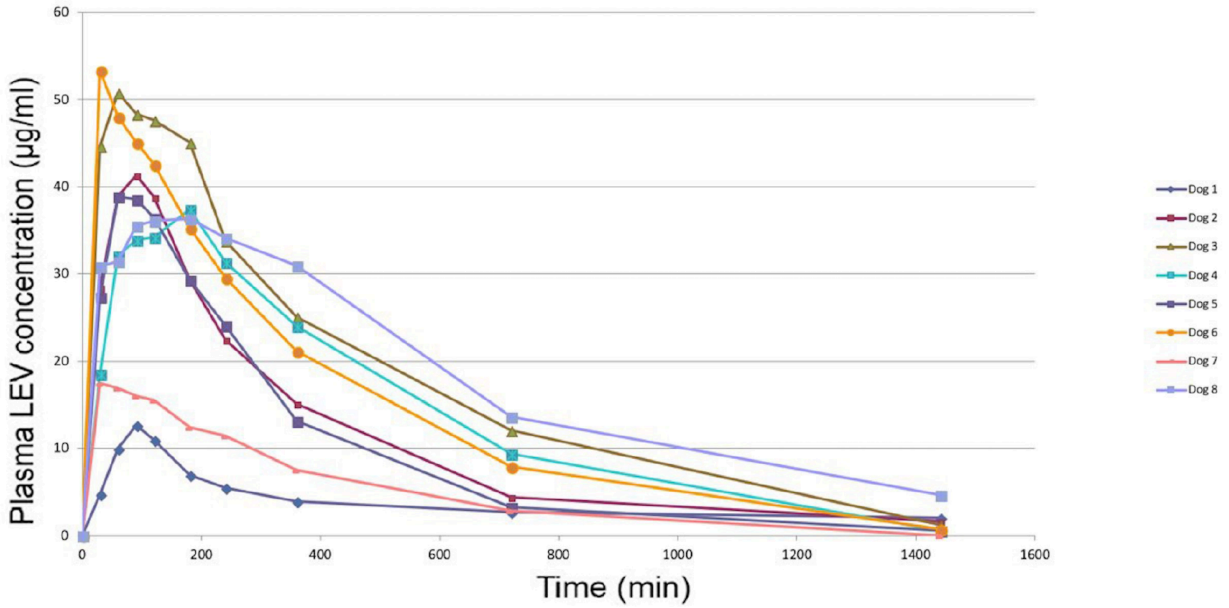

Figure 13. Plasma LEV concentrations versus time

The plot of plasma LEV concentration versus time showed a lower peak concentration and a more rapid decrease in LEV concentration over time in two patients. Pharmacokinetic analysis of the data from the eight dogs revealed a C_{max} of 36.0 ± 14.4 µg/ml, with a T_{max} of 90 ± 60 min. The t ½ was 251.7 ± 75.6 min and the AUC 227.8 ± 131.8 µg-h/ml.

Six out of eight patients (75%) experienced no further seizures during the 24-h observation period and between hospital admission and discharge. Two patients (25%), diagnosed with confirmed and suspected idiopathic epilepsy, respectively, and both with lower peak concentrations and a more rapid decrease in LEV concentration over time, were classified as "non-responders". They required further medications (constant rate infusions of diazepam in one and constant rate infusion of propofol in the other) for seizure control.

Considering the different outcomes, a post-hoc analysis was carried out with the patients grouped into "responders" (*n* = 6) and "non-responders" (*n* = 2). The results are shown in Table 3.

	C_{max} (µg/ml)	T_{max} (min)	AUC_{0-t} (µg-h/ml)	t ½ (min)
"Non-responders" (n = 2)	12.7 and 17.53	90 and 30	89.63 and 105.24	153 and 249
"Responders" (n = 6)	43.02 ± 7.27	100.2 ± 64.8	337.94 ± 83.41	268.6 ± 75.8

Table 3. Results of statistical analysis

Discussion

To our knowledge, this is the first study to evaluate LEV concentration after rectal administration in dogs presenting with CS or SE and potentially receiving concurrent therapy with other AEDs for long-term seizure control. In line with the observations reported by Peters and colleagues (Peters et al., 2014), our results show that the targeted minimum plasma LEV concentration can be achieved with rectal administration of 40 mg/kg. In the majority of cases, plasma concentrations reached the minimum targeted concentration after rapid absorption, already at the first blood sample taken 30 min after administration of the drug. A therapeutic range of LEV specific for dogs has not yet been established. The values of 5–45 µg/ml typically employed in veterinary medicine are deduced from human medicine (De Risio, 2014c). The therapeutic range is highly variable, however, even in human patients, and mainly in correlation with age (Patsalos et al., 2008).

Peters and colleagues highlighted the potential risk of lower LEV absorption after rectal administration if palpable fecal material is present in the rectum (Peters et al., 2014). The lower values of C_{max} and T_{max} for the two dogs in our series may be associated with less absorption of the drug due to the presence of fecal material. Another possible explanation for the different results is

the concurrent long-term administration of PB. Indeed, LEV undergoes predominant renal excretion as unchanged drug (47 and 58% in female and male dogs, respectively). The remaining percentage of the drug is metabolized as acid metabolites and hydroxylated metabolites by hydrolysis and oxidation, respectively. This latter route of degradation was found to be induced by PB in rats and dogs (Strolin Benedetti et al., 2004). Further investigations on dogs confirmed that chronic PB administration alters the metabolism of LEV, resulting in lower concentrations and more rapid renal clearance of LEV when administered PO (S. Moore et al., 2011; Muñana et al., 2015). In all studies performed in veterinary medicine, only 21 days of PB administration were proven sufficient to increase metabolism of LEV, so the chronic PB administration is an unlikely explanation for the results in these two dogs. Unfortunately, we did not check for the presence of fecal material since we wanted to replicate the conditions in which rectal administration is performed in clinical settings. While this could be the most plausible explanation, we are unable to determine whether the lower drug absorption was due to any fecal material potentially present at the time of administration. This issue represents a limitation of the present study. Further studies are needed to evaluate the pharmacokinetics of rectal LEV in patients concurrently receiving PB, while excluding the confounding factor of feces present in the rectum. If this assumption is confirmed, it could also be interesting to assess the feasibility and safety of higher doses of LEV administered per rectum in patients under PB therapy.

One of the two dogs classified as “non-responders” had been diagnosed with idiopathic epilepsy at the time of inclusion in the study. According to the revised definition of pharmacoresistant epilepsy issued by the International League Against Epilepsy in human medicine in 2010 (Kwan et al., 2010), this patient can be classified as pharmacoresistant, and so this condition could explain the patient’s non-responsiveness to treatment. Since seizure frequency was not recorded by the owner of the second non-responder patient diagnosed with suspected idiopathic epilepsy, it is impossible to establish whether this dog can be classified as pharmacoresistant as well. If LEV efficacy can be demonstrated in a larger number of cases, achievement of the targeted minimum LEV plasma concentration with rectal administration in epileptic dogs might allow at-home use of this formulation for better seizure control. The usage of IV/oral LEV in so-called “pulse treatment” for cluster seizures is well known (De Risio, 2014c; Packer et al., 2015). Nevertheless, in dogs experiencing seizures, the administration of oral medications may be delayed by the post-ictal phase, potentially leading to further seizure events. The rectal route of administration would avoid this delay and improve seizure control.

In our study, we formulated LEV suspension at a concentration of 200 mg/ml to reduce as much as possible the volume of medication introduced into the rectum, thus preventing induction of defection and subsequent accidental expulsion of the drug. The LEV suspension was made using pure LEV powder for scientific reasons. We also made LEV suspension from commercially available LEV

tablets and found no differences in the chemical purity of the two formulations (data available from the authors on request).

