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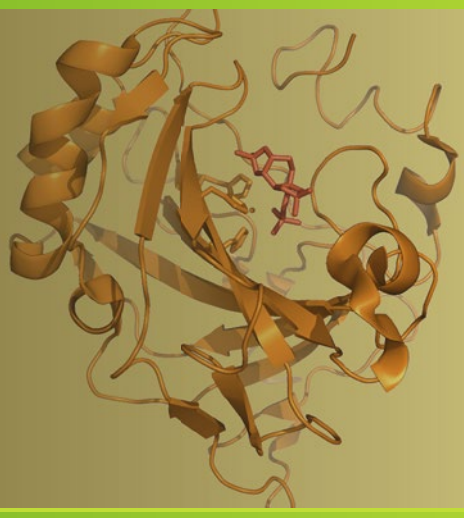

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Luisa Rocha *Editors*

Antiepileptic Drug Discovery

Novel Approaches

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Methods in Pharmacology and Toxicology

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Novel Approaches

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Preface

Despite the introduction of several novel antiepileptic medications to the clinical practice during the last 20 years, the proportion of patients failing to achieve a seizure-free status has not improved substantially. Furthermore, current antiepileptic drugs provide symptomatic control to responsive patients (anticonvulsive or antiseizure effect) but have not proven to prevent the setting and progression of the disorder (antiepileptogenesis). Leading experts have emphasized the need for paradigm shifts in the field of antiepileptic drug discovery as a key factor to arrive at truly innovative therapeutic solutions. Such innovative solutions involve a wide and multidisciplinary array of strategies, including, among others, the development of new in vitro and in vivo models of epilepsy, the exploration of new molecular targets and mechanisms of action, the integration of computer-aided approaches, and the development of advanced drug delivery systems. Even though remarkable (yet incomplete) progress has been made in the knowledge of the pathophysiology of epilepsy and the determinants of refractoriness, these advancements have so far not crystallized into more efficacious therapeutic strategies. What is more, the epilepsy field has fallen somewhat behind regarding the integration of modern drug development tools, in comparison with other complex disorders such as cancer or Alzheimer's and Parkinson's diseases.

This volume aims to provide medicinal chemists, pharmacologists, and other scientists involved in antiepileptic drug discovery with a comprehensive view of novel approaches for the development of antiepileptic therapies, with a focus on novel molecular targets for antiepileptic drugs, computer-aided approaches for the identification of new drug candidates, and therapeutic strategies to overcome refractory epilepsy. The last part illustrates the potential benefits that network pharmacology and rational drug repurposing could bring to the antiepileptic drug discovery community.

The volume is made up of 19 chapters that are briefly commented on next.

Firstly, López-Meraz et al. describe the role of proinflammatory cytokines in the neuroinflammation process associated with seizure activity. They indicate the relevance of considering these cytokines as potential therapeutic targets for the pharmacological treatment of epilepsy. Valle-Dorado et al. present a review of different anti-inflammatory drugs that may be used to avoid the neuroinflammation that results from seizure activity. These authors suggest that anti-inflammatory drugs represent a strategy to decrease the higher excitability associated with epilepsy. Mendoza-Torreblanca et al. describe some of the basic aspects of the role of synaptic vesicle protein 2A (SV2A) in epilepsy, and support that this molecule is a good molecular target for the design of new antiepileptic drugs. Interestingly, they also provide an immunochemical protocol to obtain reliable semi-quantitative data of SV2A as well as a methodology to develop SV2A molecular dynamics simulations. Zavala and Rocha explain the relevance of evaluating the pro- and anticonvulsant effects of cannabinoids, depending on the type of epilepsy and the rate of excitability. Cuellar-Herrera et al. examine the importance of the pharmacological blockade of glutamate receptors as a therapeutic strategy to control seizure activity and induce neuroprotection. Gavernet discusses the existing evidence of the potential use of carbonic anhydrase as an antiepileptic drug target, describing a structure-based procedure for the design of novel inhibitors. Joshi and Kapur

describe experimental evidence supporting the idea that neurosteroids regulate seizure activity via modulation of $GABA_A$ receptors. They also provide data that neurosteroids represent a novel target to control seizure activity in women with epilepsy. Marelli et al. describe the mechanisms involved in the neuroprotective effects induced by erythropoietin and propose that the activation of its receptor (EPO receptor) can be used as a therapeutic strategy to avoid epileptogenesis and pharmacoresistant epilepsy. The last two chapters of the first part of the volume are dedicated to treatment options different from traditional small molecule therapies. Carvajal Aguilera and Phillips Farfán review different eating regimes used to control seizure activity and the mechanisms involved in their effects. They propose that elucidating the underlying mechanisms of these therapeutic interventions could help in the development of innovative antiepileptic medications, and they provide a protocol for the preclinical study of caloric restriction. López-García et al. provide a review of different gene therapies considered as possible strategies to reduce seizure activity.

In the second part, Lara et al. present an overview of the relevance of using human brain tissue to evaluate the pharmacoresistance in epilepsy and describe protocols of different approaches to fulfill this goal: in vitro electrophysiological recordings, in vitro procedures for receptor evaluation, and genomic analyses. Rogel-Salazar and Luna-Munguia analyze the role of the blood-brain barrier in epilepsy and the relevance of considering this structure as a target to design new antiepileptic strategies. Talevi and Bruno-Blanch describe the general procedures to implement a ligand-based virtual screening campaign for the identification of novel antiepileptic drugs. Couyoupetrou et al. also discuss technical aspects of computational (ligand-based) and experimental approaches used for the early detection of substrates of efflux systems, which could assist in the development of new antiepileptic drugs addressing the transporter hypothesis of pharmacoresistant epilepsy. Similarly, Palestro and Gavernet describe structure-based approaches that can be used to predict P-glycoprotein substrates. At the end of the second part of the volume, Ruiz and Castro examine the potential use of pharmaceuticals' micro- and nanocarriers to improve central nervous system bioavailability of antiepileptic agents; they also provide an experimental procedure to study drug release from such advanced pharmaceutical vehicles in vitro.

In the last part, Kubova presents a review of the different side effects induced by the different antiepileptic drugs and points out the necessity to carry out comparative, well-designed, and long-term trials in order to identify the best pharmacological strategy to control epilepsy. This author also emphasizes some reported beneficial effects of known antiepileptic drugs on comorbid mood disorders. In the two final chapters of the book, Talevi discusses the potential of network pharmacology and drug repurposing in the field of antiepileptic drug discovery, along with some practical consideration on how these approaches could be integrated into the drug discovery process.

The editors consider this book to be an excellent tool for the study of pharmacoresistant epilepsy and the discovery of new antiepileptic drugs.

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Part I

Novel Approaches for the Treatment of Epilepsy

Chapter 1

Inflammatory Cytokines as Targets for Epilepsy Drug Therapy

María-Leonor López-Meraz, Jesús-Servando Medel-Matus, and Jerome Niquet

Abstract

Seizures and epilepsy are associated with a neuroinflammatory process. Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are acutely expressed after seizures in experimental models and, in some cases, still overexpressed later during the chronic stage. Increased levels of these cytokines have been found in the serum from epileptic patients. Pharmacological approaches in rodents support that neuroinflammation is involved in modulating seizure susceptibility, neuronal damage, and other neurological disorders associated with epilepsy. This chapter review studies suggesting that proinflammatory cytokine systems are potential therapeutic targets for the pharmacological treatment of epilepsy.

Key words Cytokines, Interleukin-1β, Seizures, Epilepsy, Neuronal damage, Comorbidities

1 Cytokines in the Central Nervous System

Although inflammation produces beneficial effects on central nervous system (CNS) like the elimination of pathogens and the activation of repair processes in the damaged area, excessive and uncontrolled inflammation can be detrimental to the brain since it produces neurotoxic factors leading to aggravation of neurodegenerative disorders [1]. Cytokines are usually considered chemical messengers between immune cells, having a crucial role in the inflammatory and immune response to injury and infection [2]. Nathan and Sporn have defined cytokines as a (glyco)protein, non-immunoglobulin in nature, released by living cells of the host, which acts nonenzymatically in picomolar to nanomolar concentrations to regulate host cell function [3]. Cytokines comprise a diverse group of proteins including interleukins, interferons, colony-stimulating factors, and peptide growth factors [3]. Research in cytokines has been complicated considering that cytokines arise from apparently unrelated cell types and display diverse and even

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opposing bioactivities. Thus, it is difficult to predict how, when, and where cytokines will play a role (even if their amino acid sequence and actions are known). The actions of a given cytokine can also be exerted by another one, and some cytokines promote the synthesis or release of other cytokines, which finally produce the biological effect [3]. The classification of cytokines can be confusing, since they are frequently named for their biological activity [2]. Cytokines can be classified in three groups: proinflammatory [such as interleukin-1 (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α)], anti-inflammatory (such as IL-4, IL-10, and IL-13), and hematopoietic cytokines (such as IL-3 and IL-5) [2].

It is now accepted that cytokines are signaling agents in the brain and serve as mediators between the immune and nerve cells; the brain is able of influencing immune processes, and this immunological response can in turn change the brain activity [2]. However, a full assessment of these complex interactions is still under study. The first reports describing a relationship between inflammation in the central nervous system and epilepsy were performed in the 1990s. Several research groups performed studies in order to characterize how seizures and epilepsy could regulate cytokine transcription, expression, or release [4–8]. In the current chapter, we examine the studies identifying the potential therapeutic properties of inflammatory cytokine systems, with an emphasis on interleukin-1β signaling.

2 Cytokine and Its Receptors After Seizures

Nowadays, it is well established that cytokines can also be synthesized and released in the brain by microglial cells, astrocytes, and some populations of neurons. It is also known that there is a rapid and strong increase in cytokine expression levels in the brain acutely following an insult or chronically in many neurodegenerative disorders. There is a large body of experimental and clinical studies that have characterized the relationship between epilepsy and cytokines. Electrically or chemically induced seizures in developing and adult rats trigger gliosis and microglia activation, along with an increase of mRNA and protein expression of proinflammatory cytokines. Data obtained from human brain tissue or blood samples, as well as meta-analysis, have also shown an association between epilepsy and neuroinflammation. This chapter reviews the studies revealing the interaction between neuroinflammation and seizures and the data identifying the specific inflammatory signaling pathways that may be potential therapeutic target for the treatment and/or prevention of seizures and their sequels.

The relationship between seizures and neuroinflammation can vary depending on the stage at which it is evaluated: during the acute phase of seizures (mainly *status epilepticus*, *SE*), the latency or

epileptogenesis phase, or the chronic phase (i.e., epilepsy, when spontaneous and recurrent seizures occur). Additionally, results often differ depending on the experimental model used to induce the convulsions or the epilepsy and the level of maturation of the brain. In the following paragraphs, we review the experimental evidence obtained from rodents.

It is known that baseline levels of cytokines in the rat brain are barely detectable in normal condition. Initial studies in seizure models demonstrated that the systemic administration of kainic acid (KA) or pentylenetetrazol (PTZ) in the adult rat triggers an upregulation of brain (cortex, hippocampus, thalamus) mRNA for cytokines, including IL-1β, TNF-α, IL-1β natural antagonist (IL-1Ra), and IL-6, 2–24 h after seizures [4, 5, 7]. A similar effect was later observed in adult rats following electrically induced SE or amygdala kindling, where it results in an upregulation of IL-1β, IL-6, TNF-α, type I IL-1 receptor (IL-1RI), and inducible nitric oxide synthase mRNA in hippocampus 2–24 h following seizures [9, 10]. Other studies also demonstrated that TNF-α and IL-6 secretion was increased in hippocampal slices prepared from rats previously injected with KA in the amygdala [6].

It is noteworthy that most of the studies indicate that IL-1β (mRNA or protein) peaks sooner than other proinflammatory cytokines. Actually, in some cases, it is the only cytokine increasing after seizures. IL-1β belongs to the IL-1 family and exerts its biological effects by acting on type I IL-1 receptor (IL-1RI) [11]. IL-1β natural antagonist (IL-1Ra) is a protein that competitively and selectively avoids the binding of IL-1β with its type I receptor [12–14].

Vezzani and colleagues also reported an increased number of IL-1β-immunoreactive cells in CA1, CA3, and dentate gyrus (DG) after seizures induced by intrahippocampal injection of KA in adult rats [8]. A similar effect was observed in adult rats after SE induced by electrical stimulation, where the number of IL-1β, IL-6, TNF-α, IL-1RI immunoreactive cells in CA1 and CA3 hippocampal areas was increased 2–48 h post-SE (acute stage) and still higher than controls 60 days post-SE [9, 15]. In adult rats, SE induced by pilocarpine produced an overexpression of IL-1β and IL-1RI cells in damaged CA3 area during the acute (18 h post-SE), latent (7 days post-SE), and chronic phases (4 months post-SE), along with triggering astrogliosis and microglia activation [16]. Recently, an upregulation of IL-1β was detected by western blot in the hippocampus from adult rats after chemical kindling induced by PTZ, along with an increase in the number of IL-1RI immunoreactive cells in the DG [17].

Chemically induced seizures cause a strong neuroinflammatory response in the developing hippocampus (P9, P15, and P21 rat pups; P, postnatal day). That response includes overexpression of astrocytes and activated microglia [18, 19], as well as an acute mRNA increase of inflammatory cytokines, such as IL-1β, IL-1Ra,

TNF-α, and IL-6 [18–20]. In 15-day-old rat pups, SE promotes an increase in IL-1β mRNA levels in hippocampus 4 h after SE that does not persist 18 h following seizures, whereas mRNA levels of IL-1Ra, IL-6, or TNF-α remain unchanged [18, 19]. Omran and collaborators showed that hippocampal mRNA and protein of IL-1β are upregulated in 11-day-old rats 2 h after lithium-pilocarpine-induced SE (acute stage), as well as during the chronic stage, when spontaneous seizures occurred [21]. Recently, it has been shown that SE induced by lithium-pilocarpine produced a transitory overexpression of IL-1β and IL-1RI in the injured hippocampus (mainly in the CA1 area, but also in dentate gyrus and hilus) 6 h following convulsions [22]. Altogether, those data suggest that neuronal damage induced by SE in the developing rat brain may involve the participation of the IL-1β/IL-1RI system. Febrile convulsions, induced by a lipopolysaccharide (LPS) and a subconvulsant dose of KA in 14-day-old rat pups, increased IL-1β levels in hypothalamus and hippocampus after seizures [23]. Additionally, the chronic expression of IL-1β in hippocampus is associated with the development of spontaneous limbic seizures after induction of febrile seizures in P11 rats [24].

On the other hand, some groups have reported alterations of the immune system in patients with epilepsy. The involvement of neuroinflammation in the pathogenesis of human epilepsy has long been debated and is controversial. Changes in the immune system vary with the type of epileptic syndrome, etiology, and antiepileptic drug treatment. Furthermore, studies in epileptic patients are limited and even showed opposite results. One of the first approaches to study the relationship between epilepsy and neuroinflammation has been the measurement of blood levels of cytokines after seizures and in different types of epilepsies [25–29]. A limitation of these clinical studies is the inherent difficulty in correlating cytokine blood level with the brain physiopathology. It has been reported that patients with mesial temporal lobe epilepsy due to hippocampal sclerosis (MTLE-HS) and patients with focal epilepsy due to benign cerebral tumors have higher levels of IL-6 serum concentration during the interictal state, as compared with controls; no differences were detected in IL-1β or TNF-α serum levels in MTLE-HS. However, elevated IL-1β concentrations were seen in patients with focal epilepsy due to tumors [27]. Other studies showed that patients with temporal lobe epilepsy (TLE) have increased serum concentrations of IL-6, but not those of IL-1β or TNF-α, immediately after seizure [25]. However, in a meta-analysis by Yu et al, no differences between plasma concentrations of IL-1β and IL-1Ra in patients with epilepsy and control subjects were identified [30]. A recent study reported that increased serum levels of IL-1β, IL-6, and IL-1Ra were detected in both patients with temporal and extratemporal lobe epilepsy; however, higher levels of IL-1β were observed in the TLE group [31]. Other studies

showed that there is an increase in IL-1β and IL-1Ra plasma levels in patients with TLE and a decrease following epilepsy surgery when patients remained free of seizures [28, 29, 32].

Ravizza and colleagues showed that the hippocampus of patients with TLE and hippocampal sclerosis have neurons and glia immunoreactive to IL-1β and IL-1RI, suggesting the activation of IL-1β/IL-1RI system in human epilepsy [33]. Hippocampal tissue from children with medial temporal lobe epilepsy also displayed higher levels of IL-1β than control patients [21]. Remarkably, the cortical tissue of patients with focal cortical dysplasia and glioneuronal tumors also showed increased expression of IL-1β and its type I receptor in neurons and glial cells, and this effect positively correlated with the frequency of seizures [34]. *See* Appendix 2 and Appendix 3.

3 Effect of Cytokines on Ictogenesis and Epileptogenesis

As indicated above, seizures and epilepsy induce a strong inflammatory response in the brain. The remaining question is whether inflammation increased seizure susceptibility. Proconvulsant and anticonvulsant effects have been reported for IL-1β, IL-6, and TNF-α. A possible explanation for this phenomenon is that cytokines play different roles through multiple pathways, depending on their concentration and availability of receptors [35]. These cytokines could be a target for modifying seizure susceptibility, but the efficacy of treatment varies with the experimental model, animal species, age of the experimental subject, type of seizure modeled, as well the time point of intervention.

Experimental evidence suggests a particularly strong proconvulsant effect of IL-1β, which makes to the IL-1β/IL1-RI system an interesting target for pursuing of anticonvulsant drugs. This effect is associated with the capacity of IL-1β to induce the production of other cytokines and growth factors [36] and changes in blood flow [37]. In adult rats, the intrahippocampal injection of IL-1β prolongs and worsens the electroencephalographic and behavioral seizures caused by KA [8, 38]. These authors demonstrated that 1 ng of IL-1β intrahippocampally injected 10 min before KA increases seizure duration (but not seizure onset or number), an effect that was blocked by the coadministration of 1 μg of IL-1R1a, indicating the involvement of the IL-1β/IL-1R1 system in ictogenesis. In this study, the effect of IL-1β was also blocked by the coinjection of 0.1 ng of 3-((+)-2-carboxypiperazin-4yl)-propyl-phosphonate, an NMDA receptor antagonist, implying that IL-1β requires the glutamatergic neurotransmission to maintain seizures [8]. The administration of the inflammatory cytokines IL-1β and high-mobility group B1 (HMGB1) protein can rapidly enhance the mean frequency of spontaneous ictal-like discharges,

reduces the latency, and prolongs the duration of the first ictal-like event in a brain slice model of temporal lobe epilepsy, supporting the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures and epileptogenesis [39]. In another report, the subchronic treatment with an inhibitor of interleukin-1 synthesis (VX-765, 200 mg/kg, i.p.) in adult rats prevented the epileptogenesis triggered by electrical kindling [33]. Marchi and colleagues confirmed the efficacy of anti-inflammatory strategies in the treatment of seizures. In their study, the pretreatment of adult rats with IL-1Ra (35 μg/kg, i.v.) 2 h before pilocarpine reduced the prevalence of lithium-pilocarpine-induced SE and delayed SE onset. These effects were associated with a decrease in the blood-brain barrier (BBB) damage [40]. As indirect evidence of proconvulsant effect of IL-1β, the intracerebral injection of IL-1Ra reduces bicuculline-induced convulsions in mice [41]. Also, transgenic mice with overexpression of IL-1Ra in astrocytes exhibit an increased seizure threshold to bicuculline-induced convulsions [38].

Systemic administration of LPS in P14 rats increased the susceptibility to seizures induced by lithium-pilocarpine, KA, or PTZ in adulthood [42]. It is noteworthy that the hippocampal IL-1β release 6 h following KA-induced seizures was higher in the LPS group; levels of TNF-α were also increased, but it did not reach statistical significance [42]. However, the effect of LPS on PTZ-induced seizures in adult rats was blocked by the concomitant intracerebroventricular administration of a TNF-α-neutralizing antibody (50 μg/5 μl), but not by postnatal treatment with IL-1Ra (10 or 50 μg/5 μl). Interestingly, it was mimicked by the intracerebroventricular injection of rat recombinant TNF-α [42]. These results suggest that an inflammatory trigger (in this case a single LPS injection) during a critical postnatal period may cause a long-lasting increase in seizure susceptibility through TNF-α [42].

IL-1β contributes to febrile convulsions induced by LPS and a subconvulsant dose of KA in 14-day-old rat pups. The treatment with IL-1β (3 or 30 ng, i.c.v.) increased the proportion of rats that experienced febrile convulsions, whereas IL-1Ra (10 μg or 15 ng, i.c.v.) had anticonvulsant effects [23] (*see* Appendix 1). Additionally, IL-1β receptor-deficient mice were resistant to febrile seizure induced by a stream of heated air [43]. These findings suggest that IL-1β signaling contributes to fever-induced hyperexcitability and seizures and represents a potential target for their prevention and management.

Kwon and collaborators tested a combination therapy of anti-inflammatory drugs in 3-week-old rats, in order to evaluate its effect on epileptogenesis and mossy fiber sprouting due to SE. The authors found that the combination of IL-1Ra (100 mg/kg) and COX-2 inhibitor (CAY 10404, 10 mg/kg) administered for 10 days following SE reduced the development of spontaneous recurrent seizures and limited the extent of mossy fibers [44].

An indirect way to identify the participation of cytokines in the development of seizures is by blocking their inflammatory activity. A good example is the transforming growth factor β (TGF-β), a cytokine activated in astrocytes by serum-derived albumin and involved in epileptogenesis. A study indicated that the inhibition of TGF-β signaling by angiotensin II type I receptor (AT1) antagonist, losartan, prevents the development of delayed recurrent spontaneous seizures in two different rat models of vascular injury. This inhibitory effect persists weeks after drug withdrawal. Thus, this treatment could be an efficient anti-epileptogenic therapy for epilepsy associated with vascular injury [45]. Another study demonstrated that the treatment with carbamazepine (a classical antiepileptic drug) or vinpocetine (a nonclassical antiseizure drug) reduces IL-1β and TNF-α expression in rat hippocampus after seizure induction. These findings suggest that the mechanisms of action of these antiepileptic drugs involve a decrease in neuroinflammation and confirm the close relationship between cytokines and seizure development [46]. The antidepressant sertraline is an effective inhibitor of tonic-clonic seizures in animal models. Recently, it was shown that sertraline markedly reduced IL-1β and TNF-α expression induced by the convulsive agent PTZ and the inoculation of LPS in the rat hippocampus. The results of this study indicate that a reduction of brain inflammatory processes may contribute to the antiseizure action of sertraline, a drug which could be used to treat depression in epileptic patients [47]. The treatment with bupivacaine, an anesthetic agent that inhibits the release of glutamate in rat cerebrocortical nerve terminals, increased seizure latency and reduced hippocampal neuronal death. These effects were accompanied by a reduction in mRNA of IL-1β, IL-6, and TNF-α in the hippocampus in a KA model of temporal lobe epilepsy in rats [48].

4 Role of Cytokines in the Pathophysiology of Seizures and Epilepsy

4.1 Neuronal Damage

As described above, proinflammatory cytokines are produced, overexpressed, and/or released in the brain (by microglia, astroglia, and even neurons) after seizures. Experimental studies showed that cytokines contribute to neuronal cell death occurring after seizures. In adult rats, the combination of IL-1Ra and VX-765 (a specific non-peptide inhibitor of IL-1β cleavage and release) decreases neuronal cell loss in the adult rat forebrain following SE induced by pilocarpine or electrical stimulation [49], suggesting that the IL-1 β/1L-1RI system is an important therapeutic target to promote neuroprotection after brain injury.

In the immature rat brain (up to 2 weeks old of age), CA1 pyramidal neurons are highly sensitive to SE-induced neuronal injury, and most of them die by necrosis [50–52]. Recently, it was

demonstrated that i.c.v. injection of IL-1β (3 ng/μl) increased SE-induced necrotic neuronal cell death in the CA1 area of the hippocampus from 14-day-old rats. IL-1β effect was blocked when coadministrated with IL-1Ra [53]. This data showed that IL-1β facilitates necrotic neuronal cell death after SE through the activation of IL-1RI [53]. Administration of the proinflammatory agent lipopolysaccharide (LPS) prior to the induction of *SE* by lithium-pilocarpine in rat pups (P7 and P14) increases neuronal cell death observed in the hippocampal CA1 area [54]. Sankar's group tested a combination of anti-inflammatory drugs to promote neuroprotection against LPS plus lithium-pilocarpine-induced SE in 2 week-old rat pups. Their findings indicate that the combination of IL-1Ra (100 mg/kg) and a COX-2 inhibitor (CAY 10404, 1 mg/kg) decreased the number of CA1 injured neurons after SE; however, the combination of minocycline, an anti-inflammatory and antagonist of microglia activation (100 mg/kg) plus CAY 10404 or IL-1Ra plus minocycline, was ineffective in decreasing neuronal damage. Interestingly, any of these drugs injected alone had neuroprotective effects against SE-induced damage [44]. These authors suggest that multiple anti-inflammatory therapies may provide a practical approach to neuroprotective and anti-epileptogenic treatment [44].

Other drugs have been found to have neuroprotective properties against seizure-induced neuronal cell death along with reducing the expression of neuroinflammation markers. In this context, the anesthetic drug bupivacaine reduces neuronal cell death and activation and production of IL-1β, IL-6, and TNF-α in the rat hippocampus after seizures induced by KA [48]. In a different study using the same model of seizures, the intraperitoneal administration of hispidulin (a flavonoid compound that inhibits glutamate release) increased seizure latency, decreased seizure score, reduced production of proinflammatory cytokines, and attenuated hippocampal neuronal cell death [55].

The mechanism by which IL-1β induces neuronal death is not fully understood. IL-1β (10 mg/ml) aggravates NMDA or glutamate-induced hippocampal neuronal death in fetal rat hippocampal neuron cultures [56]. A recent study showed that spinal glial glutamate transporters activity in neuropathic animals is attenuated by endogenous IL-1β, which in turn could increase glutamate levels and promote excitotoxicity [57]. IL-1β may aggravate neuronal death by binding to its receptor (IL-1RI) and/or affecting $GABA_A$ and NMDA receptor activities [15, 58, 59]. In vitro experiments with neuronal cultures have shown that IL-1β inhibits currents mediated by the $GABA_A$ receptor, suppresses potassium flow through voltage-dependent channels, and enhances the function of the NMDA receptors [58–62]. Also, it has been observed that IL-1β increases Ca^{2+} intracellular mobilization through the nitric oxide (NO)/cyclic guanosine

monophosphate signaling pathway [63]. These cellular effects amplify neuronal excitability and may participate in the mechanisms underlying mechanisms of seizure-induced neuronal cell death. A recent study demonstrated that IL-1β decreased GABA-evoked currents in specimens from TLE with or without hippocampal sclerosis, through the mediation of IL-1R1 and the involvement of protein kinase C and IRAK1 (the proximal kinase mediating the IL-1R1 signaling) [64]. These data support that the IL-1β/IL-1RI system is activated in human epilepsy and may contribute to seizure generation in humans by reducing GABAergic neurotransmission [64]. Altogether, these studies suggest that a better characterization of the signaling cytokine pathways, particularly the IL-1β one, in the brain may have significant therapeutic implications for the treatment of seizures and epilepsy.

4.2 Mood Comorbidities

A number of studies indicate that cytokines exhibit diverse activities in the brain and can modify systemic and CNS responses to insults, including infection, trauma, inflammation, and seizures. Variations in serum and brain levels of IL-6, TNF-α, or IL-1 have been reported and may play a key role in several neurological disorders including autism, depression, and anxiety. It has been accepted that the prevalence of mood disorders and autism is higher among patients with epilepsy than in the general population [65]. Cytokines could be the link between epilepsy and mood disorders; here we summarize the current knowledge about the participation of inflammation on seizures/mood disorder development.

Major depressive disorder (MDD) is a highly prevalent, heterogeneous, and recurrent neuropsychiatric condition characterized by a broad range of symptoms, including altered mood and cognitive functions. It has a multifactorial etiology originating from the interaction between environmental and genetic factors and presents frequent comorbidity [66]. Observations in depressed patients showed increased levels of acute-phase proteins (e.g., IL-1Ra), chemokines, adhesion molecules, and inflammatory mediators, such as cytokines [67]. Proinflammatory cytokines mimic, in animal and in vitro models, many of the neurological changes thought to be features of depression, including decreased neurogenesis, changes in the monoamine system, and neurodegeneration [66, 67]. Studies in animal models confirm the relationship between depression and cytokine expression after seizures. It has been observed that the blockade of hippocampal IL-1 receptors, by intrahippocampal injection of human IL-1Ra, attenuated the behavioral and biochemical manifestations of depression in epileptic/depressed rats [68, 69].

The most studied cytokines related with depression and epilepsy are IL-1β, IL-6, and TNF-α. There is a positive correlation between the serum and plasma level of these proinflammatory cytokines and depression [70, 71]. Higher levels of the systemic

inflammatory marker IL-6 in childhood are associated with an increased risk of developing depression and psychosis in young adulthood [72]. Furthermore, increased brain level of several cytokines has been reported. For example, IL-1β is found in the cerebrospinal fluid (CSF) of depressive patients [73], and postmortem analyses in the prefrontal cortex of depressive patients have suggested an upregulation of a variety of proinflammatory and anti-inflammatory cytokines [74]. Other studies indicated that TNF-α may induce changes in brain structure and function, contributing to the development of depression [75].

The main mechanisms involved in the pathophysiology of depression implicate the activation of the hypothalamo-pituitary-adrenocortical (HPA) axis [76, 77]. Several studies confirmed that post-SE rodents showed dysregulation of the HPA axis, along with increased plasma corticosterone levels as occurs in depression [68]. The upregulation of the HPA axis is an important feature associated with depressive signs [78, 79]. This highlights the potential for direct clinical significance of raised proinflammatory cytokines, especially TNF-α [70]. Moreover, TNF-α activates the neuronal serotonin transporters [76, 80]. During the process of depression, the uptake of serotonin is diminished. A study demonstrated that both IL-1β and TNF-α can acutely regulate the neuronal serotonin transporter activity by stimulation of serotonin uptake in both a rat embryonic raphe cell line and in mouse midbrain and striatal synaptosomes [80, 81].

In general terms, an anxiety disorder involves an excessive state of arousal characterized by feelings of apprehension, uncertainty, or fear [81]. It has been widely shown in humans and animal models that epilepsy can contribute to the development of anxiety, but the evidence of a direct participation of cytokines on the anxiety symptomatology produced by seizures has not been demonstrated. However, increases in levels of proinflammatory cytokines closely related to epilepsy have been used in some studies as a marker of anxiety disorders. Studies in humans have shown augmented circulating levels of IL-6, TNF-α, IL-1β, and IL-8 in patients with post-traumatic stress disorder (PTSD), which may be due to an insufficient regulation of the immune function [82–84]. Similarly, patients with panic disorder (PD) or PTSD exhibited significantly increased levels of IL-6, IL-1α, IL-1β, IL-8, and IFN-α compared with healthy controls [85]. The high comorbidity between anxiety disorders and depression and the similar effects of antidepressant treatment in these conditions suggest that similar neurobiological mechanisms may be affected in both disorders. Studies show that stress may produce an inflammatory response, along with increasing the levels of circulating proinflammatory cytokines. Cytokines induce changes in the HPA axis and the immune system; consequently, they act as a trigger for anxiety, in a similar fashion to depression [86].

Autism is a severe neurodevelopmental disorder with a large population prevalence, characterized by impairments in social interaction, deficits in verbal and non-verbal communication, repetitive behavior, and restricted interests [87]. It is known that there is a comorbidity between autism and epilepsy [88]. Recent experimental studies showed that the induction of MIA (maternal immune activation) in mice increases hippocampal excitability, accelerates kindling rate, prolonges increase of seizure susceptibility after kindling, and diminishes sociability (main feature of autism) in the offspring. Remarkably, epileptic and social impairments were abolished by antibodies to IL-6 and mimicked by recombinant IL-6 [89]. These data confirm previous findings about comorbidity between autism and epilepsy [88]. However, the relationship between epilepsy, autism, and neuroinflammation is complex. A recent study explored the effect of MIA induced by the prenatal exposure to IL-6 and IL-1β combination on SE induced by KA in mice. Prenatal exposure to IL-6 alone alleviated the severity of KA-induced epilepsy, while exposure to IL-6 + IL-1β increased its severity. Increased severity of epilepsy in the IL-6 + IL-1β mice correlated with the improvement of social behavior deficit (autism-like behavior) [90]. It is known that BTBR T + tf/J mice exhibit autistic-like behaviors (social interaction impairments and restricted repetitive behavior). One study that evaluated the expression of several cytokines in the brain of BTBR mice showed a significantly increased production of IL-6 [91]. On the other hand, increased IL-6 levels have been measured in the brain tissue [92, 93] and peripheral blood [94] of patients with autism spectrum disorders suggesting the cytokine profile may be altered in these conditions. Numerous studies have shown that the cytokine levels in different tissues as blood, brain, and cerebrospinal fluid of autistic subjects are altered; for example, a series of studies suggests that IL-6, TNF-α, and interferon-γ are significantly increased in autistic subjects [95].

5 Final Considerations

The evidence given above demonstrates that seizures and epilepsy are associated with a neuroinflammation process, involving, among others, gene expression, production, and release of proinflammatory cytokines. Considering the literature from experimental models and patients with epilepsy, IL-1β, IL-6, and TNF-α would be the most promising therapeutic target candidates. These cytokines facilitate seizure occurrence and epileptogenesis, seizure-induced neuronal cell death, and even neurological disorders that have high comorbidity with epilepsy. Thus, several experimental approaches are worthy of consideration: the use of small interfering RNAs (siRNAs), in order to prevent or reduce gene transcription by cleavage or degradation of specific mRNA;

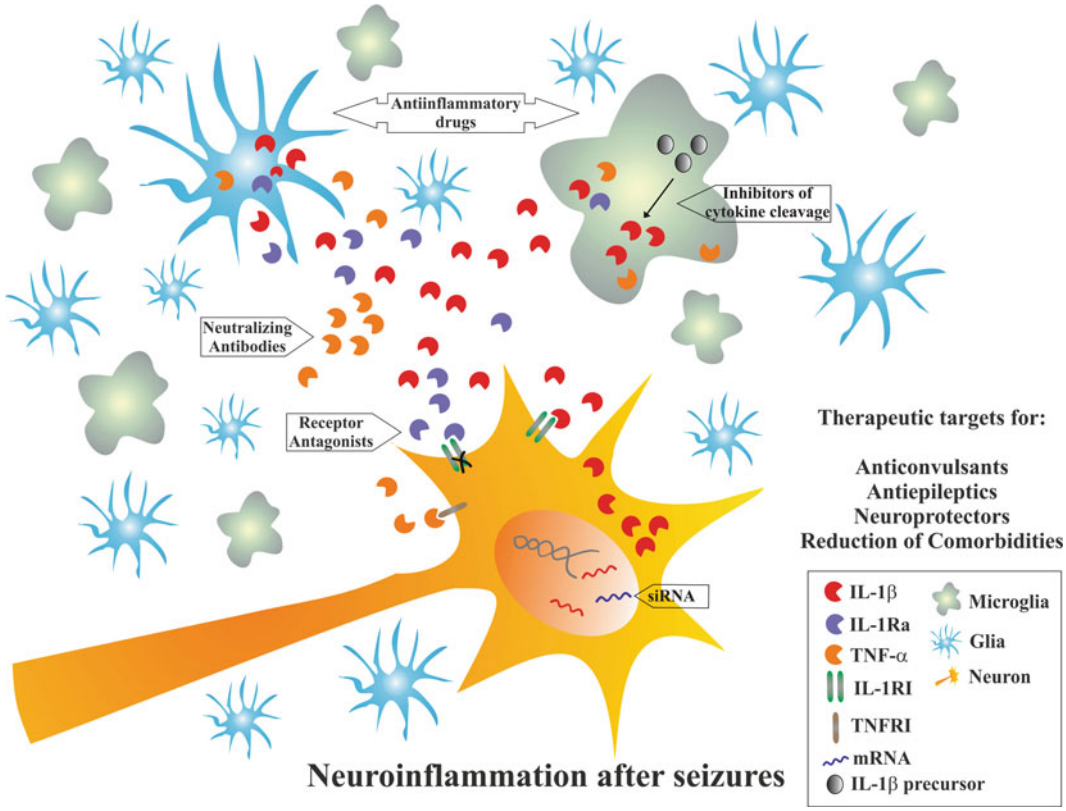

Fig. 1 Seizures and epilepsy are associated with a neuroinflammatory process that involves gliosis and microglia activation, as well as gene expression, production, and release of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, among others. These cytokines facilitate seizure occurrence and epileptogenesis, seizure-induced neuronal cell death due to seizures, and even neurological disorders that have high comorbidity with epilepsy. Experimental approaches worthy of consideration to prevent cytokine receptor activation and the corresponding intracellular signaling cascade include the use of anti-inflammatory drugs, inhibitors of cytokine cleavage, antagonists of cytokine receptors (particularly IL-1RI), neutralizing antibodies (that prevent cytokines from reaching its cellular targets and consequently its biological effects), or small interfering RNAs (siRNAs) in order to cleavage or degrade specific mRNA

antagonists of cytokine receptors, particularly IL-1RI, to prevent receptor activation and the corresponding intracellular signaling cascade; the use of neutralizing antibodies that prevent cytokines from reaching its cellular targets and consequently its biological effects; inhibitors of cytokine cleavage; or the use of anti-inflammatory drugs (Fig. 1). One aspect to consider when selecting the experimental model is the variability of the proinflammatory response among models, which is highly dependent on the molecular mechanisms involved in the generation and spread of epileptic activity. Additionally, the inflammatory response associated to seizures can vary with the age of experimental subject, with the understanding that the developing and the mature brain may

respond differently to inflammation. Also, new evidence has shown that human cytokine production varies depending on the type of epilepsy, temporal or extratemporal, with or without hippocampal sclerosis, and/or associated with tumors or not.

The current use of computational techniques such as molecular modeling and docking, among others, allow the identification of drugs targeting specifically a particular pathway. These tools could help us to discover new chemical entities or identify already known drugs, which modulate or decrease neuroinflammation, with the intention of controlling the generation of epileptic activity or preventing epilepsy and its consequences. However, these anti-inflammatory drugs will be credible therapeutic alternatives for epilepsy only if they can effectively cross the BBB to exert their effect. Considering this limitation, it is worth exploring the system of proinflammatory cytokines as a potential therapeutic target for the treatment of epilepsy.

Appendix 1: Intracerebroventricular (i.c.v) Injection of Cytokines in 14-Day-Old Rat Pups

Materials

Anesthesia system, isoflurane anesthesia system, hair clippers, scalpel, forceps, fine scissors, 25-gauge needle, stereotaxic frame adapted for neonatal rodents, heating blanket, handheld drill for skull openings, microinfusion pump, bone wax, sterile suture, and dry glass bead sterilizer.

Solutions

Picric acid saturated solution, sterile saline.

Protocol

1. Anesthetize the rat with isoflurane (3%, for induction) and shave the top of the head.
2. Place the anesthetized rat in a stereotaxic frame adapted for neonatal rodents and maintain the rat's body temperature with a heating blanket. Anesthetized rats with isoflurane (1.5–2%, for maintenance) during the surgery.

   Note: Surgery must be performed in aseptic conditions; surgical instruments must be sterilized.
3. Clean the skin with iodine solution to reduce the risk of contamination.
4. Make a small incision in the skin to expose the skull.
5. Make a small burr hole at the following coordinates: –4.0 mm posterior to bregma, –1.6 mm lateral to the midline (right side).
6. Insert a 25-gauge needle (0.5 mm) into the hole and lower it until it is at the depth of 3.5 mm below the surface of the skull.

7. Infuse drug over a period of 5 min (0.2 μl/min) using a micro-infusion pump.
   Note: Prepare stock solution of lyophilized drug by dissolving them in 1 μl of sterile saline solution.
8. Seal the hole with bone wax and close the skin with a single suture.
9. Apply the saturated picric acid solution to the wound to prevent maternal cannibalism.
10. After recovery, the pup is transferred to its mother.

Appendix 2: Determination of IL-1β and IL-1RI mRNA Expression by qRT-PCR

Materials

*mir*Vana™ PARIS™ kit (AM1556, Applied Biosystems, USA), agarose gel for electrophoresis, M-MLV reverse transcriptase (Invitrogen, USA), cDNA, primers (forward and reverse), Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Thermo Scientific, USA), nuclease-free water, spectrophotometer, electrophoresis system, and thermal cycler.

Protocol

1. Isolate RNA from brain samples with *mir*Vana™ PARIS™ kit (AM1556) following the manufacturer's recommendations (Applied Biosystems, USA).
2. Determine RNA concentration with a spectrophotometer by measuring the absorbance at 260 nm.
3. Assess RNA integrity by agarose gel electrophoresis.
4. Perform reverse transcription reactions with 1 μg of total RNA in a final volume of 20 μl, using M-MLV reverse transcriptase (Invitrogen, USA), according to the manufacturer's recommendations.
5. Perform real-time PCR reactions in triplicate as follows: each sample containing 1 μl of cDNA, 5 pmoles of each primer (forward and reverse), 10 μl of Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Thermo Scientific, USA), and nuclease-free water up to 20 μl.
   Note: Include triplicates for each sample containing primers for β-actin to normalize the data.
6. Run reactions in a thermal cycler, according to the following protocol: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 30 s at 95 °C, and 1 min at 60 °C, followed by a melting curve analysis.
   Note: Primers were designed using the Primer 3 software [96], and sequences are shown in Table 1.
7. Calculate amplification efficiencies by using lineal regression and determine changes in relative gene expression using β-actin as internal control.

Table 1

Sequence of primers used to amplify IL-1β, IL-1RI, and β-actin

	Primer sequence from 5′ to 3′	Product size (bp)
IL-1β	F TGAAGCAGCTATGGCAACTG	200
	R CTGCCTTCCTGAAGCTCTTG	
IL-1RI	F TGTGGCTGAAGAGCACAGAG	199
	R CGTGACGTTGCAGATCAGTT	
β-Actin	F AGGCTGTGCTGTCCCTGTAT	201
	R GCTGTGGTGGTGAAGCTGTA	

F forward, *R* reverse

Appendix 3: Localization of IL-1β and IL-1RI by Immunohistochemistry

Materials

Peristaltic pump, microtome, humidity chamber, histology jars, hydrophobic pen, aqueous mounting media, microwave, timer, and light microscope.

Solutions

4% phosphate-buffered paraformaldehyde, 0.1 M phosphate buffer (0.1 M PB), saline solution, 10 mM citrate solution (pH 6.0), ethanol solutions: 70, 80, 95, and 100%, blocking buffer (0.5% goat serum in 0.1 M PB).

Protocol

Tissue Processing

1. Anesthetize rats with an overdose of pentobarbital.
2. Perform transcardiac perfusion with saline solution followed by 4% phosphate-buffered (PB) paraformaldehyde.
3. Keep brain in situ at 4 °C overnight.
4. After removal of the brain, dehydrate in graded series of ethanol solutions (70%, 8 h; 80%, 2×3 h; 95%, 2×1.5 h; 100%, 2×1 h) and xylene (2×40 min) and then embed it in paraffin.
5. Section brains into 10-μm-thick coronal sections at the level of dorsal hippocampus and mount sections on gelatin-coated glass slides; keep slides at room temperature.
6. Deparaffinize (in xylene, 2×10 min) and rehydrate sections in a graded series of ethanol (100%, 2×10 min; 95%, 2×5 min; and 70%, 1×5 min) and dH_2O (2×10 min) with a final wash in 0.1 M PB (1×10 min).

Immunodetection of IL-1β and IL-1RI

1. Perform antigen retrieval procedure by heating brain sections in a 10 mM citrate solution (pH 6.0) using a microwave oven [2 min at high power and 10 min at power 20]; then allow the slides to slowly cool to room temperature for 45 min.

2. Rinse slides in dH_2O (1×10 min), wash them in 0.1 M PB (2×10 min), and incubate in a blocking buffer (0.5% goat serum in 0.1 M PB) at room temperature for 1 h.
3. Incubate slides overnight at 4 °C in a humidified chamber with primary antibody [rabbit polyclonal anti-IL-1β (ab9787, Abcam) diluted 1:500 or rabbit polyclonal anti-IL-1RI (sc 25775, Santa Cruz) diluted 1:200 with blocking buffer].
4. Wash sections in 0.1 M PB (3×10 min) and then expose them to biotinylated goat anti-rabbit (BA 1000, Vector Labs) diluted 1:1000 with the blocking solution for 2 h at room temperature.
5. Wash sections in 0.1 M PB (3×10 min) and then incubate with ABC-peroxidase complex 1:1000 (Vectastain ABC kit elite DK-6100 standard, Vector Labs) during 90 min.
6. Wash sections in 0.1 M PB (3×10 min) and reveal staining after incubation with AB peroxidase substrate kit, 3,3′-diaminobenzidine (SK-4100, Vector Labs).
7. Wash sections in 0.1 M PB (3×10 min), dehydrate it in graded series of ethanol solutions (70%, 1 min; 95%, 1 min; 100%, 1 min) and xylene (20 dips), and then mount with nonaqueous mounting medium.
8. Examine brain sections with a light microscope.

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Chapter 2

The Use of Anti-inflammatory Drugs in Epilepsy

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Abstract

Epilepsy is a brain disorder associated with neuroinflammation. Furthermore, it is known that important elements of the inflammatory process increase the susceptibility to epileptic activity. Therefore, the search of pharmacological strategies focused on decreasing both neuroinflammation and neuronal excitability associated with epilepsy is important. This chapter is a review of several drugs that may be used for this purpose.

Key words Anti-inflammatory drugs, Neuroinflammation, Seizures, Epilepsy

1 Introduction

Inflammation is the result of a series of events that arise in response to the presence of different types of agents that are noxious to the organism. It is considered as a defense mechanism that occurs to eliminate and later to repair the damage caused by any harmful agent. However, changes associated with the inflammatory process may cause tissue damage and, in the long term, lead to cell death [1]. In the central nervous system (CNS), inflammatory response, or *neuroinflammation*, is regulated by the interaction of cellular elements of the immune and nervous systems. Neuroinflammation involves the activation of astrocytes, microglia, lymphocytes, and mast cells [2]. The activation of these cells causes the release of inflammatory mediators, including cytokines, histamine, and serotonin, among others [3].

Neuroinflammation may result from central (neuroinfections, stroke, seizures, and *status epilepticus* (SE), among others) or peripheral (infections or autoimmune diseases) events. Neuroinflammation causes the activation of cells from the immune system, damage to the blood-brain barrier (BBB), neuronal death, and the beginning of a chronic state that facilitates the development of epilepsy (*epileptogenesis*) [4, 5]. Therefore, at the level of

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the CNS, it is relevant to identify cellular therapeutic targets to block the process of neuroinflammation and its consequences.

It is known that during the neuroinflammatory process microglia (brain phagocytic cells) produce different mediators such as reactive oxygen species (ROS) and increase the expression of the inducible nitric oxide synthase (iNOS), glutaminase, cytokines such as interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), cathepsin B, matrix metalloproteinases (MMP), and glutamate [6–8]. In this regard, the release of these mediators from microglia contributes to the generation of seizures by increasing neuronal excitability [9].

On the other hand, astrocytes are cells that regulate the electrochemical gradient in the CNS [10] and recognize albumin through transforming growth factor beta receptors (TGF-βRs). At this level, extravasated albumin resulting from BBB damage and/or seizures [11] produces the activation of TGF-βR and decreases the expression of the inwardly rectifying potassium channels (Kir 4.1) with a consequent reduction of astrocytes potassium (K^+) buffering capacity, subsequent neuronal hyperexcitability, and an increase of epileptiform activity [12]. Another situation that results in the decline of the buffer function of astrocytes is produced by the decoupling of gap junctions between these cells and the endothelial cells of the BBB due to the increase of IL-1β and TNF-α [13].

In the CNS, it is considered that mast cells are part of the BBB since they are in intimate contact with this structure and are rarely located in the cerebral parenchyma [14, 15]. Mast cells can migrate from the BBB to the brain parenchyma and release their components by degranulation as a consequence of different factors, such as hyperthermia, changes in pH, exposure to high levels of substance P, interleukins, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) [16–18]. The release of factors contained in mast cells, such as MMP (e. g., MMP-2, MMP-9) [19], vascular endothelial growth factor (VEGF) [20], TNF-α [21], IL-1β, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [22], may contribute to the damage of the BBB. It is possible that the epileptic activity increases the expression of ICAM-1, IL-1β, TNF-α, and IL-6 [23] as a result of mast cell activation. This idea is supported by the decrease in neuronal damage secondary to status epilepticus in animals pretreated with sodium cromoglycate, which stabilizes mast cells and thus prevents the release of their content [24].