The main limitations of the present study are the small patient series, the concomitant use of other AEDs, and the absence of a control group of patients for comparison. The designation of “responder” and “non-responder” was in reference to the combination of medications administered, and therefore it is not possible to discern any potential effect of rectal LEV administration. Therefore, we cannot conclude that rectal LEV is effective in preventing the onset of further seizures in patients with CS or SE. Nonetheless, these preliminary pharmacokinetic data are promising and are consistent with those reported by Peters and colleagues. Given the postulated enhancement of the anticonvulsive effects of benzodiazepines and the lack of side effects, such as cardiac and respiratory depression typical of other AEDs (Uges et al., 2009), LEV can offer a potentially useful add-on to the treatment of seizure activity in dogs once its efficacy has been confirmed in a greater number of cases. Further studies are needed to confirm or confute our preliminary hypothesis. A future area of focus of this project is to better evaluate the efficacy of rectal LEV in a larger number of cases.

Conclusions

In conclusion, our findings show that targeted minimum plasma LEV concentration can be reached after rectal administration of 40 mg/kg in dogs with CS or SE. These preliminary results, if confirmed, may allow for the use of rectal LEV as an additional treatment option for CS and SE in dogs.

Additional information

The present study has recently been published as:

Cagnotti G., Odore R., Gardini G., Amedeo S., Bertone I., Guerriero G., Lentini L., Dappiano E., D’Angelo A. *Pharmacokinetics of rectal levetiracetam as add-on treatment in dogs affected by cluster seizures or status epilepticus.* BMC Vet Res; 2018:14 - 189. doi: 10.11867/s12917-018-1522-0.

PROJECT 2

OPEN-LABEL CLINICAL TRIAL OF RECTALLY ADMINISTERED LEVETIRACETAM AS SUPPLEMENTAL TREATMENT IN DOGS WITH CLUSTER SEIZURES.

Background

Medical literature provides evidence for progressive loss of efficacy of benzodiazepines during prolonged seizure activity because of a functional alteration of gamma aminobutyric acid-A receptors that are internalized into the cells (Sánchez Fernández et al., 2018). This limitation has prompted investigation of other treatment options for the emergency management of these neurological conditions. Furthermore, in both human and veterinary medicine, treatment guidelines for CS and SE must balance the need for seizure control with the risk of dangerous adverse effects (eg, cardiorespiratory depression) associated with AED administration (Patterson, 2014; Sánchez Fernández et al., 2018). To enhance the anticonvulsant action of AEDs and decrease the occurrence of adverse effects, the concept of early polytherapy recently has been introduced in human medicine (Alvarez and Rossetti, 2016). Although the literature on early combined polytherapy is still limited, it is hypothesized that AEDs with different molecular mechanisms of action can be combined together so as to enhance their anticonvulsant properties (Radhakrishnan, 2016).

Because of its different mechanism of action, its favorable pharmacokinetics, and favorable safety profile, LEV has been investigated as a candidate for early combined polytherapy in human medicine. In particular, 2 experimental studies on mice and human patients have provided evidence of synergy between LEV and diazepam (Mazarati et al., 2004; Modur et al., 2009). LEV use also has gradually increased in recent years in veterinary medicine as long-term monotherapy or pulse treatment given IV or PO in CS patients (De Risio, 2014c; Packer et al., 2015). The latter treatment strategy has been proposed to avoid the induction of LEV tolerance as reported in both mice and dogs (Loscher and Schmidt, 2006; Volk et al., 2008).

The PO route can be easily employed by owners at home. However, the postictal phase in epileptic patients can impair swallowing ability, preventing use of the PO route because of aspiration risk, thus delaying the initiation of treatment. For this reason, we evaluated another route of administration of LEV in epileptic patients.

The pharmacokinetics of LEV after rectal administration in both healthy and epileptic dogs recently has been investigated. These studies indicated that administration of 40 mg/kg per rectum achieved the minimum target concentration of 5 µg/mL 10 and 30 minutes after the administration in healthy and affected dogs, respectively (Cagnotti et al., 2018; Peters et al., 2014).

Our aim was to investigate the clinical efficacy of rectally administered LEV in preventing additional seizures when administered to dogs presented for CS and SE. We hypothesized that administration of LEV rectally in addition to a standard treatment protocol would provide better control of seizure activity as compared with the standard treatment protocol alone.

Materials and methods

The study was approved by the Bioethics Committee of the University of Turin (protocol #9834, dated February 25, 2016). Written informed consent was obtained from the dog owners before enrollment in this open-label clinical trial.

Dogs

Dogs referred to the VTH of the Department of Veterinary Science, Turin, between September 2016 and May 2018 for CS or SE of any type were eligible for inclusion in the study. No age, breed, or sex limitations were applied. If the dogs were referred to the VTH for CS or SE multiple times during the study period, only the first hospitalization was considered for the purpose of the study. Status epilepticus and CS were defined according to the definitions of the IVETF consensus report (Berendt et al., 2015).

Minimum database blood tests, including complete blood count, serum biochemistry profile, serum electrolyte concentrations, blood ammonia concentration, and pre-prandial and post-prandial bile acid concentrations were performed. All dogs underwent neurological examination by a board-certified neurologist or a neurology resident under the supervision of the board-certified neurologist. Dogs were excluded if already under treatment with LEV for long-term seizure control or if further diagnostic tests indicated reactive seizures.

A diagnosis of idiopathic epilepsy was made according to the IVETF consensus report (Luisa De Risio et al., 2015), whereas a diagnosis of structural epilepsy was suspected when reactive causes of seizures were excluded, along with signalment, history, and an abnormal interictal neurological examination. Magnetic resonance imaging results, cerebrospinal fluid analysis results or both were included if available but were not required for the diagnosis of structural epilepsy.

Eight dogs included in the present study had been enrolled in a previous study evaluating the pharmacokinetics of LEV administered per rectum in dogs with CS and SE (Cagnotti et al., 2018).

Study design

At the time of presentation to the VTH, a standard care protocol comprising rectal/IV diazepam (at a dosage of 1-2 mg/kg if the patient was seizuring at presentation) followed by IV PB (4-5 mg/kg q8h) was administered to each dog. After administration of these medications, the patients were selected to receive either a single dose of rectal LEV at a dosage of 40 mg/kg in association with PB q8h (*rectal LEV group*) or no other medications except for PB q8h (*control group*). No proper randomization of patients between the 2 study groups was performed because the dog owner, through written informed consent, made the final decision for assigning the dog to the rectal LEV group or control group.

The LEV suspension employed was created and administered as previously reported (Cagnotti et al., 2018). The dogs were monitored for additional seizures for the first 24 hours after admission and until discharge. Dogs that experienced no additional seizures in the first 24 hours were defined as

responders, whereas those that showed additional seizure activity and therefore required other AEDs in the first 24 hours were classified as non-responders.

Statistical analysis

Statistical analysis was performed using commercially available software (R 3.5.2—R Core Team, 2018). Data were analyzed for normality using the Shapiro-Wilk test and were found to be nonparametric. Numerical data (age and body weight) were tested using the Wilcoxon rank sum test; categorical data (sex, reason for presentation, and seizure etiology) were tested using the test for equality of proportions or Chi-square test, where appropriate. A comparison between the number of responders versus non-responders between the 2 groups was carried out using Fisher's 2-tailed exact test. In addition, a comparison between the number of responders versus non-responders in relation to the etiology (idiopathic versus suspected or confirmed structural epilepsy) of seizure activity was performed using Fisher's 2-tailed exact test. Results were considered statistically significant at a significance level of $P < .05$.