Cyclooxygenase, which is located inside the cells, catalyzes the conversion of arachidonic acid into different mediators of inflammation that belong to the family of the prostaglandins and thromboxanes. There are two isoforms of this enzyme: the cyclooxygenase-1 (COX-1), which is expressed constitutively in most tissues, and COX-2, which is found in small amounts and it is inducible by damage such as inflammation and seizures [25]. In the brain, there is an upregulation in the expression of COX-1 and

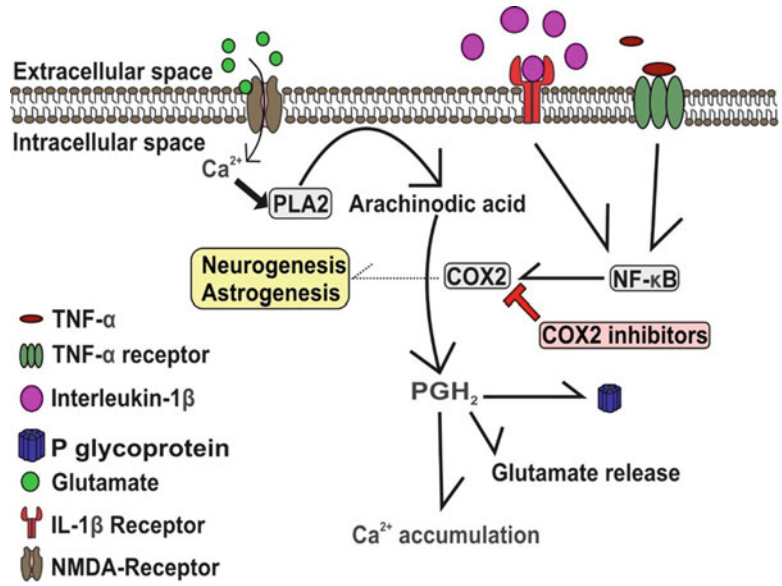

Fig. 1 COX-2 and its inhibitors in the epileptic brain. Glutamate and pro-inflammatory cytokines stimulate the expression of COX-2 in the CNS. This enzyme synthetizes PGH2, a prostaglandin that gives rise to many others; each of them results in a particular effect, such as the increase in the expression of P-glycoprotein, intracellular accumulation of Ca^{2+}, and a rise in the glutamate release, which contribute to the pathophysiology of epilepsy. *CNS* central nervous system, *Ca2+* calcium ion, *PLA2* phospholipase A2, *COX-2* cyclooxygenase-2, *TNF-α* tumor necrosis factor-α, *NMDA N*-methyl-d-aspartate, *NF-kB* nuclear factor kB, *PGH2* prostaglandin H2. The *dotted line* indicates an effect without a specific mechanism. The *red truncated arrow* indicates inhibition

COX-2 as a result of epileptic activity [26–28]. In experimental models, the use of anti-inflammatory drugs that are COX inhibitors diminishes neuroinflammation, an effect associated with a decrease in the frequency of seizures [29, 30] (Fig. 1).

In the cells, oxidative stress occurs when there is an imbalance due to an increase in free radicals and/or a decrease in antioxidants, which may cause tissue damage. As a result of epileptic activity, there is a release of calcium that favors the activation of phospholipase A_2, which in turn increases the release of arachidonic acid at the level of membrane lipids. Arachidonic acid promotes the production of ROS by different routes, thus increasing the inflammatory response [31]. The main biomolecules damaged by oxidative stress produced during epileptic seizures are membrane proteins, lipids, DNA, and RNA in susceptible regions of the CNS, such as the hippocampus and the cerebral cortex [32–35]. In different seizure models, the use of antioxidants showed a decrease in oxidative stress and neuroinflammation [36, 37].

Different neuroinflammatory processes increase neuronal excitability, which in turn produce an increase in seizures, situation that favors neuroinflammation generating a harmful vicious circle for the organism. This recurring sequence of events may contribute to the development of epilepsy [38]. This is the reason for which the search for pharmacological strategies focused on the pharmacological blockade of pro-inflammatory pathways in the CNS, which may be considered antiepileptic, is relevant [39]. Different pharmacological therapies with anti-inflammatory effects and potential antiepileptic use are discussed below.

2 Anti-inflammatory Drugs

2.1 COX Inhibitors

There is some evidence indicating that the selective inhibition of COX-2 may constitute an appropriate pharmacological strategy to prevent neuronal damage and neuroinflammation resulting from epileptic activity. Celecoxib, a selective COX-2 inhibitor, prevents microglia activation and inhibits aberrant neurogenesis as well as astrogliosis through the inhibition of the MAPK/ERK signaling pathway. These effects of celecoxib are associated with a decrease in the production of prostaglandin E [40], a decrease in neuronal excitability, an upregulation of the expression of $GABA_A$ receptors, and anti-epileptogenic effects [29]. On the other hand, it is known that the high expression of P-glycoprotein in the BBB is a mechanism that mediates drug resistance in epilepsy [41]. Experimental evidence suggests that celecoxib prevents the overexpression of P-glycoprotein induced by glutamate overexposure in endothelial cells of the BBB [42]. These studies suggest that celecoxib may represent a therapeutic strategy to prevent drug-resistant epilepsy.

Parecoxib, another selective COX-2 inhibitor, decreases the levels of prostaglandin E2 and neuronal damage in the hippocampus and the piriform cortex when administered during 18 days after lithium-pilocarpine-induced SE model. This treatment also reduces the intensity of spontaneous seizures during the development of epileptogenesis [43].

To the present, the effects resulting from the administration of acetylsalicylic acid, a nonselective COX inhibitor, are controversial. Ma et al. (2012) reported that chronic administration of aspirin after lithium-pilocarpine-induced SE decreases neuronal damage in the hippocampus as well as the frequency and duration of subsequent spontaneous seizures [30]. However, another study found that treatment with acetylsalicylic acid chronically administered before pilocarpine induction of SE increases susceptibility to seizures and does not alter the subsequent neuronal cell death and gliosis in the hippocampus [44]. These data suggest that further studies—in different experimental models using different doses and protocols of management—are needed to determine the possible anti-epileptogenic effect of acetylsalicylic acid.

Furthermore, although the administration of indomethacin, an inhibitor of COX, does not modify the presentation of SE induced by pilocarpine [45], it decreases the expression of IL-1β and TNF-α associated with this event [46]. This suggests that indomethacin may be used to decrease the inflammatory process associated with epileptic activity.

These data suggest that the decreased activity of COX by selective inhibitors may represent a therapeutic strategy to reduce seizure-induced neuroinflammation and avoid drug-resistant epilepsy.

2.2 Glucocorticoids

Seizure activity may induce vasogenic cerebral edema, which is harmful. In addition, the use of anti-inflammatory agents such as dexamethasone, a synthetic glucocorticoid steroid, is considered a therapeutic strategy for treating cerebral edema resulting from seizures [47]. It is described that the continuous administration of dexamethasone reduces the epileptic encephalopathy with continuous spike-and-wave during sleep [48] as well as the epileptic activity associated with neurocysticercosis [49].

Dexamethasone induces a significant reduction of epileptic activity secondary to intracerebral injection of penicillin [50]. However, experimental evidence indicates that dexamethasone increases cerebral edema, an effect associated with a decrease in the volume of the hippocampus and increased mortality of animals subjected to SE [51]. These controversial data can be explained from the fact that anti-inflammatory and antiepileptic effects of dexamethasone depend on the administered dose [52].

2.3 Antioxidants

Due to the increase in oxidative stress during epileptic activity, it is proposed that the use of antioxidant drugs may represent a pharmacological therapy to reduce the process of neuroinflammation associated with epilepsy (Fig. 2). In this regard, it is described that pretreatment with α-tocopherol (i.e., vitamin E) reduces the activation of astrocytes, microglia, neuronal death, and oxidative stress induced by SE in the hippocampus [36]. In addition, treatment with α-tocopherol during the subsequent period to seizure significantly reduces astrocytosis, activation of microglia, and neuronal death, effects associated with a lower oxidative stress [53]. It is suggested that the antioxidant effect of α-tocopherol may increase when it is combined with other antioxidants such as vitamin C (ascorbic acid). Vitamin C by itself reduces hippocampal injury and mortality induced by epileptic activity [54].

The administration of baicalein, another antioxidant suppressor of oxidative stress, reduces epileptiform activity and cognitive impairment in epilepsy like in tremor rat model. These effects are associated to a decrease of oxidative stress and inflammation indicators [37].

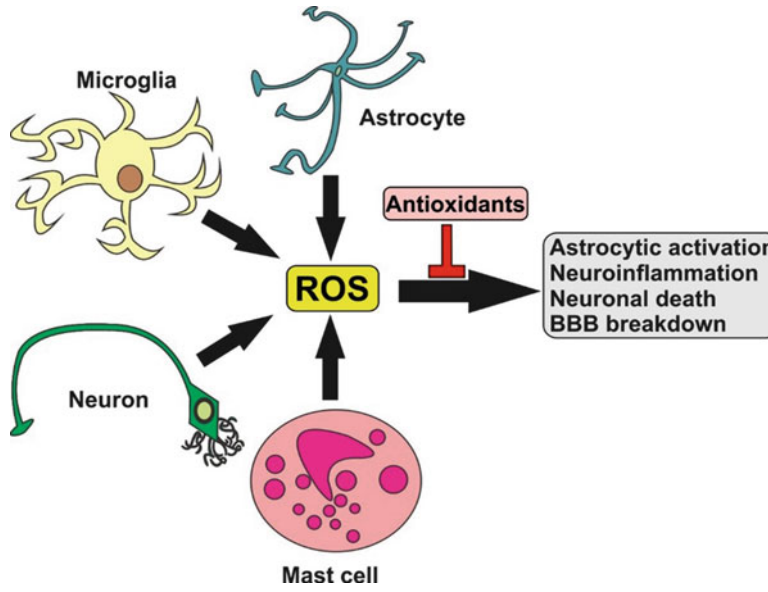

Fig. 2 ROS in epilepsy and the role of antioxidants. Cells in the CNS are able to generate ROS in response to multiple insults (e. g., hypoxia, cell death, and inflammation). ROS may alter cell function through multiple mechanisms. The presence of antioxidants, like vitamin E, controls these effects by binding to ROS and stabilizing these particles. *ROS* reactive oxygen species, *BBB* blood-brain barrier. The *red truncated arrow* indicates inhibition

The evidence described above supports the idea of using antioxidants as adjuvant therapy to prevent the neuroinflammation-associated epileptic activity.

2.4 Antimicrobial Agents

It is known that some antimicrobial agents, such as penicillins, cephalosporins, quinolones, and antimalarials, may induce proconvulsant effects and epileptiform activity in both patients with epilepsy and normal subjects [55]. In addition, many antimicrobials present pharmacokinetic interactions with some antiepileptic drugs [56]. On the other hand, it is described that tetracyclines, such as minocycline, doxycycline, and tetracycline, induce anticonvulsant effects in a dose-dependent manner on the model of partial seizures induced by 6-Hz electrical stimulation. However, at high doses, they cause toxic effects, such as motor and respiratory deficiencies and death [57].

With regard to minocycline, a second-generation tetracycline, it is known that it presents very powerful anti-inflammatory properties which are independent of its antimicrobial activity [58]. At the level of the CNS, these mechanisms of action include the suppression of the activation of microglia and a reduction in the release of pro-inflammatory cytokines [59]. Interestingly, it was found that the repeated administration of minocycline after the induction of SE decreases neuronal damage by preventing microglial

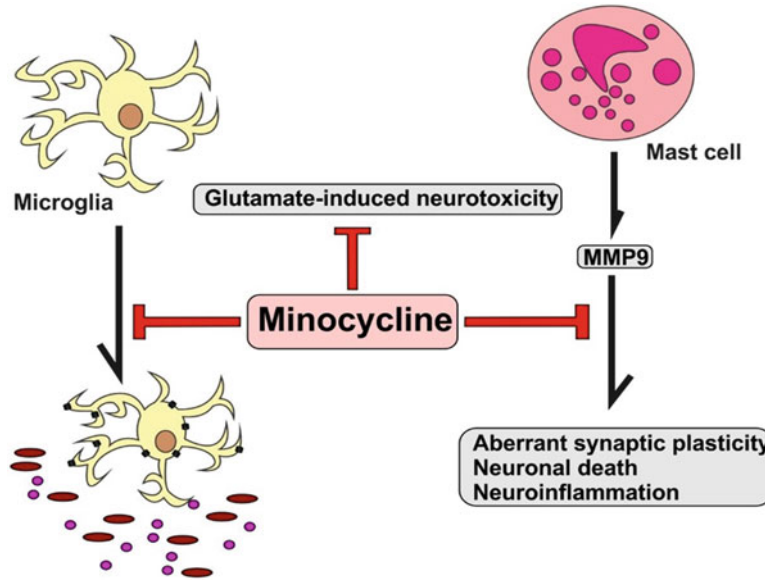

Fig. 3 Mechanisms of minocycline in epilepsy. It has been suggested that minocycline exerts its effects through the inhibition of microglial activation; moreover, it is known that it is able to inhibit MMP-9, an enzyme involved in BBB damage. Finally, it is considered that minocycline decreases glutamate excitotoxicity due to the inhibition of caspases pathway (apoptosis) and the p38 MAPK pathway. *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *MMP-9* matrix metalloproteinase-9, *MAPK* mitogen-activated protein kinase. The *red truncated arrow* indicates inhibition

activation and producing IL-1β and TNF-α in the CA1 region of the hippocampus and the adjacent neocortex. These anti-inflammatory and neuroprotective effects of minocycline resulted in the reduction of the frequency, duration, and severity of spontaneous recurrent seizures subsequent to SE [60]. Such evidence suggests the possibility of using minocycline as an anti-epileptogenic treatment for subjects who have presented SE (Fig. 3).

2.5 Anti-inflammatory Drugs with Antiepileptic Effects

Cytokines are a group of peptides and proteins that are released from the immune system in response to infectious diseases. In the CNS, cytokines are produced and released from glia or blood cells that reach the brain parenchyma as a result of BBB damage. Currently, it is known that, in addition to their immunological effects, cytokines are involved in neuronal excitability through changes in receptors coupled to ion channels, among other mechanisms. This situation results in long-term changes in brain function and facilitation of epileptic activity [61]. At present, there are drugs that prevent the production and release of pro-inflammatory cytokines and reduce epileptic activity, which represents a novel strategy for the control of this disease.

Caspases are a group of proteins that belong to the cysteine protease group, which participate mainly in the process of apoptosis. Some caspases are also involved in protein maturation of some interleukins. In particular, caspase 1, also called IL-1β converting enzyme (ICE), is involved in the maturation and secretion of some interleukins [62]. The administration of VX-765, a selective blocker of IL-1β production by inhibition of the ICE, blocks kindling-induced epileptogenesis in rats [63]. Similarly, repeated treatment with VX-765 reduces chronic epileptic activity secondary to SE as well as the expression of acute seizures in mice [9]. The combination of VX-765 with anakinra (a recombinant human IL-1 receptor antagonist) in animals previously subjected to SE by pilocarpine reduces subsequent neuronal loss and IL-1β expression in astrocytes [64]. These data indicate that the specific blocking of the synthesis of interleukins is capable of modifying the epileptic activity and subsequent neuronal damage (Fig. 4).

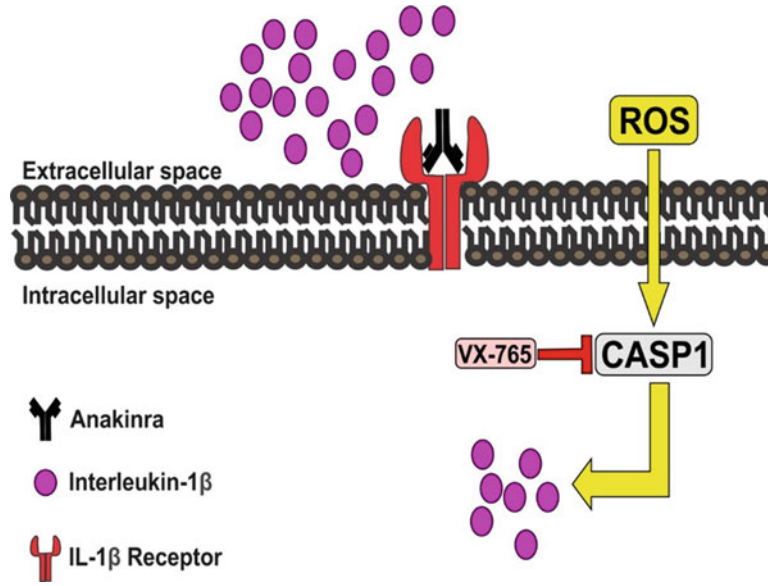

Fig. 4 Role of VX-765 and anakinra in neuroinflammation and epilepsy. ROS are molecules able to induce the expression of IL-1β through the induction of CASP1. VX-765 inhibits CASP1, diminishing IL-1β expression and, in turn, neuroinflammation. Anakinra is a selective inhibitor of IL-1β receptor; this binding allows it to diminish the effects of cytokines in target cells. *ROS* reactive oxygen species, *IL-1β* interleukin-1β, *CASP1* caspase-1 or ICE, interleukin-1β converting enzyme. The *red truncated arrow* indicates inhibition

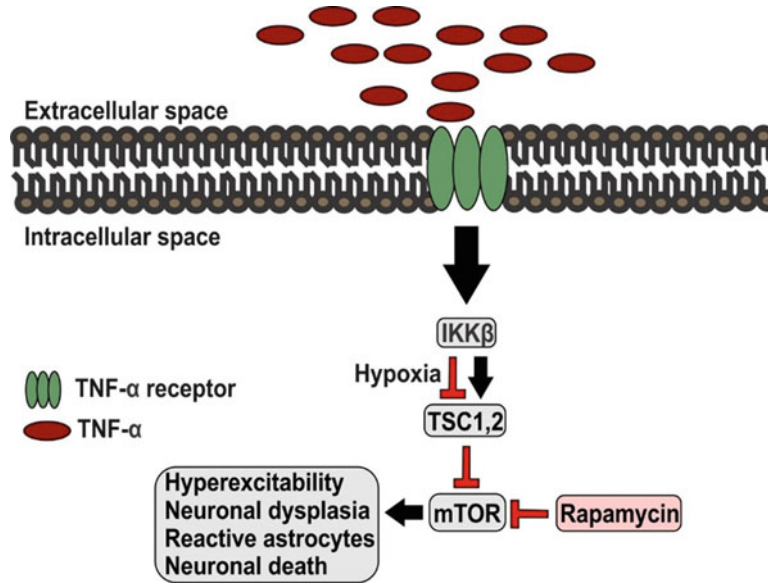

Fig. 5 Role of mTOR inhibitors in epilepsy. The mTOR pathway has an important role in the normal development of the CNS; however, when the TSC is inhibited (by mutations or another intracellular pathway), mTOR is overexpressed leading to deleterious consequences such as hyperexcitability, neuronal dysplasia, reactive astrocytes, and neuronal death. In the epileptic brain, TSC can be inhibited by TNF-α and hypoxia. Under hypoxic conditions, the activation of TNF-α receptor leads to this inhibition, and finally to the overexpression of mTOR. Rapamycin, a selective inhibitor of mTOR, has been used in the treatment of epilepsy in patients with TSC, showing convenient effects. *CNS* central nervous system, *IKKβ* inhibitor of kappa light polypeptide gene enhancer in B-cells, *TSC* tuberous sclerosis complex, *mTOR* mechanistic target of rapamycin, *TNF-α* tumor necrosis factor-α. The *red truncated arrow* indicates inhibition

The mammalian target of rapamycin (mTOR) pathway is an intracellular signaling cascade involved in multiple cellular functions, including the synthesis of proteins, cell growth, and cell proliferation as well as synaptic plasticity. It is also involved in neuronal excitability and epileptogenesis [65, 66]. Rapamycin is an immunosuppressant that specifically inhibits mTOR pathway. In addition, it decreases the expression of IL-1β and TNF-α, very likely through the inhibition of the activation of NF-kB [67] (Fig. 5). In patients, rapamycin has been found to decrease seizures associated with tuberous sclerosis, infantile spasms, seizures secondary to neonatal hypoxia, absence seizures, and temporal lobe epilepsy [68]. Results of experimental models confirm its antiepileptic effects [69]. Currently, inhibitors of the mTOR pathway are being considered as antiepileptic compounds for the control of drug-resistant epilepsy [70].

Within the process of neuroinflammation, mast cells are given little importance despite they play an important role in the process of peripheral inflammation. At the level of CNS, there is a high density of mast cells in the leptomeninges, hypothalamus, hippocampus, thalamus, and dura mater of spinal cord [71]. It is proposed that mast cells are part of the BBB and that their activation is involved in the breakdown of this structure and the subsequent process of neuroinflammation associated with brain disorders [1].

In the CNS, degranulation of mast cells produces the release of factors such as histamine, heparin, and serotonin that can produce BBB rupture and the extravasation of blood elements to the cerebral parenchyma, situation that facilitates neuroinflammation. Therefore, it is considered that the use of drugs such as sodium cromoglycate represents a strategy to stabilize mast cells [72, 73]. The stabilizing effect of cromoglycate is carried out through the blockade of Ca^{2+} channels [74] and by the phosphorylation of a 78 kDa protein located around granules of mast cells that regulates the translation of signals between the membrane and the cytoskeleton [75].

There is experimental evidence that indicates that the administration of cromoglycate prevents the degranulation of mast cells and reduces neuronal damage as well as activation of glial processes of cerebral ischemia [17, 76]. Recently, we reported that pretreatment with sodium cromoglycate resulted in a neuroprotective effect in hippocampus of rats submitted to SE induced by pilocarpine. This effect is associated with a reduced release of histamine in the hippocampus [24], which induces proconvulsant effects [77]. These experimental evidences suggest that systemic administration of cromoglycate may be a good option to avoid or reduce neuronal and BBB damage resulting from the degranulation of mast cells in response to acute events such as SE, which leads to the development of spontaneous seizures (Fig. 6).

3 Conclusion

It is clear that epilepsy is associated with neuroinflammation processes, which in turn induce neuronal damage. For this reason, the use of drugs that facilitate neuroprotection and/or which block transduction cascades associated with neuroinflammation is so relevant. Therefore, the search for combinations of these drugs with traditional antiepileptic drugs may result as a more effective strategy to control epileptic activity and its consequences.

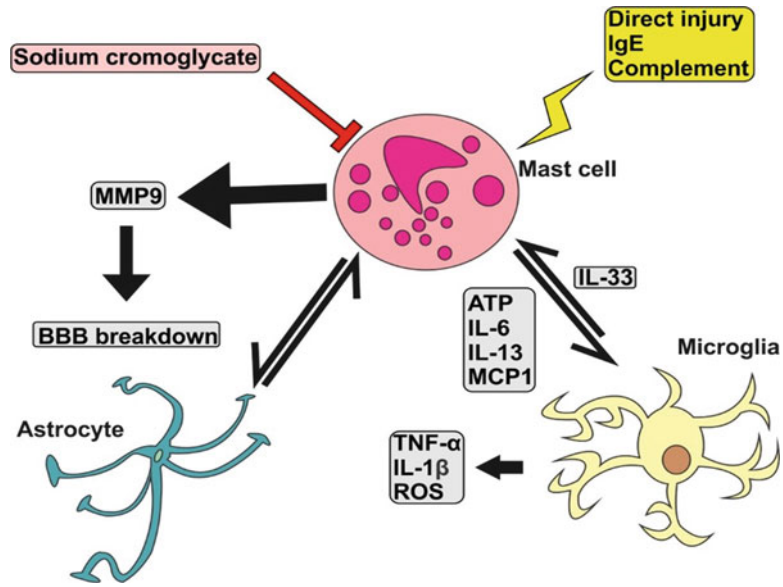

Fig. 6 Role of mast cells and their inhibition in epilepsy. Mast cells have many effects in different cells in the CNS. These target cells produce substances that stimulate mast cells, leading to a neuroinflammation cycle. The activation of microglia and astrocytes, and the BBB-breakdown are consequences of mast cell activation, which lead to negative effects in the epileptic brain. The pretreatment with sodium cromoglycate, a mast cell stabilizer, has proven beneficial effects in the litium-pilocarpine model of temporal lobe epilepsy, suggesting the possible therapeutic effect of this inhibition in epilepsy. *IgE* immunoglobulin E, *MMP9* matrix metalloproteinase-9, *BBB* blood-brain barrier, *IL-6* interleukin 6, *IL-13* interleukin-13, *IL-33* interleukin-33, *MCP1* monocyte chemo-attractant protein 1, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *ROS* reactive oxygen species. The *red truncated arrow* indicates inhibition

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Chapter 3

Carbonic Anhydrase and Epilepsy

Luciana Gavernet

Abstract

The deficiencies of current antiepileptic drugs (AEDs) demand the search of new active compounds through novel strategies of drug discovery. Particularly, the design of AEDs based on molecular targets constitutes a promising alternative to empirical screening, the traditional method to detect anticonvulsant action in new structures. In this chapter we described the advances in the dynamic field of carbonic anhydrases, with emphasis in the development of selective inhibitors as anticonvulsants. We first detailed the 3D architecture of carbonic anhydrases and the mechanism of action of classical inhibitors. Then we reviewed the known anticonvulsant drugs that present carbonic anhydrase inhibition and the progress made in the design of selective inhibitors of CAVII, the isoform implicated in the generation of febrile seizures.

Key words Epilepsy, Carbonic anhydrase, Sulfonamides, Sulfamides, Sulfamates

1 New Targets for Antiepileptic Drugs

Epilepsy is a complex chronic brain disorder. The International League Against Epilepsy (ILAE) defines and quantifies over 15 different seizure types and more than 30 epilepsy syndromes that can be originated by a variety of pathological conditions [1]. Pharmacological experiments carried out over the last decades have increased our knowledge about the physiopathology of epilepsy, and they proved that mechanisms of generation of seizures are related with the imbalance between inhibitory and excitatory conductance in brain tissues [2]. Antiepileptic drugs (AEDs) work to reconstruct this balance and avoid the seizures, but they are efficient in about 70% of the patients. The remaining 30% of the people (who cannot completely control the seizures though drug therapy) suffer from refractory or intractable epilepsy [3, 4]. On the other hand, responsive patients experience medication-related side effects, which become more evident and dangerous when lifelong medication is necessary [3].

After four decades of research, numerous compounds have been introduced to the market with notable improvements in their absorption, distribution, metabolism, excretion, and toxicity

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(ADME/Tox) properties, relative to the first generation of AEDs. However, no significant progresses have been achieved in terms of the efficacy in resistant patients [3]. This fact demanded a conscientious analysis about the strategies employed to the design of anticonvulsant compounds.

Without a doubt, the approach more extensively addressed for the discovery of new AEDs has been the phenotypic screening in acute models of seizures [5]. The US National Institute of Health, through its Anticonvulsant Screening Project, proposes an initial protocol that includes in vivo/in vitro models to identify new active molecules [5]. Among them, the most employed assays are the maximal electroshock seizure test (MES test) and the pentylenetetrazol test (scMES test) in mice and rats [6]. The MES test is associated with the electrical induction of the seizure, whereas scMES test involves a chemical stimulus to generate the convulsion. Most of the marketed AEDs are capable of suppressing the seizures induced with at least one of these two tests.

The screening methodology does not detect AEDs with a specific mechanism of action. In fact, the molecular targets of the new compounds are elucidated after the identification of the activity. Accordingly, screening methods might ignore compounds with novel mechanisms of anticonvulsant effect, if they cannot avoid the seizures caused by classical test [7]. To partially solve this limitation, other assays have been recently included in the program, which detected drugs that are ineffective into the classical models [5].

The restrictions of the empirical screening, the progress in the knowledge of the mechanisms involved in ictogenesis (or epileptogenesis), and the lack of efficiency of known AEDs in refractory patients have supported the search of alternative methods of drug discovery, like the design of anticonvulsants based on molecular targets [3, 8]. Successful examples of this rational methodology are vigabatrin, tiagabine, and perampanel [9]. Vigabatrin and tiagabine potentiate the GABA-ergic neurotransmission, whereas perampanel impedes the glutamatergic excitation [9]. However other less explored molecular targets that affect in some way over the GABA-ergic neurotransmission or glutamatergic excitation have been pointed out as new alternatives for target-based drug design [8, 10]. In this context, carbonic anhydrase has emerged as an attractive enzyme for designing new active anticonvulsant compounds [11].

2 Carbonic Anhydrases

Carbonic anhydrases (CAs) belong to a family of metalloenzymes that are responsible for the reversible hydration of carbon dioxide and bicarbonate. They have been classified into five classes (named as α, β, γ, δ, and ε CAs). The α class is the most studied and it is found mainly in vertebrates. In fact, α-CAs is the only class found in mammals [12].

Up to now, 16 isoforms of α-CAs have been identified with different catalytic activities, cell/tissue distributions, and response to inhibitors [12, 13]. There are eight cytosolic proteins (CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI, CA XIII), two mitochondrial matrix proteins (CA VA, CA VB), one secreted protein (CA VI), two glycosylphosphatidylinositol (GPI)-anchored proteins (CA IV and CA XV), and three transmembrane proteins (CA IX, CA XII, CA XIV). Isoform CA XV is not expressed in primates, and isoforms CAVIII, CAX, and CAXI have no catalytic activity since they lack of histidine residues of the active sites [11]. They are also defined as CA-related proteins (CARPs).

Extensive experimental data showed the architecture of the catalytic forms of α-CAs, especially for CAII. The active site is well conserved in all the active isoforms, and it comprises a Zn^{2+} ion located at the bottom of a half hydrophilic and half hydrophobic conical cleft. The cavity is approximately 15 Å deep and the active site is accessible to water [13]. The metal ion then is coordinated by the imidazole rings of three histidine residues with the fourth position occupied by a water molecule at acidic pH (<8) and by a hydroxide ion at higher pH (Fig. 1).

2.1 Carbonic Anhydrase and Seizures

CAs are responsible for maintaining the intra- and extracellular balance between CO_2, H^+, and HCO_3^- ions [12]. They play important functions in crucial physiological processes for both normal and pathological conditions, such as the acid-base equilibrium, CO_2 and HCO_3^- transport across membranes, electrolyte secretion, calcification, biosynthetic reactions (gluconeogenesis, lipogenesis, and ureagenesis), signal transduction, and tumorigenicity [13].

Regarding their role in the control of pH, CAs represent a versatile system to balance the acid-base concentrations at the level of the blood–brain barrier, neurons, glia, and interstitial fluid in the brain, as well at levels of the whole organism (respiratory functions, energy metabolic functions, and renal functions) [14]. Of course, the modulation of pH is attached to the movements of many other ions, so this enzyme is indirectly involved in the flux control of other solutes [11, 14].

As mentioned at the beginning of this chapter, seizures are related with the imbalance between inhibitory and excitatory conductance in brain tissues, which may be related with fast alterations in the extracellular ionic compositions [15]. For example, epileptiform activity is caused by a rise in the extracellular potassium concentration [16], and neuronal excitability is affected by changes in pH [14]. Generally, the excitability of most central neurons and neuronal network is increased by alkalosis while it was suppressed by acidosis [11, 14, 17–20]. Hyperventilation is a standard practice employed in the clinic to generate respiratory alkalosis, causing precipitation of petit mal seizures [21]. It is also employed in children to trigger febrile

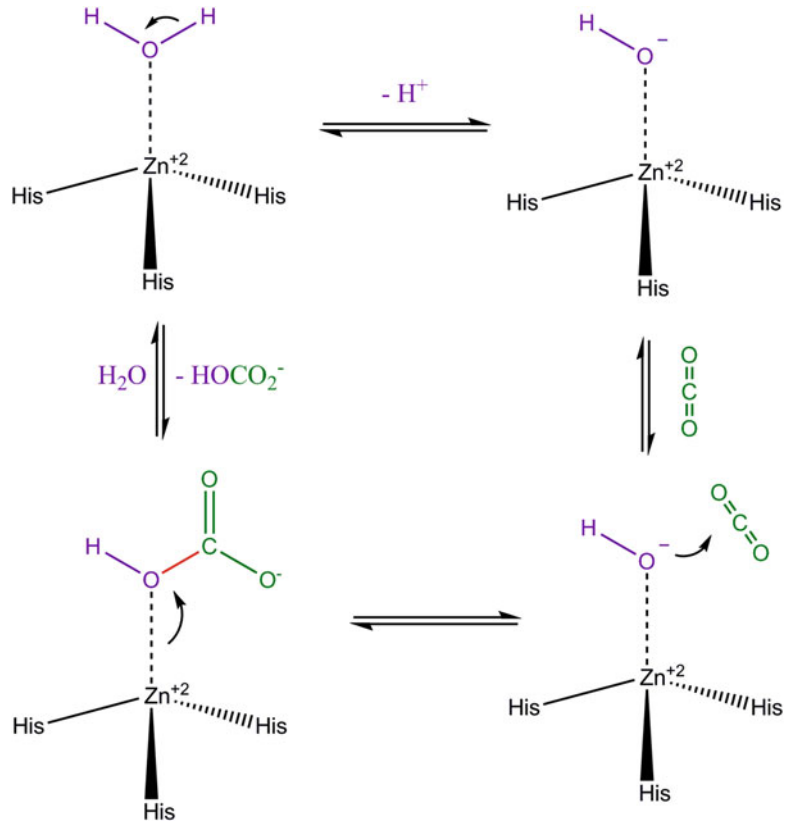

Fig. 1 Active site of catalytic α-CAs. The metal ion coordinates 3 histidine residues and a water molecule that generates the nucleophilic hydroxide anion necessary to trigger the catalytic cycle

seizures (as well in animal models of convulsions) [22, 23]. On the other hand, respiratory acidosis induced exogenously suppresses the neuronal excitability [14].

The pH is mainly balanced by the buffer CO_2/HCO_3^- (in the intracellular and extracellular space), so several links between CAs and the generation of seizures have been proposed [11]. Furthermore, the relation of CA and seizures is supported by the fact that CA inhibitors are (or were) employed to treat epilepsy. For example, in 1963, Esplin and Rosertain studied the inhibitor acetazolamide and they found that it decreased excitability in cat spinal cord [24]. After that, other authors proposed that the glial cells are more alkaline than neurons (due to higher concentrations of HCO_3^-), and the anticonvulsant action of the CA inhibitors could be related with extracellular acidosis [25]. A revision of the AEDs with inhibitory action against CA is given in the next section of this chapter.

3 CA Inhibitors as Anticonvulsant Drugs

CAs inhibitors have been the object of study in many fields of medicinal chemistry, as candidates to treat glaucoma, altitude sickness, obesity, pain, cancer, and epilepsy [12, 26–28].

Classical inhibitors of α-CAs exert their action by blocking the four position originally destined for water in the catalytic center (Fig. 1). They usually bind to the Zn^{2+} as anions, forming two different complexes depending on the presence/absence of water (Fig. 2). The most important functional group that serves as Zn^{2+} anchoring group of CA inhibitors is the sulfonamide function (and their bioisosteric partners, Fig. 2). However, it is worth pointing out that other inhibition mechanisms have been discovered for other families of compounds, such as polyamines and coumarins [12].

Some commercial AEDs are CA inhibitors [29]. However, their anticonvulsant action has not been totally attributed to the CA inhibition process in most of the compounds. In fact, the CA interactions represent a drug weakness in some cases, because it generates tolerance and/or important side effects.

Acetazolamide (Diamox®) was introduced to the market as a diuretic in 1953 (Fig. 3). Simultaneously, its anticonvulsant properties were discovered by Bergstrom et al. [30] and then other researchers probed their effect in animals and humans [31–34]. It has been employed in partial and generalized seizures, as well as in catamenial epilepsy [35, 36]. Regarding its safety, acetazolamide induces side effects as paresthesias, tinnitus, loss of appetite, and alterations of taste [37, 38]. Nowadays it is rarely indicated to treat convulsions, mainly due to the development of tolerance [39, 40]. Methazolamide is a structurally related compound that has been

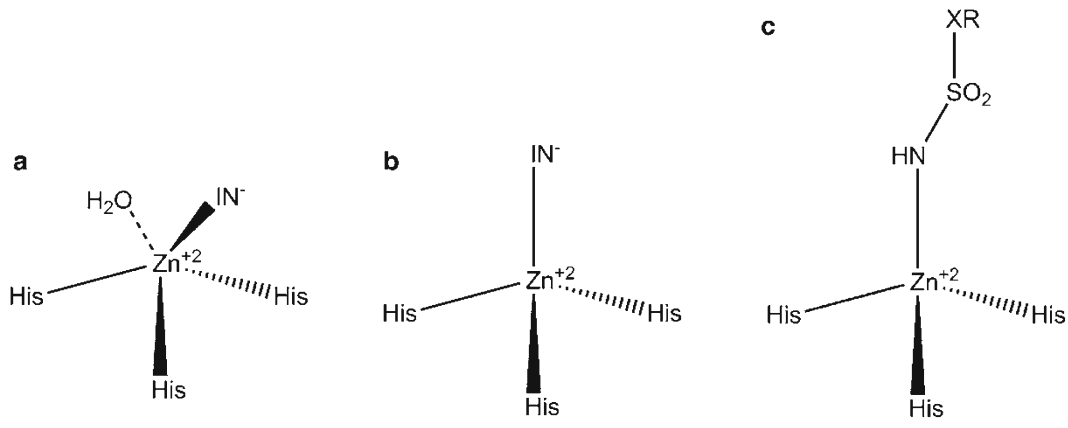

Fig. 2 Schematic representation of the interactions found for classical inhibitors and the active site [16]. (**a**) The Zn(II) ion is coordinated with a water molecule in addition to the inhibitor (IN^-). (**b**) The inhibitor (IN^-) substitutes the fourth position in the tetrahedral complex (originally available for water). (**c**) Sulfonamides (X = C atom), sulfamates (X = O atom) and sulfamides (X = N atom) bind to the zinc ion in their deprotonated form

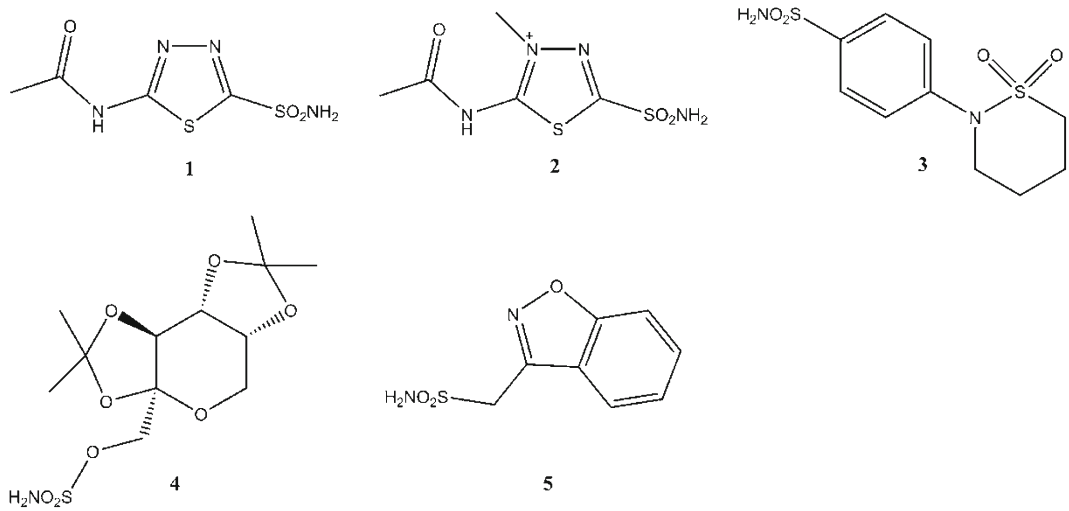

Fig. 3 Structures of anticonvulsant drugs with proved CA inhibition. **1**: Acetazolamide; **2**: Methazolamide; **3**: Sulthiame; **4**: Topiramate and **5**: Zonizamide

tested as anticonvulsant (Fig. 3), but it has gained more attention as antiglaucoma drug [29, 41, 42]. Sulthiame is another sulfonamide derivative employed as AED (Fig. 3). It is prescribed to treat partial epilepsy in children in Europe and Australia [43], but serious side effects related with deterioration in cognitive functions have been reported [44].

Topiramate (Topamax®) is a broad spectrum AED employed for the treatment of partial and generalized seizures, including Lennox–Gastaut syndrome and prophylactic treatment of migraine [45, 46]. It is also indicated in patients with bipolar and mood instability disorders, post-traumatic stress, eating disorders, addictions, and other pathologies [29, 47–50]. From a structural point of view, it is a substituted monosaccharide that contains sulfamate function (Fig. 3). The sulfamate group is a bioisosteric partner of sulfonamide moiety, and it serves as anchoring group to interact with CA active site [51].

The anticonvulsant action of topiramate is attributed to multiple mechanisms of action. It inhibits several CA isoforms [52], but it also blocks Na^+ and Ca^{2+} channels and AMPA/kainate receptors; and it enhances the GABA-ergic neurotransmission [29, 53–59]. On the other hand, CA inhibition contributes to the generation of several side effects like metabolic acidosis, hypocitraturia, hypercalciuria, and elevated urine pH, leading to an increased risk of kidney stone formation [10, 29, 60, 61].

Zonisamide is an AED effective for simple and complex partial seizures, generalized tonic–clonic seizures, myoclonic epilepsies, Lennox–Gastaut syndrome, and infantile spasms [29, 62–64]. Like acetazolamide, it has a sulfonamide function in its chemical structure

(Fig. 3), but a weaker inhibition profile: its CA-inhibiting activity in vivo is 100–1000 times weaker than acetazolamide [65]. Studies showed that its main mechanisms of anticonvulsant action are the blockade of voltage-sensitive sodium channels and the reduction of voltage-sensitive T-type calcium currents [29]. Zonisamide presents acceptable side effects like somnolence, dizziness, and weight loss but it develops tolerance very quickly [29, 65].

Regarding drugs in development, the search of new CA inhibitors as anticonvulsants is an active area of research [52, 66–70]. However, the limitations found in marketed and experimental CA inhibitors originated doubts about the potential of this target for designing efficient AEDs. Experts in the field have concluded that future inhibitors might overcome the difficulties of tolerance and toxicity by selective targeting specific isoforms involved in the pathological process [10]. Following this idea, in the last years, the drug design campaigns have focused on obtaining isoform-selective inhibitors of CAs. It represents a challenging objective because there are 13 active CAs in humans with similar architecture [12]. They share the active site characteristics (three residues of His bound to the zinc ion) and two residues highly conserved near the active site (identified as Thr 199 and Glu 106 in CAII), since they help with the coordination between water/hydroxide ion and zinc. Other common features are the physicochemical characteristics of the residues in the conical cleft, which presents a hydrophobic region opposite to one hydrophilic region [12].

However, there are environmental differences for the isozymes that provide the opportunity of designing selective inhibitors. For example, transmembrane isoforms CAIX and CAXII are targets for anticancer drugs, particularly in diseases associated with hypoxic tumors [12, 71]. Hydrophilic glycosyl sulfonamide inhibitors of CA IX and CA XII have been designed to minimize the diffusion through lipid membranes and to promote the selective inhibition of transmembrane CAs [12, 72]. Similarly, positively charged sulfonamide inhibitors were designed to minimize their transport through the lipophilic membranes [73].

There are also divergences in the identity of some amino acids in each isoform, mainly for those located at the middle and toward the exit of the active site cavity. It generates differences on the size and shape of the cleft, which finally affect the inhibition patterns of the ligands. For example, the inhibition constant (Ki) of the anticonvulsant topiramate is 210 times lower than its sulfamide analogue in CAII isoform [74]. However, this sulfamide effectively inhibits in the nanomolar range isozymes CA VA, VB, VII, XIII, and XIV [74]. The weak inhibitory properties of the sulfamide against CAII has been attributed to the unfavorable Van der Waals contacts between this ligand and one distinctive residue of CA II (Ala65) [74]. Additionally, the access to other protonation states of the sulfamide function relative to the sulfamate group has also been hypothesized as an extra source of selectivity [75].

On the other hand, the divergences in the identity of the amino acids at the entrance of the cavity provide the opportunity of designing elongated molecules that interact with the active site but also with the distinctive residues to be isoform selective [12]. This strategy is known as *the tail approach* and numerous successful examples are given in literature mainly for antiglaucoma and anticancer drugs [12]. Regarding epilepsy, there is a growing interest about the design of selective inhibitors for the isoform CAVII, which has gained attention as a promising target for AEDs.

3.1 Design of Selective CAVII Inhibitors as Anticonvulsants

As detailed previously in this chapter, CAs are responsible of preserving the intra- and extracellular balance between CO_2, H^+, and HCO_3^- ions, and these species influence the neuronal signaling in many different ways. Regarding neuronal excitability, numerous studies sustain the hypothesis that CA activity promotes depolarizing and excitatory GABA-ergic transmission through HCO_3^- currents [14, 76]. Moreover, important advancements have been recently reported about the influence of the isoform CAVII in the generation of febrile seizures via the activation of GABAA receptors [77].

Human CAVII is a cytosolic isoform composed of 263 amino acids that shares 56% of identity with the CAII isoform [78]. In contrast to ubiquitous CAII, CAVII is the unique isoform present mostly in the central nervous system. It is highly expressed in the cortex, hippocampus, and thalamus regions of humans' and rats' brain tissues and in the stomach, duodenum, colon, liver, and skeletal muscle of mice [79, 80].

In relation to CAVII inhibition, Gitto and coworkers rationally designed, synthesized, and evaluated ten isoquinoline-derived sulfonamides and other four substituted aryl sulfonamides as selective inhibitors [81]. They showed inhibitory efficacy at low nanomolar concentrations in some examples and selectivity against CAVII over CAII. The authors proposed that small substituents in the C1 position of the isoquinoline ring improve the inhibition in CAVII. Figure 4 shows the two more representative compounds (structures 1 and 2). After that, the authors tested other structurally related aryl sulfonamides, but the new compounds showed lower selectivity when compared to the previously reported ones [82].

Recently, De Luca and coworkers performed a virtual screening campaign in order to find new CAVII inhibitors [83]. They constructed two 3D pharmacophore models based on two experimentally available complexes of inhibitors with CAVII (protein data bank codes: 3ML5 and 3MDZ [84]). Later, a final pharmacophore model was made by superimposing the two structure-based hypotheses and taking out the overlapped chemical features. This pattern was employed as template for virtual screening into a focused library of 6313 sulfonamides available in the zinc database [85]. The 34 hits found were submitted to a docking simulation and finally two compounds were selected for experimental assays

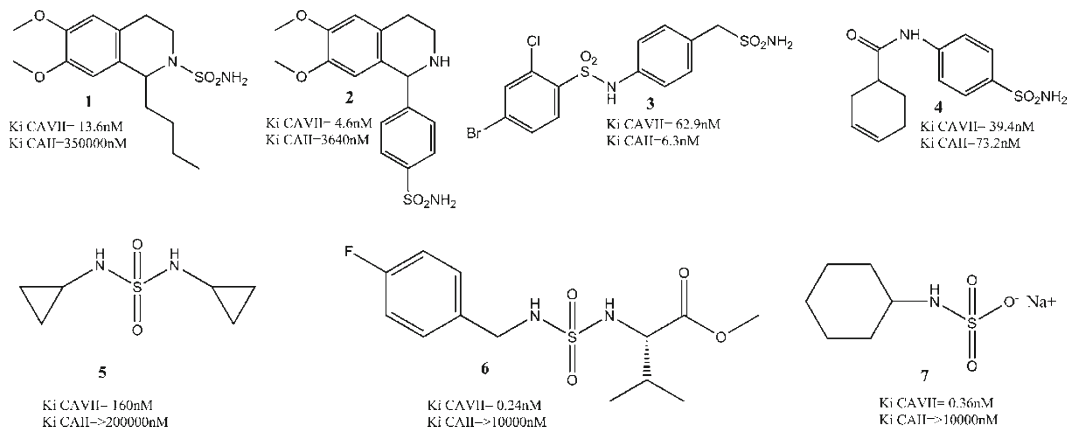

Fig. 4 Representative structures of CAVII inhibitors collected from literature [70, 81, 83, 86]

(Fig. 4, structures 3 and 4). Both molecules showed nanomolar inhibition against CAVII isoform but not selectivity against CAII.

We and other authors have also explored the applications of rational design for the discovery of new AEDs via CAVII inhibition [70, 86]. Particularly, we studied the inhibition pattern of sulfamides (–NH–SO_2–NH–), which are bioisosteres of the classic sulfonamide group [75]. Initially, we found that the *N*,*N*′-disubstitution of the sulfamide function causes a negative effect in the activity for the off-target CAII, but it provokes inhibitory action against CAVII in some cases. For example, we found that *N*,*N*′-dicyclopropylsulfamide (Fig. 4, structure 5) has a Ki value of 160 nM and it is inactive against CAII [70]. To explain the origin of the effects that may contribute to the different binding affinity, we simulated the interaction of this compound (among others) with CAII and CAVII using docking and molecular dynamics simulations.