Results

Sixty-six dogs were referred because of CS and SE to the VTH between September 2016 and May 2018. Nine of 66 patients were excluded from the study. Four patients were excluded because further investigations led to a diagnosis of reactive seizures, 4 because they were already being treated with LEV for long-term seizure control, and 1 patient because the episodes possibly related to seizure activity were witnessed only by the owner and no proper evidence of epileptic seizures could be obtained, respectively.

In total, 57 patients were included in the study: 21 dogs were assigned to the rectal LEV group and received 40 mg/kg of LEV per rectum in addition to the standard protocol, and 36 were assigned to the control group and received only the standard care protocol. Table 4 presents signalment and patient characteristics. There were no statistically significant differences in median age, sex, body weight, reason for presentation, and seizure etiology between the LEV and the control group.

A diagnosis of idiopathic epilepsy (Tier I or II confidence level using the IVETF consensus report) was present in 16 patients in the control group (44%) and in 12 patients in the rectal LEV group (57%). A structural etiology was suspected or confirmed in 20 patients (56%) in the control group and in 9 (43%) in the rectal LEV group. Table 4 presents details on the definitive diagnoses.

	Rectal LEV group	Control group
Breed	Mixbreed (8/21), French Bulldog (3/21), Boxer (2/21), Corso Dog (2/21), German Shepherd (2/21), Bloodhound (1/21), Dachshund (1/21), Pyrenean Mountain Dog (1/21), Argentine Mastiff (1/21)	Mixbreed (14/36), Border Collie (3/36), Pinscher (2/36), Yorkshire terrier (2/36), American Staffordshire (1/36), Bernese Mountain Dog (1/36), Breton (1/36), Cavalier King Charles Spaniel (1/36), Chihuahua (1/36), Dogue de Bordeaux (1/36), English Bulldog (1/36), German Shepherd (1/36), Golden Retriever (1/36), Labrador Retriever (1/36), Maltese (1/36), Poodle (1/36), Pug (1/36), Siberian Husky (1/36), Spitz (1/36)
Age	Median 75 (range 49 – 113 months)	Median 68 (range 31.5 – 93 months)
Sex	12 males (57%), 5 females (24%), 3 males neutered (14%), 1 female neutered (5%)	20 males (55%), 10 females (28%), 6 females neutered (17%)
Body weight	Median 24 (range 16 – 28.7 kg)	Median 16 (range 7.15 – 27.8 kg)
Epilepsy etiology	10 dogs tier I idiopathic epilepsy, 2 dogs tier II idiopathic epilepsy, 3 dogs intracranial neoplasia, 1 dog hemorrhagic stroke, 5 dogs suspected undefined structural epilepsy.	9 dogs tier I idiopathic epilepsy, 7 dogs tier II idiopathic epilepsy, 2 dogs undefined degenerative disease, 2 dogs meningoencephalitis of unknown origin (MUO), 1 dog hydrocephalus, 1 dog intracranial neoplasia, 16 dogs suspected undefined structural epilepsy.
Long-term AEDs	PB (5/21), PB and KBr (3/21), Imepitoin (1/21), None (12/21).	PB (10/36), PB and KBr (4/36), None (22/36).
Presentation	Generalized SE (3/21), CS (18/21)	Generalized SE (5/36), CS (31/36)

Table 4. Information on patients included in the study for each study group. No statistically significant differences were found between the 2 study groups in age, sex, body weight, reason for presentation, and seizure etiology. *The smallest* P *value obtain was .08 (sex).*

Given the small number of patients admitted with SE in both groups, statistical analysis was performed taking into consideration only patients affected by CS. Fisher's 2-tailed exact test showed a statistically significant difference ($P < .001$) between the 2 groups: the response rate was 94% (17/18) in the rectal LEV group and 48% (15/31) in the control group.

Fisher's 2-tailed exact test showed no statistically significant differences between the response rate of patients affected by idiopathic and those with suspected or confirmed structural epilepsy ($P = 1$). When the test was performed on each group separately, no statistically significant differences were found (rectal LEV group, $P = .48$; control group, $P = 1$).

Discussion

Our study provides preliminary evidence for the efficacy of rectally administered LEV when combined with a standard treatment protocol in preventing the onset of additional epileptic seizures in dogs with CS. Given the promising results of previous pharmacokinetics studies (Cagnotti et al., 2018; Peters et al., 2014), we evaluated the potential beneficial effect of rectally administered LEV combined with other AEDs in controlling seizure activity in dogs with CS. Our results show that dogs with CS treated using rectally administered LEV in addition to a standard treatment protocol consisting of diazepam and PB experienced substantially better control of seizures as compared with a control group of patients treated with AEDs using the standard treatment protocol alone. These results suggest that rectal administration could represent a viable alternative to PO administration of AEDs and therefore extend treatment options for the at-home management of these neurological emergencies before referral to a specialized veterinary clinic or hospital.

The lack of a placebo-controlled group of patients is the main limitation of our study. Of note, however, is that a reduction in placebo exposure recently has been advocated in human medicine. The SUDEP rate has been reported to be higher among placebo-treated participants of add-on treatment epilepsy trials in human medicine, suggesting that adding effective AEDs instead of placebo in epilepsy trials can decrease the risk of death of epileptic patients (Fureman et al., 2017). Death during or immediately after seizure activity has been documented in epileptic dogs as well, suggesting that the concept of SUDEP also can be extended to veterinary patients (Blades Golubovic and Rossmeisl, 2017a). The add-on administration of AEDs to a standard antiepileptic regimen therefore seems a valuable alternative to a placebo-controlled trial, even if the latter study design still represents the gold standard for treatment investigations.

In our study, allocation to the 2 groups was based on the dog owners' final decisions for their dogs to receive or not receive rectally administered LEV, and thus the lack of randomization is another study limitation. The decision to perform post hoc analysis only on the patients affected by CS was based on the few cases of SE enrolled in both study groups. However, the results obtained for this specific patient category were as promising as those obtained for both conditions, confirming the potentially beneficial effect of rectally administered LEV in addition to a standard treatment protocol.

Several patients were already being treated for long-term seizures with PB and potassium bromide. Unfortunately, information on serum concentrations of AEDs was not available or up-to-date for all patients included, and for this reason it was not taken into account in the final analysis of the study results. It is therefore impossible to evaluate the influence of these medications on patient outcome. It has been hypothesized that dogs with structural epilepsy have a higher risk of death and presumably less control of seizure activity despite treatment with AEDs (Fredsø et al., 2014; Hardy et al., 2012; Zimmermann et al., 2009). We found no statistically significant difference in response rate between dogs affected with idiopathic epilepsy (Tier I or II confidence level of the IVETF consensus report) and those with presumptive or confirmed structural epilepsy. However, the final diagnosis of structural epilepsy could not be established in all patients and, for this reason, the conclusions must be considered with caution.

In conclusion, based on our study data, rectally administered LEV combined with a standard treatment protocol seems to provide good control of seizure activity in patients with CS. Because of the low number of cases of SE included in our study, this assumption cannot be extended to SE, and further investigations are warranted.

The validity of our results should be confirmed in a double-blinded placebo-controlled clinical trial.

Additional information

The present study has recently been published as:

Cagnotti G., Odore R., Bertone I., Corona C., Dappiano E., Gardini G., Iulini B., Bellino C., D'Angelo A. *Open-label clinical trial of rectally administered levetiracetam as supplemental treatment in dogs with cluster seizures.* J Vet Intern Med; 2019 Jul;33(4):1714-1718. Doi: 10.1111/jvim.15541

PROJECT 3

ANALYSIS OF RISK FACTORS RELATED TO POOR OR FAVOURABLE OUTCOME IN DOGS WITH CLUSTER SEIZURES OR STATUS EPILEPTICUS.