The docking simulations for *N*,*N*-dicyclopropylsulfamide were performed using AutoDockTools 1.5.0 and AutoDock 4.0 docking softwares [87]. The starting CAII protein was prepared from the 0.99 Å resolution crystal structure of the CAII-sulfonamide complex deposited by Jude et al. (protein data bank code 2FOU) [88]. CA VII isoform was obtained from the complex provided by Opperman et al. (protein data bank 3MDZ) [89]. In both cases, the crystallographic water molecules, the ligand, and any cocrystallized molecule/ion were stripped. Hydrogen atoms were added using the LEaP module of AMBER11 [90].

We retained the default AutoDock parameters for all the variables but the charges of the ligands, for which AM1-BCC charges were calculated using Quacpac software [91]. We found this performs better in the docking for this particular system than the default Gasteiger charges [75]. The sulfamide was docked using

the Lamarckian genetic algorithm (LGA) in the "docking active site," defined through a grid centered on the ND2 atom of Asn67 residue for CA II and the ND2 atom of Asn64 for CA VII. In both isozymes, we employed 60, 50, and 60 grid points in X, Y, and Z dimensions respectively, with the default grid spacing (0.375 Å), and performed 50 docking runs.

The N,N'-dicyclopropylsulfamide was docked as anion, by removing the H atom of one NH group, since it is believed that classical inhibitors coordinate to the active site as negative species [75].

The conformations predicted by AutoDock for the complexes with CAII and CAVII were used as starting points for MD simulations with AMBER11 software [90]. The initial geometries were minimized (1000 cycles for the water molecules followed by 2500 cycles for the entire systems). After a 20 ps NTV equilibration period with a weak restraint (10 kcal/mol Å^2) for the complex and a NTP 200 ps without restraint, production runs larger than 12 ns were computed for each complex, for the coordinates saved every 1000 time steps. The ionizable residues were set to their normal ionization states at pH 7, except for the His residues coordinating the Zn metallocenter, which were modeled as Hid94, Hid96, and Hie119 (numbers relative to CA II). The protein atoms were surrounded by a periodic box of TIP3P32 water molecules that extended 10 Å from the protein. Counter ions were placed by the LEaP module of AMBER11 to neutralize the system [90]. The ff03 version of the all-atom AMBER force field was used to model the protein, and the GAFF force field was employed for the organic ligands [90, 92].

We derived our own nonstandard force fields for the Zn active site by means of geometry minimization (B3LYP/6-31G**, Gaussian03 software [93]), followed by calculation of the second derivatives and RESP charges for the active site supermolecule defined by three His residues, the Zn ion, and the sulfamide ligand [75].

Our molecular modeling studies suggested that the active site is more elongated in CAVII than in CAII, particularly in the hydrophilic region delimited by ASN64, HIS66, GLN69, LYS93, and GLN94 (GLN69 and LYS93 are distinctive residues for CAVII, and they are replaced by ASN67 and ILE91 in CAII). The larger space in the CAVII active site entrance allows it to accommodate bulkier sulfamides, like N,N'-disubstituted compounds. After that we design new amino acid-derived sulfamides with different alkyl/aryl chains with the purpose of improving the interactions with CAVII [86]. The idea was to construct a sulfamide with both polar and nonpolar chains, to promote the interactions with the elongated hydrophilic region close to the active site in CAVII as well with the other lipophilic half of the cavity. Snapshots of the molecular dynamic simulations previously described allowed us to access to elongated conformation of CAVII, and docking studies were also performed to analyze these new designed sulfamides. We employed the docking conditions described before for N,N'-dicyclopropylsulfamide.

However, in the simulations with the amino acid-derived sulfamides, two deprotonated forms can be constructed, since the substituents of the sulfamide function are different. Both anions were considered, and the most stable complex predicted by docking (lower binding energy) was conserved [86].

We found very interesting results in terms of CAVII activity and selectivity against CAII. Figure 4 shows one of the most promising structures (compound 6). It was also active against MES test in mice confirming the anticonvulsant activity in this classical model [94]. Compound 6 showed all animals protected in MES assay (3/3) at the lower dose tested (30 mg/kg), 4 h after administration [94].

We also studied the CA inhibition of the artificial sweetener sodium cyclamate (Fig. 4, structure 7). It showed important potency and selectivity against the off-target CAII [70]. This structure presents a sulfamate salt and it was previously tested by us in animal models of convulsion with positive results [95]. The selection of this compound was based on the results found for another sweetener, saccharine, which showed anticonvulsant action and high inhibitory potency and selectivity against CAVII [96].

4 Conclusions

Carbonic anhydrase is inhibited by drugs employed to treat several diseases, such as cancer, glaucoma, and also epilepsy. Nowadays, the main challenge in the design of CA inhibitors is to find compounds that act selectively and with high potency against specific isoforms. In this chapter, we have focused on successful examples of CAVII inhibitors, which prove its potentiality as anticonvulsant target. We are aware that more studies are needed to complete the anticonvulsant profile of the new compounds; but we consider that this isoform has much future in the treatment of febrile seizures and, perhaps, in other epileptic syndromes.

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Chapter 4

Synaptic Vesicle Protein 2A as a Novel Pharmacological Target with Broad Potential for New Antiepileptic Drugs

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Abstract

In recent years, synaptic vesicle protein 2A (SV2A) has become an attractive protein to study due to its important role in epilepsy. This protein is expressed ubiquitously throughout the brain in all nerve terminals independently of their neurotransmitter. It plays an important role in ensuring correct neurotransmission by priming vesicles for fusion. SV2A is the molecular target of the anticonvulsant drug levetiracetam (LEV), whose effects might be modulated by neuronal activity, by the pathophysiology of the nervous tissue and by SV2A expression. All of these qualities offer the advantage that some anticonvulsant drugs, by interacting with SV2A, could act on both excitatory and inhibitory synapses and simultaneously have effects restricted to epileptic tissue, resulting in the selective modulation of neuronal excitability. This chapter describes and discusses some of the basic aspects of the role of SV2A in epilepsy, addressing three main issues: SV2A function in neurotransmission, the correlation of the expression and/or distribution of the protein with treatment response and neuronal damage, and the reasons that SV2A is a good molecular target for the discovery of new antiepileptic drugs (AEDs).

Key words Synaptic vesicle proteins, SV2A, Levetiracetam, Epilepsy biomarker, Neurotransmitters release, Neurotransmission, Vesicle cycle, Synaptotagmin, Antiepileptic drugs

1 Synaptic Vesicles

Synaptic vesicles (SVs) are a specialized class of tiny (40–50 nm diameter) secretory vesicles localized in presynaptic nerve terminals. SVs have two major functions: the accumulation and storage of neurotransmitters and the release of these neurotransmitters [1]. When an action potential arrives at a nerve terminal, it causes an influx of calcium (Ca^{2+}) through voltage-gated Ca^{2+} channels, which triggers the vesicles to release their contents within a fraction of a millisecond [2]. The neurotransmitters are secreted in

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multimolecular packets (quanta), each containing several thousand transmitter molecules. At rest, nerve terminals release quanta spontaneously at a slow rate, giving rise to spontaneous miniature synaptic potentials [3].

SVs are released and reused in a simple but well-balanced cycle of exocytosis and endocytosis, its *yin* and *yang* elements [4]. The exocytosis process begins once the SVs are filled with neurotransmitters by active transport and are conveyed close to the active zone of the presynaptic plasma membrane, where they reside in a cluster ready to be recruited for exocytosis [5, 6]. Vesicle clusters appear to be homogeneous, with little sign of any morphological boundary that might demarcate the functional pools [7]. However, there are two distinct vesicle pools. One is the readily releasable pool (RRP, comprising 20% of the total vesicle population), which contains the first vesicles; these are fused upon stimulation and are refilled rapidly, almost entirely by recycling and without mobilization of the reserve pool. The second, the reserve pool, functions during depletion of the terminal and refills over a longer period [3, 7, 8].

Filled vesicles dock at the active zone where they undergo an ATP-dependent priming reaction that makes them competent for Ca^{2+}-triggered fusion. The vesicle membrane fuses with the presynaptic membrane, and the contents of the vesicle are released into the synaptic cleft [3]. There are many proteins involved in exocytosis; the most important proteins and their functions are illustrated in Fig. 1. After fusion pore opening, SVs undergo endocytosis, for which three pathways are proposed: (1) "kiss and stay" is a pathway in which vesicles are endocytosed by closure of the fusion pore and are refilled with neurotransmitters while remaining docked to the active zone, (2) "kiss and run" is a local recycling pathway that is clathrin independent but results in the mixing of vesicles with the reserve pool after endocytosis, and (3) the last pathway is that in which vesicles undergo clathrin-mediated endocytosis and are recycled either directly or via endosomes [6, 9].

Fig. 1 (continued) the vesicle over the active zone [6]. In docking stage, SNARE syntaxin is held in a closed state by the regulatory protein Munc18-1, and in that configuration, it is unable to interact with other SNARE proteins. (**b**) Priming. The configuration of the Munc 18-1/syntaxin complex is altered so that syntaxin is in its open state and assembles with synaptobrevin and SNAP-25 to form a parallel four-helix bundle that binds the vesicle in close contact with the plasma membrane [3, 6]. The SNARE proteins do not fully account for fusion of the vesicle and plasma membrane; e.g., complexin protein serves to stabilize the interaction between the synaptobrevin and syntaxin helices [3]. (**c**) Exocytosis. Upon calcium influx to the nerve terminal, the incoming calcium binds to the calcium-sensor synaptotagmin 1 (SYT-1), which in turn binds to the SNARE complex, displacing complexin and initiating pore formation [3, 5]. After fusion, the SNARE complex must be disassembled for efficient vesicle recycling to occur [5, 6]. (**d**) Endocytosis. SV2A acts in a clathrin-mediated endocytosis through its binding with SYT-1. SV2A and SYT-1 interact through first 57 amino acids of SV2A and the C2B domain of SYT-1 [34]. Y46 residue of SV2A binds to the clathrin adaptor AP2 [23]. SYT-1 has its own binding site with μ-subunit of AP-2 and with stonin 2 [23]. Then, SV2A ability to affect SYT-1 endocytosis may be through engaging μ-AP2 through its endocytosis motif Y-46 and interacting with SYT-1 bound to stonin 2 [23]. SV2A may stabilize the complex ensuring the inclusion of SYT-1 into vesicles

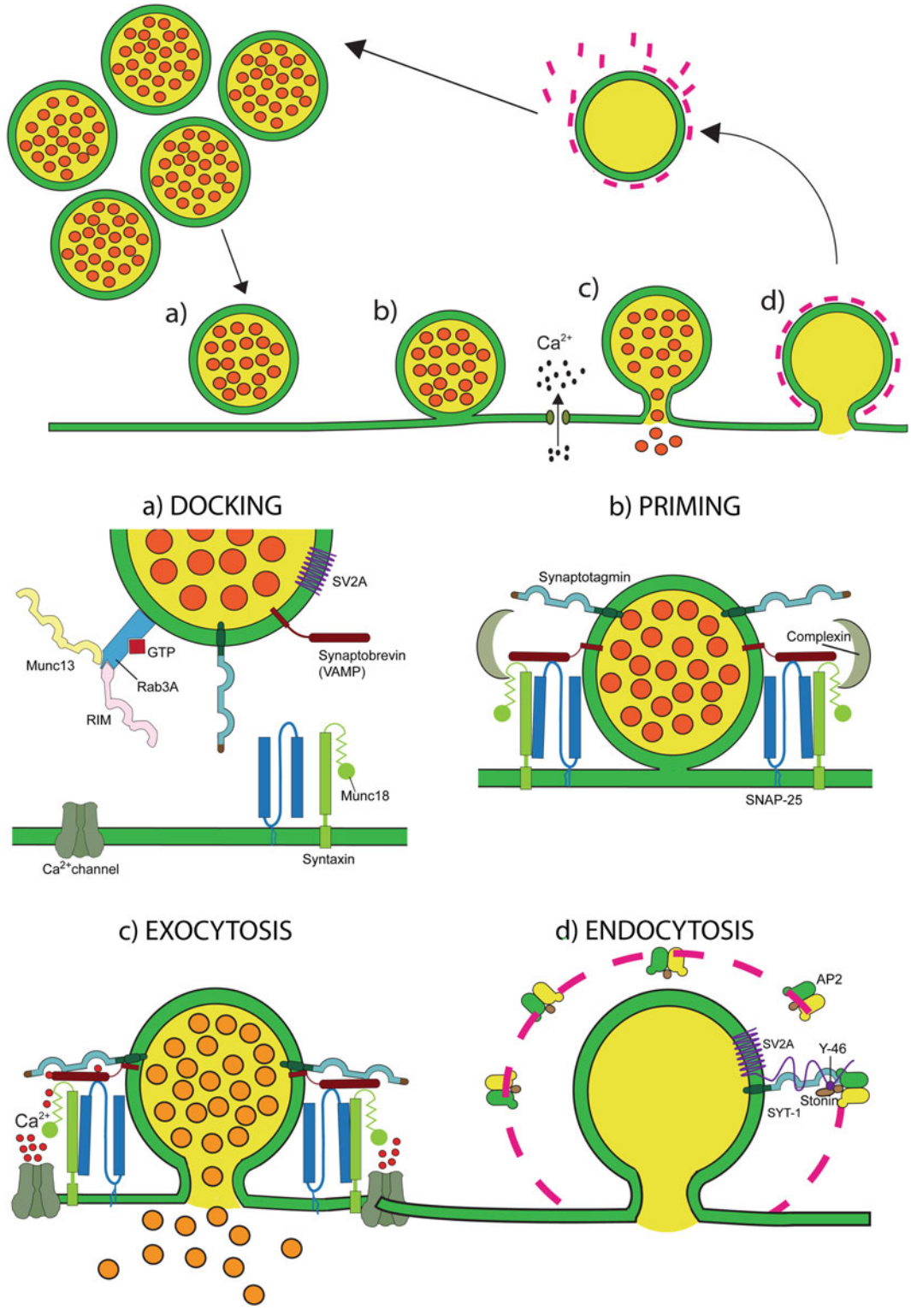

Fig. 1 The synaptic vesicle cycle; synaptic vesicles are filled with neurotransmitter and are grouped and docked (**a**) in the vicinity of active zones. Synaptic vesicles are prepared (priming; **b**) to a competent state for the fusion pore opening triggered by calcium (exocytosis; **c**). After fusion, synaptic vesicles are endocytosed and recycled through clathrin-mediated endocytosis (**d**). There are several key synaptic vesicle and plasma proteins that participate during all cycle. (**a**) Docking. The vesicle protein Rab3 binds to the cytoplasmic proteins Munc13 and RIMs, to holding

2 Synaptic Vesicle Protein 2A

Biochemists have isolated many of the key proteins of SVs. These proteins fall into two major classes: transport proteins involved in neurotransmitter uptake and trafficking proteins that participate in synaptic vesicle exocytosis, endocytosis, and recycling (several mentioned in Fig. 1) [9]. Among these proteins are those in the synaptic vesicle protein 2 (SV2) family; however, it is frequently unknown to which group each protein belongs because their specific functions have not yet been fully characterized.

Three genes in vertebrates encode this highly homologous SV2 family of transmembrane glycoproteins known as SV2A, SV2B, and SV2C. SV2A was the first isoform identified and is the most studied due to its biological and pharmacological implications [10–12]. The SV2A has an 82.7 kDa molecular weight and 742 amino acids, a long and conserved NH_2-terminus, 12 transmembrane (TM) domains, a long and conserved cytoplasmic loop, a long intravesicular loop that includes three N-linked glycosylation sites, and a short C-terminus [13–15].

The SV2A is expressed ubiquitously throughout the brain at different levels, with the exception of the facial and trigeminal nuclei [16]. As expected for a synaptic vesicle protein, SV2A is essentially absent in the neuronal soma [16]. Both in humans and in rats, SV2A immunoreactivity (IR) is present in synaptic layers throughout all subfields of the hippocampal formation. The strongest IR is observed in the stratum lucidum of CA3 (mossy fibers) and in the hilar region of the dentate gyrus (DG). Because SV2A is a protein localized in the terminal presynaptic axons of neurons, its expression is observed surrounding the dendrites or somata of the postsynaptic pyramidal neurons (CA1 and CA3), granular cells, and hilar cells [17, 18]. A protocol to detect the SV2A IR by immunohistochemistry, as well as its semiquantification using systematic random sampling and optical densitometry, is described in Appendix 1.

SV2A is expressed in both glutamatergic and GABAergic terminals; immunocytochemical studies in the rat cortex, hippocampus, and cerebellum showed that SV2A co-localizes with vesicular glutamate transporters (VGLUT) 1 and 2 and with the vesicular γ-aminobutyric acid (GABA) transporter (VGAT) [16, 19, 20]. Intriguingly, SV2A is mostly co-expressed with VGLUT1 and VGAT (82% and 96%, respectively) and less so VGLUT2 (42%); however, the distribution is almost equal among all terminals [19].

3 SV2A Protein Main Functions

The localization of SV2A in all SVs, together with its conservation across species, suggests that the protein is highly important in vesicle functioning [13]. Although it is well known that SV2A is not

indispensable for neurotransmitter release, its function is clearly essential to normal neurotransmitter release for at least the more complex vertebrate nervous systems [21, 22]. Its function remains undefined, but most evidence suggests that SV2A potentially has multiple regulatory actions at diverse points in the synaptic vesicle cycle [21, 23, 24].

3.1 SV2A Protein May Function as a Transporter

For more than a decade, enormous effort has been given to demonstrate SV2A transporter function. The first insights came in 1992 from three independent groups, all of which recognized the SV2A as a transporter because it shares significant homology with several transporter proteins [13, 14, 25]. Additionally, SV2A has residues equivalent to critical functional residues of several major facilitator superfamily (MFS) transporters. The mutagenesis approach resulted in the identification of some of these residues affecting SV2A binding with its high-affinity compound, UCB-30889 [26]. In addition, two conserved tryptophans (W300 and W666), which contribute to transport activity in some MFS proteins, are required for SV2A's function in neurotransmission [22]. Nevertheless, the finding that disruption of a canonical MFS transporter motif (R231) in SV2A does not impair neurotransmission suggests that SV2A does not act as a transporter [22].

However, there are other studies supporting SV2A transporter activity; by protein tomography, an SV2A pore opening toward the cytoplasm was identified, as well as a more open structure toward the intraluminal space. The existence of these two major alternate functional conformations sustains a valve action necessary for molecular transport [27]. Otherwise, it has been demonstrated that human SV2A, when expressed in yeast, functions as a galactose transporter. These yeast cells were able to grow on medium containing galactose, and this effect was inhibited by the addition of the drug LEV, which specifically binds to SV2A. Most importantly, direct measurement of galactose uptake shows that SV2A is able to transport extracellular galactose inside the cells [28].

Because of the variable effects of mutating residues implicated in transporter function, the lack of identification of an SV2A endogenous substrate, and the need to interpret the yeast data as implicated in the central nervous system (CNS) of vertebrates, it remains unclear whether SV2A acts as a transporter. It is still possible, however, that SV2A transports a common constituent of the vesicle environment, such as ATP, Ca^{2+}, or other ions [16, 21, 27]. The presence of two conserved, negatively charged amino acids in the first TM domain of SV2A increases the probability that it transports a positively charged substrate such as Ca^{2+} and not a negatively charged substrate such as chloride or ATP [12].

3.2 SV2A Protein Function During Synaptic Vesicle Cycle

To better elucidate SV2A function, a mouse line was created that is homozygous for the deletion of SV2A (SV2A knockout, KO) or both SV2A and SV2B double KO (DKO) [21, 29–32]. SV2A and SV2B proteins are very similar and co-expressed in largely overlapping patterns; in some studies, it was only possible to observe a disrupted electrophysiological phenotype when both SV2A and SV2B were deleted, suggesting that SV2A and SV2B are functionally redundant [21, 31].

Electrophysiological recordings in culture of autaptic hippocampal neurons from SV2A KO and SV2A/SV2B DKO mice suggest two major hypotheses about the possible mechanisms by which SV2A and SV2A/SV2B may regulate vesicular exocytosis. The first one concerns SV2A regulation of RRP, which corresponds to the vesicles that are docked and primed for fusion (see above and Fig. 1). The size of the RRP of vesicles is correlated with the synaptic release probability. In a series of experiments in hippocampal neurons, it was observed that a loss of either SV2A or SV2A/SV2B resulted in a decrease in neurotransmitter release and reduced synaptic depression, which are linked to lower initial release probability and, therefore, a reduced size of the RRP [29, 30]. Similar results were found in SV2A KO chromaffin cells [24]. No differences were found in the frequency of miniature excitatory and inhibitory postsynaptic currents or the number, composition, or structure of the synapses because the architecture of the brain was normal in the mutants [21, 29, 30].

Because a loss of SV2A did not alter the number of docked synaptic vesicles or their morphology, it is possible that SV2A acts after vesicles arrive at the active zone [21, 29, 30]. Furthermore, it was observed that brain tissue from SV2A KO contained a smaller proportion of the syntaxin protein (Fig. 1a); this finding is consistent with the interpretation that SV2A action affects the formation of the complex of soluble NSF attachment proteins (SNARE) [24, 30]. Because the formation of the SNARE complex is one of the last events before exocytosis, these results suggest that SV2A influences synaptic vesicle priming (Fig. 1b), which prepares vesicles for neurotransmitter release.

On the other hand, the second hypothesis states that SV2A is a target for residual Ca^{2+} [21, 31]. Through paired pulses and repetitive stimulation of 2 and 10 Hz, an increase in the synaptic response (relative facilitation) with subsequent progressive depression in SV2A/SV2B DKO neurons was observed. Notably, no difference in the initial release probability was detected; however, such facilitation was reversed in all frequencies by incubating SV2A/SV2B DKO neurons with ethylene glycol tetra-acetic acid (EGTA, a Ca^{2+} chelator) indicating that the difference in synaptic response was mostly due to the buildup of residual Ca^{2+} [21, 31]. In addition, the loss of SV2B in rod bipolar cells (i.e., where the major SV2 isoform is expressed) was associated with an increase in the basal Ca^{2+}

concentration in nerve terminals and with the accumulation of presynaptic Ca^{2+} during continuous stimulation [33]. These results support the role of SV2 in the regulation of cytoplasmic Ca^{2+} levels at the nerve terminal during repetitive stimulation. SV2A might act as residual Ca^{2+} target or as a Ca^{2+} transporter; as mentioned previously, the conserved presence of negatively charged residues in TM domain 1 of SV2A supports these functions [21].

Finally, SV2A also has an essential function of modulating the internalization of calcium-sensor synaptotagmin 1 (SYT-1) protein during endocytosis. Mice with a mutation in Y46 amino acid residue (an endocytosis motif) at the NH_2-terminus of SV2A show a higher proportion of both SV2A and SYT-1 on the plasma membrane (i.e., diminished internalization) and reduced binding of SV2A to multiple endocytosis-related proteins. The Y46 residue of SV2A serves as a receptor for clathrin adaptor proteins; thus, SV2A via an interaction with both SYT-1 [34] and clathrin adaptors [23] may regulate the vesicle content of SYT-1. In the absence of SV2A, the SYT-1 trafficking of SVs may become more random, leading to fewer vesicles with enough SYT-1 to trigger Ca^{2+}-stimulated fusion [23] (Fig. 1d).

Despite the somewhat disparate results, it is possible to infer multiple mechanisms of SV2A function during the neurotransmitter release process [21, 23, 24, 29]. The finding that neurotransmission is decreased but not abolished in the absence of SV2A suggests that SV2A plays an important but non-essential role in priming vesicles for fusion. The evidence indicates that SV2 proteins may act as a positive modulator of synaptic transmission by increasing the availability of secretory vesicles and thus the release probability, thereby ensuring correct neurotransmission [24, 30, 35].

4 SV2A Protein and Epilepsy

4.1 Mice Lacking SV2A

The association of epilepsy and SV2A is evidenced by the observation that the SV2A KO mouse presents spontaneous generalized seizures. These mice have a low growth rate and a high mortality rate, with the first peak after birth and a second peak between the second and third week; no animals survive after this period, indicating that SV2A is a vital protein. Organisms that survive the first peak of death begin to experience severe and progressive recurrent spontaneous seizures (RSZ) indicative of widespread CNS hyperexcitability. In contrast, the heterozygous organisms are viable and fertile; however, they have a slight decrease in body weight, a slightly higher mortality rate, and a 24% presentation rate of RSZ [21, 29, 32].

The targeted disruption of the SV2A gene results in a decrease in the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) in the CA1 and CA3 regions of the hippocampus [29, 32] as well as an increase in the frequency of

spontaneous excitatory postsynaptic currents (sEPSCs), with no change in their amplitudes [32]. The alteration of action potential-dependent glutamatergic and GABAergic synaptic transmission may cause RSZ in SV2A KO mice. Therefore, the decrease in inhibitory release may induce a reverberating hyperexcitability and subsequently potentiate glutamate transmission to produce a continuous firing of action potentials, resulting in the emergence of RSZ [32]. It is noteworthy that alteration of synaptic GABAergic inhibition in the hippocampal formation plays a critical role in the generation and propagation of seizures in several types of epilepsy in humans [36, 37].

4.2 SV2A Protein Levels in Animal Models and Patients with Epilepsy

Another important aspect in the SV2A-epilepsy relationship is the change in expression of protein levels. The CNS is highly sensitive to the up- or downregulation of diverse proteins, which may result in a diverse range of disorders, such as epilepsy [29, 38]. SV2A is not an exception; in neurons from SV2A/SV2B DKOas well as in autaptic wild type neurons with overexpression of SV2A in autaptic wild-type neurons resulted in a reduced synaptic release probability and reduced synaptic depression respectively, suggesting that excess SV2A is as detrimental to neuronal function as deficient SV2A [39].

In a genetic model of spontaneously epileptic rats, low levels of SV2A were found in all cortical layers (mainly in the frontal and entorhinal cortex) and in the inner molecular layer of the DG [40]. Similarly, in a kindling epilepsy model, a decrease in SV2A expression was observed in the early hours (acute phase), became more pronounced over days (latent period), and progressed to involve the whole hippocampus (chronic phase) [17]. In contrast, SV2A expression in pentylenetetrazol (PTZ) kindling animals was selectively elevated in the hilar region [18]. Further studies are required to clarify this contradiction.

Similarly, patients with different disorders, such as complex partial seizures (CPZ), also showed a decrease in the expression of SV2A. Individuals with focal cortical dysplasia and tuberous sclerosis complex showed a reduction in SV2A IR in the neuropil of the cortex and the hippocampus (mainly in regions with extensive neuronal loss) [41]. Furthermore, patients with temporal lobe epilepsy presented a marked loss of SV2A IR in the temporal neocortex [17, 42, 43], and those with hippocampal sclerosis exhibited a SV2A decrease throughout the hippocampus but not in the inner molecular layer of the DG [17], again in parallel with synaptic loss [43]. Finally, brain tumor patients with epilepsy (approximately 50–80 % of patients with gliomas have seizures) presented modest SV2A IR within the tumor area without changes in peritumoral tissue [38].

Interestingly, the SV2A decreased markedly in pharmacoresistant (i.e., resistant to phenytoin and phenobarbital) epileptic rats. However, through hippocampal low-frequency stimulation, a

remarkable increase in the SV2A was observed, while the seizure degree was inhibited [44]. On the other hand, glioma patients with epilepsy and a good response to LEV treatment had significantly stronger SV2A expression in tumors as well as in peritumoral tissue than did patients who did not show such a response [45]. These results suggest that the regulation of the SV2A may be an important issue for anticonvulsive treatment. Additional clinical and animal studies are required to further address this important matter.

As we describe above, the expression of the SV2A is altered under some pathological conditions such as epilepsy. Generally, in patients with CPZ as well as in epileptic animal models, the level of SV2A is decreased; indeed, this reduction has been observed in parallel with the course of the disease and through different brain structures. In addition, the regulation of the SV2A may significantly diminish the appearance, intensity, and seizure duration, and it can conceivably be a main factor in treatment response. Therefore, it is very plausible that SV2A expression can be a useful biomarker of epilepsy progression and/or a predictor of the response to treatment; in vivo SV2A quantification through positron emission tomography (PET) may be a helpful clinical tool to elucidate this.

4.3 SV2A Protein and the Anticonvulsant Drug Levetiracetam

Finally, the identification of the SV2A as a therapeutic target of the anticonvulsant drug LEV established its fundamental role in epilepsy. Photoaffinity labeling studies determined that UCB-30889 (a LEV analog with a higher affinity for SV2A) bound to a protein of ~90 kDa. This protein was only detected in brain structures but not in peripheral tissue and was abundant in synaptic vesicle fractions; this placed SV2 proteins as the primary candidates [46–48]. Afterward, it was demonstrated that brain membranes and purified SVs from mice lacking SV2A did not bind UCB 30889. These results indicated that SV2A was necessary for LEV binding. Notably, no binding was observed in the related isoforms SV2B and SV2C [49].

Currently, there is no doubt that LEV binds in a saturable, reversible, and stereospecific manner to SV2A in both rat and human brains; this also holds true for its structural analogs brivaracetam, seletracetam, and UCB-30889, which overall form the racetam family [49, 50]. LEV (S-enantiomer pyrrolidine derivative of α-ethyl-2-oxo-1-pyrrolidine acetamide IUPAC, (S)-2-(2-oxopyrrolidin-1-yl) butanamide) is a second-generation anticonvulsant drug that has demonstrated a better tolerability and improved efficiency; therefore, it has gradually become a first-choice drug. At present, LEV is indicated in the treatment of partial onset seizures with or without secondary generalization, either as monotherapy (adults and adolescents with new diagnoses) or adjunctive therapy (adults and children >1 year of age); it can also be used in the control of myoclonic seizures and primary generalized tonic-clonic seizures only as adjunctive therapy in adults and adolescents [51–53].

At the molecular level, the specific racetam-binding site on the SV2A is still unknown; however, through mutagenesis studies, 14 amino acid residues involved in LEV binding to the TM hydrophilic core of SV2A have been identified [26]. In addition, modeling, molecular dynamics, and docking simulations showed a TM consensus binding site for racetam ligands in one or two different possible conformational states of SV2A [54, 55]. Of special importance are the residues D-670, W-666, N-667, Y-462, K-694, C-697, and W-300 [14, 26, 54]. All of these TM residues may be involved in the interaction between SV2A and LEV; however, they are not necessarily involved in the ligand pocket, and they may affect other aspects, such as changing the native conformation or the stability of the protein. A protocol to perform molecular dynamics and docking simulations is described in Appendix 2.

To exert its action, LEV requires access to the intraluminal face of synaptic vesicles. Because of its hydrophilic character, this process may be slow; it is possible that LEV reaches SV2A from its luminal side during recycling and endocytosis and, later, may bind to the SV2A TM region [15, 26, 54, 55] and be released during vesicle fusion [56] (Fig. 2). LEV may antagonize the effect of the SV2A, inhibiting it from participating in its usual role of priming vesicles and, as a result, may generate a decrease in the production of RRP. This decrease in RRP may then cause a decrease in excitatory and inhibitory synaptic transmission [30, 35, 49, 56, 57]. This suggests that LEV acts like antagonist of SV2A; however, it is also possible that LEV would act as an agonist, ergo, LEV binding migth enhances a function of SV2A that inhibits abnormal bursting in epileptic circuits, a function whose loss in the SV2A KOs results in seizures [49].

The in vitro, ex vivo, and in vivo studies have corroborated that the principal mechanism of action of LEV is through the SV2A [58]. Experimental data obtained in rodent models of partial and generalized epilepsies established a strong correlation between SV2A binding affinity and the anticonvulsant potency of LEV and its derivatives [46, 49, 59]. In addition, heterozygous SV2A mice displayed increased vulnerability to seizures and accelerated epileptogenesis and a reduced anticonvulsant activity of LEV in several seizure models [58]; these effects are associated with both the occupancy and availability of SV2A [60]. These results prove that SV2A plays a crucial role in the mediation of the anticonvulsant action of LEV and suggest that the SV2A represents an important and validated target for the discovery of novel AEDs.

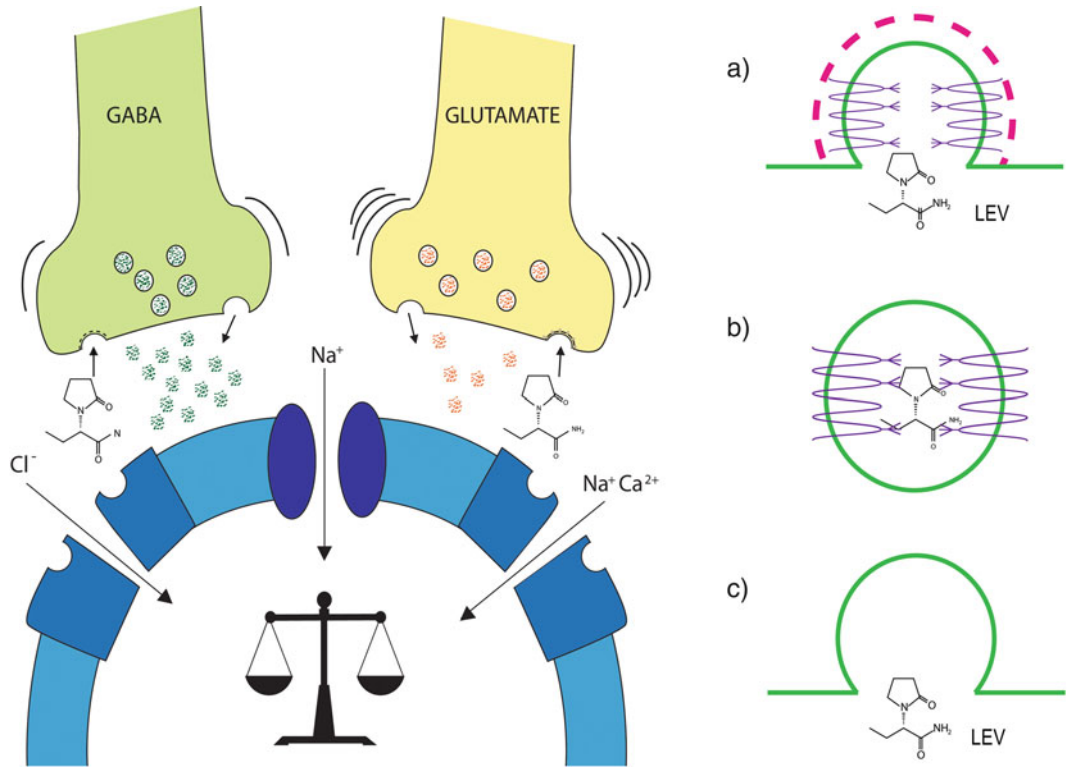

Fig. 2 Proposed LEV mechanism of action. In epileptic nerve terminal, LEV may be modulated by neuronal activity; although LEV can act on different cell populations, apparently their effects are limited to those terminals that exhibit greater activity, such as glutamatergic terminals, that union results in a selective decrease of neurotransmitter release. It is possible that LEV accesses to the intraluminal face of synaptic vesicles during endocytosis (**a**) and binds to the SV2A in its transmembrane region (**b**); later, LEV is released during vesicle fusion (**c**)

5 Is SV2A Protein a Better Target for New AEDs?

The identification of the SV2A as a therapeutic target for the anticonvulsant drug LEV opened a new and successful avenue for discovering new SV2A ligands with antiepileptic activity [49, 58]. The fact that SV2A mechanisms of action protect against different preclinical models of partial and generalized seizures suggests that SV2A is essential for neuronal synchronization associated with several types of epileptiform activity and may provide antiepileptic drug candidates with the potential for broad-spectrum clinical efficacy [58, 59].

Remarkably, recent evidence also shows that the actions of LEV may be modulated by neuronal activity [35, 56, 57, 61], by the pathophysiology of the nervous tissue [62–64] and by SV2A expression [17, 60]. Electrophysiological manipulation in hippocampal pyramidal neurons caused increases in neuronal activity and

in vesicular recycling, which were followed by a corresponding increase in the effects of LEV [56]. Additionally, although LEV can act on different cell populations, apparently their effects are limited to those terminals with high neuronal firing [61]. On this basis, it is suggested that LEV may preferably act on neurons that exhibit greater activity, such as glutamatergic terminals, under pathological conditions such as epilepsy [65] (Fig. 2).

With respect to the pathophysiology of nervous tissue, LEV seems to have little effect on healthy tissue compared with the epileptic brain [62, 63]. Intracellular slice recordings from the CA3 hippocampal region demonstrate that LEV does not influence normal glutamatergic and GABAergic transmission but decreases the epileptiform activity induced by the $GABA_A$ receptor antagonist bicuculline or the glutamate agonist NMDA (*N*-methyl-d-aspartate). This result is consistent with the previous in vivo studies that reported that LEV did not show an effect on GABAergic transmission in the normal brain [66]. In the same way, LEV did not appear to affect brain amino acid levels in healthy rats [67]; on the contrary, LEV significantly normalized some of the induced biochemical alterations in lithium-pilocarpine-treated rats [65, 68]. These results support the hypothesis that LEV could act only under pathophysiological conditions, such as in chronic models of epilepsy, where LEV has shown to have modulating effects on the levels of different neurotransmitters and excitability in diverse cell populations [65, 68–70].

In addition, these results might also explain the lack of LEV protection against seizures in the acute models of PTZ and acute maximal electroshock-induced seizure, which are traditionally used to evaluate the effectiveness of AEDs [71, 72]; it may also explain the markedly potent protection against seizures in chronic models such as mice kindled with corneal electroshocks and epilepsy models induced by pilocarpine and kainic acid [51, 71–73]. Furthermore, the pharmacological actions of LEV may depend largely on morphological and functional changes resulting from the establishment of the chronic phase of epilepsy (i.e., constant hyperexcitability and a marked imbalance between the systems of excitation and inhibition) [74, 75].

Finally, another important aspect to consider with respect to the effectiveness of LEV is SV2A expression; although it is ubiquitously distributed in the CNS, the morphological changes of nerve tissue once it becomes epileptic may change SV2A pattern of expression. As mentioned above, SV2A appears to decrease; however, it is not known whether these decreases occur throughout the whole brain, only in some structures, or only in certain cell populations. Furthermore, protein expression is closely related to the response to treatment [45], and the data from SV2A heterozygous mice show a pro-epileptic phenotype and decreased protection

against seizures [60]. Therefore, the up- or downregulation of protein levels may be associated with neuronal activity and efficacy of LEV in a directly proportional relationship.

Because SV2A is the primary target of LEV, its mechanism of action may involve effects on neurotransmitter release. The fact that SV2A is found in almost all synaptic CNS terminals suggests that LEV has the ability to act on all of these terminals; however, these effects can be selective, taking into consideration the basic aspects of epilepsy physiology (i.e., increased neuronal activity and morphological changes, including different expression levels of SV2A); therefore, the final effect of LEV would be finely conditioned by all of these aspects.

6 Conclusions

SV2A is an essential synaptic vesicle membrane protein involved in the regulation of neurotransmitter release; it is widely distributed throughout the brain with a well-defined expression pattern. In epilepsy, SV2A expression is significantly decreased in both animal models and patients. Moreover, SV2A expression diminishes with disease progression. This decreased expression might explain the lack of response to LEV treatment in some epileptic patients. Thus, if it were possible to determine the SV2A levels in vivo (e.g., using PET), this could be a good predictor of treatment response and of the progression of neuronal damage, positioning SV2A as a potential biomarker.

Additionally, SV2A is a unique protein recognized as a pharmacological target of anticonvulsant drugs. Furthermore, this protein represents an important target for new drug discovery; such drugs may act depending on the morphological and functional changes associated with the chronic phase of epilepsy; thus, these drugs may directly affect the epileptic focus and have minimal effects on the surrounding non-epileptic tissue.

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7 Appendix 1: Immunohistochemistry Protocol

7.1 Introduction

Immunohistochemistry (IHC) is a widely used histological technique that serves to determine the presence, location, and expression of biomarkers with antigenic capacity. For this, a specific antibody (Ab) is needed, which will bind to an antigen of interest and generate an antigen-Ab complex; the epitope (i.e., the region of an antigen that specifically binds an Ab) should be readily available for binding and generate an efficient interaction between antigen and Ab. The complex resulting from the immune reaction can be detected by enzymatic reactions [76, 77].

Overall, the main steps of IHC (with multiple steps) begin with the direct application of a monospecific primary Ab to the tissue section; then, a biotin-labeled secondary Ab is added [78]. Later, it is possible to apply an avidin-biotin-peroxidase complex to amplify the immunoreactivity (IR). Avidin is a glycoprotein that has high affinity for biotin and that has four biotin-binding sites; this allows for the formation of macromolecular complexes with biotinylated peroxidase and biotinylated secondary Ab, resulting in a high staining intensity [79]. Finally, chromogens are added to the sections and oxidized by peroxidase, leading to a color (brown or black) reaction [78] (Supplementary Fig. 1).

Depending on the target protein, immunoassays can be evaluated qualitatively or quantitatively; when the protein of interest is localized in cell soma and it is well defined, it is "easy" to be counted, and in this case, stereology methods can be a good option. Stereology utilizes systematic random sampling (SRS, a statistical sampling method) to unbiasedly select a representative sample of the region of interest [80, 81]. This technique can be applied to biological structures at all levels of the sampling hierarchy, beginning with a random selection of animals from the population and proceeding from high to low levels (i.e., slices, total region, each subregion of interest) [81]. The distribution pattern of the sampling sites is systematic (i.e., the distance from one sampling site to the next is constant) with a random component (**see note 1**) [80]. A sample taken in such a way allows for all units in the population to have the same probability of being sampled [82].

Nevertheless, when a protein of interest does not have a defined pattern, semiquantitative analysis is the best option. It is possible to accomplish this using densitometry, which is the quantitative measurement of optical density (OD) in light-sensitive materials, such as photographic images, due to their exposure to light [83]. Images captured through the light microscope from the IHC performed on the tissue can be processed to obtain semiquantitative data by OD as a result of the dark gradient of the images, and this can be expressed as the intensity of dark spots in a given area; usually it is a relative value expressed as OD units [83, 84].

There are several forms to quantify the IR of the proteins; however, because synaptic vesicle protein 2A (SV2A) is located on the axon terminal and presents a dotted mark with sparse nuclear or cytoplasmic presence, its quantification is complicated. Even so, we propose that reliable semiquantitative data of SV2A IR can be obtained using a combination of SRS and OD analysis.

7.2 Materials and Methods

7.2.1 Animals

Male Wistar rats (250–300 g of body weight, from Envigo Inc.) were used to evaluate SV2A IR in the hippocampus. Animals were kept in animal facilities of the Instituto Nacional de Pediatría and were maintained in controlled temperature conditions (22–24 °C) and with a light/dark cycle of 12:12 h. This protocol was approved by our Institutional Committee and conducted in accordance with the ethical principles and regulations specified in the internal guidelines of the Institution and in the Guide for Care and Use of Laboratory Animals' of the Mexican Council for Animal Care (Norma Oficial Mexicana NOM-062-ZOO-1999).

7.2.2 Tissue Sample Collection and Processing

Animals were deeply anesthetized with sodium pentobarbital (90 mg/kg). Subsequently, rats were perfused through the heart with saline solution (NaCl 0.9% in deionized water) and later with paraformaldehyde (PFA, 4%) in phosphate buffer (PB; 0.1 M, pH 7.4). Subsequently, rats were decapitated and their brains removed and postfixed at room temperature for 24 h in the same solution (PFA 4% in PB). After this time, brains were cryoprotected by immersion in buffered graded solutions of sucrose (20–30%) until they sank. Then, brains were frozen in 4-methylbutane prechilled with dry ice and stored at −80 °C until used. Subsequently, serial sagittal sections (40 μm thick) from entire hippocampus were cut using a cryostat and were collected in 24-well culture plates filled with PB [85].

7.2.3 Immunohistochemistry

The IHC procedure was performed on free-floating sections continuously stirred at room temperature. Importantly, between each reaction, three washes were performed with PB (**see note 2**) for 15 min. The process was initiated by incubating the sections with hydrogen peroxide (3% H_2O_2, Sigma; **see note 3**) diluted in PB for 10 min. After the washes, the tissue was incubated in fetal calf serum (FCS, 5% Gibco) diluted in PB for 1 h (**see note 4**). Without washing, the slices were incubated with primary SV2A Ab (1: 500; goat IgG; diluted in PB/FCS; Santa Cruz, sc-11936; **see note 5**) overnight. The next day, the sections were washed and incubated with horse anti-goat biotinylated secondary Ab (1: 500; Vector BA-9500; **see note 6**) diluted with PB/FCS for 2 h. Then, the slices were washed and incubated with avidin-biotinylated peroxidase (Vectastain ABC Elite Standard Kit, Vector PK-6100; **see note 7**) for 1 h. By the end of this period, the tissue was again washed, and the activity of peroxidase was revealed by using 3,

3′-diamino-chromogen benzidine (DAB, Vector SK-4100) for 5 min (**see note 8**). The reaction was stopped by washes with PB. Finally, the sections were mounted on gelatin-coated slides and cover slipped with Cytoseal (Richard-Allan Scientific) [85].

7.2.4 Quantification of SV2A Protein IR

To obtain an estimate of the levels of SV2A protein, a semiautomatic stereological system (MBF Bioscience) equipped with a light microscope (Olympus BX51) and digital video camera (AxioCam MRC) was used. Also used were the *Stereo Investigator 11* program and the optical fractionator probe. One of every six sections was selected, and 11 subregions of the hippocampus were drawn. In each subregion, a systematic random procedure was carried out with a sampling grid of 750 × 500 μm (**see note 9**) to capture the images with a 20× objective. The OD analysis was performed using the NIH image J v1.43 m program [86] (**see note 10**); the values were expressed as OD units, and each value of OD was corrected using background subtraction [84].

7.3 Results

Supplementary Fig. 2 illustrates the SV2A IR observed throughout all subfields of the hippocampus. SV2A staining pattern showed diffuse stains in all regions with the strongest IR present in the hilus, external molecular layer, and stratum lucidum (Supplementary Fig. 2a; stratum lucidum is not shown). As expected for SV2A, it was essentially absent in the neuronal soma of the pyramidal and granular cells (Supplementary Fig. 2b–d); however, the SV2A IR was observed surrounding their somata (Supplementary Fig. 2e, f) representing the axon terminals which contact them. The analysis of labeling intensity in the 12 regions of hippocampus showed less SV2A IR in laconosum molecular layer, in oriens and radiatum layers of CA1 field, and in granular layer showing a significant difference with SV2A IR of hilus, stratum lucidum, and external molecular layer (Supplementary Fig. 2g).

7.4 Conclusions

As we mentioned above, it is possible to obtain a reliable semiquantitative data of SV2A IR using a combination of SRS and OD analysis. The main advantage of this methodology is that it allows obtaining data from the entire structure of interest and from all subregions required for analyze; this permits a more detailed study of the phenomenon under analysis.

Note 1: With SRS, the first subject-object (i.e., rats, slices, first point in the region or subregion) is sampled at random. Then, each successive object is sampled systematically. For example, suppose there are four experimental groups with five individuals each. Each of the individuals (a total of 20) is numbered, and a random number from 1 to 4 is chosen; if the first number is 3, then 4 numbers are added each time to obtain the first group, i.e., the individuals with the numbers 3, 7, 11, 15, and 19 will form the first group. For another example, if the striatum must be analyzed,

it is first recommended that the entire region be cut extensively (40–50 μm). All the slices have to be taken into account; if there are 140 slices and only 1 in 20 are to be analyzed, then a number from 1 to 20 is randomly selected. Supposing that the first number selected is 15, 20 is added to obtain 35, and again 20 is added each time, resulting in the analysis of slices with the assigned numbers 35, 55, 75, 95, 115, and 135.

Note 2: Usually, washes are performed with PB + Triton (PB; 0.1 M, pH 7.4; Triton-X100 0.3%; Sigma); however, to detect SV2A IR, Triton was omitted because it is a nonionic detergent that breaks or denatures the cell membrane. Because SV2A is an integral membrane protein, when phosphate buffer with Triton is added, many protein are lost, and, consequently, the immunostaining is decreased.

Note 3: The H_2O_2 reduces or blocks the endogenous peroxidase activity present in the tissue, avoiding background and false positive.

Note 4: Blocking with serum from the same animal species in which the secondary Ab that was made will saturate the unspecific binding sites and thereby reduces background reactions in the tissue. Tissues after this step do not have to be washed.