Background

Seizure disorders are one of the most frequent neurological diseases encountered in dogs (Podell, 1996). Most seizures are self-limiting and stop without any acute intervention, however, seizure termination depends on specific mechanisms which may fail in some patients, leading to a lack of neuronal firing inhibition and prolonged seizure activity (Westbrook, 2013). Status epilepticus (SE) results from this lack of inhibition (Blades Golubovic and Rossmeisl, 2017b; S. R. Platt, 2014b) and it is defined as a seizure activity lasting more than 5 minutes or repetitive seizures without intercurrent normalization of consciousness (Berendt et al., 2015). Furthermore, seizures of any type may occur in groups or clusters over a number of hours or days, exceeding over the patient's typical seizure frequency. Definitions of CS in dogs have been inconsistent between publications, but currently CS are defined clinically as two or more seizures within a 24-h period with complete recovery of consciousness in the between (Berendt et al., 2015).

SE and CS represent serious neurological emergencies that require emergent diagnosis and treatment, as they are important risk factors associated with euthanasia and spontaneous death, which are the two expressions of poor outcome in epileptic patients (Arrol et al., 2012; Monteiro et al., 2012; M Saito et al., 2001; Zimmermann et al., 2009).

In human medicine, several scoring systems have been developed in order to predict the short-term outcome of epileptic patients. In particular, 2 scoring system have been focused on the assessment of adult patients with SE (Leitinger et al., 2015a; Rossetti et al., 2006). One of these, the Status Epilepticus Severity Score (STESS) aims to predict survival before treatment institution in adult patients with SE, based on previously established predicting factors such as age, history of prior seizures or epilepsy, level of consciousness, and seizure type at SE onset (Rossetti et al., 2006). Risk factors associated to outcome in canine patients affected by SE and CS have not been studied extensively in veterinary medicine and only limited data are available (Arrol et al., 2012; Berendt et al., 2007; Fredsø et al., 2014; Monteiro et al., 2012; Zimmermann et al., 2009). Based on these premises, the aim of this scientific study was to describe and evaluate the presence of risk factors (prognostics) able of predicting a positive or negative outcome in a population of dogs affected by CS or SE.

Materials and methods

Population and data collection

Medical records of dogs admitted for CS or SE to the Teaching Veterinary Hospital (VTH) of the Department of Veterinary Sciences of the University of Turin between July 2015 and February 2019 were taken into consideration for the study. All clinical charts were manually reviewed.

Only patients with confirmed SE or CS which underwent at least one neurological examination performed by a board-certified neurologist or a neurologist in training were eligible for the inclusion in the study. When patients had more than one stay with SE or CS during the period under examination, only the first was used for analysis. Data extracted from the clinical charts included: sex, neutering status, breed, size, presence of brachycephalic morphology, age at hospitalization, age at first seizure, seizure type (focal vs generalized), seizure presentation (CS or SE), history of previous seizures, long-term AEDS given (in case of epileptic patients), comorbidity, in-hospital complications, lactate and glucose blood concentration at admission, rectal temperature at admission, heart rate and respiratory pattern at admission and outcome. Outcome was defined as death in hospital versus hospital discharge.

Patients were excluded in case of lack of information in medical charts.

Defintions

Signalment and age at hospitalization

Signalment information was collected from each patient's medical records.

Patients were classified on the basis of sex and reproductive status as follows: intact males, neutered males, intact females and neutered females. Breeds and brachycephalic morphologies were defined accordingly to American Kennel Club[a] and UK Kennel Club definitions[b]. To establish the presence of brachycephalic morphology in cross breeds, phone calls were made to the owners who have been asked whether the dog's muzzle was comparable to that of a typical brachycephalic breed (e.g., pug).

Age at hospitalization was expressed in months.

Comorbidity

Comorbidity was defined as the presence of additional diseases in relation to an index disease in one individual (Valderas et al., 2009). In the present study, each disease has been classified according to the guidelines of International Classification of Diseases[c]

Presentation

SE and CS were defined according to the definitions reported by the IVETF consensus report (Berendt et al., 2015).

Type of seizure activity

Seizure type was defined as focal or generalized. Criteria for categorization as focal seizures included clinical signs (either motor, autonomic or behavioural signs, alone or in combination) reflecting the function of the areas involved by a consequent to an abnormal electrical neuronal activity within one hemisphere. Generalized epileptic seizures were defined when both sides of the body were affected by seizure activity as a result of a bilateral involvement of cerebral hemisphere. (Berendt et al., 2015). Only convulsive epileptic seizures (tonic-clonic, tonic, clonic and myoclonic) were taken into consideration for the study, due to the unavailability of an EEG confirmation of non-convulsive seizure activity.

Complications

In hospital complications were considered as secondary complications that affected patients following initial hospital admission (Warner et al., 2016).

Age at first seizure, idiopathic interval and history of previous seizures

History of prior seizures and age at first seizure (expressed in months) were considered. Moreover, as seizure onset between 6 months and 6 years is commonly assumed to be more probably associated with a diagnosis of idiopathic epilepsy (Luisa De Risio et al., 2015), that time period was defined as 'idiopathic interval'.

Previous long-term AEDs

Information regarding any antiepileptic treatment previously established, including which AEDs were given, was collected from the patients' medical record with previous history of seizures.

Lactate and glucose concentrations

According to the guidelines for the emergency management and treatment of patients affected by CS and SE adopted by the VTH, as soon as the seizure activity was controlled and the patient stabilized following referral, a venous blood sample was taken upon entry and analyzed by means of a blood gas analyzer machine (ABL825 Flex Blood Gas Analyzer, Radiometer Medical ApS, Denmark).

Lactate and glucose blood concentrations are two of the parameters obtained by the analysis.

Lactate is an anion and conjugate base to lactic acid, the product of anaerobic metabolism of glucose, clinically recognized as a valuable triage tool, prognostic indicator and potential therapeutic target (Rosenstein et al., 2018). Hyperlactemia was defined as a serum, plasma or blood lactate concentration above the relevant reference interval of 0.3 – 2.5 mmol/l in adult animals (Gillespie et al., 2017). Hyperlactemia was defined as mild to moderate between 2.6 and 6 mmol/l, while lactate

concentrations > 6 mmol/l were considered as severe hyperlactemia (Gillespie et al., 2017; Rosenstein and Hughes, 2015).
Glucose reference value ranged between 60 to 130 mg/dl (Forcada, 2017). Patient whose values were below the lower limit were considered as hypoglycemic; conversely hyperglycemia was defined when serum glucose concentration was over the upper threshold.

Rectal temperature, heart rate and respiratory pattern
Variables were classified accordingly to reference values based on size as follow: dogs weighing 4 to 10 kg were considered small and their temperature, heart and respiratory rate normal ranges were 38.5-39.2°C, 80-180 beats per minute (bpm) and 24-36 breaths per minute respectively.
Dogs weighing 11 to 25 kg were considered medium, their normal ranges were 38-39°C, 80-160 bpm and 20-32 breaths per minute. Dogs weighing > 25 kg were considered large, with reference intervals of 37.5-38.5°C, 60-80 bpm and 16-25 breaths per minute respectively (Ciaramella, 2013).
Puppies (i.e., dogs up to 9 months) were classified with different reference values. Puppies normal ranges were considered as follows: 38.5 °C for the first month and then, according to size as previously described for adults; normal heart rate up to 220 bpm and normal respiratory rate from 20 to 40 breaths per minute (Ciaramella, 2013).
Size classification was based on weight and breeds standard sizes, and references were taken from Royal canine guidelines[d].

Outcome
Poor outcome, defined as death in hospital, was considered either if caused by euthanasia or by spontaneous death. Survivors were discharged after 24 h of seizure-free or after 24 h from the discontinuation of drug-induced coma in absence of seizure activity.

Statistical analysis
Data were analyzed using commercially available software programs (Excel [Microsoft], version 16.27 [19071500]; R commander graphical interface R version 3.3.2 [2016-10-31]). Standard descriptive statistics were reported as median and interquartile range or mean and standard deviation for continuous variables and percentage and frequency for categorial variables. Continuous variables were tested for normality distribution by Shapiro-Wilk test and were found to be nonparametric. Numerical data were tested using Wilcoxon rank sum test, while categorial variables were compared using Chi-square or Fisher's 2-tailed exact tests when appropriate. Statistical significance was defined as $P < 0.05$. A P value between 0.05 and 0.10 was defined as a trend (tendency) to significance. Categorical variables included: sex and neutering status, breed, brachycephalic morphology, seizure presentation, comorbidity, complications,

idiopathic interval, history of previous seizures, previous long-term AEDs, seizure type and respiratory pattern; age at first seizure, age at hospitalization, rectal temperature, heart rate, glucose and lactate serum concentrations were instead considered as continuous variables.
Subsequently, rectal temperature, heart rate, glycemic level and blood lactate level were categorized, as previously reported, for statistical purposes.

Results

One hundred thirty-four dogs met the inclusion criteria for the present study. Due to the lack of information, 41 patients were excluded, resulting in a final population of 93 dogs. Of these, 36 (39% of patients total) were female (19 intact, 17 neutered) and 57 (61%) were male (51 intact, 6 neutered). Cross breed (32/93), German Shepherd (8/93) and French Bulldog (7/93) were the most common represented breeds, followed by 3 dogs for each of the following breeds: American Staffordshire Terrier, Boxer, Chihuahua, Corso dog. Respectively 2 dogs for Yorkshire, Border Collie, Cocker, English Bulldog, Poodle and Pekinese, and 1 dog for Australian Shepherd, Beagle, Bernese Mountain dog, Epagneul Breton, Cavalier King Charles Spaniel, Czechoslovakian Wolf, Dachshund, Dogue de Bordeaux, Pinscher, Giant Schnauzer, Golden Retriever, Labrador Retriever, Maltese, Miniature Schnauzer, Pitbull Terrier, Pomeranian Spitz, Pug, Shih tzu, Siberian Husky, St. Bernard and Weimaraner were also included. Brachycephalic dogs were 27/93 (29%). Examined dog population was distributed concerning sizes as follow: 34/93 (36%) large breed dogs, 23/93 (25%) medium breed dogs and 36/93 (39%) small breed dogs.
The median age at hospitalization was 88 months (range 48 - 134), while the median age at first seizure was 72 months (range 24 - 120). Age at first seizure was within ‘idiopathic interval’ in 46/93 dogs (49%). Four out of 93 (4%) patients experienced their first seizure before 6 months, while 43/93 (46%) after 6 years.
History of previous seizures was present in 52 out of 93 dogs (56%). Of these, 25 patients were on long-term AED treatment for seizure control. Nine-teen dogs were receiving 1 medication:
14 patients were in treatment with PB, 4 with Imepitoin and 1 dog was having Gabapentin treatment; four dogs were having 2 AEDs given: 2 patients were in treatment with PB and LEV, 1 patient with PB and Carbamazepine, and 1 patient with PB and KBr. Only 2 dogs were receiving three AEDs: PB, Imepitoin and Diazepam in one case and PB, KBr and LEV in the other case.
The mean period between age at first seizure and age at hospitalization was 12 months in patients already on antiepileptic treatment and 15 months in patients not receiving any AEDs.
Twenty-one out of 93 patients (23%) presented with SE while 72/93 (77%) with CS. Generalized and focal seizures occurred in 87/93 (94%) and 6/93 (6%) dogs, respectively.
Comorbidities were identified in 27 out of 93 (29%) dogs, while in-hospital complications occurred in 6/93 (6%) patients.

The median glucose serum concentration was 109 mg/dl (range 96 - 127); 20 out of 93 (21%) dogs were hyperglycemic (median 142.5 mg/dl; range 135 - 162), while 9/93 (10%) were hypoglycemic (median 49 mg/ dl; range 35 - 53).

Median lactate serum concentration was 2.3 mmol/l (range 1.4 - 3.9). Thirty-three out of 93 (35%) dogs were mildly hyperlactatemic (median 3.9 mmol/l; range 3.1 - 4.6 mmol/l) while 9/93 (10%) displayed severe hyperlactatemia (median 10.5 mmol/l; range 8 - 14.8).

The median temperature of the examined population was 38.6°C (range 38.2 - 39.2°C). Hyperthermic patients (median 39.6°C; range 39.1 - 40°C) were 34/93 (37%) while 17/93 (18%) suffered from hypothermia (median 38°C; range 37.8 - 38.2°C).

The median heart frequency was 120 bpm (range 100 - 140); tachycardic dogs (median 120 bpm; range 100 - 140) were 33/93 (35%) while bradycardia (median 71 bpm; range 67.5 - 72.5) was detected in 4/93 (4%) dogs.

Seventy-one out of 93 (76%) dogs experienced tachypnea, while bradypnea was present in 6/93 (7%) patients. One dog (1%) presented with dyspneic respiratory pattern.

Twenty-one out of 93 (23%) patients died during the hospitalization, 5 of them died of spontaneous death (cardiorespiratory arrest) and 16 from euthanasia, while 72 (77%) patients were discharged alive from hospital.

The results of variable comparisons between survivors and non-survivors is reported in Table 5.

Age at the first seizure was statistically different between survivors (median 61 months, range 24 - 100) and non survivors (median 120 months, range 43 - 156) (p-value 0.05). When comparing the outcome of patients whose first seizure occurred inside or outside the idiopathic interval a trend towards an association between poor outcome and age < 6 months or > 6 years was detected (p-value 0.09). Two (50%) of 4 patients which had their first episode before 6 months, died in hospital, while 13 dogs out of 43 (30%) which experienced their first seizure after 6 years of age, died in hospital. Six (13%) out of 46 dogs for which age at first seizure was between the idiopathic interval died during hospitalization.

The presence of in hospital complications was as well statistically associated with a poor outcome (OR 7.9, IC: 1.05-95.15, p-value 0.02).

Cardiac frequency resulted statistically different between survivors and non survivors, with a median of 110 bpm (range 100 - 130) in dogs discharged alive and a median of 132 bpm (range 112 - 150) in dogs dead before discharge (p-value 0.02). However, this significance disappeared when heart rate categories were created (p-value 0.75).

The median rectal temperatures were 38.5°C (range 38.2 - 39°C) and 38.9°C (range 38.5 - 40°C) in survivors and non survivors respectively, and this difference resulted to be statistically significant (p-value 0.02). However, after categorizing temperature in low, normal and high according to size, hyperthermic patients were correlated to fatal outcome only with a trend (p-value 0.08).

Finally, epileptic dogs not in treatment with long-term AEDs were associated with a risk of poor outcome compared to epileptic patients already receiving AEDs (p-value 0.01; OR 11.5, IC: 1.38-545.73). No other correlations were found.