Note 5: One should be especially careful with the choice of primary Ab because, depending on the sequence to which the antibodies bind (epitope), it is possible to ensure that the IR observed corresponds only to SV2A protein (not another SV2 isoform). There are different epitopes that can be detected by IHC for the SV2 proteins, e.g., the amino terminal and the large intraluminal loop. The first 46 amino acids of the amino terminal region of the SV2A are absent in the SV2B isoform; instead, the SV2C isoform shares 60% amino acid sequence homology. Therefore, an antibody that binds to amino terminal region is able to detect both SV2A and SV2B isoforms. However, the anatomical distribution of SV2 proteins should be useful to discriminate between these two isoforms. For instance, in the rat hippocampus, the main expression of SV2 protein corresponds to the SV2A isoform, followed by SV2B and with little or almost no expression of SV2C. Then, an antibody which binds to the amino terminal region of the SV2 protein would be a good choice for labeling SV2A in the hippocampal area.

Note 6: The secondary antibody should be made against the same species as the primary antibody and has to be obtained from a species that is different from the source of the primary antibody.

Note 7: For this, the avidin-biotinylated peroxidase complex must be incubated 30 min before its use. This complex binds to the biotinylated secondary antibody, allowing for the amplification of the IR, increasing the specificity and sensitivity of the signal at the site of antigen localization.

Note 8: To detect the peroxidase activity (from the avidin-biotin-peroxidase complex), three substances must be added:

H_2O_2, DAB, and buffer. The buffer serves to stabilize the oxide reduction reactions. The peroxidase enzyme (Px) reacts with its specific substrate and forms a complex enzyme substrate ($Px \cdot H_2O_2$). DAB is a donor substance that loses electrons when it interacts with the $Px \cdot H_2O_2$ complex, resulting in DAB oxidation and polymerization. This forms a brown precipitate, and because it is highly insoluble, it sticks to the site of the immunoreaction. Adding nickel darkens the DAB staining, resulting in a gray-blackish reaction product [87].

Note 9: In the *Stereo Investigator 11* program with the optical fractionator workflow, it is possible to draw and to sample all the subregions of interest. To do this, one must set up the information about an experimental subject and trace the regions of interest. After this step, the "SRS grid layout" must be defined; this grid helps the program to automatically select the sites to be sampled. The first site is selected at random (by the program); then, depending of the grid size, the rest of the sites are located with a uniform distance between sampling sites. It is recommended that at least three sites per section/subregion be sampled.

Note 10: Perform the following steps. (1) Open the image in the program: select the "File" menu, and click the "open" option; this opens a window to choose the saved image. Select the image and click "open." (2) Define the bits to display the image: click on the "Image" menu, and click the "type" and "8 bit" options. (3) Calibrate: click the "analyze" menu, choose the "calibrate" option, then click the "function: uncalibrated OD" and "unit: Gray value," and select "Global calibration, OK, list." (4) Analyze the image: select the "Analyze" menu, search and click the "tools" option, select "ROI manager," and draw a piece of the area of interest with the drawing tools. Once drawn, select "Add" to add each data for each area. To observe the data of the OD of each area selected, click on "measure"; this will cause a window to appear with a chart containing the OD of each region.

8 Appendix 2: Synaptic Vesicle Protein 2A Molecular Dynamics Simulation

8.1 Introduction

Molecular dynamics simulation (MDS) is a technique that uses the classical physics equations to describe the atomic behavior of bio/molecule(s) in a given condition. In fact, there are examples where MDS has demonstrated to be predictive, e.g., human immunodeficiency virus (HIV) integrase crystallographic structure was used to perform a MDS, and the results demonstrated that the protein exhibited a cryptic motif that was used to find specific organic molecules to inhibit it. Then, a molecule was found and subsequently approved by the US Food and Drug Administration to be used as inhibitor of this protein [88]. MDS is at present widely used in drug discovery [89, 90], thanks to the advances in computing power that

allows simulating bigger systems for longer time periods. It is also important to point out that algorithms for calculation are improving constantly. We advise the reader to find the mathematical and physiochemical fundament of MDS to know more about this technique, advantages, and limitations. References [90] and [91] can be a good starting point to discover more about MDS.

In more technical details, MDS treats all atoms as rigid spheres interacting between them by a set of numbers called force field (FF). FF has defined all atom types, bonds, angles, dihedrals, impropers, charges, and van der Waals values, for solving the classical movement equations and describing the trajectory of a molecule in a certain condition.

One of the most important things to start a simulation of a biomolecule is to be sure that the structure is complete and correct. It is extremely important to be certain about the geometry, atomic spatial disposition, and completeness of the molecule. When this annex was written, the Protein Data Bank (PDB), the most important repository to obtain crystallographic structures of biological systems, had 113,130 total structures. These structures include proteins, nucleic acids, and protein complexes, and not all of them are complete [92]. Since some systems show flexible motifs, X-ray crystallography is not sensitive enough for solving more flexible motifs, and many atomic models are not complete. Although nuclear magnetic resonance is a possible solution to know the structure of a biomolecule, it has a limitation: the size of the system. However, to solve the problem of uncompleted systems, there are some other bioinformatic tools that can help to model a complete system, such as homology modeling. It is possible to find homology modeling servers on the Internet to generate a complete model of a biomolecule. Iterative Threading ASSEmbly Refinement (I-TASSER) is one of the most used to predict the structure of a protein from its sequence [93, 94]. I-TASSER uses PDB structures and applies several threading algorithms to perform the structure prediction of a protein.

To carry out a MDS, it is necessary to have software to solve the classical motion equations. There are several freely accessible (under open source released under the GNU General Public License) such as GROMACS (GROningen Machine for Chemical Simulations) [95, 96] or NAMD (Nanoscale Molecular Dynamics) [97]; they are free of charge for noncommercial use by individuals and academic institutions. There are also commercial packages such as CHARMM (Chemistry at HARvard Molecular Mechanics) [98] or AMBER (Assisted Model Building with Energy Refinement) [99]. All packages are useful and include tools to set up, simulate, and analyze a system. NAMD uses VMD (Visual Molecular Dynamics) [100] to perform a setup graphically. AMBER has also a graphical interface. In contrast, GROMACS or CHARMM needs text files as inputs. Other differences are found, e.g., in the way of coupling pressure in

simulations at constant pressure and temperature. CHARMM and NAMD can perform more coupling schemes than AMBER. This annex does not pretend to be a summary of advantages for each program to perform MDS. We refer the reader to the original papers and manuals of each program for more information.

As mentioned above, FF is the set of parameters that describe the possible intra- and intermolecular interactions. Some FF are used to improve atomic description in different systems, e.g., Optimized Potentials for Liquid Simulations (OPLS) [101–103]. OPLS is an FF developed for liquids, being capable of reproducing properties such as vapor pressure, density, and heat of vaporization. OPLS is also a FF frequently used for proteins and lipids. We refer the reader to review the Ruso and Piñeiro's chapter [104], which contains useful information about MDS with membranes.

For starting a MDS, it is necessary to define a box called unit cell (where the biomolecule is introduced). The unit cell is the virtual flask where the system will be simulated and depends directly on the geometry and the nature of the molecule of interest. Periodic boundary conditions (PBC) are used in MDS to reproduce the behavior of a system in bulk. There are several unit cells that can fill the space for reproducing the bulk behavior as in an experiment. Some are cubic, orthorhombic, dodecahedron, or even hexagonal prism; cubic and dodecahedron unit cells can be used for globular proteins in solution; however, dodecahedron unit cell saves 30% of water molecules in comparison with cubic, which implies a faster simulation owing to the reduced quantity of particles in the system.

The trajectory is the sequence of conformations (frames) that the biomolecule visits in the simulation; in other words, it is the "movie" that shows how the atoms move in that condition. Much information can be obtained from a trajectory. Some properties to measure in function of time are:

(a) *The radius of gyration* (Rg) which is the weighted scalar length of each atom from the center of mass of the global structure. It gives a rough measure for the compactness of a macromolecule.

(b) *Root-mean-square deviation* (RMSD) which is a global measurement for the scalar atomic distance between two structures. It is typically employed to assess global protein structural changes.

(c) *The root-mean-square fluc*tuations (RMSF) of the atomic positions throughout the trajectory indicate what are the most flexible parts of the system.

(d) The solvent accessible surface area (SAS) of a molecule is a measure of the area of the system that is exposed to the solvent.

(e) *Numbers of hydrogen bonds* (HB), based on geometrical criteria, donor-acceptor distance cutoff <3.5 Å, and donor-H-acceptor angle <30°, are the most typical values for determining if a group of atoms form an HB.

(f) Secondary structure, which can be calculated using the do_dssp program, is based on the geometrical criteria defined in the Dictionary of Secondary Structure of Proteins [105].

Other analyses are cluster analysis and principal component analysis (PCA). First, all frames can be clustered in function of RMSD to know what the most visited configuration in trajectory is. GROMACS has several clustering algorithms. GROMOS algorithm uses a cutoff for choosing the nearest neighbors and eliminates it from the pool of clusters [106]. Doing this iteratively with the rest of structures gives a set of average structure of each cluster. It is necessary to calculate the RMSD after fitting to define the distance between structures. The PCA determinates the predominant movements that a molecule takes in simulation. That helps to know if an atomic displacement is responsible for a determined function. It is possible to obtain the correlation matrix and diagonalize it to get the eigenvectors and eigenvalues. Eigenvectors are projected in structure to observe these atomic displacements. The less eigenvalues, the less global movements that describe the system; it means there are few atomic displacements that describe the behavior of the system. Please refer to the website [107] where there are very easy lecture of what PCA is.

Summarizing, there are several things necessary to implement a MDS: first, a complete system with biological sense, the FF that will describe the system correctly, and the program to perform the simulations and analyze them. Then, several analyses can be done to evaluate how the molecule evolves in simulation, measuring its properties in function of time. Next, we will develop and analyze an MDS of synaptic vesicle protein 2A (SV2A).

8.2 Materials and Methods

8.2.1 SV2A Modeling

For studying the SV2A, we started with protein primary sequence obtained under NP_476558.2 code from the National Center of Biotechnology (NCBI) and UniProt (Q02563). Once with the correct sequence, it was send to I-TASSER server [93, 94] to obtain a model of SV2A protein. The model was confirmed and completed with other models predicted by independent algorithms; the CPHmodels-3.2 [108] and 3D-JIGSAWv3.0 [109] servers were used to confirm the reproducibility of the SV2A 3D model provided by I-TASSER.

8.2.2 SV2A Molecular Dynamics Simulations

We chose GROMACS program to perform the SV2A simulation because it is a scriptable program, and we have developed many in-house tools to perform the analysis of MDS trajectory. In Supplementary Fig. 3, the basic pipeline to carry out a MDS is

described. The simulations were performed by applying the half-ε double-pair list method [110] to ensure the compatibility of the Berger united-atom parameters used for the lipids with the OPLS-AA (all atom) FF [101–103] employed for the protein and ions.

Protein NH_2-terminus was carefully placed out the membrane, while the transmembrane (TM) helices were placed inside of it. The system was then solvated using simple point charge water molecule model. Upon removing the solvent molecules found in the hydrophobic region of the lipid bilayer, 19 Na^+ ions were added to neutralize the system. The simulation box contained one molecule of SV2A, 342 POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine) molecules, 47,671 waters, and 19 Na^+ ions, leading to a total of 172,298 atoms (Supplementary Fig. 4).

Firstly, a minimization for 500 steps using the steepest descent method prior the generation of MDS trajectories was made. Then, the solvent-protein and lipid-protein contacts were optimized by a 5 ns long simulation with harmonic constraints in the heavy atoms of the protein. The force constants of these positional restraints were sequentially reduced. Then, an unrestrained 50 ns long production trajectory was generated. All the simulations were performed using a time step of 2 fs. In all simulations, the pressure was controlled at 1 bar using a semi-isotropic Parrinello-Rahman barostat [111] to allow independent modifications of the box dimensions in the XY plane and in the *Z*-axis. The temperature was kept constant at 310 K by a Nosé-Hoover thermostat [112].

The LINCS (Linear Constraint Solver) algorithm [113] was employed to remove the bond vibrations. The particle mesh Ewald method [114] coupled to periodic boundary conditions was used to treat the long-range electrostatics using a direct space cutoff of 1.2 nm and a grid spacing of 0.15 nm. The van der Waals interactions were computed using PBC coupled to a spherical cutoff of 1.2 nm. A replicate of MDS was carried out in order to confirm the results of the first MDS, with the same initial configuration but with different initial velocities allowing to find the reproducibility in both simulations. All the MDS were done with the program GROMACS [8, 9] (version 4.6.1) [115].

8.3 Results

In this section, we will focus on cluster analysis and PCA of two, original and replicate, SV2A MDS. Details of the Rg, RMSD, RMSF, SAS, and HB results have previously been reported [116].

8.3.1 SV2A Cluster Analysis

Using a cutoff radius of 0.3 nm, the protein conformations observed throughout the original trajectory were distributed into ten different clusters (Supplementary Fig. 5). The most populated five clusters correspond to 95.61 % of structures (51.73 %, 21.34 %, 8.84 %, 7.50 %, and 6.20 %, respectively). The Cluster 1 is the most probable configuration that SV2A adopts in the membrane. The principal differences between clusters were, first, in the orientation

of extravesicular domain and, second, in the intravesicular NH_2-terminal domain, with a sequence of loops that can adopt several conformations. The most conserved part of the protein structure is the TM region.

8.3.2 SV2A Principal Component Analysis

The variance-covariance matrix of atomic motions showed which atoms move together in a concerted way. We calculated the diagonalized covariance matrix for the α-carbons of SV2A. Eigenvalues revealed that only 2–3 components were relevant for the two trajectories. The projection of the original trajectory on the two principal eigenvectors clearly indicates that the protein experiences a serious structural evolution during the first 16 ns of the trajectory. The protein structure seemed to reach a more stable structure in the last part of the trajectory.

The PCA results of the two independent simulations (T1 original and T2 replica) show that first eigenvector from T1 has significant projections on two first eigenvectors of T2. Thus, the movement along T1 is larger and also less specific. While the TM domain remains quite stable, the most mobile parts of the protein, i.e., the extramembranal domains, take different conformations along both trajectories (Supplementary Fig. 6). More specifically, the ATP-binding motif (in extravesicular loop of SV2A) shows a different conformation along both trajectories. The NH_2-terminal displacement seems to conserve better its geometry in T2 than in T1. Besides, the extraluminal loop exhibits a larger displacement in T1 than in T2. The covariance matrix reveals that these three atomic displacements are anticorrelated with the TM region. Just a few residues, including some in the N-terminal domain, are correlated.

8.4 Conclusions

Using MDS technique allows studying complex systems such as explicit solvated TM proteins obtaining results in atomic detail. SV2A protein was inserted in a POPC membrane, solvated and simulated for 50 ns. Clusterization showed a principal average structure of the protein and PCA demonstrated stability in TM domains and some correlated movements between the extra- and intravesicular motifs; additionally with those movements, the ATP-binding motif moves correlated with them. It is necessary to continue with this work extending the simulation; if there are some extra conformational changes, they will be seen in longer simulation time. Given the pharmacological importance of SV2A, knowing its conformation is essential to direct further experimental work to discover new potential anticonvulsant ligands.

Finally, we invite you to prove this technique in your own system. There are manuals and tutorials that will help you to set up and simulate your system. Try it on!

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Chapter 5

Do Cannabinoids Represent a Good Therapeutic Strategy for Epilepsy?

Cecilia Zavala-Tecuapetla and Luisa Rocha

Abstract

The medical use of cannabinoids has been proposed for the control of epilepsy. At present, several studies have focused on investigating how cannabinoids can regulate the expression of epileptic seizures as well as the epileptogenesis process. Some of them suggest that cannabinoids may represent a therapeutic approach for different types of epilepsy. However, experimental evidence indicates that the effects of cannabinoids depend on several experimental and pathological conditions. In this chapter, we provide an overview of these preclinical and clinical research.

Key words Endocannabinoid system, Anandamide (AEA), 2-Arachidonoyl glycerol (2-AG), Δ9-Tetrahydrocannabinol (Δ9-THC), Cannabidiol (CBD), CB1/CB2 receptors, Seizures, Epilepsy

1 Introduction

During centuries cannabis plants have been used for both medicinal and recreational uses as described in Chinese, Indian, and Arab pharmacopeias [1]. Presently, the medical use of *Cannabis* extracts has been approved in some European countries [2].

Cannabis plants present a mixture of chemical constituents, the C_{21} terpenophenolic compounds also called phytocannabinoids. Detailed chemical analysis has allowed the identification of about 70 molecular species of these phytocannabinoids [3] whose amounts depend on each plant and environmental conditions [4, 5]. The most important phytocannabinoids are the psychoactive Δ9-tetrahydrocannabinol (Δ9-THC) and the non-psychoactive cannabidiol (CBD). CBD was isolated in 1940, and its structure was elucidated in 1963 [6, 7]. The Δ9-THC was isolated by Yechiel Gaoni and Raphael Mechoulam [8] and was shown to account for the psychotropic effects of cannabis preparations in rhesus monkeys [9]. During the late 1980s, it was found that Δ9-THC exerts its effects through the activation of two G-protein-coupled receptors: cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2)

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receptors [10, 11]. Thereafter, the endocannabinoids (eCBs) anandamide and 2-arachidonoylglycerol were identified as the endogenous ligands of CB1 and CB2 receptors [12–14].

A growing body of evidence supports that the eCB system is involved in several functions of the brain and that *Cannabis* and phytocannabinoids represent pharmacological strategies to induce neuroprotection and control of disorders such as epilepsy, migraine, and pain [15]. In the case of epilepsy, experimental evidence indicates a key role for eCB system in the modulation of neuronal excitability. The present review focuses on providing a better understanding of how and when pharmacological interventions with cannabinoids or phytocannabinoids may control epilepsy.

2 The Endocannabinoid System

The eCB system has a crucial role in different brain functions including cerebral development, cognition, learning, memory, motor behavior, appetite regulation, temperature regulation, and pain [16]. Experimental evidence indicates that the role of eCB in the regulation of physiological responses depends on the gender [17]. The eCB system consists of cannabinoid receptors, their endogenous lipid ligands (eCBs), and the enzymatic machinery for their biosynthesis, cellular uptake, release, and degradation [18].

2.1 Endocannabinoids

The first eCBs identified in the central nervous system (CNS) [19, 20] were the hydrophobic ligands N-arachidonoyl ethanolamide (anandamide, AEA) [12] and 2-arachidonoyl glycerol (2-AG) [13, 14]. The synthesis of these eCBs depends on specific enzymes using membrane phospholipids as precursors. The *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) is the enzyme responsible for the synthesis of AEA and other *N*-acylethanolamines [21], whereas different diacylglycerol lipases (DAGLs) are involved in the synthesis of 2-AG [22]. The effects mediated by eCBs are limited by their fast catabolism. The enzyme fatty acid amide hydrolase (FAAH) catabolizes AEA [23]. Monoacylglycerol lipase (MAGL) and serine hydrolase α/β--hydrolase domain 6 (ABHD) induce degradation of 2-AG in the brain [24–26]. Carrier-mediated transport systems are involved in clearing eCBs from the extracellular space [27–29], and their subsequent enzymatic degradation can proceed through either hydrolysis or oxidation [24, 30, 31].

Unlike other neuromodulators and traditional vesicular neurotransmitters, eCBs are believed to be synthesized "on demand" by changes in neural activity [32]. The synthesis of eCBs in postsynaptic neurons can be triggered by the increase in intracellular Ca^{2+} concentration subsequent to depolarization and activation of voltage-gated Ca^{2+} channels [33–37] and the activation of certain

Gαq/11 protein-coupled receptors [38–40]. Other studies suggest that intracellular storage organelles might accumulate pre-synthesized eCBs [41, 42].

2.2 Endocannabinoid Receptors

CB1 and CB2 receptors belong to the large superfamily of heptahelical G-protein-coupled receptors (GPCR) and couple to Gi/Go proteins. The CB2 receptor is predominately expressed in the immune system [43] and has very limited expression in the CNS. By contrast, the CB1 receptors are highly expressed at presynaptic levels in the brain, and its activation is implicated in inhibition of the synaptic neurotransmission [44–47]. Concerning this notion, it is known that the activation of presynaptic CB1 receptors reduces the release of neurotransmitters like glutamate and γ-aminobutyric acid (GABA) [48] as a consequence of the inhibition of Ca^{2+} channels and activation of K^+ channels [49–54], a situation that may modify the neuronal excitability [55].

Activation of CB1 receptors promotes its interaction with Go proteins, resulting in guanosine diphosphate/guanosine triphosphate exchange and subsequent dissociation of α and βγ subunits with a consequent reduction of adenylate cyclase and cyclic adenosine monophosphate production [56]; inhibition N-, P/Q-, and L-type voltage-gated Ca^{2+} channels [20, 46, 57, 58]; stimulation of A type K^+ channels [44, 59, 60], activation of G-protein-coupled inwardly rectifying K^+ channels [61, 62]; and inhibition of the vesicular release machinery [63].

While the CB1 receptor is responsible for the vast majority of the currently known effects of cannabinoids and eCBs in the CNS, it is worth noting that additional cannabinoid receptors may exist. The cannabinoid-sensitive receptor G-protein-coupled receptor 55 (GPR55), identified as a novel cannabinoid receptor that couples to Gα13 protein [64], is activated by some phytocannabinoids such as Δ9-THC. In the brain, GPR55 is present in the caudate, putamen, hippocampus, thalamus, pons, cerebellum, frontal cortex, and thalamus [64]. In human embryonic kidney cells, the activation of GPR55 triggers the release of intracellular Ca^{2+} from endoplasmic reticulum stores via a pathway dependent on Ras homolog gene family member A (RhoA), phospholipase C, and inositol 1,4.5-trisphosphate receptor [65]. The increases of intracellular Ca^{2+} levels that result from the activation of GPR55 by L-α-lysophosphatidylinositol (LPI, an endogenous agonist) augment the probability of vesicular release of glutamate at excitatory hippocampal synapses [66, 67]. These results support a relevant role of GPR55 in cerebral excitability.

3 Phytocannabinoids

CBD and Δ9-THC represent the most important phytocannabinoids contained in the *Cannabis* plants [3]. Δ9-THC is a partial agonist of CB1 receptors that induces most of the behavioral,

cognitive, and psychotropic effects of *Cannabis*. The mechanisms by which Δ9-THC induces these effects also involve the activation and desensitization of the transient receptor potential (TRP) channels of ankyrin type 1 (TRPA1) and vanilloids type 1 (TRPV1) and type 2 (TRPV2) [68–70].

CBD is considered a "multitarget" drug because of its interaction with many other non-eCB signaling systems. It acts as an agonist of TRPV1, TRPV2, and TRPA1 [68, 70–72], 5-hydroxytryptamine1α receptors [73], and glycine receptors [74]. CBD acts as an antagonist of TRP melastatin type-8 channels [69], T-type voltage-gated Ca^{2+} channels [75], and GPR55 receptors [76]. Also, it exerts dynamic control over intracellular Ca^{2+} stores [77, 78] and inhibits the uptake and enzymatic degradation of AEA via FAAH [79].

CBD may potentiate some effects induced by Δ9-THC such as analgesia, antiemesis, and anti-inflammation, but it also reduces Δ9-THC-induced psychoactive effects (impaired working memory, sedation, tachycardia, and paranoia) [80–82]. Cannabis products with a high content of CBD induce greater tolerability and lower incidence of psychosis when compared with those with high content of Δ9-THC [83].

4 Effects of Cannabinoids on Seizure Activity and Epilepsy

Several studies indicate that eCBs and cannabinoids play an important role in epilepsy. Here, we summarize evidence from preclinical and clinical studies focused on clarifying this situation.

Concerning experimental models of acute seizure activity, it is described that the i.c.v. administration of arachidonyl-2-chloroethylamide (ACEA, a CB1 receptor agonist) decreases the frequency of penicillin-induced epileptiform activity in rats, an effect blocked by AM-251 (a CB1 receptor antagonist) [84]. Compounds like Δ9-THC, WIN55,212-2, CBD, and AEA and their analog O-1812 induce anticonvulsant effects in the maximal electroshock seizure model [85, 86]. In in vitro models, the activation of CB1 receptors with agonists (methanandamide, 2-AG, AEA, or WIN 55,212-2) reduces the epileptiform activity induced by low or omission of Mg^{2+} and high K^{+} [87–89]. The cannabinoid agonist HU210 reduces the epileptiform synchronization in hippocampus induced by kainic acid administration, an effect avoided with the pretreatment with rimonabant, a CB1 receptor antagonist [90]. This group of evidence reveals that cannabinoids may modify both focal and generalized seizures blocking neuronal hypersynchronization associated with epileptic activity.

Studies reveal the participation of cannabinoids in the expression of seizure activity and the epileptogenesis process. Δ9-THC and the cannabinoid agonist WIN55,212 abolish spontaneous

epileptic seizures subsequent to pilocarpine-induced SE. Conversely, the administration of the CB1 receptor antagonist SR141716A increases both seizure duration and frequency [91]. The administration of WIN 55,212-2 during 15 days after pilocarpine-induced SE reduces the severity, duration, and frequency of spontaneous recurrent seizures, an effect associated with the preservation of GABAergic neurons, as well as absence of changes in the oxidative stress and expression of NMDA receptor subunits [92]. In the kindling model, the activation of CB1 receptors has been proposed to delay the acquisition of generalized seizures, whereas the inhibition of the enzymatic degradation of AEA did not affect the epileptogenesis process but reduces the neurogenesis associated to it [93]. All these findings support the idea that activation of CB1 receptors can suppress recurrent excitation during epileptogenesis.

Studies indicate that the activation of CB1 receptors can augment or reduce the seizure termination and duration, a situation that depends on the neuronal subpopulation [94]. CB1 receptors are also expressed in astrocytes [95, 96], and their activation is involved in the maintenance of epileptiform discharge [97].

eCBs may interact with other neurotransmitters and neuromodulators. Using the pentylenetetrazol-induced clonic seizure model, it was found that opioids are able to modulate the anticonvulsant effects of cannabinoids [98, 99]. In glutamatergic neurons, activation of CB1 receptors reduces the excitatory neurotransmission and the susceptibility to seizure activity [94, 100]. In experimental models of temporal lobe epilepsy (TLE), the activation of CB1 receptors with agonists (WIN 55,212-2, AEA, or 2-AG) decreases the epileptiform activity, the EPSCs evoked by glutamate, and the excitatory events evoked after antidromic electrical stimulation of mossy fibers in hilus [101].

Several experiments have focused on determining the role of eCB system on seizure activity by enhancing the availability of eCBs. Inhibition of AEA hydrolysis with URB-597, a FAAH inhibitor, results in anticonvulsive effects in the PTZ-induced seizures [102]. The inhibition of the 2-AG hydrolysis using WWL123 (an antagonist of ABHD6) reduces spontaneous seizures in R6/2 mice (a genetic model of juvenile Huntington's disease seizures) and PTZ-induced tonic-clonic convulsions [103]. Also, the increased levels of 2-AG that result of inhibition of degrading enzyme MAGL have been associated with a delay in the development of the kindling process [104]. The reduced metabolism of eCBs induced by the combination of AM404 (inhibitor of endocannabinoid reuptake) and URB597 (inhibitor of FAAH) results in decreased kainic acid-induced SE in guinea pigs [105]. These studies indicate that the blockage of specific enzymes can represent a new strategy to augment the anticonvulsant effects of eCBs.

In WAG/Rij rats, a genetic animal model of absence seizures, the administration of AEA or WIN55,212-2 (CB1 receptor agonists) reduces the seizure activity, while rimonabant (a CB1 receptor antagonist) increases it [106]. These results suggest that attenuated eCB function may contribute to the generation and maintenance of absence seizures.

5 eCBs and CB1 Receptors in Experimental Models of Seizure Activity and Epilepsy

Several studies indicate that seizure activity and epilepsy modify the eCB system and CB1 receptors. Concerning this issue, it is known that pilocarpine-induced SE increases 2-AG and CB1 receptor expression in hippocampus [91]. Acute seizures induced by kainic acid produce a rapid augmentation of AEA synthesis in the hippocampus and activation of CB1 receptors [107]. Studies indicate that seizure-induced changes in eCBs are age specific. Kainic acid-induced seizures in young rats augment the tissue content of AEA and their biosynthetic enzyme (NAPE-PLD) in the hippocampus, while adult rats present elevated tissue content of 2-AG and its biosynthetic enzyme DAGL [108]. Kindling-induced seizures augment CB1 receptor density in the pyramidal cell layer of the hippocampus [94]. Similar findings have been reported for different mouse models of epilepsy [109, 110]. In contrast, other studies indicate a low expression of CB1 receptors in certain neuronal subpopulation [111, 112]. These contradictory results can be explained by the different epilepsy models used and the period of evaluation after induction of seizures.

Upregulation of CB1 receptors in hippocampus is detected in mice with TLE subsequent to pilocarpine-induced SE [101]. Using the same experimental model of TLE in rats, it was found that spontaneous recurrent seizures are associated with a redistribution of CB1 receptors and changes in expression, binding, and G-protein activation in hippocampus [113]. This situation might depend on the time course of the SE-induced epileptogenesis process [111]. This group of evidence leads to suggest that the redistribution of CB1 receptors is associated with the cerebral plasticity involved in the epileptogenesis process.

6 eCBs and CB1 Receptors in Patients with Epilepsy

In dogs with idiopathic epilepsy, high concentrations of AEA were found in the cerebrospinal fluid, a situation that correlates with the severity of seizures and duration of the disease [114]. This study suggests an important activation of the cannabinoid systems as result of seizure activity. However, the low levels of AEA detected

in the cerebrospinal fluid of drug-naïve patients with TLE do not support this hypothesis [115].

Positron emission tomography (PET) imaging revealed increased availability of CB1 receptors in the ipsilateral temporal lobe of patients with TLE, a situation that was more evident in those subjects evaluated within short term after the last seizure and presenting higher number of seizures. These patients also show a decreased availability of CB1 receptors in the ipsilateral superior insular cortex, a condition that may restrict the seizure propagation [116]. However, it is important to consider that in vivo studies using PET imaging cannot avoid the presence of endogenous ligands and the enhanced availability of CB1 receptors can be associated with an increase in their number or affinity, or it is a consequence of low extracellular levels of eCBs.

The evaluation of hippocampal tissue obtained from patients with refractory TLE indicates a reduced expression of cannabinoid receptor-interacting protein-1a (CRIP1a) mRNA and the metabolic enzymes DGAL-α (enzyme involved in the synthesis of 2-AG). There is also a decrease in the mRNA and protein expression of CB1 receptors, mainly at glutamatergic axons, but not in GABAergic boutons, in the dentate gyrus [117]. Considering that CB1 receptors reduce the excitatory neurotransmission in glutamatergic neurons [94, 100], their lower expression at glutamatergic axons can facilitate the excitatory neurotransmission in the epileptic hippocampus. In contrast, CB1 receptors are preserved in dentate gyrus and CA1 region of patients with TLE, suggesting increased expression of these receptors in the GABAergic sprouting axons [118]. These results indicate that the disruption of the inhibitory effects of eCB system on GABAergic transmission in hippocampus of patients with TLE may facilitate the seizure activity.

Concerning TRPV1, studies revealed no significant changes in their expression in hippocampus of animals submitted to repetitive seizures [94]. However, patients with pharmacoresistant temporal lobe epilepsy show increased TRPV1 expression in the hippocampus [119]. Considering that cannabinoids may act as agonists of TRPV1, TRPV2, and TRPA1 [68, 70–72], the activation of these receptors by eCBs may contribute to the modulation of synaptic plasticity in human epileptic hippocampus.

7 The Phytocannabinoids and Epilepsy

There are new well-documented cases reporting remarkably strong beneficial effects of cannabinoids on seizure activity. This situation has triggered an upsurge in exploiting medical marijuana in patients with refractory epilepsy.

CBD is the major constituent of marijuana; it lacks psychoactive side effects and does not act as a CB1 receptor agonist. CBD induces anticonvulsant effects in the seizure activity induced by maximal electroshock test, pentylenetetrazol, pilocarpine-induced temporal lobe seizures, and penicillin [120–123]. However, CBD does not modify the seizure activity induced by cortical administration of cobalt [124]. In kindled rats, CBD reduces the seizure susceptibility and reduces the afterdischarge amplitude, duration, and propagation [125]. Clinical studies also support the anticonvulsant effect of CBD [126]. On the other hand, results obtained from in vitro and in vivo models indicate that cannabidivarin and Δ9-tetrahydrocannabivarin represent the two most important phytocannabinoids with therapeutic potential as anticonvulsant agents [127–131]. At present it is evident that CBD and other phytocannabinoids exert their antiseizure effects at CB1 receptors and other pharmacological targets [128].

In patients with Dravet syndrome, in which epilepsy is usually refractory to standard antiepileptic drugs, medical marijuana with a high CBD/Δ9-THC ratio has been successful to reduce the seizure activity [132]. CBD reduces the seizure frequency in patients with Lennox–Gastaut syndrome, who experience multiple refractory seizures everyday in spite of antiepileptic drugs [133]. Epidiolex (GW Pharmaceuticals), a new phytocannabinoid obtained from *Cannabis* extracts that contains about 98% of CBD and 2% of other cannabinoids, is now approved as a drug to be evaluated in pediatric patients with Dravet and Lennox–Gastaut syndromes [134]. However, proper controlled clinical trials are necessary to establish efficacy and safety of these phytocannabinoids in patients with epilepsy. In addition, future studies have to explore the cellular mechanisms and the signaling pathways involved in the anticonvulsant effects of CBD and other phytocannabinoids.

8 Is the Administration of Cannabinoids a Good Option to Control Epilepsy in Humans?

It is clear that epilepsy modifies the eCB system (e.g., CB1 receptors). However, as many other neuromodulatory systems, the activation of CB1 receptors can augment or reduce the seizure termination and duration, a situation that depends on the neuronal subpopulation and the experimental model used. Concerning this issue, cannabinoids may induce excitatory effects if CB1 receptors are overexpressed in GABAergic neurons. In contrast, the overexpression of these receptors in glutamatergic neurons can produce inhibitory effects. Therefore, the findings obtained from the evaluation of CB1 receptors in patients with epilepsy using PET or in in vitro conditions have to include a clear identification of the cells

in which those changes are produced. In addition, it is relevant to demonstrate that CB1 receptors are functionally active. This situation will help in the clarification of the mechanisms that underlie the anticonvulsant effects of cannabis and cannabinoids in different types of human epilepsy. It will also facilitate the establishment of compounds with therapeutic efficacy to reduce the seizure activity.

Although the results obtained from experimental models are relevant to understand the role of eCBs in epilepsy, they do not reproduce totally the pathological conditions of the human epilepsy. Therefore, the analysis of cerebral tissue obtained from patients with pharmacoresistant epilepsy and submitted to epilepsy surgery is essential to clarify if cannabinoids represent a good therapeutic strategy for epilepsy.

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Chapter 6

Glutamate Receptors as Targets for Novel Antiepileptic Drug Therapy

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Abstract

In recent years, extensive research has been directed towards the development of novel therapeutic targets in epilepsy. A number of studies have reported that glutamate plays an important role in seizure generation and spread. Glutamate is the predominant excitatory neurotransmitter in the brain, exerting its action through ionotropic and metabotropic receptors. Notably, a significant increase of extracellular glutamate release was observed in hippocampus during the ictal, postictal, and interictal period of patients with pharmacoresistant epilepsy undergoing surgery (During and Spencer, Lancet 341:1607–1610, 1993; Cavus et al., Epilepsia 49:1358–1366, 2008). These results indicate a strong association between increased glutamate and epileptiform activity. The chronic increase in extracellular glutamate release favors neuronal hyperexcitability and subsequent neuronal damage. Thus, the pharmacological blockade of glutamatergic signaling represents an attractive alternative for the control of seizures and neuroprotection. In particular, glutamate receptors are attractive targets for novel antiepileptic drugs because different studies have demonstrated that its agonists and antagonists reduce excessive excitatory responses providing neuroprotection and causing seizure suppression. We provide a brief description of the glutamate agonists and antagonists with potential effects on these receptors and the results obtained in both preclinical models of epilepsy and during clinical trials.

Key words Epilepsy, Glutamate receptors, Antiepileptic drugs, Anticonvulsive, Neuroprotection, Preclinical models, Clinical trials

1 Ionotropic Glutamate Receptors (iGluRs)

The iGluRs provide rapid postsynaptic depolarization in response to synaptic release of glutamate, and have a major role in neural transmission, synaptic plasticity, and learning and memory [1–3]. The receptors consist of three distinct families: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate (KA) [4, 5] (Fig. 1).

Alan Talevi and Luisa Rocha (eds.), *Antiepileptic Drug Discovery: Novel Approaches*, Methods in Pharmacology and Toxicology, DOI 10.1007/978-1-4939-6355-3_6,

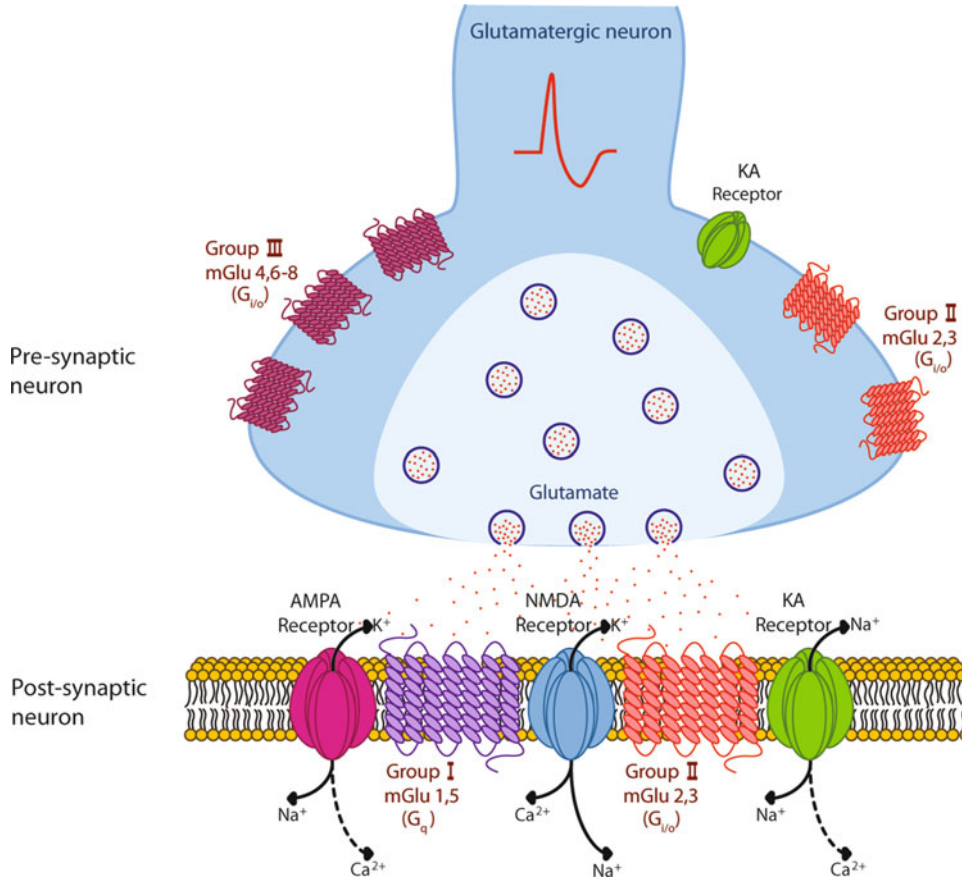


Fig. 1 Glutamatergic synapse. After presynaptic depolarization, glutamate content is released into the synaptic space. This glutamate interacts with postsynaptic ionotropic receptors (NMDA, AMPA, and KA) as well as the KA receptor presynaptically. The intensity or continuity of *arrows* is associated with the permeability to Na^+, K^+, and Ca^{2+}. In addition, glutamate can exert its action through metabotropic receptors (mGlu) located on the presynaptic (group II and III) and postsynaptic terminal (group I and II). The activation of glutamate receptors increases neuronal excitability, which makes them an attractive therapeutic target for the treatment of epileptic seizures

An increase in the excitatory synaptic transmission activation for iGluRs has been reported to induce epileptiform discharges, and initiation and spread of seizure activity in different animal models of epilepsy [6–9]. Although few studies of receptors are available in patients with epilepsy, the evidence suggests that there are changes in up- or down-regulation of iGluRs in tissue of sclerotic hippocampus and its immediate surroundings [10–14].

Based on this evidence, the use of antagonists acting on all three iGluRs subtypes has been investigated for antiseizure activity in preclinical models of epilepsy and clinical trials.

1.1 NMDA Receptors (NMDARs)

NMDARs are coupled to high-conductance cationic channels permeable to K^+, Na^+, and Ca^{2+}, and they are blocked by Mg^{2+} at resting potential (Fig. 1). These receptors exist in multiple forms

(NR1, $NR2_{A–D}$, $NR3_{A–B}$), which have different physiological and pharmacological properties and are differentially distributed throughout the brain [15].

1.1.1 Antagonists in Preclinical Models of Epilepsy

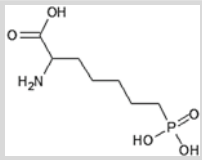
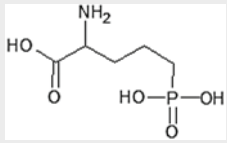
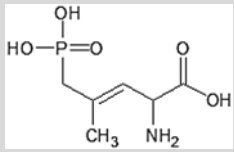
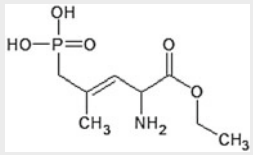
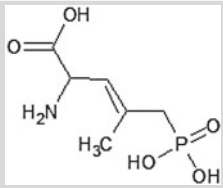
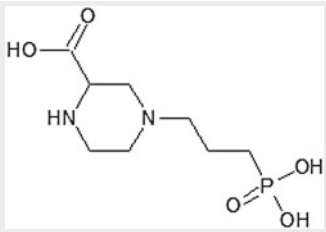
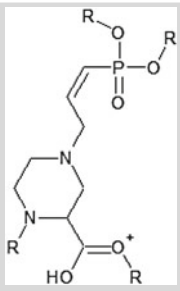
The NMDARs' competitive antagonists such as the AP-7, CGP 40116, D-CPPene, APV, CPP, and CGP 39551 have been demonstrated to possess a broad spectrum of anticonvulsant activity in diverse animal models of generalized tonic-clonic seizures (pentylenetetrazol (PTZ), 4-aminopyridine (4-AP), NMDA, maximal electroshock stimulation (MES), bicuculline, and cocaine), *status epilepticus* (SE) models (pilocarpine and continuous electrical stimulation of the hippocampus), and genetic animal models (sound-induced seizures in DBA/2 mice, genetically epilepsy-prone (GEP) rats, and genetic absence epilepsy GAERS rats) (Table 1) [16–36]. Only the APV has been reported to reduce mortality in NMDA-induced seizure in mice [37]. Regarding animal models of focal epilepsy the CPP, AP-7, CGP 39551, CGP 37849, D-CPPene, and CGP 40116 reduce the propagation of secondarily generalized motor seizures during the development of the amygdaloid and hippocampal kindling in rat [38–40]. However, the anticonvulsant effects were weaker once the rats were kindled [41–46]. In vitro studies showed that only AP-7 and APV reduce epileptiform activity induced by low Mg^{2+}, penicillin, and picrotoxin models in hippocampal neurons [47, 48].

On the other hand, the non-competitive NMDAR antagonists such as ketamine, dizocilpine (MK-801), remacemide, dextromethorphan, memantine, and ifenprodil ($NR2_B$ selective antagonists) showed anticonvulsant effects in animal models of generalized tonic-clonic seizures (NMDA, cocaine, bicuculline, PTZ, 4-AP, MES, and ethanol-withdrawal seizures) (Table 1) [16, 23, 37, 49–62]. In these same animal models only memantine prevented tonic but not clonic seizures [50, 52, 63, 64]. In SE animal models induced by chemical compounds (kainic acid and pilocarpine) or hippocampal continuous electrical stimulation, drugs like ketamine, dizocilpine, remacemide, and ifenprodil exerted anticonvulsant effects and significantly decreased seizure-related brain damage [31, 65–71].

Interestingly the daily administration of dizocilpine, ifenprodil, remacemide, dextromethorphan, and ketamine prevented or increased the latency to development of amygdaline and chemical kindling in rodents [72–76]. On the other hand, studies in genetic seizure models (sound-induced seizure in DBA/2 mice, GEP rats, and model of absence epilepsy in WAG/rij rats) showed that ketamine, dextromethorphan, ifenprodil, and dizocilpine have anticonvulsant effects [77–81]. Regarding studies of in vitro models, remacemide, ketamine, dizocilpine, and memantine suppressed or blocked epileptiform activity in preparations of cultured hippocampal neurons and slices (hippocampal and cortical) in rats [23, 82–87].