	Patients total (93)	**Survivors (72)**	**Non-survivors (21)**	**p-value**
N	93 (100%)	72 (77%)	21 (23%)	
Gender				0.85
Male	51 (55%)	41 (57%)	10 (48%)	
Male neutered	6 (7%)	4 (6%)	2 (9%)	
Female	19 (20%)	14 (19%)	5 (24%)	
Female neutered	17 (18%)	13 (18%)	4 (19%)	
Breed				1.00
Crossbreds	32 (34%)	25 (35%)	7 (33%)	
Purebreeds	61 (66%)	47 (65%)	14 (67%)	
Size				0.21
Small size	36 (39%)	28 (39%)	8 (38%)	
Medium size	23 (25%)	15 (21%)	8 (38%)	
Large size	34 (36%)	29 (40%)	5 (24%)	
Brachycephalic morphology	27 (29%)	23 (32%)	4 (19%)	0.25
History of seizures	52 (56%)	42 (58%)	10 (48%)	0.38
Long-term AEDs*	25 (48%)	24 (57%)	1 (10%)	**0.01**
Monotherapy	19 (76%)	19 (79%)	0 (0%)	
Polytherapy	6 (24%)	5 (21%)	1 (100%)	
Presentation				0.18
SE	21 (23%)	14 (19%)	7 (33%)	
CS	72 (77%)	58 (81%)	14 (67%)	
Seizure type				0.72
Generalized	87 (94%)	67 (93%)	20 (95%)	
Focal	6 (6%)	5 (7%)	1 (5%)	
Comorbidities	27 (29%)	19 (26%)	8 (38%)	0.30
Disease count				0.94
One comorbidity	20 (74%)	14 (74%)	6 (75%)	
Two comorbidities	7 (26%)	5 (26%)	2 (25%)	
Disease type				

Cardiocirculatory system	8	5	3	
Respiratory system	4	2	2	
Genito-urinary system	1	1	0	
Infectious and parasitic diseases	2	1	1	
Gastrointestinal system	3	2	1	
Endocrine, metabolic and nutritional diseases	3	2	1	
Neoplastic diseases	3	1	2	
Ocular diseases	6	6	0	
Ear diseases	1	1	0	
Neurological diseases	1	1	0	
Muscoloskeletal system	1	1	0	
Traumatic, toxic and other external causes of diseases	1	1	0	
Complications	6 (6%)	2 (3%)	4 (19%)	**0.02**
Antibiotic therapy	3	2	1	
Acute lung injury	1	0	1	
Acute kidney insufficiency	1	0	1	
Cardiac arrest	1	0	1	
Atrial fibrillation	1	0	1	
Age at first seizure	72 (24 – 120)	61 (24 – 100)	120 (43 – 156)	**0.05**
First seizure in "idiopathic interval"	46 (49%)	40 (56%)	6 (29%)	**0.09**
Age at hospitalization	88 (48 – 134)	84 (47 – 127)	129 (48 – 156)	0.13
Serum glucose concentrations				0.65
Normoglycemia	64 (69%)	51 (71%)	13 (62%)	
Hypoglycemia	9 (10%)	6 (8%)	3 (14%)	
Hyperglycemia	20 (21%)	15 (21%)	5 (24%)	
Serum lactate concentrations				0.61
Normolactemia	51 (55%)	38 (53%)	13 (62%)	
Mild-moderate hyperlactemia	33 (35%)	26 (36%)	7 (33%)	
Severe hyperlactemia	9 (10%)	8 (11%)	1 (5%)	
Rectal temperature				**0.08**
Normothermia	42 (45%)	36 (50%)	6 (29%)	
Hypothermia	17 (18%)	14 (19%)	3 (14%)	
Hyperthermia	34 (37%)	22 (31%)	12 (57%)	
Heart rate				0.75

Within normal range	56 (60%)	42 (58%)	14 (67%)	
Bradycardia	4 (4%)	3 (4%)	1 (5%)	
Tachycardia	33 (36%)	27 (38%)	6 (28%)	
Respiratory pattern				0.31
Eupnoea	15 (16%)	12 (17%)	3 (14%)	
Dispnoea	1 (1%)	0 (0%)	1 (5%)	
Bradypnoea	6 (6%)	5 (7%)	1 (5%)	
Tachypnoea	71 (77%)	55 (76%)	16 (76%)	

Table 5. Variables of interest compared to outcome in the whole cohort. **Long term AEDs percentage refers to patient with previous history of seizures, the totality, the survivors and the non survivors only, depending on the column.*

After the evaluation of risk factors for poor outcome, post hoc analyses were performed in order to evaluate specific associations between variables examined and presentation. No statistically significant correlations were found, except for hypoglycemia, that resulted to be associated with SE presentation ($p = 0.03$).

Discussion

The aim of the present retrospective study was to evaluate early assessable parameters and their potential link with the outcome in canine patients affected by CS and SE.

Management of CS and SE is currently based on recommended guidelines deduced from clinical experience and from results of human or rodent studies (Patterson, 2014; S. R. Platt, 2014b). In veterinary medicine, only few scientific papers have been published on the evaluation of different treatment options in case of SE (Charalambous et al., 2019, 2017; Hardy et al., 2012). To date, there are no guidelines based on clinical history and evaluation of vital signs of patients that would guide a clinician's decision whether a patient should be treated more or less aggressively, whose decision has an impact on both the patient health but also on the owner's finances. To the authors knowledge, literature on risk factors for death or euthanasia in epileptic dogs is scant, and information on dogs specifically affected by CS and SE are even more limited (Arrol et al., 2012; Bateman and Parent, 1999; Monteiro et al., 2012; M Saito et al., 2001; Zimmermann et al., 2009). The results of the present study allowed the identification of several risk factors associated with a poor prognosis in dogs affected by CS or SE.

A higher age at seizure onset resulted to be significantly correlated with poor outcome. When the association between poor outcome and first seizure outside or within the idiopathic interval was evaluated, a trend was detected. This could be explained by the underlying etiology of seizure activity. Indeed, when seizure activity is detected outside the defined idiopathic interval, it is possible to assume that patients are more probably affected by structural epilepsy, even in the absence of

deficits on interictal neurological examination (Smith et al., 2008) and this etiology has been reported to be associated with a worse outcome (Fredsø et al., 2014; Hardy et al., 2012; Zimmermann et al., 2009). However, it has to be noted that in this study also patients with reactive seizures were included, whose occurrence is independent of age.

Considering only patients with history of seizures, patients without previous antiepileptic treatment were associated with a poor outcome. In human medicine, evidence exists to indicate that early initiation of seizure treatment improves the outcome of human patients affected by SE (Chen and Wasterlain, 2006; Rossetti and Lowenstein, 2011; Shorvon, 2001) and this concept can be reasonably extended to veterinary patients (S. R. Platt, 2014b). In fact, patients who have a greater number of seizures prior to initiation of treatment are more likely to develop pharmacoresistant epilepsy, due to kindling effects: indeed it has been shown that seizure activity itself can determine the development of changes in the brain, causing a synaptic reorganization which results in an increase of excitatory neuronal circuits and consequent seizures (Volk, 2014).

The absence of AEDs treatment in case of history of seizures could also be due to the fact that the first episode occurred shortly before the moment of hospitalization. However, that does not seem true. Indeed, the mean value of the time periods between the first seizure and the hospitalization in patients with history of seizures who were on AEDs was similar to that of the same category of patients who were not receiving any antiepileptic treatment. Therefore, not being on medications despite prior seizures could be the consequence of a less responsible management by the owner such as the effect of a low frequency of seizure episodes.

Nevertheless, polytherapy has been associated with a higher risk of SUDEP in human medicine, even if it has also been postulated that this association may just be a marker of the severity of epilepsy (Shorvon and Tomson, 2011). In the present study, no statistically significant differences were found comparing outcome of epileptic dogs receiving one or more AEDs. This result should be interpreted cautiously due to the low number of cases in this category.

The likelihood of short-term poor outcome was almost eight-fold higher in patients who experienced in-hospital complications. Infections, cardiac disfunction and respiratory failure are reported as complications of prognostic importance in SE outcome in human literature (Hawkes and Hocker, 2018; Sutter et al., 2013). Moreover a retrospective cohort study showed that the likelihood of experiencing systemic complications in human patients is two-fold higher in those who required infusion of an anesthetic agent for induced coma and pneumonia was the most common medical complications in both groups (Hawkes et al., 2019). It is reasonable to assume that complications can worsen the outcome of an already severe neurological condition by worsening the clinical status of patients. However, due to the low number of patients experiencing in-hospital complications, these results should be interpreted cautiously.

Among clinical vital signs recorded in emergency setting, the only statistically significant identified variable was rectal temperature. In particular, increased rectal temperature was associated to poor

outcome with a tendency to significance. Hyperthermia can result from severe muscular contraction secondary to seizure activity: the longer the duration of the seizure activity, the more the body temperature rises, and the clinical condition gets worse. Moreover prolonged muscle contractions and hyperthermia can lead to rhabdomyolysis and myoglobinuria that, sometimes in combination with hypotension and severe metabolic acidosis, may compromise renal function (Blades Golubovic and Rossmeisl, 2017b; Patterson, 2014; S. R. Platt, 2014b). However, it has to be noticed that in the present study, no distinction was made between hyperthermia and fever.

Despite the lack of data supporting this hypothesis, we further supposed that brachycephalic dogs' respiratory tract morphology usually causing the peculiar brachycephalic airway obstructive syndrome could predispose the subjects to respiratory distress and consequent hypoxia, possibly predisposing to fatal outcome. However, no significant correlation was found.

Despite the evidence that SE is a more severe condition than CS (Patterson, 2014; S. R. Platt, 2014b, 2014a) when investigating prognostic ability of presentation, no significative correlation with the outcome were found.

Comorbidies as risk factors for poor outcome were evaluated because they represent a variable of interest in a scoring system developed in human medicine for patients affected by SE. Indeed, the Epidemiology-based Mortality Score in Status epilepticus was developed in 2015 with the aim of assessing the severity of SE based on seizure etiology, comorbidities, EEG features, patient's level of consciousness and age. Available methods for measuring comorbidity in human medicine are either classified as a "disease count" (when the authors solely used an enumeration of the number of conditions present) or as an "index". Several human indices have been developed; some of them rates comorbidity burden by using a system that assesses the effect of the pathology on specific body systems, others that develops a list of clearly defined diagnoses. In both cases the conditions are further described by pathophysiologic severity rankings (Charlson et al., 1987; De Groot et al., 2003; Thompson et al., 2015). Although data in human literature show that indexes are more accurate than a simple count, this kind of indices are not defined for veterinary medicine. As the concurrent diseases detected in our canine population were mostly different from that included in human index lists, and because of the lack of detailed medical reports that evaluate the severity and pathophysiology of each pathology considered; a simple count was made. In the present study, additional diseases were counted and correlations with outcome were analyzed but none association was found, neither when distinguishing dogs who do have comorbidities (one or more) from those who do not, nor when diseases count has been put into practice. Finally, a new emerging human construct is that of patient complexity: morbidity burden is influenced not only by health-related features, but also by socioeconomic, cultural, environmental, and patient behavior characteristics (Valderas et al., 2009). This perspective is real in veterinary field as well, where the management of pets is extremely variable from owner to owner. However, capturing and measuring this variety of factors that interact with diseases remains a challenge in both human and veterinary patients.

The inclusion of seizure etiology in the variables examined would have certainly enhanced the value of our study, but very often seizure causes can only be detected after neurologic examination and ancillary diagnostic exams, requiring a delay up to several hours or days from admission, that may be critical for the therapeutic management. Indeed, the purpose of this study was to evaluate only clinical and demographic features that could be assessed upon admission in hospital. In this regard, history of prior seizures has been taken into consideration following human footsteps: the Status Epilepticus Severity Score is a prognostic score for human patients affected by SE which relay on four significant outcome predictors: age, history of seizures, seizure type and level of consciousness (Rossetti et al., 2008, 2006). In this scoring system, history of prior seizures is considered as a surrogate of acute symptomatic etiology (Trinka et al., 2015) and no history of seizures is a risk factor for death. In the present study no similar correlations were found, but the predicting power of this features could be limited by the fact that acute symptomatic etiology does not include malignant tumors or other progressive symptomatic causes that might induce a high mortality risk as well (Rossetti et al., 2008).

Also age is associated to outcome in STESS human scoring system, identifying a cut off age of 65 years, beyond which a higher risk of short-term mortality is present (Rossetti et al., 2008). That was not highlighted by our study, where no significative association between age and outcome was found. A bias that could have influenced this result is the fact that smaller breeds generally live longer than heavier breeds, so that age burden could be different among breeds and sizes (Greer et al., 2007). Furthermore, a previous investigation determined that life span of purebred dogs compared with crossbreed dogs is decreased in all weight categories, suggesting that selective breeding of dogs over time for phenotypic traits such as body size had accelerated aging, regardless of the effect of size alone (Patronek et al., 1997). In the present study approximately one-third of the dogs were crossbreeds, and similar percentages of crossbreed dogs resulted either among dogs who died in hospital or within patients discharged alive. Furthermore, no significative differences between distributions of small and large breed dogs were present if considering patients total, survivors or non-survivors only. Therefore, these confounding factors are not highlighted.

The majority of dogs included in our study experienced generalized convulsive seizures and no correlations were found between type and outcome. However, the present study does not consider further semeiologic subdivision: indeed, generalized seizures can occur as tonic-clonic, clonic, tonic, atonic or absence; while focal seizures can be distinguished in motor, behavioral and autonomic (Berendt et al., 2015). The specific weight of different clinical manifestation of seizure activity on outcome was not assessed in the present study. Moreover, SE can be further classified as either convulsive or non convulsive. Although in human scoring system non-convulsive generalized SE is associated with a worse outcome (Rossetti et al., 2008) this type of seizure activity can be diagnosed only by means of EEG monitoring, a tool not available in many veterinary structures. For this reason, this variable was not taken into consideration in the present study.