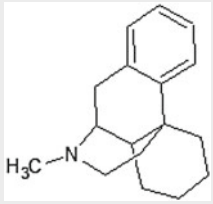
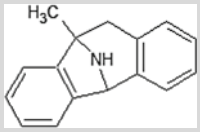
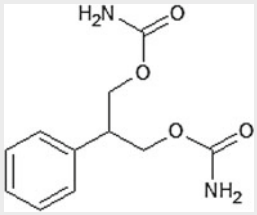
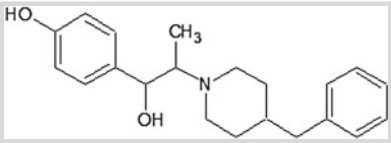
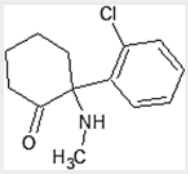
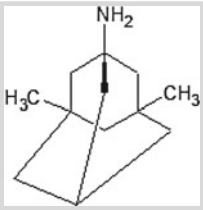
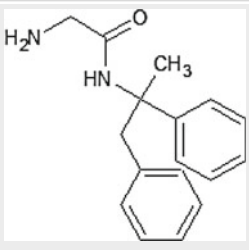
Table 1

Molecular structure of the antagonists of the ionotropic glutamate receptors

Structure	IUPAC name	Traditional name
NMDA antagonists		
	2-Amino-7-phosphonoheptanoic acid	AP-7
	2-Amino-5-phosphonopentanoic acid	APV
	(*E*,2*R*)-2-Amino-4-methyl-5-phosphonopent-3-enoic acid	CGP-37849
	(*R*,*E*)-(4-Amino-5-ethoxy-2-methyl-5-oxopent-2-en-1-yl)phosphonic acid	CGP-39551
	D-(*E*)-2-Amino-4-methyl-5-phosphono3-pentenoic acid	CGP-40116
	3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid	CPP
	(*R*)-4-[(*E*)-3-Phosphonoprop-2-enyl] piperazine-2-carboxylic acid	D-CPPene

(continued)

Table 1
(continued)

Structure	IUPAC name	Traditional name
	(9α,13α,14α)-3-Methoxy-17-methylmorphinan	Dextro methorphan
	[5*R*,10*S*]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine	Dizocilpine
	(3-Carbamoyloxy-2-phenylpropyl) carbamate	Felbamate
	4-[2-(4-Benzylpiperidin-1-yl)-1-hydroxypropyl]phenol	Ifenprodil
	(*RS*)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone	Ketamine
	3,5-Dimethyladamantan-1-amine	Memantine
	*N*-(1-Methyl-1,2-diphenylethyl) glycinamide	Remacemide
AMPA antagonists		

(continued)

Table 1
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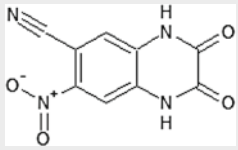
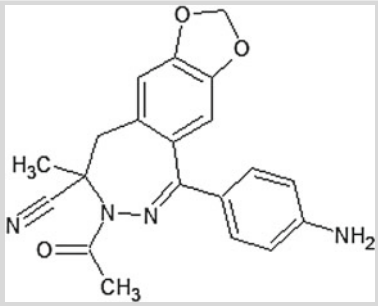
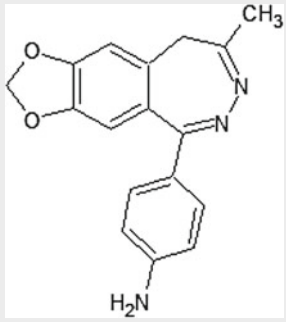
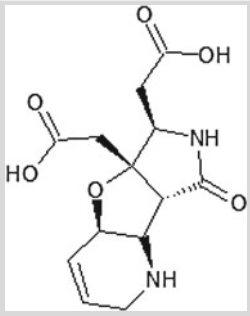
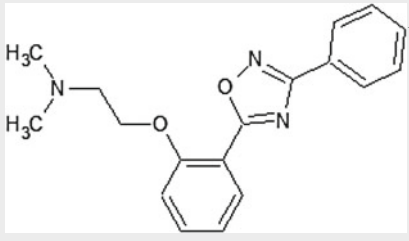
Structure	IUPAC name	Traditional name
	(6-Cyano-7-nitroquinoxaline-2,3-dione)	CNQX
	7-Acetyl-5-(4-aminophenyl)-8-methyl-9H-[1,3]dioxolo[4,5-h][2,3]benzodiazepine-8-carbonitrile	EGIS-8332
	4-(8-Methyl-9*H*-[1,3]dioxolo[4,5-h][2,3]benzodiazepin-5-yl)aniline	GYKI-52466
	(*R*)-2-Amino-2-(4-methoxyphenyl) ethanol	IKM-159
	*N*,*N*-Dimethyl-2-[2-(3-phenyl-1,2,4-oxadiazol-5-yl)phenoxy] ethanamine	Irampanel

(continued)

Table 1
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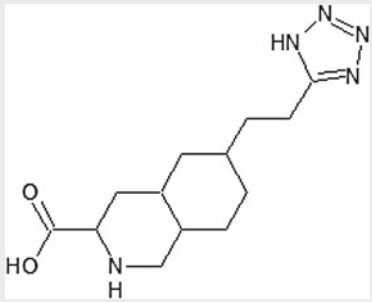
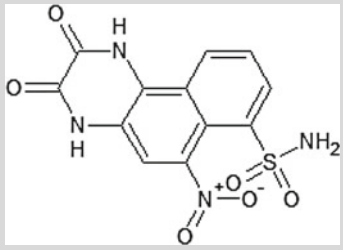
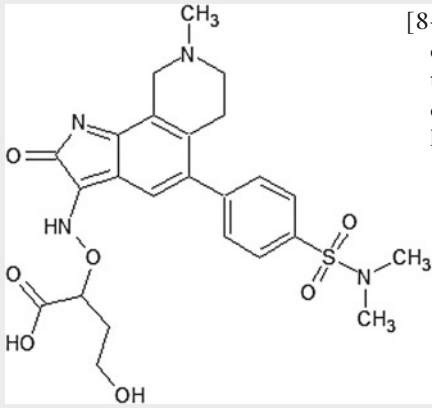
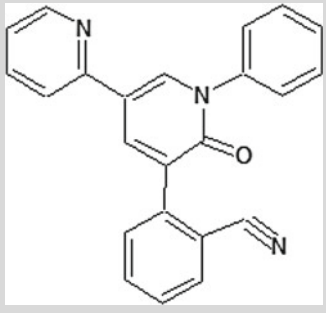
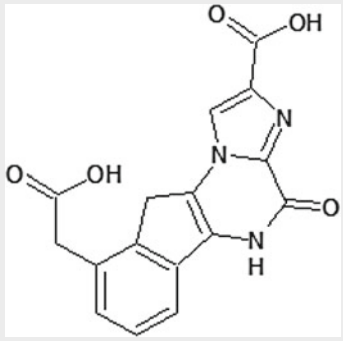
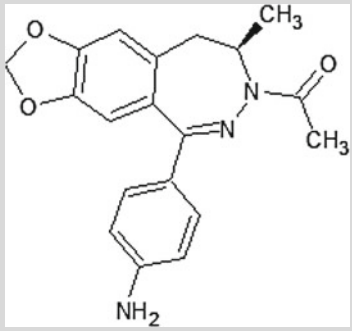
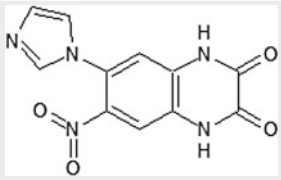
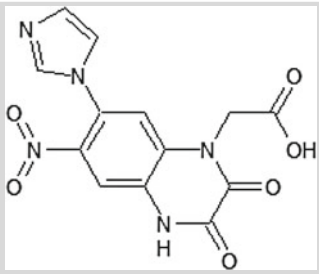
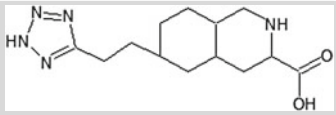
Structure	IUPAC name	Traditional name
	(3*RS*,4a*RS*,6*RS*,8a*RS*)-6-[2-(1(2) H-Tetrazole-5-yl)ethyl] decahydroisoquinoline-3-carboxylic acid)	LY215490
	(2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione	NBQX
	[8-Methyl-5-(4-(*N*,*N*-dimethylsulfamoyl)phenyl)-6,7,8,9-tetrahydro-1H-pyrrolo[3,2-*h*]-isoquinoline2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime]	NS1209
	(2-(2-Oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl) benzonitrile)	Perampanel

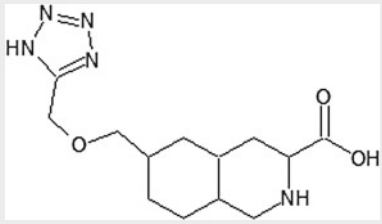
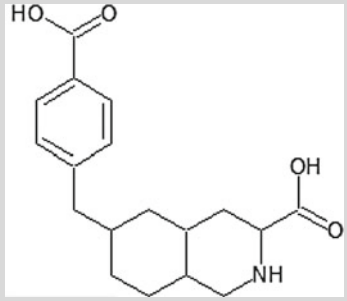
(continued)

Table 1
(continued)

Structure	IUPAC name	Traditional name
	9-Carboxymethyl-imidazo-[1-2a] indeno[1-2e]pyrazin-4-one-2-carboxylic acid	RPR117824
	(8*R*)-7-Acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7*H*-1,3-dioxolo[4,5-h] [2,3] benzodiazepine	Talampanel
	[6-(1H-Imidazol-1-yl)-7-nitro-2,3-(1H,4H)-quinoxalinedionehydrochloride	YM90K
	[2,3-Dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydroquinoxalin-1-yl]-acetic acidmonohydrate	YM872
Kainate antagonists		
	(3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1H-Tetrazol-5-yl)ethyl]decahydro-3-isoquinolinecarboxylic acid	LY293558

(continued)

Table 1
(continued)

Structure	IUPAC name	Traditional name
	(3*S*,4a*R*,6*S*,8a*R*)-6-(((1H-Tetrazol-5-ylmethyl)oxy)methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid	LY37770
	(3*S*,4a*S*,6*S*,8a*R*)-6-[(4-Carboxyphenyl)methyl]-1,2,3,4,4a,5,6,7,8,8a,decahydroisoquinoline-3-carboxylic acid	LY382884

ACD/ChemSketch 11.0 was used to draw the molecules

In spite of the anticonvulsant and neuroprotective effects shown by NMDAR antagonist in preclinical studies, the disadvantage of some of these antagonists is that they may produce prominent side effects in kindled animals, such as hyperlocomotion and stereotypies [45]. In addition, NMDAR antagonists reported adverse events as confusion, difficulty in concentration, agitation, ataxia, and catatonia [88, 89]. Another important limitation of these drugs is that they have also induced proconvulsant effects [90–94]. Also, drugs as memantine and dizocilpine can modulate dopaminergic system, serotonergic system, and cholinergic neurotransmission with similar potency to that acting on NMDARs.

1.1.2 Antagonists in Clinical Trials

Ketamine

Initially ketamine was reported to be effective in stopping the refractory SE (RSE), either convulsive or non-convulsive, by intravenous or oral administration, without apparent adverse effects in children and adults [95, 96]. However, a multicenter study on the use of intravenous ketamine reported serious complications, such as sepsis, shock, organ failure, and pneumonia. These complications were not related to the dose or duration of exposure to ketamine, but were certainly frequent when compared to the administration of other anesthetic drugs used for RSE [97]. This study reported that ketamine induced supraventricular tachycardia and atrial fibrillation in 7% of patients. Another study reported psychiatric symptoms (hallucinations, delirium, a floating sensation, and blurred vision),

intracranial pressure increase, salivation, intraocular pressure, and arrhythmia at doses from 0.45 to 10 mg/kg/h and in some cases neurotoxicity [98]. Moreover, diffuse cerebellar and cerebral atrophy were detected 3 months after ketamine infusion (7.5 mg/kg/h) in an adult patient with RSE. The finding of this study suggests that damage was consistent with NMDA receptor antagonist neurotoxicity.

Although ketamine administered in healthy volunteers does not induce seizures [99], and preliminary reports suggest that ketamine may be an efficacious agent for the control of RSE, further studies are needed to establish doses of prolonged ketamine therapy and adverse events at long term.

Felbamate

Felbamate is an NMDA receptor blocker slightly selective for $NR1_A/NR2_B$ subunits and corresponds to the group of the second-generation antiepileptic drugs. Clinical studies have demonstrated its efficacy as polytherapy or monotherapy in the treatment of drug-refractory partial seizures with or without generalization and in absence seizures at doses from 45 to 300 mg/kg for oral administration [100–102]. Besides, in pediatric patients with Lennox-Gastaut syndrome, its administration reduced the frequency of atonic and tonic-clonic seizures in a dose-dependent manner [103]. Reported adverse events are ataxia, sedation, insomnia, dizziness, anorexia, vomiting, nausea, weight loss, diplopia, dysphagia, irritability, and headache [104]. The use of felbamate has been limited because aplastic anemia and hepatic failure have been reported to occur in some patients [105]. Based on these evidences, felbamate should be administered under a careful and strict control of laboratory parameters for hematological and hepatic dysfunctions.

DCPP-ene

In one study, DCPP-ene was tolerated at doses from 10 to 900 mg for oral administration with an elimination half-life of approximately 4 h in healthy volunteers and patients with intractable complex partial seizures [106]. However, seizure number remained unchanged in 50% of patients. One patient showed improvement in the first week, but thereafter DCPP-ene treatment was discontinued because the patient developed RSE. DCPP-ene was discontinued in all patients because they presented adverse effects that included poor concentration, sedation, ataxia, depression, dysarthria, amnesia, and unilateral choreo-athetosis in one patient [106].

Dextromethorphan

Dextromethorphan considerably improved the electrographic activity and stopped seizures in children with drug refractory epilepsy and in one neonatal patient with myoclonic encephalopathy in doses from 20 to 42 mg/kg/day (orally administered by tube) [107, 108]. Also, it has been reported that doses from 6 to 20 mg decrease the frequency and duration of seizures in patients with medically refractory temporal lobe epilepsy for oral administration

[109]. However, increase of epileptiform abnormalities in the EEG without behavioral concomitants has been reported and led to discontinuing of this therapy [107]. To date it is not known whether dextromethorphan aggravates seizures, so more studies are needed to assess side effects and dosage regimen, before being a drug of first choice in the clinic.

Remacemide

In a multicenter study in adult patients with refractory epilepsy, the seizure frequency decreased by 50% during treatment with remacemide at a dose of 800 mg/day (oral administration) as compared to the placebo group [110]. However, when compared to carbamazepine in the treatment of newly diagnosed patients with epilepsy, it was observed that remacemide was less effective [111]. It is absorbed rapidly in the gastrointestinal tract, and reaches plasma peak in 1 h with elimination half-life of 3–4 h [110]. The undesired effects were fatigue, dizziness, nausea, and diplopia [112].

In general, remacemide has shown a good profile of safety and tolerability; however, its effectiveness in the treatment of epilepsy is questionable, and for the moment further evaluation on seizure control is required.

1.2 AMPA Receptors (AMPARs)

These receptors are configured by subunits GluR1–4 and are permeable to K^+, Na^+, and in some cases also Ca^{2+} (Fig. 1). AMPARs are the most abundant ionotropic glutamate receptors in the mammalian brain. These are the predominant mediators of excitatory neurotransmission in the central nervous system. In addition, AMPARs play a pivotal role in seizure generation and spread. The use of AMPAR antagonists broadly protected against seizures in diverse in vitro and in vivo models (Table 1, (Fig. 2)).

1.2.1 Antagonists in Preclinical Models of Epilepsy

Studies of the AMPAR antagonists in experimental models of SE showed that the CNQX was an effective anticonvulsant drug suppressing both electrographic and behavioral seizures induced in rats by electrical stimulation of the perforant path [113]. However, 4 ± 5 h later, electrographic spikes and behavioral convulsions reappeared [113]. Other AMPAR antagonists (NS1209 and IKM-159) blocked SE induced by electrical stimulation of the amygdala, kainic acid, and in vitro models of SE [114, 115].

Competitive AMPAR antagonists such as NBQX, YM872, YM90K, RPR117824, and LY215490 showed neuroprotective and anticonvulsant effects on preclinical models of partial and generalized epilepsy induced by MES, PTZ, kindling, bicuculline, strychnine, and 4-AP [116–122].

On the other hand, the non-competitive AMPAR antagonists such as the GYKI 52466, BIIR561 CL (irampanel), and EGIS-8332 provide potent anticonvulsant protection in models of amygdala-kindling, bicuculline, PTZ, picrotoxin, MES, AMPA-induced seizures, and sound-induced seizures in DBA/2 mice [123–125].

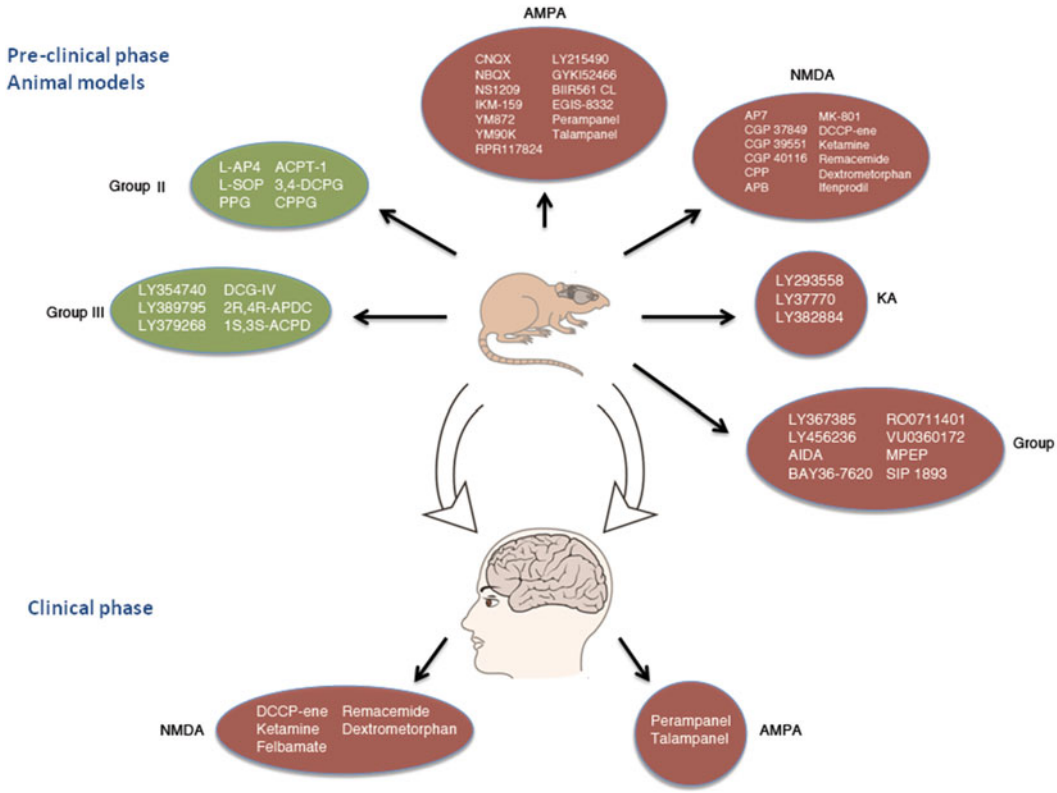


Fig. 2 Drugs with potential effects on ionotropic and metabotropic receptors in preclinical models of epilepsy and clinical trials. Note that clinical trials have only confirmed the effectiveness of ionotropic NMDAR and AMPAR antagonists in epilepsy. However, their use in humans should be contemplated with extreme caution

Other AMPAR antagonists as perampanel (E2007) and talampanel (GYKI 53773, LY-300164) showed to be anticonvulsant and neuroprotective in MES, PTZ, amygdala-kindled, audiogenic, and 6 Hz seizure test models [126, 127]. However, both drugs were inactive in genetic absence epilepsy (GAERS and WAG/Rij models) [126, 127].

Although competitive and non-competitive antagonists have shown neuroprotective, anticonvulsant, and antiepileptic effects in several animal models, the great disadvantage of some of these antagonists is that they are not orally active, only intravenously [128]. Another important limitation of these drugs is their too short half-life, so the administration for therapeutic use must be continuous, causing increasing plasma concentration that might unfavorably influence their safety profile [126]. The main adverse effects reported in animals were motor impairment, sedation, and mild-to-moderate ataxia. Besides they have a weak anticonvulsive activity in genetic models of absence epilepsy [126, 127].

1.2.2 Antagonist Clinical Trials

Despite this promising scenario, only some compounds reach clinical trials. Here, we present the results of two non-competitive AMPAR antagonists talampanel and perampanel.

Talampanel

Talampanel was well tolerated and absorbed in single doses of 3–100 mg for oral administration in healthy volunteers. The mean time to reach peak concentration is approximately 2 h, with an elimination half-life of approximately 6.5 h [129]. The most commonly reported adverse events were dizziness, sedation, and ataxia [129].

In patients with refractory partial seizures with or without secondary generalization, talampanel demonstrated anticonvulsant efficacy as monotherapy and as an adjunctive to antiepileptic drugs (AEDs) reducing seizure frequency [130]. The dose of 50 or 75 mg for oral administration was well tolerated; it was found that peak plasma concentrations were reached from 1 to 3 h, with a mean elimination half-life of 3 h [129]. Despite the anticonvulsive effect in animal models, talampanel in clinical trials showed an inadequate seizure control and short half-life time and, when used in combination with carbamazepine, it increased plasmatic concentration [129]. The adverse events that occurred around plasma peak concentration included mild-to-moderate ataxia, dizziness, headache, and drowsiness [129, 130]. To date talampanel is not used in patients with epilepsy. In other diseases such as glioblastoma and amyotrophic lateral sclerosis additional trials have been realized to evaluate its effect in proliferation and migration in these diseases [131, 132].

Perampanel

More recently, the potent non-competitive AMPAR antagonist perampanel showed favorable pharmacologic properties, good oral bioavailability, safety, and tolerability. In healthy human subjects and in patients with epilepsy it was shown that the time to reach peak concentration was 0.5–2.5 h given once daily through oral administration, with an elimination half-life of approximately 105 h, 70% of which was excreted in the feces and nearly 30% in the urine [133]. Phase II studies in patients with partial-onset seizures showed a reduction in seizure frequency with perampanel as adjunctive therapy with other AEDs in doses from 2 to 12 mg/day for oral administration [133]. Phase III studies showed that a single dose of 12 mg/day for oral administration significantly reduced seizure frequency, and increased responder rate in treatment-resistant patients, which was maintained over 1-year follow-up with good tolerance [134]. The adverse events that occurred were dizziness, headache, drowsiness, and fatigue [133]. Overall, perampanel has a favorable pharmacologic profile for therapeutic use. It is important to consider that extensive half-life be able to induce severe adverse reactions; so far there is no information. It is therefore important that future studies assess possible adverse effects.

1.3 Kainate Receptors (KARs)

KARs are integrated by subunits GluK5-7 and KA1-2 and are permeable to K^+, Na^+, and Ca^{2+} [139]. They act postsynaptically in principal neurons and interneurons, as well as presynaptically to modulate GABA and glutamate release [135–140] (Fig. 1).

KARs have received attention as potential drug targets due to their modulatory role at both postsynaptic and presynaptic levels (Fig. 2).

1.3.1 Antagonists in Preclinical Models of Epilepsy

KAR antagonists such as LY293558, LY37770, and LY382884 showed to reduce epileptiform activity in vitro models of electrical stimulation in hippocampal slices, and to interrupt seizures in vivo in pilocarpine, (RS)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA), and picrotoxin models (Table 1) [141–143]. However, it has not been approved for therapeutic use in humans. The reasons are lack of antibodies for recognizing specific subunits to distinguish KARs from AMPARs and lack of specific agonists and antagonists at both pre- and postsynaptic sites. Several studies have demonstrated an important subset of drugs that antagonize the GluR5 subunit inducing an anticonvulsant and neuroprotective effect [141, 142, 144, 145]. In the future it is essential to identify new anticonvulsant compounds acting on KARs with clinical potential.

2 Metabotropic Receptors

The metabotropic glutamate receptors (mGluRs) are a family of G-protein-coupled receptors that provide important modulatory control of glutamatergic transmission. The mGluRs have proved to have presynaptic and postsynaptic roles. Postsynaptically they modulate membrane properties by second messenger interactions and presynaptically they control synaptic release [146]. Based on signal transduction mechanisms, pharmacological profile, and receptor protein, mGluRs are classified into three groups: group I (mGluR1 and 5), group II (mGluR2 and 3), and group III (mGluR4, 6, 7, and 8) (Fig. 1).

Studies suggest as a role for mGluRs in epileptogenesis and seizure-induced damage; these evidences proposed that mGluRs may be considered a potential target for the treatment for epilepsy [147] (Fig. 2).

2.1 Group I (mGluR1 and mGluR5)

Group I mGluRs are located postsynaptically and act on Gq protein to stimulate phosphoinositide hydrolysis and phospholipase C. These receptors are excitatory, acting to enhance neurotransmitter release, potentiate ionotropic glutamate receptor responses, and modulate various depolarizing currents [148]. The activation of group I by antagonists attenuated neuronal damage and seizures [149].

2.1.1 Antagonists in Preclinical Models of Epilepsy

The treatment with antagonists of mGluR1 (LY367385, LY456236, AIDA, and BAY 36-7620) has shown anticonvulsant effects and has reduced neuronal damage in generalized convulsive seizure (sound seizure in DBA/2 mice and tonic seizures in PTZ), limbic seizure (6 Hz focal seizure model, amygdala kindled), and absence-like seizure models (Table 2) [150–153]. However, the antagonist LY456236 did not inhibit clonic seizures produced by PTZ [152].

On the other hand, studies in genetic models of absence seizures (WAG/Rij rats model) have shown that systemic treatment with positive allosteric modulators of mGlu1 or mGlu5 (RO0711401 and VU0360172) reduces spontaneous-occurring spike-and-wave discharges in these animals (Table 2) [154, 155].

Otherwise, the antagonist mGluR5 (MPEP) inhibits spike-and-wave discharges in sound-induced seizures in DBA/2 mice [156]. In addition, other mGluR5 antagonist, the MTEP, has anticonvulsant effect in models of PTZ and hippocampal stimulation in 12- and 18-day-old rats. However, it did not induce anticonvulsant effect in 25-day-old animals [157, 158].

On the other hand, SIB 1893, a non-competitive antagonist of mGluR5, combined with sub-effective doses of oxcarbazepine reduced seizure and after-discharge durations in amygdala-kindling model [159]. This evidence suggests that these antagonists of group I metabotropic receptors could be developed as new antiepileptic drugs, particularly for patients that are refractory to current medication.

2.2 Group II (mGluR2 and mGluR3) and Group III (mGlu4, mGluR6, mGluR7, and mGluR8)

Groups II and III are expressed primarily at presynaptic level and bind to Gi/Go protein to inhibit cAMP. They generally act to reduce glutamate release, and may also inhibit high threshold calcium channels, activate potassium channels, and directly inhibit transmitter release machinery. Both groups have anticonvulsant and neuroprotective effects in preclinical models of epilepsy.

2.2.1 Group II (mGluR2 and mGluR3)

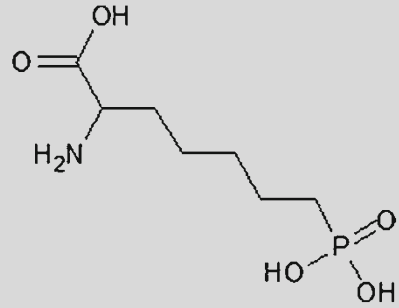
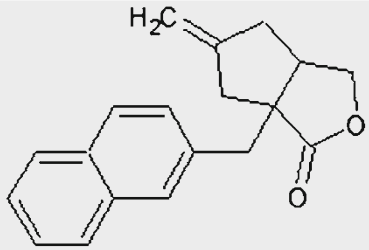
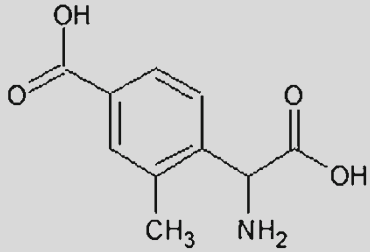
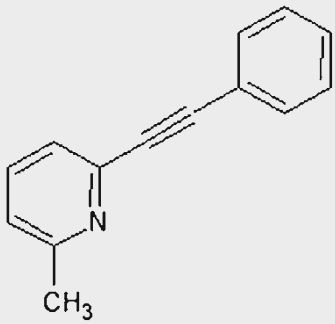
Agonists in Preclinical Models of Epilepsy

Group II mGluR agonists such as LY354740, LY389795, LY379268, DCG-IV, and 2R,4R-APDC have proved anticonvulsant and neuroprotective effects in models of limbic and generalized motor seizures such as amygdala-kindled seizures, PTZ, picrotoxin, kainic acid model, pilocarpine, and ACPD-induced model (Table 2) [160–165]. LY354740 inhibited spontaneous epileptiform discharges which developed in rat Mg^{2+}-free medium cortical slices, and it potentiated the anticonvulsant activity of diazepam in PTZ. However, it had no effect on NMDA-induced convulsions [162].

In genetic models, LY389795 and LY379268 inhibited sound-induced clonic seizures in DBA/2 mice, and absence seizures in lethargic lh/lh mice [165]. However, neither of these agonists inhibited sound-induced seizures in GEP rats nor absence seizures

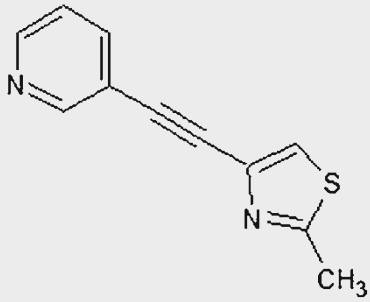
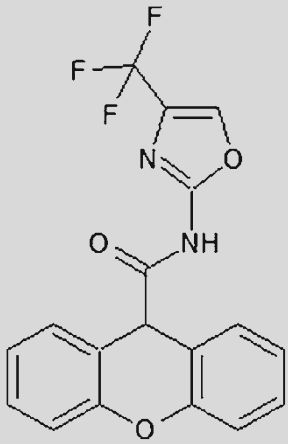
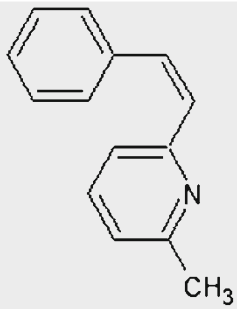
Table 2

Molecular structure of agonists and antagonists of metabotropic glutamate receptors

Structure	IUPAC name	Traditional name
Group I (mGluR1 and mGluR5)		
	2-Amino-7-phosphonoheptanoic acid	AP-7
	(3a*S*,6a*S*)-5-Methylidene-6a-[(naphthalen-2-yl)methyl]-hexahydro-1H-cyclopenta[c]furan-1-one	BAY-367620
	(*S*)-(+)-A-amino-a-methylbenzeneaceticacid	LY-367385
	2-Methyl-6-(phenylethynyl)pyridine, Hydrochloride	MPEP

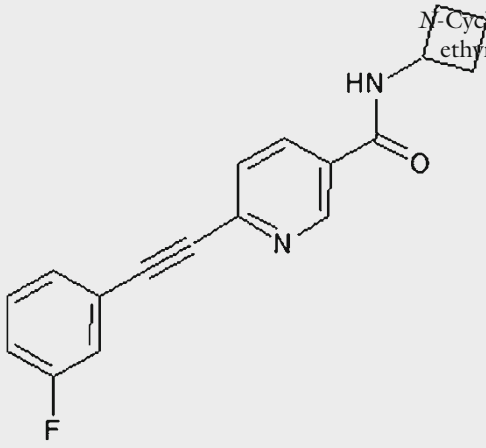
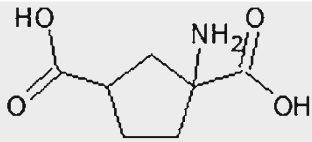
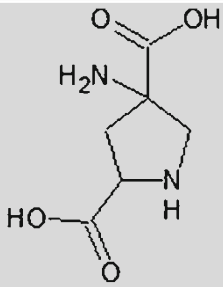
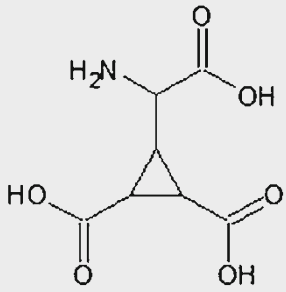
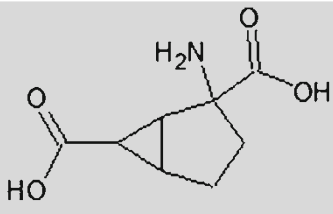
(continued)

Table 2
(continued)

Structure	IUPAC name	Traditional name
	3-[2-(2-Methyl-1,3-thiazol-4-yl)ethynyl] pyridine hydrochloride	MTEP
	*N*-[4-(Trifluoromethyl)-1,3-oxazol-2-yl]-9H-xanthene-9-carboxamide	Ro0711401
	2-Methyl-6-[(*E*)-2-phenylethenyl]pyridine	SIB-1893

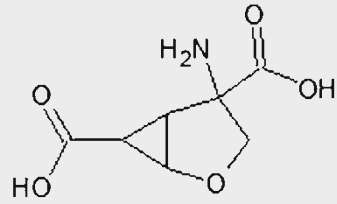
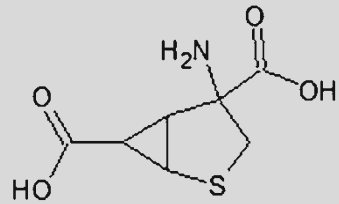
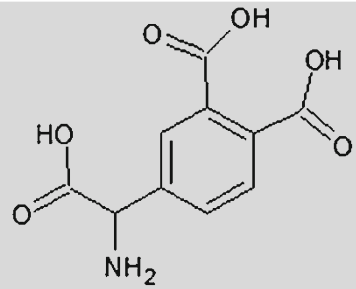
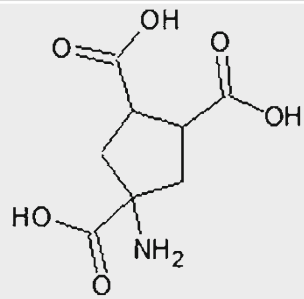
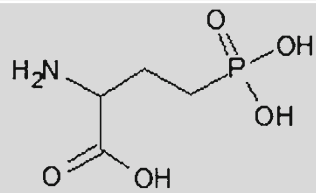
(continued)

Table 2
(continued)

Structure	IUPAC name	Traditional name
	*N*-Cyclobutyl-6-[2-(3-fluorophenyl) ethynyl]pyridine-3-carboxamide	VU0360172
Group II (mGluR2 and mGluR3)		
	(1*S*,3*S*)-1-Aminocyclopentane-1,3-dicarboxylic acid	1S,3S-ACPD
	(2*R*,4*R*)-4-Amino-2,4-pyrrolidinedicarboxylic acid	2R,4R-APDC
	(2*S*,2′*R*,3′*R*)-2-(2′,3′-Dicarboxycyclopropyl)glycin	DCG-IV
	(1*S*,2*S*,5*R*,6*S*)-2-Aminobicyclo[3.1.0] hexane-2,6-dicarboxylic acid	LY354740

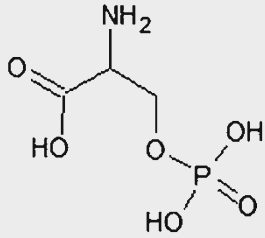
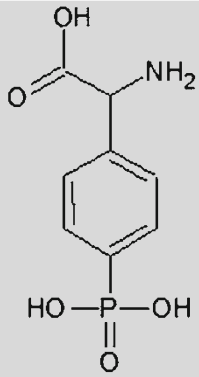
(continued)

Table 2
(continued)

Structure	IUPAC name	Traditional name
	(1*S*,2*R*,5*R*,6*R*)-2-Amino-4-oxabicyclo[3.1.0]hexane-2,6-dicarboxylic acid	LY379268
	(4*S*,6*S*)-4-Amino-2-thiabicyclo[3.1.0]hexane-4,6-dicarboxylic acid	LY389795
Group III (mGlu4, mGluR6, mGluR7, and mGluR8)		
	3,4-Dicarboxyphenylglycine	3,4-DCPG
	(1*S*,3*R*,4*S*)-1-Aminocyclopentane-1,3,4-tricarboxylic acid	ACPT-1
	(2*S*)-2-Amino-4-phosphonobutanoic acid	L-AP4

(continued)

Table 2
(continued)

Structure	IUPAC name	Traditional name
	L-Serine-*O*-phosphate	L-SOP
	(*RS*)-4-Phosphonophenylglycine	PPG

ACD/ChemSketch 11.0 was used to draw the molecules

in WAG/Rij rats [165, 166]. On the other hand, the 1S,3S-ACPD suppressed sound-induced seizures in GEP rats when injected directly into the inferior colliculus [167].

2.2.2 Group III (mGlu4, mGluR6, mGluR7, and mGluR8)

Agonists in Preclinical Models of Epilepsy

The agonists L-AP4 and L-SOP are antiepileptogenic and anticonvulsant in experimental models of amygdala- and PTZ-induced kindled as well as the limbic seizures induced by 3,5-DHPG (Table 2) [168–171].

Preferential agonists of mGluR8 such as PPG and 3,4-DCPG and the agonist of mGluR4 ACPT-1 have shown anticonvulsant effect against sound-induced seizures in DBA/2 mice and MES (Table 2) [165, 172–174]. L-SOP gives only partial protection in sound-induced clonic seizures in DBA/2 mice [172]. However, it suppressed all phases of sound-induced seizures in GEP rats when injected into the inferior colliculus [167].

PPG, ACPT-1, and L-AP4 have neuroprotective effects against kainic acid and NMDA-induced excitotoxicity in cultured cortical and hippocampal neurons [174–176], while PPG and 3,4-DCPG showed anticonvulsant effects against seizures induced by bilateral intracerebroventricular infusion of DL-homocysteic acid in immature 12-day-old rats [177, 178].

Although preclinical data positively support the therapeutic potential of mGluR ligands in epilepsy they have not been tested in clinical trials. One reason is that some agonists (L-AP4 and L-SOP) may also induce proconvulsant effects [179]. These have been observed depending not only on the animal model of epilepsy used but also on timing and dosage of the drug delivery.

Dietrich et al. [180] reported that surgical specimens of hippocampi of epileptic patients with sclerosis showed loss of function of the L-AP4 agonist. This study suggests that the loss of inhibition mediated by group III mGluRs is an important factor contributing to hyperexcitability in hippocampus of patients with epilepsy [180].

The systemic injection of the selective mGlu4 receptor-positive allosteric modulator PHCCC enhanced the number of spike-wave discharges in WAG/Rij rats. These data support the hypothesis that activation of mGluR4 receptors participates in the generation of absence-type seizures which can be exacerbated with the use of a positive allosteric modulator [154]. Moreover, mGluR4 antagonist CPPG significantly attenuated the PTZ-induced absence seizures [181].

Future research should focus more on the specific physiological and pathological conditions under which each receptor subtype participates, which may be the key for fully understanding their therapeutic potential.

3 Conclusion

The development of drugs that target ionotropic and metabotropic glutamate receptors has recently gained new interest for its potential use in epilepsy. The efficacy of these drugs in preclinical models is evaluated by their anticonvulsive, antiepileptic, and neuroprotective effects on (1) generalized tonic-clonic seizures, (2) partial or focal seizures, (3) convulsive and non-convulsive SE, and (4) genetic epilepsy. Clinical trials have only confirmed the effectiveness of ionotropic receptor antagonists (NMDARs and AMPARs) in epilepsy. However, their use in humans should be contemplated with extreme caution due to their unfavorable pharmacokinetic profile, efficacy, adverse effects, and tolerability.

Regarding drugs that target metabotropic receptors, they have not been approved yet for clinical trials. The main problems are that some of them are not orally active, lack of specific agonists and antagonists, there are not enough studies in animal models of the adverse effects, short duration of action, and the fact that they have induced proconvulsant effects in some animal models. It is important to consider that at present, there is insufficient evidence that alterations in mGluRs have a role in pharmacoresistance in patients with epilepsy. We are currently studying mGluR functional activity and transduction cascade in surgically resected tissue of patients with pharmacoresistant epilepsy. These studies will allow us to

investigate the influence of clinical factors on the mGluRs, and may provide the scientific basis for these alterations in epilepsy, thus leading to know whether they can be used as a therapeutic target to treat epilepsy in the future. In conclusion, great progresses have been achieved and the recent results focus again attention on these compounds as therapeutic targets in epilepsy.

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Chapter 7

Neurosteroid Regulation of Seizures: Role of $GABA_A$ Receptor Plasticity

Suchitra Joshi and Jaideep Kapur

Abstract

Neurosteroids, which are derived from adrenal or gonadal steroid hormones or synthesized de novo from cholesterol in the brain, regulate neuronal excitability. Neurosteroids suppress seizures, and this action is particularly important in women with epilepsy, who may experience seizure exacerbation due to hormonal fluctuations associated with their menstrual cycle, called catamenial epilepsy. Neurosteroids exert their anticonvulsant action by increasing $GABA_A$ receptor-mediated fast inhibitory neurotransmission. However, the $GABA_A$ receptors expressed in epilepsy have altered subunit composition, such that higher concentrations of neurosteroids are required to enhance the inhibition. Despite advances in our understanding of the neurosteroid regulation of seizures via modulation of $GABA_A$ receptors, our knowledge of whether endogenous neurosteroid synthesis is altered by seizures or in epilepsy is incomplete. Furthermore, the molecular mechanisms that trigger the down-regulation of neurosteroid-sensitive $GABA_A$ receptors in epilepsy are not well understood. Insights into these aspects of neurosteroids and $GABA_A$ receptors could provide novel targets for the development of therapies aimed at a better management of seizures in women with epilepsy.

Key words Neurosteroids, $GABA_A$ receptor, Epilepsy, Catamenial epilepsy

1 Introduction

The pioneering work of Seyle (1942) first demonstrated the anesthetic and anticonvulsant actions of reproductive and adrenal steroid hormones [1]. Neurosteroids, a term used to describe neuroactive derivatives of steroid hormones [2], have since been a focus of intense research. The discovery of an interaction between neurosteroids and γ-aminobutyric acid type-A ($GABA_A$) receptors provided a molecular target for the action of neurosteroids in the brain [3, 4]. Kokate and colleagues later discovered that the rank order potency of the anticonvulsant action of neurosteroids was similar to their potency for activating $GABA_A$ receptors [5]. The anticonvulsant action of neurosteroids through modulation of $GABA_A$ receptors has been extensively studied in clinical settings and in experimental animals. In this chapter, we review the advances

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in our understanding of the neurosteroid regulation of seizures, with a particular focus on the neurosteroid control of seizures in women with epilepsy.

2 Neurosteroid Synthesis in the Brain

Allopregnanolone (THP, 3α 5α-tetrahydroprogesterone) and tetrahydrodeoxycorticosterone (THDOC) are the two major neurosteroids; they are derived from gonadal and adrenal steroid hormones and can readily cross the blood-brain barrier (Fig. 1). However, circulating steroid hormones are not the only source for neurosteroids in the brain; neurosteroids were found to be present in the brain of animals that underwent adrenalectomy and gonadectomy or after pharmacological suppression of the adrenal and gonadal secretions [6, 7]. Furthermore, neurosteroid levels in the brain also exhibited a circadian cycle that was unrelated to the steroid levels in the blood [8]. These findings indicate that the brain may be able to synthesize neurosteroids from a precursor other than the circulating steroid hormones. Subsequent studies have indeed established that the brain is not only a target of neurosteroids but also a neurosteroidogenic organ (Fig. 1) [9, 10].

De novo synthesis of neurosteroids in the brain utilizes cholesterol as a precursor. The enzyme StAR or translocator protein (TSPO), also called as peripheral benzodiazepine receptor, is located on the outer mitochondria membrane and transports cholesterol from the cytoplasm to the inner mitochondrial compartment [11–13]. The enzyme P450 side-chain cleavage then converts cholesterol to pregnanolone in the first and rate-limiting step of neurosteroid biosynthesis [14]. The subsequent enzymatic reactions occur in the cytoplasm, where pregnanolone is first converted to progesterone by the enzyme 5β-hydroxysteroid dehydrogenase and then to THP via an intermediate compound, dihydroprogesterone. The enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase regulate the conversion of progesterone to dihydroprogesterone and then to THP, respectively. Circulating steroid hormones can also be used in this biosynthetic pathway to synthesize neurosteroids.

The neurosteroid synthetic enzymes are expressed in the brain across amphibians, birds, and mammals [9]. Immunohistochemical studies and in situ hybridization analyses have revealed a CNS-wide distribution of the mRNA and/or protein of these enzymes beginning from the early postnatal age [9, 15–20]. Thus, brain neurosteroid synthesis occurs throughout the life-span of an animal. Excitatory neurons in the cortex, hippocampus, amygdala, thalamus, and hypothalamus express the enzymes involved in neurosteroid synthesis [16, 18–25]. Interestingly, GABAergic interneurons in the hippocampus are devoid of neurosteroid synthetic enzymes [26]. Thus, there may be an autocrine mechanism to regulate the excitability of the principal neurons.

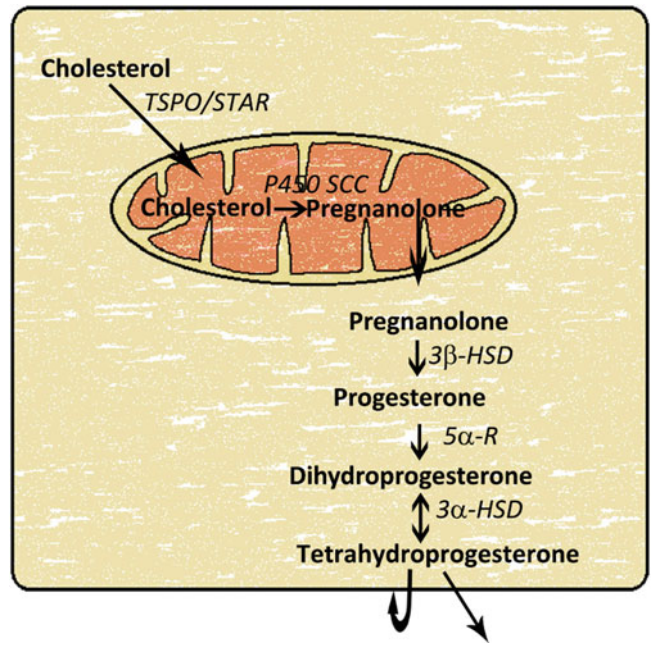

Fig. 1 A schematic representation of endogenous neurosteroid synthesis in the brain from cholesterol. Cholesterol is transferred from the cytoplasm to the inner mitochondrial membrane by StAR (steroidogenic acute regulatory protein), also called as translocator protein (TSPO), or peripheral benzodiazepine receptors. In the inner mitochondrial membrane, cholesterol, in a rate-limiting step, is converted to pregnanolone by the activity of the enzyme cytochrome P450 side-chain cleavage (P450scc). Pregnanolone is then converted to progesterone in the cytoplasm by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). In the subsequent reactions, progesterone is converted to dihydroprogesterone by the enzyme 5α-reductase (5α-R). This is the second rate-limiting step in neurosteroid synthesis from cholesterol. Finally, dihydroprogesterone is converted to tetrahydroprogesterone (THP), also called allopregnanolone, by the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD). The enzyme 3α-HSD has bidirectional activity and can also convert THP to dihydroprogesterone. Allopregnanolone then can activate $GABA_A$ receptors expressed in the same cell or surrounding cells

3 Neurosteroid Modulation of $GABA_A$ Receptors

3.1 *$GABA_A$ Receptors*

Neurosteroids are potent modulators of $GABA_A$ receptors, which are ligand-gated chloride channels that mediate inhibitory neurotransmission in the brain and spinal cord [3, 27–32]. These pentameric receptors are composed of membrane-spanning subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, π, ε, and θ) that are encoded by 19 genes [33–35]. A majority of the receptors are composed of 2 α, 2 β, and a γ, δ or ε subunits [35]. The receptors containing a γ subunit are generally, but not exclusively, expressed at the synapses [36–39], whereas γ subunit-lacking receptors, such as those containing a δ

subunit, are exclusively extrasynaptic [38, 40]. There is also a region- and neuron-specific preferential subunit assembly; for example, the δ subunits expressed on hippocampal DGCs, thalamic neurons, and cerebellar granule neurons assemble with the α4 and α6 subunits [41–43]. In contrast, the δ subunit-containing $GABA_A$ receptors expressed on hilar interneurons contain the α1 subunits [44, 45].

The subunits of $GABA_A$ receptors consist of a large "N" terminal extracellular domain, four transmembrane domains (TM1-4), and a small extracellular "C" terminal region. The TM2 domains line the chloride channel, and the sites for binding to GABA and benzodiazepines are present in the "N" terminal extracellular loop [46, 47]. The neurosteroid-binding sites are located within the transmembrane domains of the α and β subunits [48] and are distinct from those for GABA, benzodiazepines, and barbiturates. Neurosteroids exhibit an enantio-selectivity in activating $GABA_A$ receptors, which indicates the stringent structural requirements of the neurosteroid-binding site [49]. Furthermore, due to their lipophilic nature, neurosteroids can access the binding site either from inside the cell or through lateral diffusion across the membrane [50]. Neurosteroids have a dual mode of action on $GABA_A$ receptors: in nanomolar concentrations, found under physiological conditions, neurosteroids exert an allosteric effect on GABARs, whereas at micromolar concentrations, neurosteroids act like an agonist [51]. The residues T236 and Q241 on the α1 subunits are proposed to bind to neurosteroids; T236 is involved in direct activation, whereas Q241 is involved in potentiation and activation [48]. A third site, which is yet not fully characterized, is involved in the interaction with sulfated steroids [52], which generally reduce $GABA_A$ receptor-mediated inhibitory neurotransmission [53–55].

Studies using recombinant receptors have revealed that neurosteroids act on receptors composed of many different subunit combinations; however, certain subunits confer higher neurosteroid sensitivity on the $GABA_A$ receptor [51, 56–58]. For example, the δ subunit-containing receptors have a higher neurosteroid sensitivity than other $GABA_A$ receptor subtypes [57, 59], and certain behavioral effects of neurosteroids are diminished in mice lacking δ subunit expression [60]. Thus, neurosteroids regulate the excitability of hippocampal DGCs, thalamic neurons, and cerebellar granule neurons, which express high levels of the δ subunit-containing $GABA_A$ receptors.

Neurosteroids increase the conductance of $GABA_A$ receptors. In cultured spinal cord neurons, two conductance states of $GABA_A$ receptors were found to be present based on the properties of single-channel recordings: a main conductance state and a smaller sub-conductance state [61]. In the presence of neurosteroids, channel openings to the main conductance state were more frequent. Neurosteroids also enhance the GABA efficacy of δ subunit-containing receptors [62].

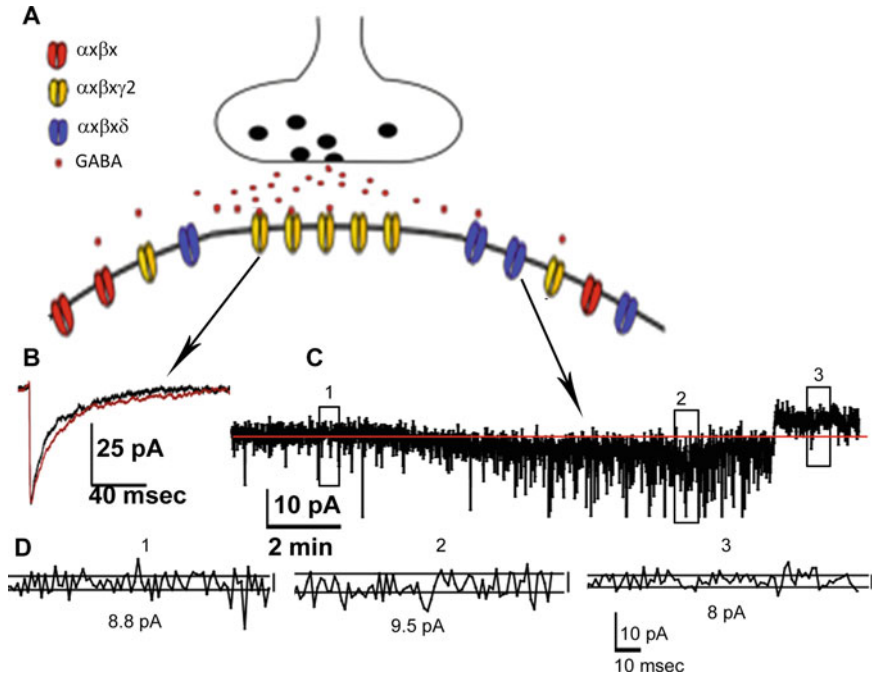

Fig. 2 A schematic representation of the neurosteroid modulation of synaptic and extrasynaptic $GABA_A$ receptor-mediated inhibition (**a**). The synaptic γ2 subunit-containing $GABA_A$ receptors mediate rapid phasic inhibition in response to GABA released from the presynaptic terminal. This type of current can be recorded as sIPSCs or mIPSCs. Neurosteroids at physiological concentrations prolong the decay of synaptic current (**b**). The decay of averaged mIPSCs recorded from hippocampal DGCs is prolonged after (*red*) application of 10 nM allopregnanolone compared to the decay before allopregnanolone application (*black*). The extrasynaptic $GABA_A$ receptors mediate the tonic current in response to GABA spilled from the synaptic cleft or released by glia. The tonic current is typically recorded in terms of shifts in the holding current or as membrane noise (RMS noise) (**c**), which is enhanced following application of agents that activate the receptors and reduced by agents that block the receptors. Please note the increase in holding current following bath application of allopregnanolone (10 nM, *arrow*, THP). Furthermore, application of $GABA_A$ receptor antagonists such as picrotoxin (50 μM) blocked the synaptic currents and reduced the holding current. The traces at the bottom show RMS noise from areas 1, 2, and 3 on the current trace; note the increase in the RMS noise from 8.8 to 9.5 pA following allopregnanolone application and the reduction in the RMS noise to 8 pA following picrotoxin application (**d**)

3.2 Neurosteroid Modulation of Synaptic and Tonic Inhibition

Neurosteroids modulate both the fast synaptic and slow tonic inhibition. The synaptic $GABA_A$ receptors mediate a fast inhibition in response to release of GABA from the presynaptic terminal (Fig. 2). On the other hand, extrasynaptic $GABA_A$ receptors mediate a slow persistent background inhibition called tonic inhibition [63, 64] and play an important role in regulating the excitability [65, 66]. The tonic inhibition is typically measured as the change in holding current in response to drugs that modulate $GABA_A$ receptors. Another measure of tonic current is the change in membrane noise (root mean square noise, RMS noise), which reflects opening and closing of the channels, in response to $GABA_A$ receptor-modulatory

drugs [64, 65, 67]. The application of low nanomolar concentrations of neurosteroids prolongs the decay of mIPSCs. Similar concentrations of neurosteroids also enhance the holding current and RMS noise, indicative of augmentation of tonic current (Fig. 2).