Neither glucose nor lactate serum level resulted to be statistically associated with outcome. Plasma lactate concentration has been previously evaluated as a predictors of gastric necrosis and outcome in dogs with gastric dilatation-volvulus (Beer et al., 2013; Zacher et al., 2010). In a recent study, lactate level was evaluated in association with base excess. This combination has been shown to be superior in that regard to either lactate or base excess assessment alone (Beer et al., 2013).
Future studies could therefore investigate the prognostic weight of the two variables analyzed simultaneously. Moreover, lactate concentration can increase for several reasons which might have been confounding factors in our study: indeed, hyperlactatemia can be associated with underlying disease such as microcirculatory dysfunctions, which can occur in sepsis and mitochondrial respiratory distress and many drugs, such as glucocorticoids, and toxins have been reported to induce hyperlactatemia in people. Ethylene glycol, a possible etiology of reactive seizures, can promote lactate formation during its metabolisms (Gillespie et al., 2017). As in the present study reactive seizures have not been excluded, it is not possible to rule out that extrinsic conditions or underlying diseases could be the cause of the increase lactate serum concentration evaluated in some cases.
Mortality and survival rate of CS and SE detected in this retrospective study are in line with previous studies with similar inclusion criteria (Bateman and Parent, 1999). However, other studies describe a higher in-hospital mortality of 38.5% (Zimmermann et al., 2009) even if this latter result is mostly based on a higher rate of euthanasia (33% of hospitalized dogs). Indeed, percentages of short term mortality due to spontaneous death are almost equal: 5.4% and 5.1% of the dogs died spontaneously respectively in the present study and in the one by Zimmermann and colleagues. Bateman et al. described relative percentages similar as well, with 2.1% of dogs died spontaneously, and 23.2% dogs euthanized (Bateman and Parent, 1999; Zimmermann et al., 2009).
Our results confirm also the slight predilection of male dogs for epileptic seizure reported in literature (Berendt et al., 2007; Podell et al., 1995). Despite the presence of several studies reporting conflicting data regarding the predisposition to seizures of spayed females rather than sexually intact females (Bateman and Parent, 1999; Zimmermann et al., 2009), no correlations that could demonstrate a significantly higher risk of developing seizures in intact female compared to neutered ones were found in the present study. If on one hand data from veterinary literature display that spayed females had a significantly higher risk of developing epileptic seizures, on the other hand sex hormones influence on seizure activity is well described in humans. Hormonal associated (catamenial) epilepsy is defined as seizures occurring principally o exclusively in any one phase of the menstrual cycle (Foldvary-Schaefer and Falcone, 2003; Rosciszewska et al., 1986). Estradiol is believed to be proconvulsive while progesterone appears to have protective effects against seizures, although recent literature is contradictory (Scharfman and MacLusky, 2006; Velíšková and DeSantis, 2013). Although this phenomenon may not be seen in canine patients due to the difference in

the species' sexual cycle, Merveene et al. described an association between specific periods of the estrous cycle (during heat and during a specific point at the end of diestrus) and seizure onset in a population of 45 intact females with idiopathic epilepsy (Van Meervenne et al., 2015).

Hypoglycemia at presentation was correlated with SE presentation. It is well established that energy deprivation via either hypoxia or hypoglycemia often results in coma and neuronal death and it is sometimes associated with onset of seizure activity (Auer, 1986; Sang et al., 2007). Dogs suffering from reactive seizures (whose frequent cause is hypoglycemia) were reported to have 1.57 higher odds for developing SE than dogs with idiopathic epilepsy (Platt and Haag, 2002). The results of our study seem to support the idea of hypoglycemia as a detrimental factor for SE development.

Nevertheless, human literature reports as well that several systemic changes can occur during SE, including a decrease in glucose blood concentration (Rossetti and Lowenstein, 2011). From this point of view, hypoglycemia may not be a cause but a consequence. In contrast with previous findings, in vitro recordings show that reduced levels of glucose might also affect seizure duration, reducing frequency and amplitude in the seizure-like discharge by respectively 50% and 25% (Kirchner et al., 2006). According to the ketogenic diet rationale, increasing the extent of calories restriction resulted in improved seizure control in epileptic mice, even if the to date the mechanisms underlying its clinical efficacy still remain unknown (Bough and Rho, 2007). To summarize, the role of glucose in epileptic seizures pathophysiology and specifically in SE is currently debated.

No significative association was found between CS and seizure onset in the 'idiopathic interval'. This result seems to be in conflict with data previously reported (Bateman and Parent, 1999; Monteiro et al., 2012) where CS presentation was described as frequent in dogs with idiopathic epilepsy.

The main limitation of this study is that either euthanasia or spontaneous death are considered as poor outcome. The degree to which the frequency of euthanasia outweighs ''natural'' death in veterinary medicine poses a unique challenge to models developed on mortality outcome as it is mainly influenced by owner (Hayes et al., 2010). Moreover, some bias may be intrinsic to the retrospective design because patients with incomplete data had to be excluded and retrospective studies results have been defined as not as reliable as prospective studies results (Euser et al., 2009). Thus, a complete prospective study using a proper registry would be needed in the future, and it should ideally be a multicenter study in order to include more patients.

As to establish the presence of brachycephalic morphology in crossbreed dogs, owners have been asked by phone calls whether the dog's muzzle was comparable to that of a famous brachycephalic breed (e.g. pug), the subjective interpretation of the owner may have been a limitation.

In addition, the owner's attitude, financial situation and lifestyle may have influenced the greater or lesser effectiveness of AEDs, such as detection of previous history of seizures and euthanasia decision.

Conclusion

In conclusion, this study attempted to identify risk factors associated with short term outcome of dogs with CS and SE. Age at first seizure, previous long-term AEDs and in-hospital complications were the only factors that resulted to be statistically different between dogs who survived and those who did not. Increased body temperature was found to be correlated as well with a poor outcome, even if a significative difference was not found. Moreover, given the purpose of the study, it would be better to seek early evaluable factors associated with development of complications (such as the possibility of hyperthermia as a predictor of acute renal failure complication), than complications themselves. Finally, statistically significant correlations with the same specific variables considered by human scoring system, such as age at hospitalization and history of previous seizures, have not been shown. Although it is possible that such human risk factors have no meaning in the canine species, larger studies with greater effective sample sizes would be needed to further investigate these parameters. Many diagnosis-independent veterinary scores have been developed in recent years and they can be used to benchmark performance and establish protocols for triage and therapeutic management. Inf acts, by using appropriate reference scores, an objective measurement of the illness severity could be given and the analysis of treatment effect could be significantly improved (Hayes et al., 2010).

According to this, the present study was intended to be preliminary to the creation of an ad hoc scoring system, similar to those currently in use in human medicine, to be used upon entry in patients suffering from seizure disorders. A possible future research field in this direction is the application of recent Machine Learning techniques. These, exploiting the collected data, would lead to the creation of an appropriate algorithm able to predict, with of course some degree of uncertainty, the outcome. The use of Artificial Intelligence for biological and medical issues is in fact a growing field and it is obtaining promising results.

Footnotes

[a] https://www.akc.org/

[b] https://www.thekennelclub.org.uk/

[c] https://www.who.int/classifications/icd/en/

[d] https://www.royalcanin.com/us

Additional information

The present study will be submitted to a peer-reviewed scientific journal shortly.

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N°	Breed	Sex	Weight (Kg)	Age at first seizure (months)	Age at inclusion (months)	Seizure frequency	AEDs and dosages (mg/kg)	Age at initiation of anti-epileptic treatment (months)	Suspected/Confirmed Diagnosis
1	Bloodhound	F	40	27	56	Monthly (CS)	PB (6.25 q12h) KBR (20 q12h)	28	Idiopathic epilepsy
2	Mixbreed	F	28.7	92	105	Monthly	PB (2.61 q12h)	104	Suspected structural epilepsy
3	Pyrenean mountain dog	M	52	124	126	1 epileptic seizure 3 months before presentation	Not in treatment	-	Suspected structural epilepsy
4	Argentine Mastiff	F	44.3	43	43	-	Not in treatment	-	Suspected idiopathic epilepsy
5	Boxer	F	26	69	75	CS 6 months before inclusion, then unknown	PB (3.46 q12h) KBR (26.92 q24h)	69	Structural epilepsy (suspected glioma)
6	German Sheperd	M	38	63	75	Monthly	Imepitoin (15.78 q12h)	63	Suspected idiopathic epilepsy
7	Mixbreed	M	24	38	60	CS 7 months before inclusion, then unknown	PB (3.75 q12h)	40	Suspected idiopathic epilepsy
8	German Sheperd	F	30	94	94	-	Not in treatment	-	Structural epilepsy (suspected glioma)

Additional file 1. Information on signalment and history of patients included in Project 1