The effect of neurosteroids on synaptic and tonic currents has largely been studied using exogenous application of known concentrations of THP or THDOC. The way that endogenous neurosteroids may shape the GABAergic inhibition is not well understood. Keller and colleagues treated acutely isolated spinal cord slices with finasteride to block endogenous neurosteroid synthesis [68]. The decay of mIPSCs recorded from lamina II neurons of finasteride-treated slices was shorter than that recorded from control slices [68]. These findings revealed that endogenously synthesized neurosteroids indeed increase the total charge transfer by $GABA_A$ receptors.

3.3 Effect of Neurosteroids on the Expression of $GABA_A$ Receptors

In addition to the acute effects of neurosteroids, prolonged exposure to neurosteroids changes the expression of $GABA_A$ receptors [69, 70]. Estrus cycle-linked hormonal fluctuations influence δ subunit expression; high progesterone levels are associated with increased δ subunit expression and vice versa, whereas the expression of γ2 subunit follows an opposite trend [71, 72]. The alterations in δ subunit expression were blocked by finasteride [73]. The expression of α4 subunits on the other hand was increased following neurosteroid withdrawal via mechanisms involving Egr3 [74]. Changes in the reproductive hormones and neurosteroid levels at the onset of puberty, during pregnancy, and postpartum also induce alterations in $GABA_A$ receptor expression [75, 76]. The expression of the δ subunit increases at late stages of pregnancy, whereas that of the γ2 subunit decreases [75]. The expression of the δ and γ2 subunits returns to the basal levels postpartum, whereas that of the α4 subunit increases [75]. The long-term use of contraceptives also increases the expression of the γ2 subunit [77], which could influence seizure management in women with epilepsy.

3.4 Reduced Neurosteroid Modulation of $GABA_A$ Receptors in DGCs of Epileptic Animals

The hippocampus is a major site of seizure generation and propagation [78]. Hippocampal DGCs are highly inhibited and act as a "gate" to prevent the spread of activity into the hippocampus proper, which consists of intrinsically excitable neurons of the CA1–CA3 subfields [79, 80]. An impaired $GABA_A$ receptor-mediated inhibition of DGCs could compromise the dentate "gating" function and recruit the hippocampus proper [81, 82].

The GABAergic inhibition of DGCs plays an important role in maintaining the "gating" function of DGCs; however, the efficacy of GABAergic inhibition of DGCs is reduced in epilepsy. The whole-cell GABA-evoked currents of DGCs were found to be larger in epileptic animals [83, 84], the amplitude of sIPSCs and mIPSCs enhanced [85–87], and the tonic current unaltered [39, 88]. However, the neurosteroid modulation of GABA-evoked whole-cell currents, synaptic currents, and tonic current was

diminished in DGCs of epileptic animals [39, 83, 85, 88]. Nanomolar concentrations of allopregnanolone prolonged the decay of mIPSCs recorded from DGCs of naïve animals, but not in the DGCs of epileptic animals [85]. In addition, a fraction of hilar interneurons was also lost in epileptic animals [89–92]. In association with the reduced neurosteroid sensitivity of $GABA_A$ receptors expressed on DGCs of epileptic animals, there was a loss of neurosteroid control of the excitability of DGCs [45].

The diminution in neurosteroid modulation of synaptic and tonic currents is due to alterations in the expression and characteristics of $GABA_A$ receptors in experimental animal models and [39, 45, 88, 93–98] in human epilepsy [99–101]. Most prominently, the expression of the α1 and δ subunits is reduced at the mRNA and protein levels in the DGCs of epileptic animals [39, 45, 88, 93–95]. Immunohistochemical studies have revealed that the expression of the δ subunit decreases rapidly following status epilepticus and remains low throughout during the period of recurrent spontaneous seizures [39, 45, 88, 94, 95]. Because the tonic current regulates neuronal excitability, this early reduction in δ subunit expression likely contributes to the breakdown of the dentate gating function. In contrast to the reduced δ subunit expression on DGCs, the δ subunit immunoreactivity on interneurons was shown to be upregulated following status epilepticus, and the tonic current was augmented [45, 102]. Taken together, the alterations in δ subunit expression following status epilepticus likely increase the excitability of DGCs while simultaneously reducing the excitability of interneurons. The molecular mechanisms triggering the down-regulation of δ subunit expression following status epilepticus are not fully understood. We have found that the activation of NMDA receptors and increased phosphorylation of ERK1/2 reduced δ subunit expression in cultured hippocampal neurons [103]. NMDARs are activated [104] and the phosphorylation of ERK1/2 is also increased during status epilepticus [105–107], and they may contribute to the subsequent reduction in δ subunit expression.

The expression of α1 subunits, which are present in a majority of synaptic receptors expressed on DGCs, was also shown to be reduced in epileptic animals [45, 93–95], and this reduction was triggered by activation of the JAK/STAT pathway [98]. Furthermore, the reduced α1 subunit expression may contribute to the development of the seizure phenotype, as preventing α1 subunit down-regulation suppressed epileptogenesis [93].

There is a compensatory upregulation of α4 and γ2 subunit expression in DGCs of epileptic animals [45, 88, 98]. Electron microscopic studies have revealed increased expression of α4γ2 subunit-containing $GABA_A$ receptors in DGCs of epileptic animals [39, 85]. We have also found that a larger fraction of α4 subunits co-precipitated with the γ2 subunits in DGCs of epileptic animals

[88], indicative of altered subunit assembly. However, the α4γ2 subunit-containing $GABA_A$ receptors have lower affinity to neurosteroids than α1γ2 or α4δ subunit-containing receptors, and the compensatory changes are not sufficient to restore the neurosteroid modulation of synaptic and tonic currents [39, 85, 88]. The upregulation of α4 subunits in epilepsy is triggered by mechanisms involving the transcription factor Egr3 [108]. Thus, although α4βxδ and α1βxγ2 subunit-containing receptors are expressed in naïve animals, the expression of the partnering subunits is differentially regulated and novel receptors with reduced neurosteroid affinity are expressed in epileptic animals [39, 85, 88, 98].

It is important to note that the reduction in δ subunit expression following status epilepticus occurs before the compensatory changes are seen. Thus, there may be a period of heightened susceptibility following status epilepticus. Studies using voltage-sensitive dyes have found that the "gating" function of DGCs is transiently compromised in slices obtained from post-status epilepticus animals [81]. However, it is unlikely that the "gating" function of DGCs is fully restored in vivo following status epilepticus.

In addition to the differential alterations in the expression of $GABA_A$ receptor subunits, phosphorylation of $GABA_A$ receptors also appears to influence neurosteroid modulation. In one study, inhibition of PKC or PKA reduced the ability of neurosteroids to prolong the decay of mIPSCs recorded from CA1 pyramidal neurons [109], whereas in another study, phosphorylation of the β3 subunit by PKC diminished the neurosteroid modulation of GABAergic inhibition [110].

There may also be a feedback regulation of $GABA_A$ receptor phosphorylation by neurosteroids. Neurosteroids trigger the phosphorylation of the α4 subunit residue S443 by PKC and increase the cell surface stability of $GABA_A$ receptors [111]. We have shown that the kinetics of trafficking of the δ and γ2 subunit-containing receptors are distinct and that BDNF and PKC activation may play a role in regulating the surface expression of δ subunit-containing $GABA_A$ receptors [112, 113]. Thus, stimuli that change the protein kinase activity/expression may also influence the surface expression of $GABA_A$ receptors and in turn affect their neurosteroid modulation.

4 Neurosteroid Regulation of Seizures

4.1 Seizure Suppression by Neurosteroids

Progesterone and its neurosteroid derivative allopregnanolone exert anticonvulsant actions [114–117]. Finasteride blocks the seizure suppression by progesterone [118], and the anticonvulsant effects of progesterone are abolished in animals that do not express the enzyme 5α-reductase [119]. In contrast, finasteride did not affect the efficacy of allopregnanolone to suppress seizures [118].

These findings indicate that the anticonvulsant effects of progesterone are mediated through neurosteroids. The neurosteroid suppression of seizures has been confirmed in multiple animal models, in which treatment with progesterone, allopregnanolone, or THDOC blocked single seizures or prolonged seizures of status epilepticus (SE) evoked by chemoconvulsants such as PTZ, picrotoxin, bicuculline, kainic acid, and pilocarpine, as well as seizures triggered by electrical stimulation [5, 120–127]. Treatment with progesterone and neurosteroids also increased the threshold for chemical or electrical kindling [128–132]. Thus, neurosteroids can efficiently suppress limbic seizures.

Neurosteroids also regulate the occurrence of recurrent spontaneous seizures, which are a hallmark of epilepsy. Neurosteroid treatment is effective in suppressing recurrent spontaneous seizures. In one study, treatment with ganaxolone controlled antiepileptic drug-withdrawal-induced seizures in adult patients [133] and infantile spasms in pediatric patients [134]. In another study, ganaxolone was effective in treating drug refractory seizures in pediatric and adult patients [135].

4.2 Catamenial Epilepsy

Neurosteroid regulation of recurrent spontaneous seizures is exemplified in women with epilepsy. A third of women of reproductive age with epilepsy may experience clustering of seizures, a condition called catamenial epilepsy [114, 115, 136–141]. Catamenial seizures are most prevalent in focal epilepsy, but are also observed in symptomatic, idiopathic, or cryptopathic epilepsy with or without a focus [139, 142, 143].

The levels of progesterone and estrogen fluctuate during the menstrual cycle; progesterone is secreted by the corpus luteum, and its levels remain high until the end of the menstrual cycle. On the other hand, estrogen levels peak mid-cycle and decrease following ovulation. Based on the period in which seizure exacerbation is observed, catamenial seizures can be divided into three types [140, 141]. In type I catamenial epilepsy, which is the most prevalent form, seizures cluster during the perimenstrual period, which is caused by the declining progesterone levels. On the other hand, seizure exacerbation in type II catamenial epilepsy occurs around ovulation. These seizures are triggered by high levels of estrogen, which typically exert proconvulsant effects [114, 115, 144–146]. Type III catamenial seizures are observed in women with anovulatory cycles and can occur mid-cycle or at the end of the cycle. Further, in women with epilepsy, the seizure frequency can change during puberty and pregnancy and around menopause [147–150]. We propose that these changes occur due to alterations in the endogenous hormonal milieu and the $GABA_A$ receptor plasticity. Seizures are more frequent when progesterone levels decrease in women with epilepsy [151, 152]. Similarly, in experimental animals, serum progesterone or allopregnanolone levels have an inverse

correlation with seizure frequency [71, 153]. Rats in proestrus/estrus stages have higher allopregnanolone levels compared to rats in the diestrus stage, and the susceptibility to kainate-induced seizures is higher in animals in the diestrus stage [153].

In order to obtain insight into the mechanisms of catamenial seizures, Reddy and Rogawsky developed an animal model of catamenial epilepsy [115, 154–156]. In this model, circulating progesterone levels were elevated by administration of pregnant mare's serum gonadotropin (PMSG) followed by human chorionic gonadotropin (β-HCG), and a neurosteroid withdrawal condition was induced by finasteride, an inhibitor of the enzyme 5α-reductase [154]. Finasteride-treated animals were more susceptible to PTZ-induced seizures compared to the animals that were not treated with finasteride or animals that did not receive PMSG and β-HCG. The heightened seizure susceptibility following finasteride treatment was also observed in the kindling of PMSG- and β-HCG-treated animals [157]. Thus, formerly elevated progesterone levels were necessary to observe finasteride-induced seizure precipitation.

These studies had some limitations. The effect of neurosteroid depletion on spontaneous seizures was not determined in these studies. Second, the $GABA_A$ receptors expressed in the hippocampi of epileptic animals have lower neurosteroid affinity; thus, a reduction in neurosteroid levels in epileptic animals is likely to have greater effects on neuronal excitation than those observed in naïve animals. Finally, Reddy and colleagues determined the effect of neurosteroid withdrawal only under the condition of high progesterone levels. Neurosteroids are synthesized in the brain independent of circulating progesterone, but the above study did not determine the effect of depleting de novo-synthesized neurosteroids on seizures. To address these issues, we determined the role of endogenous neurosteroid synthesis in the regulation of recurrent spontaneous seizures in epileptic animals (Fig. 3) [158]. Finasteride-induced neurosteroid withdrawal caused a dramatic increase in the frequency of spontaneous seizures in female epileptic animals. The increase in seizure frequency was more pronounced in animals with elevated progesterone levels than in animals in which progesterone levels were not elevated or maintained at a lower range. Administration of allopregnanolone blocked the increase in seizure frequency, indicating that endogenous neurosteroids or neurosteroids synthesized from circulating progesterone kept seizures suppressed [158]. In agreement with the findings in epileptic animals, an anecdotal study has revealed neurosteroid withdrawal-triggered seizure exacerbation in a woman with epilepsy after the start of finasteride treatment for male-pattern hair loss [159].

The findings in experimental animals led to a central hypothesis that catamenial seizures occur because of the development of tolerance to progesterone followed by a withdrawal. The question

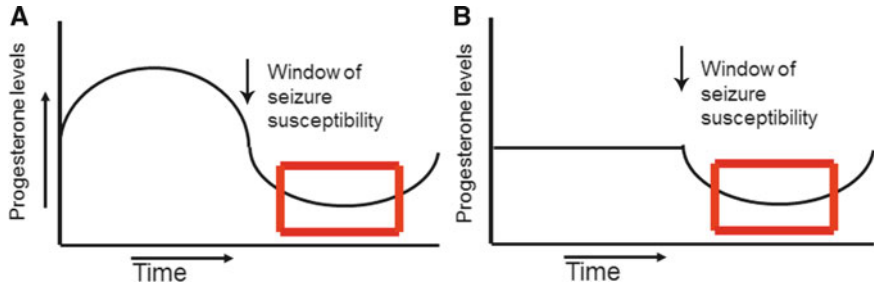

Fig. 3 Administration of finasteride (marked by *downward arrow*) to female epileptic animals either with elevated progesterone levels (**a**) or with maintained low progesterone levels (**b**) induces neurosteroid withdrawal and creates a window of increased seizure susceptibility (*red box*). The seizure precipitation following finasteride was more drastic in animals with elevated progesterone levels compared to that in animals with low progesterone levels [158]

of whether supplementing progesterone exogenously to prevent the decrease that occurs during the perimenstrual period suppression of catamenial seizures was addressed. In the early clinical trials progesterone treatment appeared to regulate catamenial seizures [152, 160, 161]. However, in a recent NINDS-supported phase III multicenter, double-blind clinical trial, progesterone was no more effective than placebo in controlling either catamenial or non-catamenial seizures [162]. A post hoc analysis suggested that there may be seizure suppression in women with type I catamenial epilepsy, in which seizure precipitation occurs due to a decrease in progesterone levels during the perimenstrual period [163]. The reasons behind the failure of this clinical trial could be multi-fold. First, the expression of $GABA_A$ receptors, which are the targets of neurosteroids, is altered in epilepsy such that receptors with lower affinity to neurosteroids are expressed. Second, progesterone can also exert additional effects on the brain through activation of progesterone receptors [164]. The effects of progesterone receptor activation on seizures and the plasticity of neurotransmitter receptors are not well understood.

It is noteworthy that androgens also regulate seizures through modulation of $GABA_A$ receptors [165, 166]. Testosterone derivatives can suppress seizures evoked by chemoconvulsants such as PTZ, pilocarpine, and 4-amino pyridine or by electrical stimulation [121, 167]. In one clinical study, testosterone treatment reduced the frequency of seizures in an individual with post-traumatic epilepsy [168]. Our preliminary studies showed that the blockade of endogenous neurosteroid synthesis also increases the frequency of spontaneous seizures in male epileptic animals [117]. Thus, although the neurosteroid regulation of seizures is pronounced in females due to cyclic hormonal fluctuations, the ongoing neurosteroid synthesis also regulates the seizure frequency in male epileptic animals.

4.3 Neurosteroid Regulation of Epileptogenesis

In acquired epilepsy, spontaneous seizures develop as a consequence of an inciting stimulus, such as febrile seizures, status epilepticus, or brain trauma [169–171]. Preliminary studies in experimental animals suggest that neurosteroids may play a role in suppressing epileptogenesis, the process through which a normal brain is transformed into an epileptic brain [172–174]. Status epilepticus is a commonly used epileptogenic stimulus in experimental animals, and blockade of neurosteroid synthesis following status epilepticus accelerated the development of spontaneous seizures [172]. These preliminary findings suggest that endogenous neurosteroids may resist epileptogenesis or suppress the seizure phenotype. Neurosteroid synthesis may be increased following status epilepticus, as there is a transient increase in the expression of the enzyme cytochrome P450scc in the hippocampi of animals that experience pilocarpine-induced status epilepticus [172]. However, further studies are warranted to characterize the changes in endogenous neurosteroid synthesis and to determine brain neurosteroid levels following status epilepticus to understand whether endogenous neurosteroid synthesis is altered during epileptogenesis.

4.4 Stress and Seizures

Because stress hormones are precursors for some neurosteroids, they may also affect seizures. In experimental animals, acute stress was shown to elevate neurosteroid levels in the brain and plasma [7, 124, 175] and upregulate the expression of enzymes involved in neurosteroid synthesis [176]. In accordance with an anticonvulsant action of stress steroids, exposure to acute stress exerts anticonvulsant action and elevates the seizure threshold [124, 177–179]. Stress also increases the levels of THP and upregulates the expression of 5α-reductase and cytochrome P450scc in the fetal brain [180]. However, in contrast to the anticonvulsant effects of acute stress observed in adult animals, exposure to early life stress appears to enhance the vulnerability to limbic epileptogenesis in adulthood [181, 182]. The secretion of corticosterone, which can alter the homeostatic plasticity, may explain these long-term effects of early life stressors.

In contrast to the protective effect of acute stress observed in naïve animals, stress is a self-reported factor associated with increased seizure frequency in epilepsy patients [183–185]. Altered expression of $GABA_A$ receptors in epilepsy may explain the differential effects of stress on the naïve brain vs. the effects on an epileptic brain.

5 Conclusions

Neurosteroids are synthesized in the brain and exert an anticonvulsant action in an autocrine manner. The rapid effects of neurosteroids on the brain are mediated by increasing $GABA_A$ receptor-mediated inhibitory neurotransmission. The diminished

neurosteroid sensitivity of $GABA_A$ receptors in epilepsy leads to a predisposition to hypersynchrony. The reduced expression of δ subunit-containing $GABA_A$ receptors in epilepsy is of particular significance because these receptors are a target of neurosteroids at low nanomolar concentrations and regulate neuronal excitability. Neurosteroid regulation of seizures is manifested in women with epilepsy who are at a risk of experiencing catamenial seizure exacerbation. The mechanisms underlying catamenial seizure exacerbation involve cyclic fluctuations in the neurosteroid levels and the plasticity of the $GABA_A$ receptors. However, our knowledge of the mechanisms that regulate the expression and/or function of neurosteroid synthetic enzymes, particularly in epilepsy and following exposure to an epileptogenic stimulus, is incomplete. Understanding the molecular mechanisms that regulate the expression of neurosteroidogenic enzymes under pathophysiological conditions may open up novel avenues for therapeutic interventions. Furthermore, studies are also needed to understand the mechanisms that regulate the expression and surface membrane trafficking of the δ subunit-containing $GABA_A$ receptors, such that strategies can be devised to block the reduction in their expression in epilepsy.

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Chapter 8

Erythropoietin as Potential Neuroprotective and Antiepileptogenic Agent in Epilepsy and Refractory Epilepsy

Amalia Marelli, Liliana Czornyj, Luisa Rocha, and Alberto Lazarowski

Abstract

Erythropoietin (EPO) can mediate neuroprotective effects by limiting the damage and death of cells that are still alive. This effect depends on the activation of both receptor to EPO (EPO-R), which undergoes a classic dimeric conformation called $(EPO\text{-}R)_2$, and the EPOR-β common receptor (βCR). The interaction between EPO and EPO-R can prevent and repair tissue damage induced by hypoxia and neuroinflammation. The current chapter is focused on presenting the information necessary to support the hypothesis that EPO administration and/or EPO-R activation can represent a new therapeutic strategy to prevent the development of pharmacoresistant epilepsy after hypoxic events. This pharmacological effect can prevent the overexpression of multidrug resistant proteins, particularly P-glycoprotein (P-gp), which is an event induced by hypoxia and producing refractory epilepsy. The administration of high doses of EPO could also reduce the brain damage that results from seizure activity seen in the epileptogenic process subsequent to status epilepticus and perhaps avoids sudden unexpected death in epilepsy (SUDEP).

Key words Refractory epilepsy, P-glycoprotein, EPO receptor, Erythropoietin

1 Hypoxia

Oxygen is the chemical element that is most vital to the human body. In the brain, the interstitial oxygen levels range from approximately 1 to 5% [1]. The concept of ischemia, which is derived from the Greek words "isceiu" (brake) and "aima" (blood), was introduced by Virchow R. in 1858 to explain an imbalance between tissue demand for oxygen and blood flow [2] and its consequences. The final stage of this condition is associated with a loss in energy efficiency secondary to an altered mitochondrial aerobic metabolism. In contrast, hypoxia is a state in which there is insufficient oxygen available in the blood and/or in tissues due to an inadequate transport of this gas to the tissues [3].

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The brain has a very strong demand for oxygen and glucose to maintain its structural and functional integrity. To function normally, the brain depends on a continuous and adequate supply of oxygen. If the delivery of oxygen to the brain is sufficiently compromised, individuals can present with loss of consciousness (within seconds), neuronal death (within minutes), and conditions such as coma, seizures, cognitive impairment, and other neurological disabilities (longer periods). Hypoxia can result in significant dysfunction of sensitive brain areas such as the hippocampus [4].

The cascade of events activated by hypoxia begins immediately in response to energy deprivation and may induce neurotoxicity via the overstimulation of glutamate receptors, the excessive accumulation of intracellular Na^+ and Ca^{2+}, mitochondrial damage, and eventual neuronal death in the areas that undergo ischemia. In addition, the surrounding area undergoes inflammation and subsequent apoptotic death within the few hours and in the subsequent days following the ischemic episode [5].

Hypoxia also induces changes that facilitate the depolarization of brain cells within milliseconds and results in changes in neurotransmitters [6, 7]. Indeed, hypobaric hypoxia has been associated with alterations in dopaminergic and glutamatergic neurotransmission [8, 9] and a reduction in $GABA_A$ receptor binding [10], which can modify neuronal excitability.

As an adaptive response, hypoxia leads to the activation of hypoxia-inducible factor 1 (HIF-1), which is involved in the expression of transcriptional mechanisms to maintain cellular and systemic oxygen homeostasis. The targets of HIF-1 include genes that code for proteins implicated in vasomotor control, angiogenesis, erythropoiesis, iron metabolism, proliferation mechanisms, energy metabolism, and cell death. HIF-1 is a heterodimeric transcription factor that typically consists of the constitutively expressed 120 kDa HIF-1α subunit and the 91–94 kDa HIF-1β subunit. The other molecules termed HIF-2α and HIF-3α can also form a complex with HIF-1β. In the brain, HIF-1α expression appears to be induced by hypoxia in neurons, astrocytes, ependymal cells, and possibly endothelial cells. HIF-2α is expressed in glia and capillary endothelial cells but not in neurons [11, 12].

The expression of the HIF-1 protein is regulated by the proteolysis of its α subunit *via* oxygen-dependent proteolysis. Under normoxic conditions, the binding of the von Hippel-Lindau tumor suppressor (VHL) protein to the oxygen-dependent degradation domain activates the ubiquitin-proteasome pathway and the subsequent degradation of HIF-1α. This event is regulated by three different isoenzymes of the (HEME) proteins, termed prolyl-hydroxylase domain enzymes (PHD1-3). These proteins are characterized by a highly conserved common C-terminal domain Fe^{2+}-dioxygenase, which is also known as factor-inhibiting HIF-1 (FIH-1). Under hypoxic conditions, the activity of PHDs is inhibited. The HIF-1α that accumulates dimerizes with HIF-1β

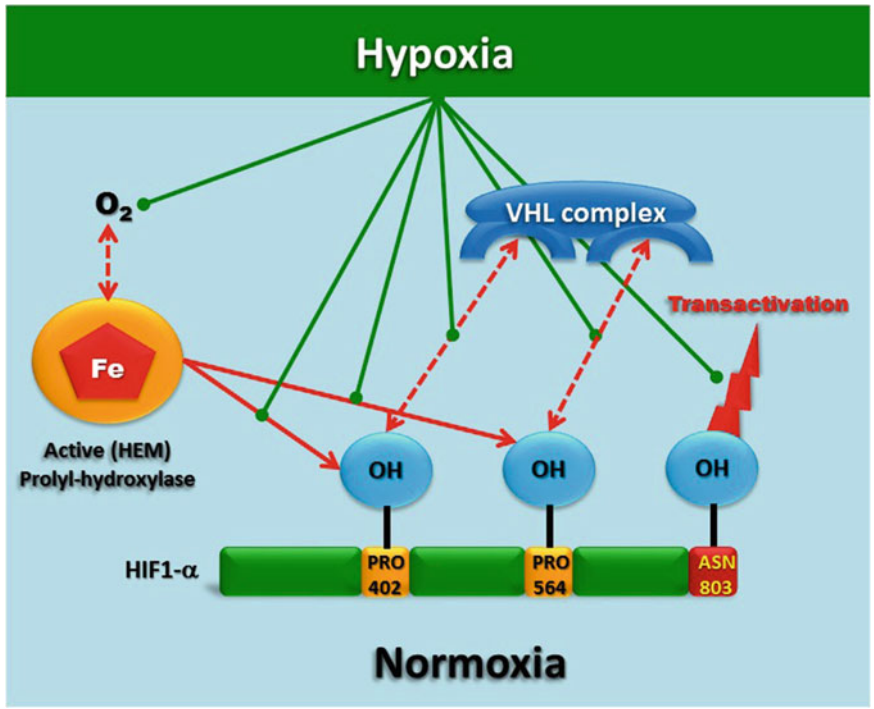

Fig. 1 Under normoxic conditions, HIF prolyl-hydroxylases induce the hydroxylation of the α subunits of HIF at proline residues 402 and 564. This event allows their identification and ubiquitination by the von Hippel-Lindau (VHL) complex and their subsequent degradation. Under hypoxic conditions, these effects are blocked because of the inhibition of HIF prolyl-hydroxylase due to the low levels of oxygen. Additional hydroxylation of the 803-asparaginyl group by factor-inhibiting HIF (FIH) blocks its binding to transcriptional coactivators

and activates the transcription of more than 100 target genes whose protein products are involved in critical developmental and physiological processes, metabolic adaptation, and cellular anti-apoptosis [13, 14] (Fig. 1).

2 Hypoxia and Erythropoietin (EPO)

Erythropoietin (EPO) is a glycoprotein composed of 165 amino acids (30.4-kDa). EPO has two disulfide bridges linking cysteines 7–161 and cysteines 29–33 and has a peptide core and four carbohydrate chains that are important for its stability in the peripheral circulation [15]. EPO is primarily produced in hepatocytes during the fetal period. In adults, EPO is produced in the interstitial fibroblasts of the kidney. EPO acts on its receptor (EPO-R), which is normally expressed in the erythroid precursor cells in the bone marrow [16]. Under certain circumstances, nucleated cells of different organs such as the liver, spleen, lung, testis, and brain can express EPO and EPO-R [17, 18].

Cloned in 1985 [19] and approved by the FDA in 1989, human recombinant EPO has been demonstrated to be safe and is highly effective in treating anemia in adults and children with renal failure, cancer, and prematurity [20]. In the bone marrow, EPO regulates the differentiation of red blood cells by inhibiting the apoptosis of erythroid progenitors, which becomes more evident under conditions of hypoxia [15]. At present, the EPO/EPO-R system is known to be activated by hypoxia in several organs [21–24] and induces protective effects [25]. The molecular regulation of EPO expression under hypoxic conditions is primarily mediated by the action of HIF-1α and HIF-1β [26]. However, under chronic conditions of hypoxia, HIF-2α is able to activate the EPO gene, though only in conjunction with other regulatory sequences in the vicinity of the "HIF-responsive-elements" in the DNA promoter region [27, 28].

Previous studies have indicated that the administration of EPO in patients with renal anemia results in the improvement of cognition, which is commonly assumed to be a consequence of the physiological increase in oxygenation that is secondary to the higher levels of hemoglobin [29–32]. At present, it is known that EPO acts directly on the brain; in particular, high levels of EPO and EPO-R have been observed in hypoxia-sensitive cerebral regions such as the hippocampus and telencephalon [33, 34]. In the brain, EPO is highly expressed in astrocytes and has low expression in neurons, whereas EPO-R is expressed in endothelial cells, microglia, astrocytes, oligodendrocytes, and neurons. EPO is also believed to act in both an autocrine and paracrine manner [35].

EPO is considered to have neuroprotective and therapeutic effects in different neurological disorders as stroke, ischemia, multiple sclerosis, schizophrenia, retinopathy, Parkinson's disease, brain trauma, spinal cord injury, attention deficit hyperactivity disorder, and epilepsy [20, 36–38]. The neuroprotective effects induced by EPO are achieved at concentrations between 0.01 and 10 U ml^{-1} [39–41]. However, experimental evidence indicates that under repetitive or severe hypoxic conditions, it is necessary to administer high doses of exogenous EPO to prevent secondary damage caused by neuroinflammatory responses [42].

The neuroprotection induced by EPO has been associated with the following effects in the CNS: (1) anti-glutamate and trophic effects (dendritic branching; axonal outgrowth; synaptic activity); (2) effects on neuronal progenitors that facilitate neurogenesis and migration through the activation of neurotrophic factors; (3) antioxidant activity; (4) antiapoptotic activity; (5) anti-inflammatory activity; (6) BBB protection and angiogenesis; (7) the maintenance of intact genomic DNA; and (8) the modulation of microglia [43–45] (Fig. 2).

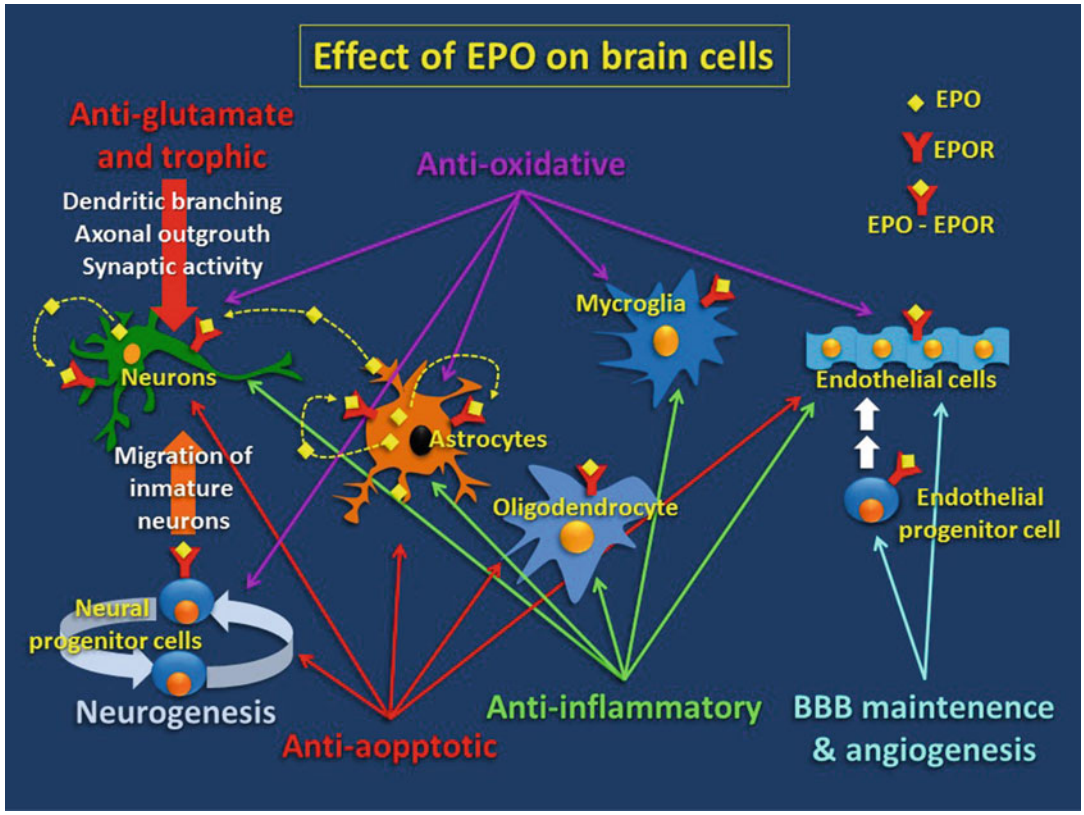

Fig. 2 Representation of the six most important effects of EPO in brain cells. All brain cells express EPO-R depending on the rate of maturation and on the conditions of hypoxia or inflammation

3 Epilepsy, Pharmacoresistance, and Hypoxia

Epilepsy is the second most common chronic neurological disorder after stroke, with a high incidence in the first decade of life and after the age of 60 years. Different types of epilepsy are recognized by the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE) [46]. Epilepsy is a consequence of different conditions, such as genetic factors, inherited epileptic channelopathies, alterations in neurotransmitters, metabolic disorders, brain malformations, brain tumors, and cerebral trauma [47]. At present, antiepileptic drugs (AEDs) are the first line of treatment for controlling seizures in many patients with epilepsy. However, approximately 35 % of patients present with seizures are resistant to therapy [48].

Repetitive seizures are known to augment the expression of P-glycoprotein (P-gp) in the BBB. This condition is associated with a pharmacoresistant phenotype because P-gp extrudes AEDs from the brain into the blood [49–52]. At the neuronal level, P-gp has been associated with membrane depolarization and seizure activity as a consequence of increasing amounts of intracellular free

radicals and Ca^{2+} [53]. P-gp overexpression is also associated with changes in plasma membrane characteristics. Indeed, cells with P-gp overexpression have low plasma membrane electrical potential (from –10 to –20 mV), which facilitates neuronal depolarization and the reduced (~30%) binding of drugs. These changes can also modify both the distribution of lipids and the target availability, which could have additional important consequences associated with the drug resistance phenotype [54–57]. According to these observations, therapies targeting the inhibition of P-gp function have been suggested to control pharmacoresistant epilepsy [58] and induce neuroprotection [59].

Patients with refractory epilepsy are also known to have a high risk of presenting with Sudden Unexpected Death in Epilepsy (SUDEP) [60]. In addition to the changes in the brain that are associated with pharmacoresistant epilepsy, these patients may show left ventricular dysfunction and signs of heart ischemia [61, 62]. Previous studies from our group have indicated that the repetitive seizure activity is associated with P-gp overexpression in the heart, which may result in changes in cardiomyocyte function and a high predisposition to SUDEP [63].

The enhanced glutamatergic neurotransmission induces P-gp overexpression at the BBB and in neurons *via* pro-inflammatory pathways; this process depends on cyclooxygenase-2 (COX-2) [64]. Experimental evidence indicates that both seizure activity and hypoxia enhance glutamate release, and facilitate excitotoxicity, membrane depolarization, and subsequent epileptic seizures [65–68]. In contrast, the clinical conditions that facilitate epilepsy (status epilepticus, stroke, and neonatal hypoxia) are also associated with hypoxia [69–71]. For example, during status epilepticus, cerebral oxygenation decreases phasically as a consequence of decreased cerebral blood flow and arterial blood pressure [72]. Indeed, the duration of the hypoxic-ischemic condition during seizure activity can exacerbate the subsequent brain damage [73].

The idea that seizure activity represents a condition that produces acute brain hypoxia and ischemia is well recognized [74, 75]. In relation to this finding, seizure activity in experimental models and in patients with pharmacoresistant epilepsy is known to induce the overexpression of factors associated with hypoxia such as the vascular endothelial growth factor (VEGF) a HIF-1α response gene [76–79]. Furthermore, the overexpression of HIF-1α and VEGF in neurons has been proposed to be a mechanism that induces neuroprotective effects in epilepsy [80].

Hypoxia induces alterations similar to what is observed in epilepsy (Fig. 3). Hypoxic conditions also lead to the overexpression of the MDR-1 gene that encodes P-gp [81]. Similarly, a progressive increase in cerebral P-gp expression is induced by intermittent hypoxia [82]. Previous studies from our group have indicated that MDR-1 gene overexpression in the brain is induced by two different experimental conditions of brain hypoxia: cortical devascularization

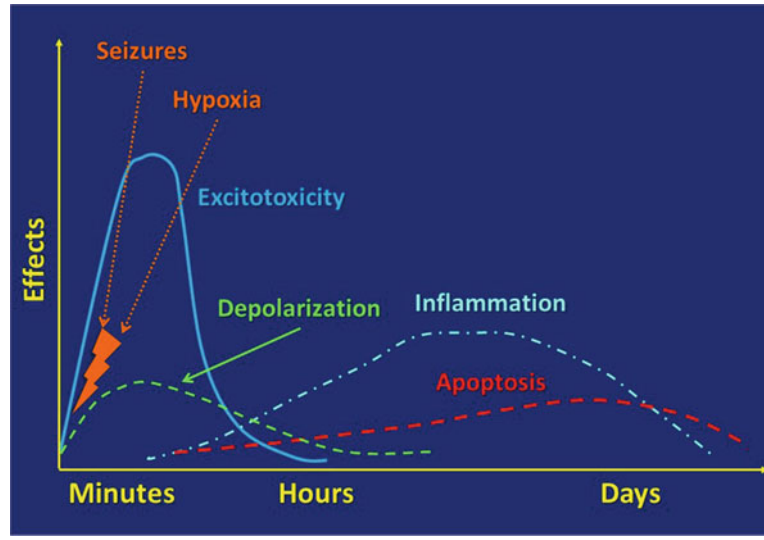

Fig. 3 Time course of different events seen in the brain during hypoxia and seizure activity

and cerebral $CoCl_2$ injection [83, 84]. However, the neuroinflammatory process that occurs secondary to cerebral hypoxia also induces the expression of MDR-1 gene with persistent P-gp overexpression, which is probably associated with the previous activation of HIF-1α. According to these findings, the activation of HIF-1α may contribute to neuroinflammation and pharmacoresistance subsequent to seizure activity [85, 86].

4 Effects of EPO on Seizure Activity, Pharmacoresistant Epilepsy, and SUDEP

EPO administration significantly suppresses pentylenetetrazol-induced seizures as well as the status epilepticus and epileptogenesis induced by pilocarpine [87–89]. Additionally, treatment with recombinant human Epo (rHu-EPO) decreases neuronal damage and apoptosis in the hippocampus and induces cardioprotection subsequent to status epilepticus. Several of these effects are mediated by the activation of the JAK2/STAT5 pathway [90, 91]. According to these observations, EPO has been suggested to represent a new strategy for preventing epilepsy and its consequences in susceptible individuals [92]. However, other studies have indicated that EPO augments neuronal activity via the activation of Ca^{2+} channels [93] and increases free cytosolic concentrations of Ca^{2+} [94]. It is possible that the anticonvulsant and neuroprotective effects induced by EPO are produced under specific conditions of cerebral excitability (Fig. 4).

Using an experimental model of focal brain hypoxia induced by $CoCl_2$, we found an overexpression of P-gp, EPO-R, and HIF-1α in the ischemic brain regions [84], whereas the intranasal

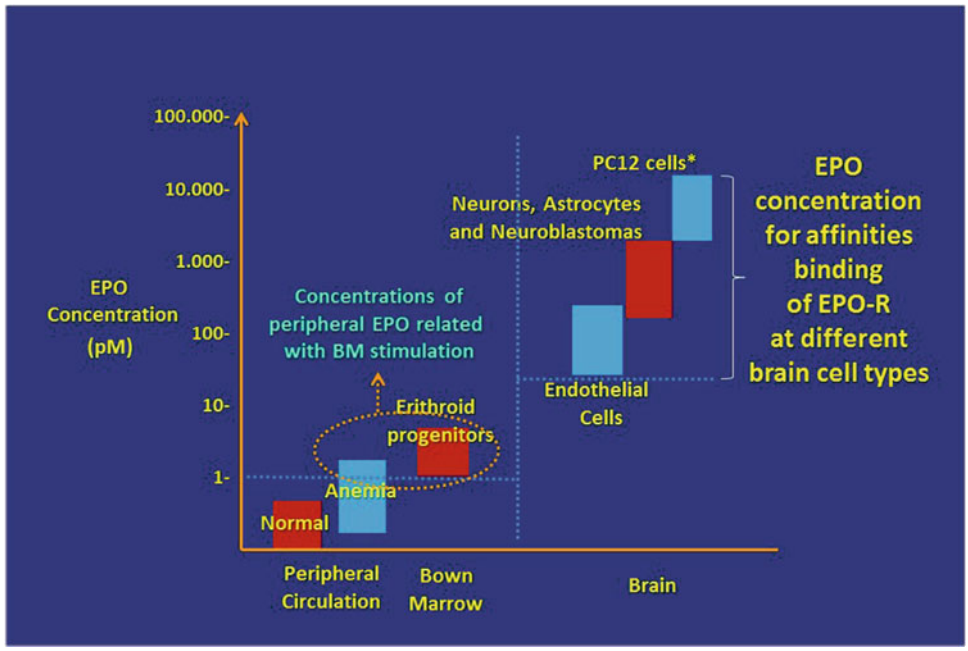

Fig. 4 Representation of the different concentrations of EPO needed to activate peripheral or bone marrow (BM) cells *vs.* brain cells. PC12 cells are adrenal pheochromocytoma cells that may differentiate into cells that resemble sympathetic neurons when cultured in the presence of factors such as nerve growth factor (NGF)

administration of rHu-EPO induced neuroprotective effects with a high score of recovery of motor function in this model [37, 95, 96]. The intranasal administration of EPO was also found to protect cardiomyocytes during heart ischemia [97] as well as neurons and cardiomyocytes in epileptic rats [98]. It is possible that the protective effects induced by EPO improve with the overexpression of EPO-R and the transductional modulation of microglia, Akt1, Bad, and caspases [45].

The protective effect induced by EPO in ischemic events has been associated with a reduction in glutamate-mediated excitotoxicity as a consequence of reduced glutamate release from synaptic vesicles [99, 100]. This event may also reduce seizure susceptibility [101]. However, additional experiments are necessary to support this hypothesis.

5 Does EPO Represent a Neuroprotective Therapeutic Strategy to Prevent Pharmacoresistance in Epilepsy?

The neuroprotective effects of EPO are associated with its interaction with kinases. The activation of EPO-R induces the phosphorylation of Janus tyrosine kinase 2 (JAK-2), thereby inhibiting

apoptosis and ultimately fostering the survival and maturation of erythrocytes. The β common receptor (βCR), also called "tissue-protective EPO receptor," activates JAK-2, which subsequently engages secondary signaling pathways associated with mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and nuclear factor-kB (NF-kB) [42] (Fig. 5). The neuroprotective effects induced by EPO can also be mediated by the activation of the βCR/JAK2/Akt signaling pathway. Indeed, the inhibition of JAK2 activation or Akt abolishes the protective effects mediated by EPO [102].

The "signal transduction and activator of transcription 5a and 5b" (STAT5) pathways that induce the expression of various anti-apoptotic genes are involved in βCR signaling. However, experimental evidence indicates that the STAT5 pathways are not essential for neuroprotection by EPO [103, 104]. The activation of pro-survival protein kinases by βCR signaling also inhibits mitochondrial permeability, which is a major event in cell death [105] (Fig. 5).

To determine whether the exogenous administration of EPO can be considered a therapeutic strategy to induce neuroprotection and avoid pharmacoresistant epilepsy and other cerebral disorders, it is important to analyze different situations. First, different studies have suggested that P-gp overexpression is a consequence of enhanced glutamatergic neurotransmission, which is also evident

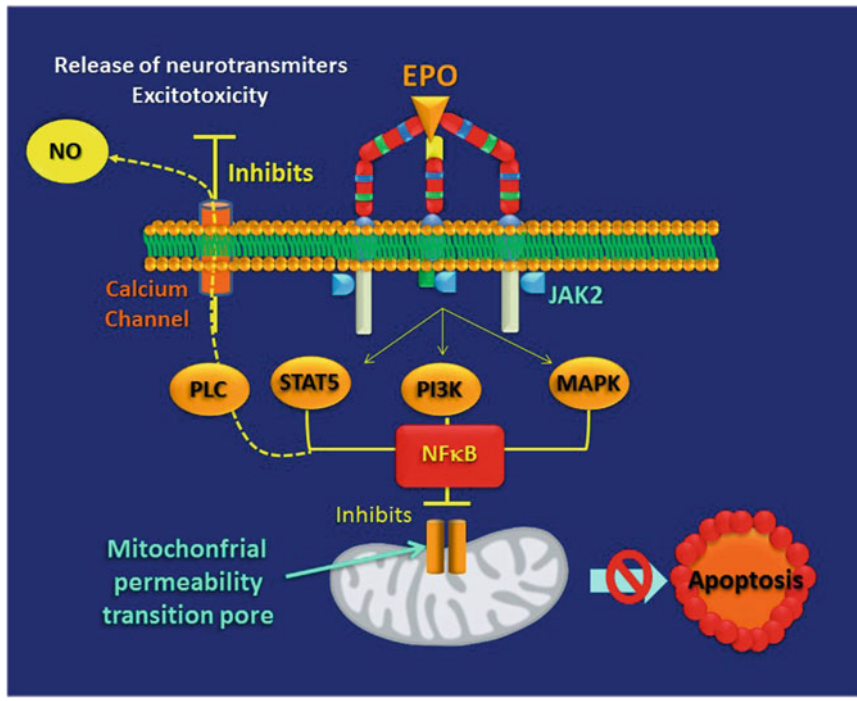

Fig. 5 Representation of the transductional mechanisms involved in the neuroprotective effects induced by EPO

in hypoxia and neuroinflammatory events [106–108]. Therefore, the neuroinflammation and hypoxia caused by stroke, cerebral hypoxia, and status epilepticus (causing continuous hypoxic stress) may contribute to the overexpression of P-gp and facilitate pharmacoresistant epilepsy [109].

In contrast, the activation of EPO-R has been suggested to induce neuroprotection by reducing the release of glutamate through the modulation of voltage-insensitive calcium channels *via* phospholipase C [100]. In addition, EPO reduces the mRNA and protein expression of pro-inflammatory molecules (HIF-1α, iNOS, COX-2, and caspase-9). According to these findings, EPO administration and EPO-R activation may reduce the neuroinflammatory processes induced by seizure activity [110, 111] and prevent P-gp overexpression, which is a key event in pharmacoresistant epilepsy. It is also important to determine whether intranasal EPO administration prevents new seizures and the development of pharmacoresistant epilepsy after brain hypoxia-ischemia insults. In addition, it is essential to elucidate whether the function of EPO-R is modified as a consequence of acute neurological disorders associated with hypoxia.

6 Conclusions and Remarks

While is true that further studies are necessary to support this hypothesis, all of these considerations strongly suggest that the hypoxia associated with repetitive seizures can induce the overexpression of HIF-1α, EPO-R, and P-gp in the heart and brain. P-gp overexpression may be related to a higher risk of developing pharmacoresistant epilepsy and/or SUDEP. The administration of rhEPO or any EPO-modified moiety without hematopoietic effects can represent a new therapeutic strategy to prevent these conditions. Finally, the intranasal administration of rhEPO could also induce a protective effect in the brain at the beginning of the epileptogenesis process without causing systemic side effects.

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Chapter 9

Caloric Restriction and Dietary Treatments of Epilepsy: Mechanistic Insights for Drug Discovery

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Abstract

Based on the observation that fasting reduces the number and severity of convulsive seizures, a few different eating regimes have emerged to treat epilepsy. These are the ketogenic diet, medium-chain triglyceride regime, modified Atkins diet and low glycemic index treatment, all of which have been used clinically. These nutritional procedures are effective and have few adverse collateral actions. A related diet is caloric restriction, which can be accomplished by several methods. Caloric restriction has been shown to possess anticonvulsive and, most importantly, antiepileptogenic properties. But there are no clinical studies exploring if caloric restriction by itself reduces the number and severity of seizures. However, there is some data suggesting that caloric restriction improves the efficacy of the ketogenic and modified Atkins diets. Its mechanism(s) of action remain(s) mysterious, although several possibilities have been suggested. Each of its proposed mechanisms of action can be targeted for the discovery of drugs that prevent or modify epilepsy. This chapter briefly describes every one of the food modifications in current clinical use or that has been studied using animal models. Much evidence indicating that caloric restriction induced by food restriction or intermittent fasting is antiepileptic or antiepileptogenic is discussed. The possible mechanisms of action of the ketogenic diet and caloric restriction are presented in short. Lastly, a brief method for performing a type of caloric restriction is detailed.

Key words Epilepsy, Epileptogenesis, Ketogenic diet, Medium-chain triglyceride regime, Modified Atkins diet, Low glycemic index treatment, Intermittent fasting, Caloric restriction, Prevention/prophylaxis

1 Introduction

It has been known for a long time that seizures can be treated by fasting. Scientific investigation of the effects of short-term starvation and diet changes for the treatment of epilepsy started in the twentieth century. Since then lots of data have been accumulated showing that most eating regimes designed to effectively reduce the frequency and severity of convulsions have a relatively benign side effect profile [1–4].

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At least one of these diets, the ketogenic diet, could actually prevent or cure epilepsy by modifying its generation (antiepileptogenic effect), since it has long-term disease alteration properties in animals and humans [5–7]. Moreover, there is evidence that this same nutritional procedure might enhance the efficiency of anticonvulsive medications [8–10]. In most patients, these food modifications are started after antiseizure drugs, which can be decreased (in number or dose) without problems [4, 11, 12]. This likely improves the quality of life by reducing adverse drug side effects and suggests that no medications would have been needed if the nutritional procedure was started sooner. Indeed, there is evidence indicating that early dietary treatment is an effective option for the management of convulsions [12–14]. Thus, there should be more studies, especially in the clinical setting and at the earliest possible opportunity, for a possible nonpharmacological treatment of epilepsy with different eating regimes.

1.1 Ketogenic Diet (KD)

Although since ancient times it is known that fasting reduces the number and severity of seizures, it was not until the early twentieth century that a diet was introduced to mimic its effects. This was the KD; which is rich in fats, low in carbohydrates and adequate in proteins [15, 16]. Typically, a 4 to 1 ratio of fats to carbohydrates plus proteins is used; however, this can be modified to increase compliance or to achieve better seizure control [15, 16]. Its name stems from the fact that people subjected to it have increased ketogenesis, referring to augmented ketone body production [16, 17]. These ketone bodies (acetone, acetoacetic acid and β-hydroxybutyric acid) are an alternative fuel source [17]. In fact, a primary objective of the KD is to generate ketosis [17], which refers to ketonemia (a higher level of ketone bodies in blood) plus ketonuria (secretion of ketone bodies in urine).

The KD and the other eating regimes which emerged after it, all cause some degree of ketosis [18]; suggesting that a threshold level of ketone bodies might be necessary for seizure control. In fact, the ketone bodies could alter neuronal excitability *per se* (see possible mechanisms of action below). If only a certain amount of ketosis is necessary to manage epilepsy it would explain why ketone body concentration is not sufficiently correlated with the anticonvulsive effect of the KD [19, 20]. But it is important to note that ketosis is due to low blood glucose levels, which could also be responsible in part for the effects of the KD [21–25]. Therefore, the beneficial actions of the KD might depend on increased ketosis decreased glucose levels or both.

The KD is quite effective in patients; about half show a >50% reduction in seizure frequency and a third a >90% decrease [3, 15]. However, the KD is rather restrictive and has poor palatability; thus other eating regimes were introduced to improve patient compliance while maintaining efficacy. As expected, the different

diets are more liberal and have either the same or slightly less effectiveness. The nutritional procedures which have been used in patients, other than the KD, are the medium-chain triglyceride (MCT) regime, modified Atkins diet (MAD) and low glycemic index (LGI) treatment. Some KD and MAD protocols intentionally include a low to moderate degree (10–25%) of caloric restriction (CR), since it possibly increases their efficacy [15, 16]. In addition, the different eating manipulations could accidentally introduce some CR due to poor palatability or limited food choice [26]. Thus, clinical studies ought to be performed to examine whether CR has anticonvulsive and/or antiepileptogenic properties [27–29] in any of its implementations: intermittent fasting (IF or EOF for "every other day feeding"), periodic fasting (PF) and food restriction (FR).

1.2 MCT Regime

The KD consists mostly of long-chain triglycerides [15, 16, 18, 26]; thus, the first diet alternative to the KD used medium-chain triglycerides to obtain about 50% of the total daily energy [16, 18, 26, 30, 31]. Medium-chain triglycerides (such as caprylic, capric and lauric acids) are found in coconut and palm kernel oils. They allow greater amounts of proteins and carbohydrates to be consumed due to fact that the medium-chain triglycerides are more ketogenic [15, 16, 18, 26, 30, 31]. Furthermore, the amount of medium-chain triglycerides can be adjusted to attain good tolerance and ketosis [32]. The MCT regime is just as helpful as the KD for seizure management [18, 32, 33]. However, there is evidence that starting (at least during the first 3 months) with a more strict nutritional procedure (*i.e.,* with more limit to the consumption of carbohydrates, in this case the KD) is likely to be better. Moreover, patients can be successfully transitioned from a strict diet to a more liberal one [18].

It is important to note that the goal of the MCT diet is to allow more consumption of carbohydrates and proteins in an effort to increase treatment palatability and compliance. Since the MCT regime is more ketogenic than the KD, it may be tempting to try to augment the level of ketosis. However, such a maneuver does not improve treatment effectiveness [19, 20, 34]. Even so, it might be advisable to achieve ketosis quicker with a fasting period [4, 16]. There have been several studies investigating the effects of specific medium-chain triglycerides on different epilepsy models; all of them showed that they possess anticonvulsive properties [35–41]. The oral administration of small-chain or medium-chain ketone esters may also be beneficial [42]. Therefore, the MCT diet could be simplified further to a simple mixture of particular medium-chain triglycerides and/or ketone esters. However, a reduction in glucose concentration and/or metabolic adaptation may be needed for the beneficial effect of the different food modifications [21–25, 43–45]. It is unclear if supplementation of medium-chain fatty acids and/or ketone esters could also achieve these effects.

1.3 MAD

Another alternative for the KD is the MAD, which only limits carbohydrate intake: 10 g/day in children and 15–20 g/day in adults initially; the portions can be increased depending on seizure control and tolerability [15, 16, 18, 30]. The MAD encourages ingestion of fats, liberalizes intake of proteins and can include a moderate degree of CR [15, 16, 18, 30]. Due to its relative simplicity it is usually started in an outpatient setting [15, 16, 18, 30]. Its efficiency for the treatment of epilepsy is similar to the KD [15, 16, 18, 30]. Some reports showed that the type of fat or the lipid to nonlipid (carbohydrates and/or proteins) ratio did not affect the efficacy of the KD [34, 46–50]. Based on this evidence, the general assumption is that the MCT regime, MAD or LGI treatment all have the same effects as the KD. Thus, animal model studies with these nutritional practices are extremely scarce. Nonetheless, these diets all restrict ingestion of carbohydrates, thus lowering blood glucose levels, which produces ketosis. It is clear that at least one of these two (lowering blood glucose or ketosis) should be responsible for their similar efficacy in managing epilepsy.

1.4 LGI Treatment

An additional option is the LGI manipulation, which also limits carbohydrate ingestion to about 40–60 g per day [15, 16, 18, 30]. In terms of the total daily calories: around 60–70% are from fats, 20–30% from proteins and 10% from carbohydrates [15, 16, 18, 30]. However, the carbohydrates consumed must have a low (<50) glycemic index [15, 16, 18, 30]. This index measures the rise in glucose concentration in systemic circulation resulting from the consumption of a specific food, as compared to the ingestion of pure glucose [15, 16, 18]. The LGI procedure is based on the decrease in blood glucose that is also characteristic of the KD and on animal studies showing that diminished circulating glucose produces anticonvulsive effects [21–25]. This scheme produces some degree of ketosis as a consequence of reducing systemic glucose; however its effectiveness is correlated better with lower serum glucose than with the level of ketone bodies [51].

The most important aspects of these diets could be combined. In any of them, low glycemic index carbohydrates should be preferentially consumed. Similarly, medium-chain triglycerides ought to be abundant in food choices. Finally, some degree of intentional CR is probably beneficial in the long term [15, 16, 52]. Since the data show that starting with a stringent eating regime is likely better, a calorie-restricted KD becomes the first choice to attempt for at least the first 3 months [18]. At any point, if needed, the patients can be successfully transitioned to a less strict nutritional practice [18].

1.5 CR

There is a great deal of naming issues regarding CR in the literature [53], reflecting the conflicts in this field. CR is traditionally defined as any maneuver that reduces the amount of calories which are ingested [27, 53]. However, many studies involving CR either

cause undernutrition [53] or do not truly restrict the quantity of calories eaten [27], yet beneficial effects are still observed; thus the role of reduced calories is controversial [54]. CR has gained attention because it is a natural method for expanding the life span in a broad range of animals [16, 27, 29, 53–59]. CR also raises healthspan: general well-being is improved due to the prevention or delay of age-related pathologies [16, 27, 54, 56, 57, 59].

CR can be performed as IF, PF or FR, with or without mineral and vitamin supplements. IF usually means eating every other day: i.e., fasting for 24 h, followed by 24 h where food is allowed either *ad libitum* or constrained to prevent its overconsumption [27, 53, 55, 57]. PF is very similar, it generally involves fasting for 2 days or longer every 2 or more weeks [57]. Lastly, FR refers to a daily calorie limit; although this procedure can also have other names [27, 53, 55, 57]. A debate in the literature is whether any of these maneuvers produces deficits in micronutrients, implying that minerals and vitamins should be supplemented, except perhaps if the degree of CR is ≤20% [27, 53, 55].

What follows is some of the data showing that FR has anticonvulsive properties. A study reported that 10% FR for 25 days significantly augmented the dose of pentylenetetrazole (PTZ) which was needed to produce seizures in 47-day-old Sprague-Dawley rats [60]. Another report established that 15% FR for 10 weeks significantly reduced the proportion of animals showing seizures in 14-week-old epileptic mice [61]. The effect was lost in 70-day-old mice, but was recovered by increasing the degree of FR to 30% [61]. An additional investigation showed that a KD restrained to 80–90% of the suggested daily calories or an equally constrained normal diet for ≥28 days reduced excitability of the hippocampal dentate gyrus in 65–67-day-old Sprague-Dawley rats [62]. Moreover, the calorie-restricted KD showed a profile that was consistent with an antiepileptogenic effect [62]. One more study in 6–7-month-old rats bred to possess a fast kindling rate determined that 2 months of FR, such that the animals lost 20% of the body weight they had when fed *ad libitum*, reduced hyperactivity and possibly stress due to restraint [63]. Although the rats had a longer after-discharge duration on the first day of kindling, FR slowed down their kindling acquisition [63]. Interestingly, in rats reared to have a slow kindling rate FR diminished the extent of tonic clonic seizures and also decreased after-discharge intervals [63]. Lastly, 15% FR for a month in 51–day-old Wistar rats augmented the after-discharge threshold and tended to reduce its duration, but did not alter any other kindling variable [64]. Importantly, this anticonvulsive action was not due to changes in blood levels of glucose or β-hydroxybutyrate [64]. However, clinical studies to test whether FR by itself reduces seizure number or severity are lacking.

Similarly, there is some evidence that IF also has anticonvulsive effects. One investigation showed that IF for 10 weeks in 2-month-old Wistar rats decreased the duration of *status epilepticus* (SE), diminished the mortality due to SE, increased the duration of the latent period, reduced the severity of convulsions and lowered the proportion of rats with SE or that later had seizures [65]. One more study reported that IF for 11–13 days decreased seizure severity and duration, as well as augmenting the time to convulsion onset after kainic acid [66]. However, IF did not affect seizures produced by PTZ; moreover, IF increased convulsion vulnerability in the 6 Hz and maximal electroshock tests [66]. In contrast, a KD increased the current necessary to obtain seizures (was protective) in the 6 Hz test, had no effect in the maximal electroshock test and augmented the severity of convulsions (was harmful) due to kainic acid [66]. Conversely, IF for 4 weeks before PTZ treatment (35 mg/kg every other day for another 4 weeks) or IF both previous to and during PTZ administration (8 weeks total) diminished convulsion severity and reduced the number of injured neurons in the CA1 area of the hippocampus in adult Wistar rats [67]. All IF diets, even IF only during the 4 weeks of PTZ injections, lowered the quantity of damaged neurons in the hippocampal CA3 area and reduced the amount of apoptotic neurons in CA1 plus CA3 [67]. Neurons in both areas seemed to be healthier in all IF groups, since they had augmented volume [67]. A relatively short duration of IF (11–13 days) did not affect seizures resulting from a single constant infusion of PTZ [66], but a longer IF period (4–8 weeks) was protective even when PTZ was injected multiple times [67]. These contradictory outcomes suggest that diet duration is a critical variable, possibly due to metabolic adaptation. In a similar manner, IF protected neurons from damage due to PTZ administration [67] but not against pilocarpine treatment [65]. This difference was likely due to the fact that PTZ did not produce SE [67], suggesting that IF may not defend neurons from massive damage. Nonetheless, there is much evidence that CR is neuroprotective against excitotoxicity [68–73]. Additionally, IF for 12 weeks diminished the number of pilocarpine-injured neurons in the subventricular zone and piriform cortex of 6–7-month-old Wistar rats [71]. However, IF did not protect pyramidal CA1–CA3 neurons from damage by pilocarpine [65]. The difference may be due to the duration of SE: the former study stopped SE after 2 h [71] and the latter report after 3 h [65]. Indeed, the latter study suggested that IF controls epileptogenesis and defends against SE lasting 1 h [65]. Another possible explanation is that distinct brain areas respond differently to CR [67, 74]. In spite of the data showing that CR has anticonvulsive and/or antiepileptogenic effects there are no clinical investigations into the effects of CR by itself. Nevertheless, there is evidence indicating that CR augments the efficacy of the KD [16, 28] and MAD [15].

2 Possible Mechanisms of Action

Given that a calorie-restricted KD has both anticonvulsive and antiepileptogenic properties [62], a potential route for the discovery of drugs that may also have these same capacities is to target one or more of its many potential mechanisms of action. Many of them will be mentioned briefly within the following paragraphs. However, two general points need to be made before: (1) both CR and the KD likely have pleiotropic effects and (2) they do not necessarily share mechanisms, as has been shown [62, 66].

2.1 KD

Individual KD components might be at least partly responsible for its actions, such as medium-chain triglycerides [35–41]. As aforementioned, ketone bodies *per se* could alter neuronal excitability [75–78]. However, there is controversy over whether the concentration at which they have their effect is really achieved in patients [76] and/or if there is a species difference in their production [78]. In this line, it is important to mention that β-hydroxybutyric acid also acts as a signaling molecule [79, 80], protects the neural tissue from damage [79–82] and likely takes part in the regulation of food intake [81]. It is unknown to what extent the other ketone bodies also share these properties. It is also wise to point out that β-hydroxybutyric acid is a weak inhibitor of histone deacetylases [79, 83], similarly to some small-chain fatty acids [84]. Therefore, the KD may have epigenetic consequences possibly explaining its long-term effects even after diet termination.

It has been shown that plasma-free fatty acids, including polyunsaturated fatty acids, are augmented by the KD [15, 75, 85–87]. The latter are hypothesized to contribute to the results produced by the KD [15, 75, 85–87]. The KD severely reduces carbohydrate intake; thus, blood glucose levels also diminish, which may have important mechanistic implications [21–25]. Actually, a large group of conjectures regarding the effects of the KD focus on energy metabolism. A hypothesis is that ionic gradients are modified by the KD, increasing the resting membrane potential and reducing epileptic activity [88]. A different conjecture states that the KD activates ATP-dependent potassium channels (K_{ATP}) to inhibit neuronal activity [89, 90]; perhaps by lowering glucose and provoking ATP release through pannexin channels, subsequently ATP converted to adenosine binds its receptor opening K_{ATP} channels [90]. Another hypothesis is that the KD acts by inhibiting glycolysis, tested using 2-deoxyglucose [91–93] and fructose-1,6-diphosphate [94–98], which have broad anticonvulsive actions. Similarly, the final hypothesis is that the KD alleviates metabolic or energy shortages produced by excessive neuronal activity, for example by augmenting anaplerotic reactions [16, 37, 43, 99–101].

Other conjectures about the mechanism of the KD suggest that it affects neurotransmitters or neuroactive substances. Regarding the former, the KD may increase synthesis of γ-aminobutyric acid [102] or hinder glutamate uptake into synaptic vesicles, diminishing its release by inhibition of vesicular glutamate transporters [103, 104]. The KD has been shown to affect several neuroactive molecules, such as cholecystokinin, cortisol, galanin, ghrelin, insulin, leptin and neuropeptide Y, among others [7, 76]. For instance, the KD reduces insulin and either augments or decreases leptin [7, 76]. These changes are thought to impact the nervous system in several ways [7, 76], including activation or inhibition of intracellular signaling pathways. Thus, a last hypothesis is that the KD hinders the mammalian (mechanistic) target of rapamycin (mTOR) signaling cascade [105], since its function seems to be essential for genetic and acquired epilepsies [106–108].

2.2 CR

The mechanisms that could underlie the effects of CR are similarly broad and pleiotropic. As seen before, the effects of CR seem to depend on the species and tissue studied, the age of the animal, the duration of CR, etc. Thus, the actions of CR may partly depend on increased ketone bodies in mice [61, 109], but not in rats [60, 62, 110–114]. Similarly, CR appears to reduce blood glucose levels in mice, but it is unclear if blood glucose levels are decreased in rats or if they remain unaffected [110, 111, 113, 114]. In fact, the antiepileptogenic action of CR might require augmented ketone bodies, diminished glucose [64] or animals inbred for seizure susceptibility [61, 63]. On the other hand, the anticonvulsive effect of CR seems not to depend on blood glucose or ketone bodies [64].

Unlike the KD, CR appears not to affect ionic gradients, the membrane potential or K_{ATP} channels. However, although somewhat neglected, CR seems to inhibit glycolysis [115–118]. To be expected, CR does not modify anaplerosis or other possible deficits produced by seizures. Although not stated before, both the KD and CR augment mitochondrial biogenesis and decrease oxidative damage [16, 17, 26, 27, 57, 58, 89], as well as diminishing inflammation [5, 17, 27, 57, 58, 119]. CR could also increase glutamic acid decarboxylase expression and activity [24, 110]. However, enhanced activity of this enzyme did not result in augmented γ-aminobutyric acid levels. Likewise, increased enzyme function was observed in mice subjected to CR with different seizure susceptibility, suggesting that these phenomena are not related [24].

Not surprisingly, CR induces many systemic effects including changes in neuroactive compounds. Some of these molecules are also influenced by the KD: ghrelin, insulin, leptin and neuropeptide Y [27, 57, 58, 120]. Indeed, the neuroendocrine network that regulates appetite could be essential for the beneficial action of CR [57]. Other substances appear to be exclusively modified by CR,

such as the thyroid hormone triiodothyronine as well as growth hormone [57, 120]. Obviously, these changes engage or disengage many intracellular signaling pathways. Among them, two that may participate in the mechanisms of action of CR are the mTOR and sirtuin cascades [56, 58, 59, 64]. Hormesis is another hypothesis that has been proposed as a method of operation for CR [58, 59]. This refers to a phenomenon whereby the response to an agent is different according to its intensity; for example if a chemical is toxic at high concentrations but beneficial at low levels [58]. It was not until relatively recently that a framework was proposed to associate the many possible mechanisms of action of CR [58].

3 CR Protocol

The protocol below is designed to carry out a study using CR in animals, but similar methods could be used in humans. Animals of any species, genotype (*i.e.,* transgenic, knockout, wild type) and age can be used. Any type, degree or duration of CR is feasible; however, the following section contains a brief and simple method focused on performing low to moderate FR (5–20%) based on the body weight of controls in group-housed weanling rats. The reasons for this choice are as follows: our laboratory is familiar with this technique; it is more economical not to supplement minerals and vitamins, thus it is best to perform a low degree of FR; it is more convenient to group-house the animals to avoid possible problems of social isolation.

If working with animals, decide the species, strain and age (refer to note 1). Choose the type, degree and duration of CR to execute (note 2). Mineral and vitamin supplementation might be needed if the extent of CR is ≥20% [27]. Finally, select the food and water the animals will be receiving; usually commercial chow and tap water are used.

3.1 Method

1. It may be a good idea to fast animals for 4–12 h, so they all initiate at a similar metabolic set point [60, 62, 64, 66, 112, 114].
2. Regardless of their age, the animals obtained are usually quite different in weight. Therefore, it is best to weight-match the subjects, either before the fast or after, to form the groups [60, 64, 66, 111–114]. They should be divided into at least two groups: controls eating the diet *ad libitum* and those that are subjected to FR. If forming two groups, the lightest animal of all goes to one group (A), the next lightest to the other group (B), the following lightest to the first group (A) and so on. As a fictional example, suppose we have eight animals that weigh: 150, 152, 154, 156, 158, 160, 162 and 164 g; group A would be those weighing 150, 154, 158 and 162 g, while group B

would be the ones that weigh 152, 156, 160 and 164 g. Control or experimental conditions should be assigned at random to the groups. Document the body weights of all subjects and the group they belong to.

3. In animal studies, decide if they will be singly housed or group housed (refer to note 3).
4. Weigh an amount of food in excess of the amount you expect the animals to eat and give it to the controls. For the experimental animals, follow the following formula:

$$\text{weight of food}\left(\text{g}\right)=\frac{\text{body weight of control}\left(\text{g}\right)\times 0.3\left(\frac{\text{kcal}}{\text{g}}\right)\times \text{degree of FR}}{\text{kilocalories in each gram of food}\left(\frac{\text{kcal}}{\text{g}}\right)}$$

where 0.3 is the recommended daily amount of kilocalories that a rat should eat per gram of body weight [121]. The units of kilocalories/gram are canceled since they appear both above and below; thus we are left with grams. In the example above, let us say that group A is the control rats, group B the experimental animals, 15% FR and 3.5 kcal per gram of food: (a) if the rats are housed in groups: $(150+154+158+162)\times 0.3\times 0.85/3.5=624\times 0.3\times 0.85/3.5=45.5$ g of food for the rats in group B. (b) if the rats are singly housed: $150\times 0.3\times 0.85/3.5=10.9$ g of food for the rat weighing 152 g in group B, 11.2 g of food for the rat weighing 156 g in group B and so forth. Give the animals water in excess of the amount you expect them to drink. Make and keep a record of the amount of food (optionally, also of the quantity of water) given to each group.

5. Make sure the animals are housed in the appropriate conditions. For rats, this is usually a 12:12 h light dark period (which can be reversed, such that their night is our day), 22–24 °C and 40–60% relative humidity [122].
6. The second and every subsequent day, including weekends and holidays, document the weight of the animals and the leftover food from the previous day (optionally also measure their water consumption). Just as above, give the controls surplus food, calculate the amount of food for the CR group and record the quantities. Be sure to leave excess water for all groups (optionally, document this amount).

3.2 Notes

1. Choosing the species, strain and age of animals is not a trivial concern. In fact, these are probably very important factors that determine the effects of FR. For example, rats and mice seem to respond differently: FR increases blood β-hydroxybutyrate levels in mice [61, 109], but does not affect them in rats [60,

110–114], with the exception of one report [62]. Similarly, the KD [123] and CR [124] show strain-specific actions. Related to this, distinct tissues respond differently to CR [125]; indeed, diverse brain areas are not affected equally by CR [74]. Finally, the antiepileptic effects of FR are observed better in younger animals [60, 61].

2. Other very important variables are the type and duration of CR. As mentioned before, CR can be implemented as IF, PF or FR. One problem often seen in the IF protocol is a behavioral adaptation to the diet, such that the subjects overeat on days they have access to food [27, 55, 68]. Thus, a maneuver could be implemented to avoid this, such as limiting the amount of food available on feeding days [55].

   On the other hand, the issues inherent to FR are: (1) some CR techniques do not actually restrain the amount of calories ingested or do so for only a certain period of time [27]; (2) to the contrary, certain CR methods likely cause malnutrition and this is especially true for high degrees of restriction [53]. Thus, the results might actually be due to a deficiency of macro- or micronutrients. However, there is controversy whether and to what extent these issues require attention [27, 53, 55].

   There are several ways to actually implement FR; it can be based on: the food intake of controls the day before, the body weight of controls the same day, achieving and maintaining a certain reduction in body weight, etc. If the body weight of *ad libitum* fed animals is used as a reference to calculate the amount of food that the restricted group obtains, as in the present protocol, pay careful attention not to exceed the recent food intake of controls. For example, the formula above gives 218.6 g for five rats weighing 600 g each, which likely exceeds their *ad libitum* food intake. If this is the case, switching to a different method of executing FR will be required.

   As for the duration, it was shown that 10–12 days were needed for the KD to increase the voltage necessary to produce a minimal convulsive response [43, 126]. After about 20 days a new threshold was reached and at around 30 days the effect was maximal [126]. Thus, 10–30 days is the minimal duration for the KD and its effect improves throughout this period. The maximal duration of the KD is unclear, especially since there is controversy concerning its effects on glucose intolerance, insulin resistance and liver disease [127–133]. However, the KD is maintained for years in humans without major ill effects [4, 134]. There are no reports on the long-term effect of the KD or its related diets in animal models of epilepsy, as far as we know.

3. Rats and mice are social animals that can become depressed, sick or even die when isolated from others of their kind [135]. If you decide to keep them one per cage (singly housed) it is

suggested to use cages with wire mesh walls to allow animals to see, smell and hear each other.

Keeping the animals group housed avoids the stress of social isolation, but introduces other problems: the individual intakes of food and water are unknown, dominant animals may eat or drink more than the others and there are issues with the statistical design of the study. The only way to resolve the first problem is to perform single housing. However, the second issue can be alleviated by placing more than one source of food and water. The final issue relates to the smallest element that can receive its own treatment (experimental unit); since all animals in a cage receive the same restricted diet then the true sample size is the number of cages regardless of the quantity of animals within [136, 137]. Thus, two per cage is an ideal number for studies using diets and group-housed animals.

4 Final Remarks

As was mentioned before, all diet therapies for epilepsy lower blood glucose and produce ketosis, suggesting that one or both of these effects are required for their antiepileptic action. On the other hand, the anticonvulsive effect of CR seems not to require reduced glucose or increased ketones in the blood. However, the pleiotropic mechanisms of action responsible for the effects of the KD and CR (as well as the other procedures) are not necessarily the same, although they do share some of them (glycolytic inhibition, augmented mitochondrial biogenesis and decreased oxidative damage as well as diminished inflammation, among others). More research is needed, especially in the clinical setting regarding the possible reduction in the number and severity of seizures using only CR.

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Chapter 10

Gene Therapy in Epilepsy

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Abstract

The genetic modification of cell cultures and their transplantation into the brain is an effective ex vivo gene therapy. This transfer of genes via the genetic engineering of viruses or plasmids and subsequent transfection into cells that will express transgenes in the central nervous system (CNS) may allow specific treatment in epileptogenic foci while sparing healthy brain tissue, and minimize the side effects of antiepileptic drug treatment. Prime modification candidates are neuropeptide Y (NPY) and galanin, which are important modulators of neuronal excitability. These neurotransmitters exhibit an inhibitory effect on neuronal activity and provide anticonvulsant effects in animal models. Galanin also exhibits neuroprotective properties. Other modification candidates are adenosine, which acts as an endogenous anticonvulsant, and the glial cell line-derived neurotrophic factor (GDNF), which exerts neuroprotective and anticonvulsive actions. Recombinant adeno-associated viral vectors can release any of these agents because of their neuronal tropism, lack of toxicity, and stable persistence in neurons. This chapter provides an overview of gene therapy methods, and reviews several studies that used neural and non-neuronal cell transplants as a basis for expanding our understanding of diseases that affect the CNS and possible therapeutic alternatives.

Key words Transfection, Viral vectors, Gene therapy, Neuropeptide Y, Galanin, Neural transplant

1 Introduction

Gene therapy is a therapeutic strategy to replace a defective gene with its functional counterpart and restore the operation of a specific cell population. Gene therapy uses nucleic acids tactically, instead of drugs, to correct the pathological state of cells via modification of their genome [1]. Therefore, molecular knowledge of the disease is essential to hit specific targets [2]. Depending on the target cell, two types of gene therapy exist: the first genetically modifies germ cells (i.e., those that are involved in the formation of eggs and sperm), so that this information will be transmitted to offspring. Another type is the somatic gene therapy where the genetic endowment is introduced in non-germ or somatic cells, so genetic modification is not transmitted to offspring [3]. Gene therapy can be performed in vivo or ex vivo. In vivo gene therapy

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introduces the genetic material directly into the cells of the organism without prior manipulation in vitro. Its disadvantages are a lower level of control on gene transfer and difficulties in achieving a high degree of tissue specificity. Ex vivo gene therapy removes target cells from the patient, and these cells are isolated and grown in culture medium for transfection. Effectively transfected cells reproduce in culture and are replaced in the patient.

Recent efforts attempted to develop techniques to insert target genes for the treatment of central nervous system (CNS) disorders. The results in animal models treated with gene therapy have created new possibilities in the treatment of multifactorial diseases, such as epilepsy, which led to a rapid development in the manipulation of gene expression in brain cells [4].

Epilepsy is the third most common neurological disease worldwide. It is a CNS disease that is characterized by spontaneous and recurring seizures. Antiepileptic drugs (AEDs) are the first treatment choice, but these drugs are effective in only 60–70% of the individuals [5–7]. Patients who continue to suffer seizures despite more than two changes in their drug regimen are considered for surgical resection of the epileptic focus. However, this option is invasive, it is associated with dysfunctional effects, and only 10% of this population are acceptable candidates for surgical treatment [8]. The death of GABAergic interneurons and plastic changes in the hippocampus have been reported in temporal lobe epilepsy [9]. This cell loss alters the levels of neurotransmitters, in this case dopamine and γ-aminobutyric acid (GABA). Cell therapy for epilepsy proposes the use of replacement cells or ex vivo gene therapy transplantation to administer a substance of interest to the damaged CNS [10]. The objectives of gene therapy in epilepsy are to obtain a sustained anticonvulsant effect (as an antiepileptogenic drug that blocks disease progression, avoids epileptic focus propagation [8], and restores neurotransmitter levels) and the possible integration of transplanted cells into damaged circuits to restore the functions that are lost during the disease progression [11].

2 Gene Therapy Methods

As discussed already, two main methods are used in gene therapy: ex vivo, which involves the removal of tissue cells, in vitro modification using a retroviral vector, and the replacement into the body. In vivo gene therapy consists of the manipulation of the corrective gene within the patient rather than the replication of cultured cells [12].

2.1 Ex Vivo Gene Transfer

The first step in ex vivo gene transfer is obtaining tissue cells from the target organ. Tissue cells are disrupted and seeded in appropriate cell culture conditions in vitro. Cells are transfected with a therapeutic gene using a suitable vector, which should be selected

for its ability to stably and persistently express the exogenous gene. Selected cells are amplified and collected for reimplantation in the organism. Allogeneic cell lines may also be used in cases where the organ or tissue of interest cannot be easily removed or provide cells for in vitro proliferation. The ex vivo method is the most used technique because rejection is minimal [12].

2.2 In Vivo Gene Transfer

This method is based on the systematic management of the genetic construct of interest. DNA may be administered directly, but the use of a vector that facilitates the process of gene transfer and allows entry and intracellular localization, resulting in a functional gene, is a more common method. A noninvasive procedure is also important for the use of specific vectors that allow gene delivery to target cells in a specific organ or tissue. This method is used in cells that are difficult to extract and implant, such as CNS cells [13] (Fig. 1).

2.3 Introduction of the Transgene

A transgene, which is also called a genetic construct, is defined as a set of one or more genes and regulatory sequences that control its expression. The transgene exerts the therapeutic function for gene therapy. Most research on this topic was performed in animal models, but there are clinical trials in humans [14].

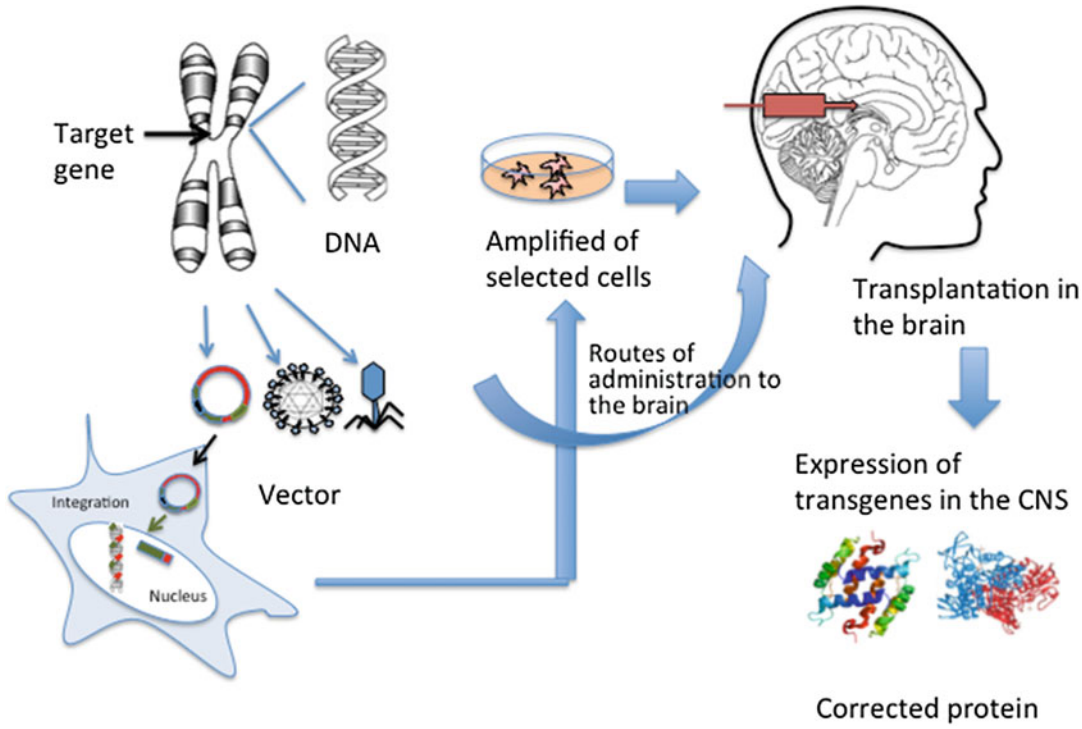

Fig. 1 General model for gene transfer microinjection or misting mediated by different types of vectors. Viral methods: adenovirus, retrovirus, adeno-associated virus, herpes virus; nonviral methods: DNA-liposomes, naked DNA

2.4 Direct Methods of Transfection

2.4.1 Chemical Systems

Calcium phosphate transfection. This technique was first used in the 1960s with good transfection efficiency; it is based on the precipitation of exogenous DNA, with the resulting DNA-Ca^{2+} precipitate entering the cell via endocytosis. Transfection efficiency reaches 10% of the cells, but it is generally transient. This technique does not cause toxicity in cells, but the transgene expression is low. It is used only in cell cultures and ex vivo applications.

2.4.2 Physical Systems

These techniques involve the injection of DNA into the cell nucleus. This direct introduction of DNA avoids cytoplasmic and lysosomal degradation. Injection is performed using a micromanipulator and an inverted microscope for visualization. Cells with inserted genetic material that survive exhibit high expression efficiency. This technique requires isolated cells, and it is often used ex vivo. The germline gene therapy method injects DNA into embryos, and it is performed in vivo.

Microinjection (*electroporation*). A gene construct is introduced into the cells by an electric shock that causes the formation of pores in their membranes through which the transgenes enter. It is especially suitable for cells with high proliferation rate. However, many cells die as a consequence of the electric shock. Another disadvantage of this technique is that it cannot be used in many cell types.

Naked DNA. It consists in the introduction, usually by intramuscular injection, of DNA in a saline solution or serum to achieve the expression of a transgene. Expression has been observed in thymus, skin, heart muscle, and skeletal muscle. The mechanism of naked DNA uptake is not fully understood but it is postulated to involve the nuclear pore complex. This technique has a low percentage of transfected cells, no integration of the transfected material into the genome once injected, and no replication in the genome. This is a system used for performing gene therapy in vivo [12, 15].

2.4.3 Direct Methods Mediated by Vectors

A wide array of vectors has been used to deliver genes into the nervous system. Several researchers use nonviral vectors composed of naked DNA. These vectors exhibit several problems (Table 1) related to delivery, short-term expression, and immune reactivity against the vector. Viruses may be delivered locally, and many, but not all, are transported to the cell body by axons via retrograde transport mechanisms afetr the infection of nerve terminals. This retrograde transport is particularly attractive for neuropathic pain therapies, as described in the following sections. Though different efficiencies are obtained depending on the type and strain of virus, the herpes simplex virus (HSV) is a highly efficient vehicle for the delivery of exogenous genetic material in humans. Retrograde transport provides means of targeting specific nodes after intradermal infection or other routes of administration.

Table 1
Vector features used in gene therapy

	Retrovirus	Adenovirus	HSV	AAV	Liposomes	Naked DNA
Insert size	8 kb	35 kb	>20 kb	<4 kb	>20 kb	>20 kb
Title (CFU/ml)	107	1011	1010	109	Undefined	Undefined
Integration	Yes	No	No	Uncertain	No	No
Sustained expression	Variable	Transient	Transient	Variable	Transient/variable	Transient
Distribution in vivo	Low	High	High	High	Variable	Muscle
Quiescent cells	Only lentivirus	Yes	Yes	Yes	Yes	Yes
Disadvantages	Insertional mutagenesis/ leukemia	Immunogenic, complex manipulation	Poor ability to insert	limited insert capacity	Immunogenic	Complex administration route

HSV herpes simplex virus, *AAV* adeno-associated virus. Modified from Zorzano, 2003

Nonviral Vectors

The ability of liposomes (structures surrounded by a lipid membrane to resemble eukaryotic cells) to deliver DNA into cells has been known since 1965, but it was not until 1980 that suitable transfection efficiency was achieved.

Cationic liposomes are artificial vesicles prepared with cationic lipids which are particularly adequate to interact with negatively charged DNA. Many lipids are used to form these liposomes, and different lipid mixtures are being tested. These liposomes are recommended for in vitro transfections. Among the advantages of liposomes we can mention the protection of the transgene from degradation until it reaches the nucleus, the unlimited DNA size, the possibility of targeting specific receptors in tissues (e.g., the lipid layer), low immunogenicity, and the ability to transfect nondividing cells. The disadvantages include low transfection efficiency, transient expression, some cellular toxicity, possible inhibition by serum components, and the inability for gene therapy in neurological disorders [13].

Viral Vectors

This methodology began in 1968, when it was discovered that viruses were capable of infecting mammalian cells. The development of packaging cells in 1989, protein composition that is required for functionality is removed from the viral genome, marked an important stage in this methodology, and reduced the degree of virus pathogenicity. Numerous genes and sequences are removed to allow the virus to capture the exogenous DNA. These viral vectors may be used in vivo and ex vivo, and several viruses are used for gene therapy (Table 1, Fig. 2).

Retroviruses. These RNA viruses integrate a relatively large amount (up to 8 kb) of therapeutic genes, but packaging cells are needed to create these vectors. Virus DNA is transferred into the packaging cells using the calcium phosphate technique. A second transduction is performed in which the gene construct of interest is introduced, and the virus is injected into the host; subsequently, it integrates its DNA into the genome to express the inserted gene. No immune response is produced because the host does not express virus proteins. This technique exhibits a high efficiency transduction and expression, and it is a well-studied system. However, this method only infects host cells that are dividing, viral concentrations are low, and integration into the genome is random. Vectors based on more complex genomes are called lentiviruses. Lentiviruses deliver durable transgene expression but require integration. Axonal transport of these vectors is inefficient, and standard retroviruses require cell division for gene delivery. Lentiviruses are integrated into nondividing cells, and they may be pseudotyped with rabies G protein to enhance retrograde delivery [16, 17]. However, these viruses may disrupt normal genes, including tumor suppressor functions, or activate oncogenes [18, 19].

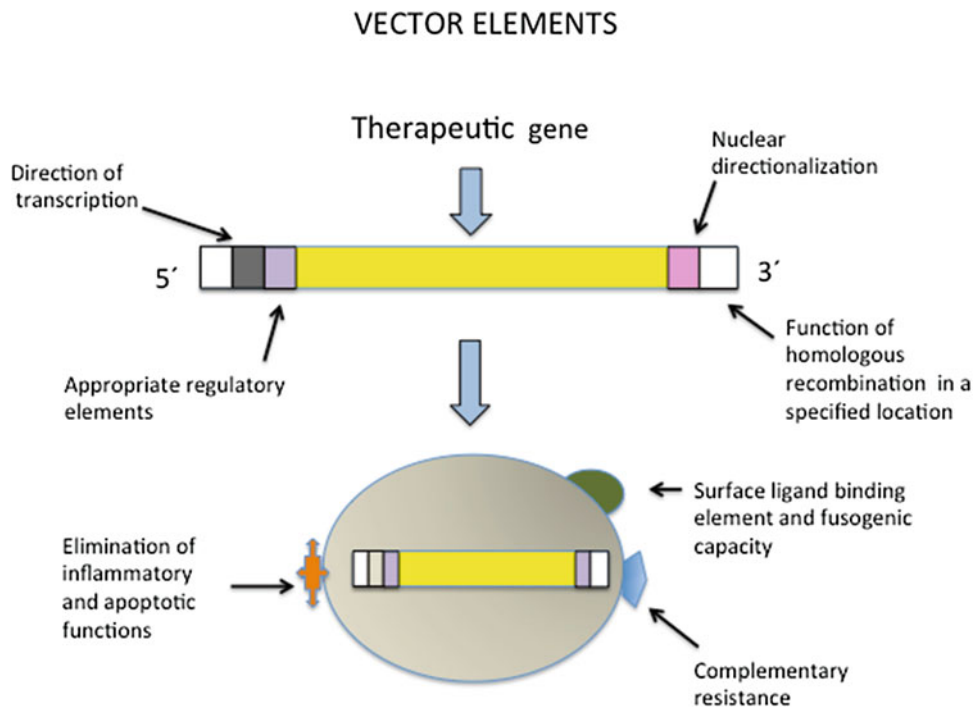

Fig. 2 Diagram of an ideal gene therapy vector. A vector should possess a number of properties to be used for therapy in an efficient manner. It is also important that the vector does not cause an immune response or other deleterious responses [63]

Vectors that stably persist as episomes are preferred for peripheral nerve applications.

Adenoviruses. Adenoviruses are a family of DNA viruses that cause different infections in humans, mainly in the respiratory tract. Approximately 7.5 kb of exogenous DNA may be inserted into these viruses. Serotype 5 is generally used in gene therapy, but 42 different serotypes infect humans. Integration of the virus genetic material into the host is needed for replication. The transgene is introduced into the genome of the cell, but cellular division of infected cells is not required for replication. The advantage of an adenovirus vector is the high efficiency of transduction and expression of the introduced gene construct, although the expression is transient (a few weeks). Periodic treatments are required, which is a disadvantage because adenoviruses produce cellular and inflammatory immune responses [15].

Adenoviral vectors do not require integration and undergo retrograde transport, but these vectors recruit inflammatory cells because of viral gene expression [20, 21], and they do not persist for a long term in sensory neurons. The immunogenicity of adenoviral vectors has relegated their exploitation to vaccine development and use as oncolytic vectors for cancer treatment.

Adeno-associated virus (*AAV*). AAVs are DNA-containing parvoviruses that require coinfection with an adenovirus to multiply. AAVs are vectors that combine the advantages of retroviral and adenoviral infection, and they can integrate small exogenous DNA (only 5 kb). The primary advantages are that AAVs integrate their DNA into the cell during replication, and transduction (which is highly effective) is stable in the target cell. AAVs also infect dividing and nondividing cells, which is highly important for gene therapy in vivo. AAVs vectors are not involved in any human disease, and the risk of an immune response to the production of viral proteins is minimized. AAVs were efficiently transduced into nerve tissue, retrogradely transported efficiently at least in mice [22–24], achieving long-term transgenes expression. As stated before, one limitation of these vectors is the payload capacity (3–4 kb), and only single moderate-sized genes are generally accommodated. Therefore, virus doses that are required to achieve effective transduction are very high compared to other vectors. Further, AAVs vectors do not adequately target specific cell types for infection, especially neurons. Antibodies readily neutralize AAV, and repeat dosing is generally not possible. High vector dosing in the periphery and the loading of genomes into cells may induce DNA damage responses that lead to inflammation [25, 26]. The disadvantages of AAV have limited the study of this type of vectors, which are not as well studied as retroviruses and adenoviruses [27].

Herpesvirus (*HSV*). HSVs are DNA viruses that can be used to target neurons. The advantage of HSVs is their DNA size, which allows the insertion of various therapeutic genes with regulatory regions. One disadvantage is the need to remove sequences that encode the lytic virus proteins, which kill infected cells.

Some characteristics of the ideal vector for gene therapy include the incorporation and regulated expression of one or more genes for clinical application during an appropriate time, specificity in gene transfer, to be unrecognizable to the immune system, absence of a stable inflammatory response, and easy obtention [12]. The vector designed from HSV allows the insertion of approximately 20 kb, and exhibits a strong neuronal tropism. This vector spreads through the nervous system, which allows a wide distribution of the transfected gene [28]. However, the main limitations are HSV cytotoxicity and its ability to induce cellular immune responses [29]. The HSV-1 variant is used. This vector deletes genes that are involved in viral replication, which reduces neurotoxicity [30]. A specific promoter for tyrosine hydroxylase is also used, which increases the specificity of neuronal transfection [31].

2.5 Genetic Repair Using Oligonucleotides

Oligonucleotides are short sequences of nucleic acids that bind to specific sequences of DNA (triplex-forming DNA) or RNA (RNA-DNA heteroduplex formation). These sequences are complementary to specific sequences of a given messenger RNA (sense

sequence) and form a double-stranded sense-antisense heteroduplex that blocks the translation of the genetic message into the protein. This strategy is used to repair genes that are altered by a well-known point mutation. There are selectively activated cellular mechanisms of DNA repair at the site of the mutation. Specific oligonucleotides are designed adjacent to genomic sequences at the mutation site, and the oligonucleotide carries, on one end, an agent that is capable of damaging the DNA by forming a covalent bond with the nucleotide responsible for the mutation. Selective nucleotide mutation triggers cellular DNA repair processes, which leads to structural and functional gene correction [1].

3 Route of Administration to the Brain

The genetic modification of cell cultures and transplantation into the brain is an effective ex vivo gene therapy. The transfer of genes via genetic engineering of viruses or plasmids and subsequent transfection into cells expresses transgenes in the CNS [32]. The first treatment emerged from adenosine deaminase deficiency. Gene therapy via the intracerebral delivery of a combination of genes was the best option for the treatment of certain CNS disorders, since the blood-brain barrier hindered the access to the CNS [32]. Intranasal administration is a viable option for the expression of transgenes in neural cells. This method achieved CP10PK HSV gene transfection in hippocampal neurons using this same virus as vehicle. However, the level of transgene expression was limited because this route of administration is not the most suitable for therapies that require transfection in specific areas of the brain, unless the vectors are developed with promoters [33]. Stereotactic surgery is a more efficient technique for the delivery of therapeutic genes into specific brain areas because high levels of transgene expression are achieved after the injection of a viral vector [34]. This approach is the most common gene delivery method to the brain, having the lowest population of antigen-presenting cells and lymphatic system in the CNS [15]. However, damage to the blood-brain barrier permits penetration of active lymphocytes and immune response cells, and this transfer technique must be redefined. Other factors should be considered when gene delivery is successful, such as the level and stability of transgene expression and the ability to regulate the expression of the transgene [35]. Lentiviral vectors are used in gene therapy because of their ability to integrate up to a 9 kb chromosome of the host and to transfect most brain cell types, which facilitates the expression of transgenes [15, 35].

Primate and human immunodeficiency viruses (HIVs) belong to the lentivirus family. Therefore, it is important to consider extreme biosecurity measures compared to other viral vectors, to prevent recombination events that could generate a virus capable of replication.

Biosecurity measures include the elimination of virulence genes in packaging plasmids, the introduction of genes involved in capsid assembly using separate plasmids to reduce the possibility of recombination, and the induction of vector auto-inactivation to suppress viral transcriptional activity [27].

The use of recombinant adenovirus (rAAV) vectors for the delivery of transgenes in the brain does not produce toxicity. These vectors also elicit a low immune response and exhibit efficient cell transduction in the brain in animal models. These adenovirus serotypes were isolated from humans and primates, and some of the rAAVs that were cloned and packaged into recombinant vectors exhibited tropism for neuronal types and various brain areas [36]. Injection of high concentrations of viral particles to the brain promoted transgene expression and dissemination, and achieved effective cell transduction [37]. The combined use of rAAV2 vectors and stereotactic surgery achieved transfection in selective foci, such as the hilum or hippocampal CA1 areas [38]. Several rAAV serotypes were characterized recently, and these vectors primarily exhibited neuronal tropism. Therefore, it is important to continue the development of vectors that are more efficient and exhibit more tropism for glial cells, including improved expression cassettes, greater storage capacity, and the characterization of specific promoters for greater expression in certain cell types. Several promoters were used to restrict rAAV expression in melanin-concentrating neurons in the hypothalamus. However, promoters have not been isolated yet, and expression is restricted to neuronal subclasses in the hippocampus, such as hilar GABAergic neurons or principal neurons in the dentate gyrus. This limitation is a particularly difficult challenge for rAAV vectors because of the promoter activity contained within the inverted terminal repeats [39]. The development of treatments for each neurological disorder is important to optimize the efficiency of the systems for gene delivery.

4 Gene Therapy Strategies for Epilepsy

Although a large part of the epilepsies are of genetic origin, genetic targets are not the best candidates for gene therapy. A single mutant gene rarely causes the disease since epilepsy is the result of the inheritance of two or more susceptibility genes [40]. In these cases, the pathology often affects a large part of the brain, which would require widespread gene transfer. These features pose two big obstacles for gene therapy. The first obstacle is the need to transfer multiple genes into diseased cells, and most viral vectors (with the notable exception of HSV) have a small genome that hosts only one gene at a time. The second obstacle is the need for widespread expression of the therapeutic gene(s): currently available gene

therapy methods mostly provide localized effects. These reasons suggest that focal epilepsies, particularly temporal lobe epilepsy, are better candidates for gene therapy. In these cases, seizures often originate in a restricted brain area, which allows local delivery of the vector using stereotaxic surgery. Epilepsy caused by focal lesions often has an identifiable cause from a damaging insult (e.g., head trauma, status epilepticus (SE), stroke, brain infection), which sets a cascade of neurobiological events in motion. The development and extension of tissue that is capable of generating spontaneous seizures would result in the development of an epileptic condition and/or the progression of the epilepsy.

Epileptogenesis (i.e., the transformation of a normal brain into an epileptic brain) in humans and animals is associated with progressive pathological abnormalities, such as cell death (the most prominent is a loss of neurons in the hippocampus termed "hippocampal sclerosis"), axonal and dendritic plasticity, neurogenesis, neuroinflammation, and alterations in ion channels and synaptic properties. The physiopathology of epilepsy is well studied in animal models and surgically resected tissue, and several candidate genes have been identified as potential therapeutic targets [41]. Gene therapy allows specific targeting of the epileptogenic region, which spares the surrounding healthy tissue and minimizes the side effects of antiepileptic drug treatment.

4.1 Gene Therapy in Animal Models of Epilepsy

Research on gene therapy for epilepsy was conducted essentially in several types of models, primarily chemical convulsant (pilocarpine or kainate) evoked SE and electrical stimulation (electrical kindling). SE models induce an epileptogenic insult that is followed by a latency period during which the animals are apparently well, followed by spontaneous recurrent seizures (SRSs), i.e., epilepsy. This situation closely mimics the situation in humans with acquired structural epilepsies [42]. SE models allow the exploration of interventions at different levels: antiepileptogenic (prevention of development of epilepsy in subjects who are at risk after an epileptogenic insult), antiseizure (reduction of frequency and/or severity of seizures), disease-modifying (alteration of the natural history of the disease) and repair of the affected regions, where cells may be modified to produce inhibitory neurotransmitters, such as GABA.

Increased GABA levels in the epileptogenic area are favorable on physiological behaviors because they reduce neuronal excitability and the occurrence of seizures. Löscher et al. [43] observed these effects following the transplantation of fetal GABAergic neurons in the substantia nigra, which transiently decreased seizures in the kindling model in rats [41, 43]. This observation led to the development of the transfection of glutamate decarboxylase (GAD) in in vitro and in vivo models to increase GABA levels (Table 2). Ruppert et al. [44] used a retroviral expression system to obtain complementary DNA (cDNA) and induce the expression of different GAD isoforms in rat

Table 2
Gene therapy used in epilepsy models

Animal model	Gene therapy	Description	Reference
Tetanus toxin into the motor cortex	Optogenetic strategy In vivo Ex vivo	Overexpress the light-activated chloride-pump, halorhodopsin, in pyramidal cells	Walker et al. (2013) [64], Tønnesen et al. (2009) [65]
Kainate-induced seizures	Expression of microRNA targeting adenosine kinase (miR-ADK) In vivo	Silencing of ADK expression using an alternative RNA interference-mediated mechanism in astrocytes	Young et al. (2014) [66]
Kainate-induced seizures	AAV-GS and AAV-EAAT2. In vivo	Overexpression of glutamine synthetase (GS) or excitatory amino acid transporter 2 (EAAT2) Synthesis of GABA	Young et al. (2014) [66]
Limbic kindling	GABAergic cell transfer (GAD 65 and GAD 67)	Reduced the incidence of severe limbic motor seizures, by granule cell stimulation Expression of GAD 65 and 67 Transient increased GABA levels	Gernert et al. (2002) [46]
Kindling	AAV-mediated delivery of an antisense RNA specific to NMDAR1	Influence NMDA receptor function both in vitro and in vivo	Haberman et al. (2002) [51]
Pilocarpine-induced status	Replication-defective HSV-1 vector expressing FGF-2 and BDNF	Attenuated the ongoing cell loss, favored the proliferation of early progenitors, and led to the production of cells that entered the neuronal lineage of differentiation	Simonato and Zucchini (2010) [56], Simonato et al. (2006) [57]
Kainic-induced epilepsy	NSCs expanded from E14 MGE	Reduced frequency, duration and severity of seizures 3 months after graft	Waldau et al. (2010) [67]
Kindled seizures	AAV Vector-derived NPY	Reduced the generalization of seizures from their site of onset, delayed acquisition of fully kindled seizures, and afforded neuroprotection	Noe et al. (2007) [68]

AAV adeno-associated virus vectors, *NMDAR1* *N*-methyl-d-aspartic acid receptor 1, *RNA* ribonucleic acid, *NSC* neural stem cell, *MGE* medial ganglionic eminence

fibroblasts. These experiments increased enzymatic synthesis and GABA activity, which was released into the surrounding medium by the modified cells. These results demonstrated that the expression of GAD from cDNA was effective for the synthesis of GABA in transplanted cells although not in cells of neuronal origin [44]. Sacchettoni et al. [45] used Moloney murine leukemia virus expressing the GAD-67 enzyme under the control of the GFAP promoter as a replication-defective vector. The clones were transfected into fibroblast cell lines and astrocytes and both expressed functional enzymes. However, only fibroblasts produced GABA in the extracellular medium. Gernert et al. [46] transplanted immortalized cortical neurons with genetically modified GAD-65 into the piriform cortex of rats with amygdala kindling. The GABA-producing cells implanted in potential brain targets enhanced the seizure threshold and augmented the latency to the first generalized convulsion during the kindling process [46]. Liu et al. [47] used vectors derived from human foamy virus (HFV) to transfect the cDNA of a specific GAD isoform into primary cultures of hippocampal neurons, which may have a potential therapeutic value in the treatment of neurological diseases. Another study investigated kainic acid-induced seizures (5 mg/kg, stage V in Racine scale) in rats that previously received transplants of a cell line transfected with human cDNA encoding GAD67 (M213-2O CL4) into the substantia nigra. The results demonstrated that the group transplanted with M213-2O CL4 cells required a higher dose of kainic acid to induce seizure activity, whereas the latency to convulsion increased, and the behavioral manifestation was reduced compared to rats with untransfected cell transplantation [48].

One target for inhibitory mechanisms is the $GABA_A$ receptor, which is the main receptor subtype for the principal inhibitory neurotransmitter. This receptor is a pentameric chloride-permeable channel. The expression of $GABA_A$ alpha-1 subunits is decreased and alpha-4 subunits expression is increased in the granule cells of the hippocampus of epileptic rats, which generates receptors that rapidly desensitize and favor the generation of seizures. Raol et al. [49] used an AVV to achieve the expression of the GABRA1 gene (GABA receptor subunit alpha-1) under control of the GABRA4 promoter (GABA receptor subunit alpha-4). The vector was injected into the dentate gyrus of rats, and SE was produced using pilocarpine. The results revealed an increased expression of GABRA1 mRNA and protein after 2 weeks of SE, and also a three-fold increase during the interictal period and a decrease in the number of rats that developed spontaneous seizures by 60 % during the first 4 weeks after SE. These results demonstrate the importance of the composition of GABA receptor subunits for the development of circuits and epilepsy, and the role of GABRA1 expression in hippocampal inhibitory function [49]. The *N*-methyl-d-aspartate (NMDA) receptor also provides a potential target for gene therapy in focal seizures. During et al. [50] constructed an

AAV to synthesize the NR1 subunit of the NMDA receptor. Gene expression persisted for at least 5 months after AAV administration, and it was associated with a strong antiseizure activity and neuroprotective effect in a mouse model of kainate-induced epilepsy and stroke.

Haberman et al. [51] cloned a cDNA fragment (729 bp) of the NMDAR1 gene (NMDA receptor) in an antisense orientation into an AAV that contained a promoter regulated by tetracycline (AAV-TTAK-NMDAR1). The vector was used for the transfection of a primary culture of cortical neurons. The decreased expression of NMDA receptors significantly reduced the evoked currents [51].

Neuropeptides are important modulators of neuronal activity in the mammalian CNS. Neuropeptide Y (NPY) is a widely distributed polypeptide in the CNS, and it has become a focus of attention in the epilepsy field because of the changes in the expression of this protein and its receptors in brain regions that are involved in seizure initiation and propagation. Hippocampal interneurons and NPY-overexpressing axons are observed in patients with intractable temporal lobe epilepsy. The expression of NPY Y2 receptors on presynaptic neurons and pyramidal granular cells inhibits glutamate release and the presence of seizures. Richichi et al. [52] designed a rAAV vector to overexpress rat NPY in neurons and obtained an increase of the expression of this protein in the hippocampus, a reduction in kainic acid-induced seizures and an increase in the latency of seizures.

Cholecystokinin (CCK) was first discovered in the gastrointestinal tract, although it is one of the most abundant neuropeptides in the CNS. This neuropeptide was identified as a central regulator of neuronal circuits, and CCK and its receptors are implicated in the neurobiology of feeding, memory, nociception, exploratory behavior, and anticonvulsant activity. CCK is also associated with neuropsychiatric disorders. Zhang et al. [53] evaluated the potential for CNS gene transfer using lipofectin-mediated plasmids encoding CCK. Intracerebroventricular transfection was performed in rats with audiogenic seizures, and a high analgesic response to peripheral electrical stimulation was observed. Previous studies demonstrated that low CCK levels may vary the neurochemistry of audiogenic seizures in rats and underlie the high analgesic responses pursuant to its anti-opioid properties. CCK expression corrected the development of seizure susceptibility for 1 week. These results suggest that gene transfer mediated by lipofectin may be useful in studies of brain function, modification of behavior, and gene therapy for the CNS. This strategy may result in the transient expression of a foreign gene with functional consequences in the adult brain, but it has the advantage of not recombining with the endogenous virus [54].

The endogenous neuropeptide galanin generally exerts an inhibitory action on the CNS by increasing potassium conductance or

reducing calcium conductance, and the magnitude of this inhibition is sufficient to suppress seizure activity in the hippocampus. Haberman et al. [51] proposed a gene therapy for temporal lobe epilepsy using the transfection of constructs with fibronectin and galanin adenovirus (AAV-FIB-GAL). AAV-FIB-GAL vectors protected the hippocampal hilar neurons from kainic acid-induced cell death, but unilateral secretion did not alter the course of electrographic seizures after kainic acid administration [51]. Other authors achieved significantly attenuated seizures induced by kainic acid using the same constructs (AAV-FIB-GAL) and prevented the electrographic seizure activity, which abolished limbic seizure activity and the bilateral constitutive secretion of galanin in the rat piriform cortex. These studies demonstrated that the modulation of this gene influenced the behavior of limbic seizure activity [55].

The flexibility to transfect exogenous genes achieving protein expression for the treatment of disease, such as protein ICP10PK of herpes simplex virus type 2 (HSV-2), is one advantage of gene therapy. This protein inhibits caspase-dependent apoptosis and protects against several neurotoxic stimuli, including viral infection, treatment with an inhibitor of protein kinase C, osmolar environment disruption, loss of carrier in trophic growth, and excitotoxicity. Notably, neurons that survived gene therapy retained their synaptic function [55].

Laing et al. [33] demonstrated that gene transfection of ICP10PK using HSV2 as a vehicle had antiapoptotic activity in rat hippocampal cultures and mice with kainic acid-induced seizures. This method may be used to target neurons that are inaccessible to surgical techniques. ICP10PK-mediated protection is likely involved in the inhibition of reactive oxygen species (ROS), astrogliosis, and microglial activation induced by kainic acid.

Genetic interventions have attempted to prevent epilepsy after an insult based on the hypothesis that impairment in the levels of neurotrophic factors (NTFs), such as FGF-2 (fibroblast growth factor-2) and BDNF (brain-derived neurotrophic factor), may be a factor in epilepsy pathogenesis. FGF-2 and BDNF protect neurons from damage. FGF-2 is a potent proliferation factor for neural stem cells, and BDNF favors differentiation into neurons [56].

Simonato et al. [56, 57] used a replication-defective HSV-1 vector expressing FGF-2 and BDNF to test the hypothesis that local supplementation of these NTFs would attenuate seizure-induced damage and inhibit epileptogenesis. This vector was injected into one damaged hippocampus 4 days after a chemically induced SE in rats (i.e., during latency and after the establishment of damage). These conditions are similar to a person who is in the latency period after the occurrence of an epileptogenic insult and preceding the onset of spontaneous seizures [58]. Expression of the transgenes was bilateral (because of the retrograde transport of the vector to the contralateral hippocampus) and transient (approximately 2 weeks).

However, short-term expression is an advantage in these specific settings because NTFs trigger plastic changes that remain detectable when the vectors are no longer expressed, and their long-term expression may be detrimental for brain function. Therefore, this approach allowed modification of the microenvironment via the generation of cells that were capable of constitutively, but transiently, secreting FGF-2 and BDNF. This treatment slightly attenuated the ongoing cell loss, favored the proliferation of early progenitors, and led to the production of cells that entered the neuronal lineage of differentiation. All untreated animals displayed hippocampal sclerosis and spontaneous seizures 1 month after SE. These studies provide evidence that gene therapy is an option in epilepsy prevention in at-risk patients. The disease-modifying effect was striking, and the result may be interpreted as truly antiepileptogenic.

Injuries, such as head trauma, ischemia, tumors, and neuroinfections, also generate epilepsy. Brain areas damaged by stroke and seizures express high levels of heat shock protein 72 kDa (HSP72). This protein is a marker of stress, but its participation in the survival of neurons after damage is controversial. Yenari et al. [32] found a neuroprotective effect by inducing the expression of the HSP72 protein in areas damaged by stroke, inhibiting the development of seizures. The vectors used for the transfection of an animal model of kainate-induced focal cerebral ischemia were constructed from HSV.

4.2 Gene Therapy in the Clinic

The process used to develop new gene therapies relies upon experimental validation in model systems. In the case of the epilepsies, the establishment of models has itself been hindered by clinical and genetic heterogeneity. In fact, due to the numerous ways in which recurrent seizures arise and the different types of seizures that can manifest, epilepsy may be considered as a group of diseases, each one potentially requiring different models and strategies for development of maximally effective treatment. Until now, antiepileptic drugs have been devised primarily through seizure models in rodents. On the other hand, preclinical results of Phase I to Phase III clinical trials are highly encouraging and demonstrate that gene therapy does not present a general increase in risk factors associated with the technique compared to other surgical methods for other neurological disorders, including Alzheimer's, Parkinson's, and metabolic diseases [59]. Given the complexity of the pathophysiology of epilepsy, patients with partial epilepsies selected for surgical resection of the epileptogenic area are ideal candidates for gene therapy: the pathology of their illness is focal; the optimal medical treatment has failed to produce the desired results; and the success of surgery in leading centers (>70% seizure-free at 1 year) supports the idea that local and sustained release of an inhibitory molecule might be sufficient to "silence" hyperactivity. In a certain way, tissue resection represents the most extreme form of cellular "silencing," and gene therapy may provide a realistic alternative [60–62].

Data from in vivo experiments using AAV vectors suggest that this method of gene delivery is a more practicable approach to progress to clinical trials. An early proof of principle study demonstrated that gene transfer using AAV vectors in resected human tissue slices resulted in an appreciable level of transduction of cells in the epileptic tissue [61]. No clinical trial has been conducted using this technology. However, the therapeutic approach in epilepsy is directed to interrupt abnormal seizure activity rather than reintroduce a cell population that was lost, which was often the focus of gene therapy for neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases [59, 63–65].

In summary, the experimental and clinical data from other neurological diseases demonstrate the feasibility of gene therapy for epilepsy [66–68]. The genetic modification of cell cultures and transplantation into the brain is an effective ex vivo gene therapy. This transfer of genes using the genetic engineering of viruses or plasmids and subsequent transfection into cells achieves the expression of transgenes in the CNS. Cell therapy and ex vivo gene therapy have increased our knowledge of plasticity mechanisms and the factors that promote cellular integration in the CNS. Basic and clinical studies suggest that temporal lobe epilepsy, which is clearly refractory to traditional pharmacological approaches, is an ideal candidate for gene therapy, which will significantly impact disease management in the coming decade. There is no doubt that accurate verification of safety and scale-up studies are needed before beginning studies in humans, but gene therapy experience in humans with other diseases is encouraging [42].

Acknowledgments

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Part II

Innovative Solutions for the Screening of New Antiepileptics Drugs and Addressing Multi-Drug Resistant Epilepsy

Chapter 11

Human Brain Tissue as a Model for the Study of Epilepsy

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Abstract

Epilepsy surgery is widely used to treat pharmacoresistant epilepsy. This therapeutic strategy offers the opportunity to study human brain tissue in vitro. We describe three approaches that represent an excellent opportunity for the study of the underlying mechanisms of pharmacoresistant epilepsy: in vitro electrophysiological recordings, in vitro procedures for receptor evaluation, and genomic analyses. These procedures could allow individual diagnosis and personalized treatment for patients with pharmacoresistant epilepsy.

Key words Temporal lobe epilepsy, Human brain tissue, Electrophysiology, Receptor analysis, Genomic analysis

1 Introduction

The human epileptic brain exhibits unique network, cellular, and molecular properties. Therefore, using brain tissue from patients with mesial temporal lobe epilepsy (MTLE) is a first-rate opportunity to characterize the electrophysiological, histological, molecular, and genetic properties in this pathological condition. There is an increase of interest in the study of epilepsy directly in human tissue, estimated by the number of publications on this topic between 1976 and 2005 [1] (Fig. 1).

Epilepsy is one of the most common neurologic disorders [2]. It is estimated that approximately 50 million people is affected with epilepsy worldwide [3]. Despite the severity and high prevalence of this disorder [4], the underlying neurobiology mechanisms associated with epilepsy are poorly understood. Indeed, most of what is known about epilepsy has been obtained from the research done in animal models. Studying human brain tissue in vitro offers a crucial opportunity for the understanding of the disease. The increment of epilepsy surgery [5] and its effectiveness to reduce pharmacoresistant epilepsy [6, 7] has increased the availability of brain tissue from

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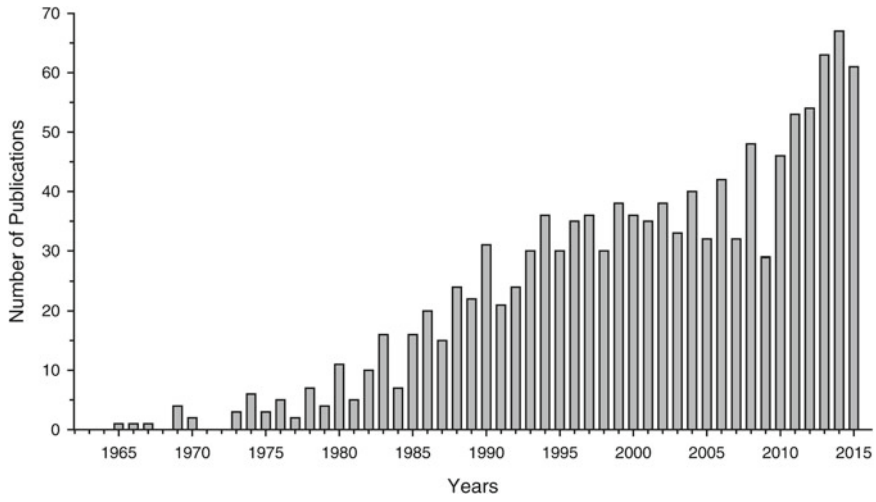

Fig. 1 Histogram showing the number of publications containing "human brain tissue" in PubMed search

patients with this disorder. In this review, we focus in the relevance of evaluating the human epileptic tissue using the following approaches: electrophysiology, receptor analysis, and genetic evaluation.

2 Epilepsy and In Vitro Electrophysiology

The first topic to be addressed is the electrophysiological approach to study the brain tissue obtained from patients with pharmacoresistant epilepsy. This situation allows us to explore directly the activity of human single neurons and networks of brain structures of patients with drug-resistant epilepsy (Fig. 2).

Once the focus has been identified, surgery often involves the resection of specific brain structures involved in the generation and propagation of the seizure activity [6]. It is essential to proceed fast in order to preserve the tissue in optimal conditions. After brain tissue samples have been excised, it is optimal to place them immediately in ice-cold (4 °C) artificial cerebrospinal fluid (ACSF) gassed with a 95% O_2–5% CO_2 mixture. The ACSF composition used for slice perfusion during electrophysiological experiments varies, but it generally includes (in mM): NaCl 124–129, KCl 2–4, $CaCl_2$ 1.6–2.4, $MgSO_4$ 1.3–2, NaH_2PO_4 1.24–1.25, $NaHCO_3$ 21–26, and glucose 10 [1, 8]. When tissue transportation takes long periods (30 min), it is advised to use ACSF reduced in Na^+ (to prevent hypoxia-induced Na^+ influx) and α-tocopherol in addition (as a free radical scavenger) [9].

The thickness of brain slices varies depending if the procedure involves the evaluation of individual neurons or networks. Commonly it goes from 300 to 750 μm [8–14], although it is recommended a maximum of 600 μm for a proper slice oxygenation [1].

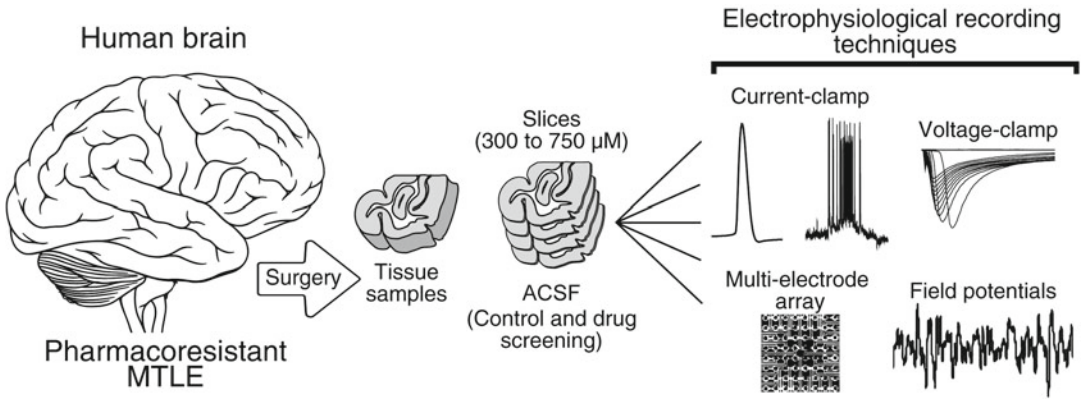

Fig. 2 Schematic representation of the standard protocol used to evaluate electrophysiological activity in brain tissue obtained from patients with pharmacoresistant epilepsy. After slicing, the tissue is available for different techniques of electrophysiological recording (*right panels*); each one provides distinct kind of information depending on the aim of the study. For example, current-clamp allows the study of epileptiform activity and action potential dynamics of individual neurons; voltage-clamp could be used to analyze currents and channel properties; multielectrode arrays provide the opportunity of simultaneously stimulating and recording field potentials, and/or action potentials in different areas of the tissue; field potentials are used to evaluate multiple neuron activity of during epileptiform activity

It is important to consider that some types of epilepsy, such as mesial temporal lobe epilepsy (MTLE), are associated with neuronal damage and hippocampal sclerosis [15–17]. This condition makes the tissue difficult to be penetrated by the pipette or electrode. Also, the usual method to visualize neurons in patch-clamp mode (infrared differential interference contrast optics) could be difficult to implement.

Reports about the evaluation of human cortex unitary activity in vivo exist since 1956 [18]. Extracellular field potentials or unitary neuron recordings in vitro are valuable alternatives for the study of the underlying cellular mechanisms of epilepsy depending on the main goal of the study. Field potential recordings are used to study extracellular activity of network synchronization activity in hippocampal preparations [11] and synaptic plasticity properties [19]. This approach allows the description of spontaneous and rhythmic activity initiated in the subiculum, a condition that resembles the epileptiform discharges recorded by intracranial electrodes [20]. Concurrent to field potential recordings, other measurements as extracellular ionic concentrations can be assessed [21].

The evaluation of unitary extracellular neuronal activity requires the incubation of the human brain tissue immediately after its resection [12]. The study of Schwartzkroin and Prince in 1976 represents the first evaluation of intracellular recording of neurons from the human cortex neurons [22]. These authors employed the same criteria used for assessing neurons of animal

cortex and used sharp electrodes (20–50 MΩ). The results obtained demonstrated that human cortical neurons present stable resting membrane potential (RMP), overshooting action potentials, and absence of rhythmic high-frequency spike firing. Although the number of neurons recorded was small, the authors stood out that the in vitro electrophysiology techniques can be used to obtain valuable data from human brain tissue [20, 22–24].

The firing properties of human neurons evaluated by in vitro electrophysiology methods have shown to be similar to those identified in rodents and other species [1, 24, 25]. Other important approach represents the evaluation of neurotransmitter effects. For example, when GABA is applied to the brain slice, it may exert inhibitory effects crucial for controlling cerebral excitability [10]. This situation is perturbed in the cerebral tissue of patients with pharmacoresistant epilepsy [26, 27]. The in vitro evaluation of the effects of other neurotransmitters such as acetylcholine, adenosine, histamine, norepinephrine, and serotonin reveals their effects and their contribution in the neuronal activity modulation [28].

The electrophysiological activity should be correlated with the cell morphology. Cellular labeling with fluorescent dyes as Lucifer yellow [23] or biocytin [24, 27] are techniques available for morphological reconstructions after the electrophysiological evaluation of the tissue. Morphological data of neurons in epileptic tissue should be carefully compared with data obtained from autopsies [29, 30]. Biocytin-filled neurons in combination with immunochemistry [26] provide more information about the expression of particular proteins.

There are some approaches that reduce the clamp problems such as the dissociation of the cells or neuronal cultures [31]. The isolation of neurons allows the characterization of essential events for the neuronal excitability, such as the transient currents in neocortical neurons [32]. The evaluation of astrocytes in cultures from medically intractable TLE allowed to establish that cells from the seizure focus exhibited action potential-like events in response to electrical stimulation [33].

Another new approach is the evaluation of electrical activity by multielectrode arrays (MEAs). This procedure allows the analysis of spatiotemporal dynamics of the epileptiform activity in specific networks, including the areas of seizure initiation and propagation. It also facilitates the analysis of effects induced by antiepileptic drugs at cellular and network levels, field potentials, and action potentials of multiple neurons from diverse regions of the tissue [34].

One of the most interesting perspectives of the evaluation of the cerebral tissue obtained from patients with pharmacoresistant epilepsy using in vitro electrophysiology is the investigation on the mechanisms underlying the pharmacoresistance to antiepileptic drugs. For example, previous studies demonstrated that the blockage of sodium channels induced by carbamazepine (i.e., use-dependent

blocker of voltage-dependent sodium channels) is not detected in tissue obtained from some patients with pharmacoresistant epilepsy [35]. In another study, the modulation of Na^+ currents induced by valproic acid was tested in isolated neurons from patients with and without hippocampal sclerosis. The cellular response to valproic acid was consistent with previous reports in animal models. In spite of the important damage found in patients with mesial sclerosis, they did not demonstrate differences in the biophysical properties of the voltage-gated sodium currents, when compared with the non-sclerotic tissue [36].

It is evident that the in vitro electrophysiology approaches can contribute to trace the underlying cellular mechanisms of epilepsy and the screening of new antiepileptic drugs and their effects. This approach can be used to determine the final diagnosis and subsequent individual therapy. However, it presents relevant limitations such as the scarce spontaneous epileptiform activity found in human brain slices possibly due to the isolation of complex networks from other brain areas.

3 Evaluation of Receptor Changes in Human Brain Tissue

A receptor is a cellular macromolecule, or an assembly of macromolecules, that is involved in chemical signaling between and within cells [37]. Receptors should present at least the following characteristics to identify them: (1) Binding, specific, and saturable ligand binding are hallmarks of receptors. (2) Affinity, the ability of a drug to bind to the specific receptor to form the drug-receptor complex. The dissociation constant (Kd) or affinity of most receptors for their ligands is in the nanomolar or subnanomolar range. (3) Ligand binding to the receptors should be able to activate signaling pathways to trigger physiological responses [38].

The evaluation of the tissue of patients with epilepsy represents a great opportunity to investigate receptor changes associated with this disorder such as number, affinity, binding, and coupling to transduction mechanisms. The knowledge obtained allows the design of new therapeutic strategies to strengthen or inhibit transductional mechanisms [39]. Receptors in the cerebral tissue of patients with epilepsy have been evaluated through different in vitro and in vivo techniques. The method of choice depends upon the aim of the study.

Western blot is a useful procedure to determine changes in the protein expression of the receptors [40]. In general, the methodology for Western blot is as follows: samples of human brain tissue are suspended in lysate buffer containing protease inhibitor cocktail including 50 mM HEPES buffer, 0.05% Triton X-100, 0.62 mM phenylmethylsulfonyl fluoride, and 20 mM sodium molybdate. Proteins from tissue are isolated by

electrophoresis using 8 % SDS-polyacrylamide Tris-glycine gels. Once separated, the proteins are transferred to a membrane of nitrocellulose in which each protein is located in specific bands according to the molecular weight. The membrane is then incubated with specific antibodies to label the protein of interest. The thickness and density of the band corresponds to the amount of protein present [40, 41]. Although the results obtained from Western blot experiments give information about the protein expression of specific receptors, they do not give data about the capacity of these proteins to bind specific agonists and induce transductional mechanisms.

Some in vivo and in vitro techniques are useful to determine receptor binding changes in the brain of patients with epilepsy. Positron emission tomography (PET) is an in vivo procedure that let us identify the distribution and density of specific receptors in specific brain areas of patients with epilepsy (Fig. 3a). This procedure involves the systemic administration of ligands that may label particular receptors. However, be cautious regarding the interpretation of results due to the presence of endogenous ligands in the brain that may compete with the exogenous ligands.

In vitro autoradiography is a useful procedure to label specific receptors and proteins involved in the transductional mechanisms in brain sections obtained from patients with pharmacoresistant epilepsy and submitted to epilepsy surgery. In vitro autoradiography procedures avoid endogenous ligands and give information about the anatomical localization of the receptors (Fig. 3b) [42].

For in vitro autoradiography, brain sections are exposed to ligands labeled with a radioactive isotope and subsequently exposed to a film [43, 44]. The more commonly used isotopes for in vitro autoradiography are the following: ^{3}H gives the highest resolution and is used to label proteins, nucleic acids, and other molecules frequently involved in binding assays; ^{14}C replaces the nonradioactive carbon in order to trace chemical and biochemical reactions involving pharmacological substances; and ^{35}S is used to label proteins, nucleic acids, and amino acids containing a thiol group [45].

The in vitro autoradiography experiments can be carried out using brain sections of patients with pharmacoresistant epilepsy. Initially, the brain sections are washed to remove endogenous ligands and then incubated in a solution containing the specific radioligand at a concentration necessary to occupy 50 % of receptors (Kd) [39]. Nonspecific binding has to be detected in parallel sections from the same tissue and incubated under similar experimental conditions but including a high concentration of a nonlabeled ligand (a concentration 500–1000 higher than that used for the ^{3}H-ligand). Specific binding is obtained from the difference between nonspecific and total binding. After incubation, the sections are rinsed in buffer, dipped into distilled water, and dried under cold air stream. The dried sections are exposed to a sensitive

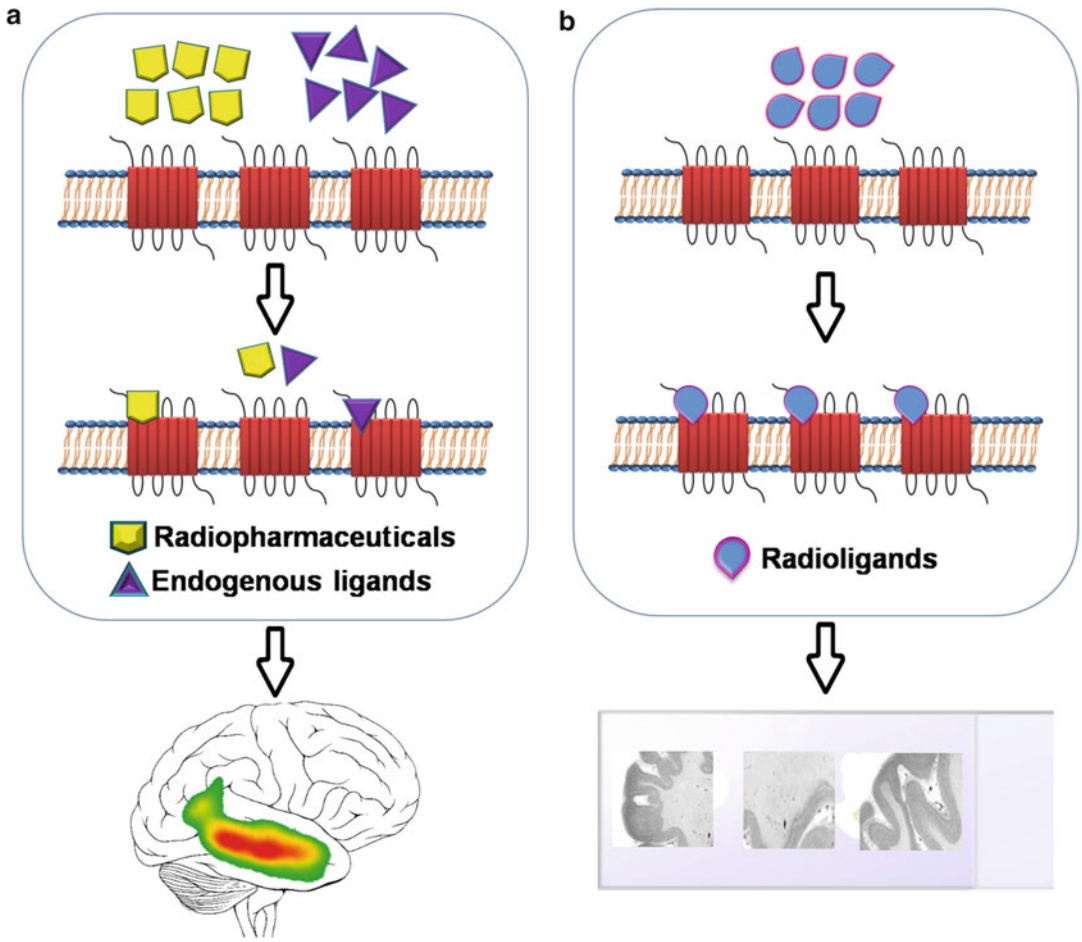

Fig. 3 Schematic representation of in vivo positron emission tomography (PET) (**a**) and in vitro autoradiography (**b**) procedures. Notice that in PET radiolabeled ligands compete with the endogenous ligands for the binding site in the receptors. In contrast, for autoradiography, the radioligand binds directly to the receptors because the endogenous ligands were previously washed

film together with standards of the isotope used. After the exposure period, the film is developed and used to determine optical densities of specific brain areas. The optical density is converted to radioactivity values (dpm/mm^2) and then to amount of receptors (fmol/mg protein) [46–48].

The in vitro autoradiography allows to determine the distribution of receptors labeled with a specific ligand in selected brain areas of patients with pharmacoresistant epilepsy [43, 49]. The results obtained will depend on the affinity and number of receptors labeled. However, in vitro autoradiography is not an appropriate procedure to determine specific characteristics of the receptor binding like maximal binding (Bmax) and affinity (Kd).

Binding of an agonist to its receptor induces conformational changes in the receptor molecule. This situation leads to activation

of the G protein, a process during which the α subunit replaces the GDP with GTP. Functional autoradiography is a technique used to know if the receptor is capable to activate this reaction and subsequent transductional signaling pathways. During the functional assay, the tissue is exposed to [^{35}S]-GTPγS that is resistant to hydrolysis. This situation provokes that [^{35}S]-GTPγS replaces the GDP upon receptor activation and remains irreversibly bound to the G proteins [39, 42].

To perform functional autoradiography, the sections are initially dipped in assay buffer (50 mM Tris, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 Mm NaCl, pH 7.4) at 25 °C for 10 min and then incubated with 2 mM GDP in assay buffer for 30 min at 25 °C. Thereafter, the process involves different assays in parallel sections under different conditions (Table 1). The specific binding results from the difference between the values obtained from the basal and nonspecific binding assays. At the end of each assay, slides are rinsed twice, 2 min each in 50 mM Tris–HCl buffer and once in deionized H_2O at 4 °C. Slices are dried overnight and exposed to film for 5 days in film cassettes containing [^{14}C] microscales. A standard curve is generated from [^{14}C] microscales values. Optical density readings are converted into nanocuries of [^{35}S] per milligram of tissue. Net agonist-stimulated [^{35}S]-GTPγS binding is calculated by subtracting basal binding (obtained in absence of agonist) from agonist-stimulated binding [46, 50].

A main limitation of the [^{35}S]-GTPγS autoradiography is that not all the G proteins can be detected with this method due to their low number or efficiency. In addition, it results difficult to identify the types of G proteins labeled with [^{35}S]-GTPγS [42, 51].

Binding assays in membranes are valuable procedures to determine important receptor characteristics such as Bmax (propor-

Table 1
Experimental conditions to implement functional autoradiography

Basal activity	G proteins are evaluated in presence of 2 mM GDP and 40 pM [^{35}S]-GTPγS. No agonists are included in the assay
Agonist-stimulated [^{35}S]-GTPγS	This step is carry out in presence of 2 mM GDP, 40 pM [^{35}S]-GTPγS, and a receptor agonist at a concentration necessary to produce maximal stimulation (μM) of the G proteins. The [^{35}S]-GTPγS labels the G proteins activated as consequence of the agonist
Antagonist blockade of agonist-stimulated [^{35}S]-GTPγS	A parallel assay has to be focused to evaluate the specificity of the receptor that activates the labeled G protein. The assay is carry out according to the conditions described for the agonist-stimulated [^{35}S]-GTPγS but containing a specific antagonist at a concentration that blocks >90% of the receptor-induced G protein activation
Nonspecific binding	The assay is similar to the agonist-stimulated [^{35}S]-GTPγS but with an excess of unlabelled GTPγS (10 μM).

tional to the number of receptors present in the brain tissue), Kd (equilibrium dissociation constant), Emax (concentration of an agonist to produce maximal stimulation), and potency of stimulation (EC_{50}).

Binding assays can be used to determine changes in Bmax and Kd, and [^{3}H] is the most frequently used isotope for this purpose. For this procedure, brain tissue (~50 mg) is thawed and homogenized into a mixture of ice-cold 50 mM Tris–HCl and 1 mM EGTA, pH 7.4. After centrifugation (13,000 rpm, 20 min, 4 °C), the pellet is resuspended in 10 ml of 50 mM Tris–HCl buffer solution (pH 7.4) and centrifuged again. The resulting pellet (crude membranes) is suspended in incubation buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4). Receptor binding assays are performed in triplicate in 0.5 ml of incubation buffer containing membranes (30–70 μg protein) and in presence of different concentrations of the radioligand with and without a non-labeled ligand. Specific binding is determined by subtracting the binding in the presence of non-labeled ligand from total binding. Incubations are terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethylenimine. Filters are washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4), dried, and then immersed in Sigma-Fluor™ scintillation cocktail (Sigma). Radioactivity is determined using a Beckman LS6000SC liquid scintillation counter. Data are expressed in fmol/mg of tissue.

Binding assays in membranes can be also used to evaluate the ligand-induced activation of G proteins subsequent to the receptor stimulation (Emax and EC_{50}). The binding assays are as follows: brain tissue (~50 mg) is thawed and individually homogenized in 10 mM Tris–HCl solution containing 1 mM EGTA (pH 7.4) and centrifuged (10 min, 1800 rpm, 4 °C). The supernatants are collected and centrifuged at 13,000 rpm (20 min, 4 °C) and the pellet obtained is incubated (30 min, 30 °C) in 5 ml of assay buffer (50 mM Tris–HCl, 100 mM NaCl, 0.2 mM EDTA, pH 7.4). The incubation is stopped with the addition of 10 ml of ice-cold buffer. The obtained membranes are incubated (60 min, 60 °C) in 0.5 ml of assay buffer containing 0.02% bovine serum albumin, [^{35}S]-GTPγS (50 pM), and increasing concentrations (10^{-10}–10^{-6} M) of a selective receptor agonist in the presence of excess GDP (10 μM). Basal binding is measured in the absence of the tested compound. Nonspecific binding is determined in the presence of 20 μM unlabeled GTPγS and subtracted from total binding to calculate the specific binding. The reaction is initiated by adding [^{35}S]-GTPγS and terminated by filtration of the samples through Whatman GF/B glass fiber filters. Filters are washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4), dried, and dipped in Sigma-Fluor™. Finally, bound radioactivity is determined using a liquid scintillation counter. Data are subjected to nonlinear regression analysis of concentration effect curves performed by Prism (GraphPad Software) to determine Emax

and EC_{50} values. Data are expressed in percentage of stimulation or in fmol/mg of protein [52].

One important limitation of the binding assays is that the changes evaluated correspond to big areas of the cerebral tissue. This procedure does not allow the evaluation of small brain areas.

4 Genomic Analysis of Brain Tissue from Patients with Pharmacoresistant Epilepsy

According to the dogma of molecular biology (Fig. 4), nucleic acids (DNA and RNA) and proteins, through the processes of replication, transcription, and translation, are key to perform genomic analyses. These represent the main tools in research, diagnosis, evaluation, treatment, and prognosis of many diseases, including various types of epilepsies. At present, genetic alterations are considered relevant in the development of epilepsy [53] and various types of seizures, such as juvenile myoclonic epilepsy [54]. It is important to consider that some of the studies that demonstrate the role of genes in the development of epilepsy and the associated pathological processes (e.g., drug resistance and hippocampal sclerosis) have been carried out using tissue obtained from patients subjected to epilepsy surgery [55].

When the cerebral tissue is removed from the human body, the neuronal cells begin to die. During this process, there is an activation of specific caspases that in turn activate endo- and exonucleases responsible for the degradation of RNA [56]. In contrast with DNA, which has a double-stranded arrangement and a chemical structure that favors its preservation, RNA is more labile and sensitive to degradation due to its single-stranded conformation while the presence of ribose facilitates the reaction with oxygen [57]. Therefore, the experimental conditions to manipulate postsurgical

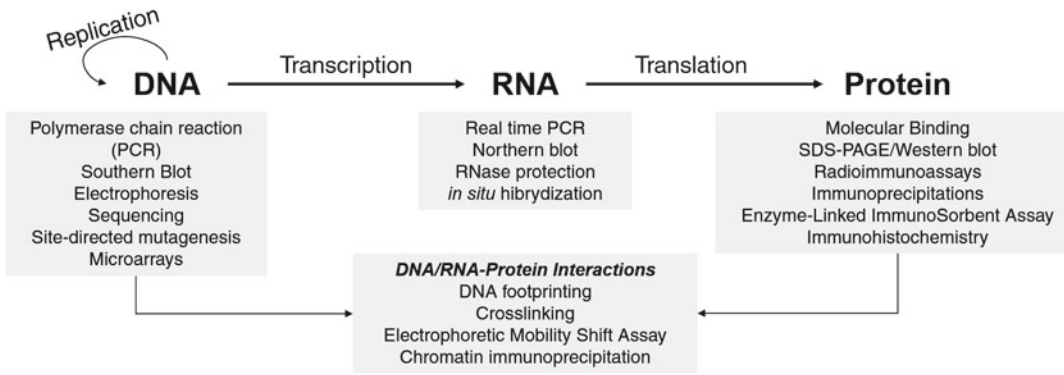

Fig. 4 Central dogma of molecular biology. Notice the different methodological approaches that can be carried out to evaluate DNA, RNA, and protein expression

tissue are essential to maintain the quality of proteins and nucleic acids and give validity and reliability to the results obtained [58].

In practice, after surgical resection, the epileptic tissue should be immediately frozen and stored at −80 °C for the subsequent mRNA evaluation. Low temperatures (−60 °C or lower) prevent the activation of caspases and hence the degradation of nucleic acids and proteins [59]. However, RNA seems to maintain some reactivity at low temperatures. RNases and some ribozymes are still active at −20 °C and −70 °C, respectively [60, 61]. At present, there are commercial compounds such as RNAlater® (Qiagen) which are useful to stabilize and protect the structure of cellular RNA and prevent its oxidation. Formalin fixation with paraffin embedding represents other procedure for mRNA preservation and maintaining histology [62]. With this procedure, the proteins are kept whole and the DNA can be isolated with very good quality for several years after [63–65]. Other important factor for consideration is the period of storage. The integrity of nucleic acids and proteins are compromised when the tissue is stored for long periods after the surgical resection.

Some considerations are relevant to keep in mind when isolating nucleic acids or proteins obtained from cerebral tissue: (1) one must work on ice and under complete sterile conditions; (2) the use of inhibitors of endo- and exonucleases in the case of nucleic acids is essential, and protease inhibitors in the case of proteins, (3) the material must be treated with diethylpyrocarbonate (DEPC) to 0.1 %, which is a potent inhibitor of RNase [66].

The nucleic acid isolation of cerebral tissue from patients with epilepsy is often achieved by common protocols. However, isolation of DNA, RNA, and proteins has to be done separately if we want to preserve the quality of the molecules. For DNA isolation, it is necessary to use extraction with organic solvents such as ethanol or acetone. This procedure preserves the DNA of high molecular weight. The RNA is isolated by phenol–chloroform extraction in addition to commercial solutions of guanidine isothiocyanate and ammonium thiocyanate (e.g., TrizolThermoFisher Sci.). Proteins are isolated through different buffers containing detergent and ionic salts. Once the RNA is obtained, it is necessary to confirm its purity and integrity. The purity is determined by commonly used spectrophotometric assays, according to the ratio 260/280 nm. Using this procedure, an index of 1.8 for DNA and RNA 2 indicates high purity, whereas lower values indicate contamination with proteins or organic solvents. The integrity is determined by electrophoretic assays using 1 % agarose gel. Using this procedure, a single band must be observed for DNA (no sweep), while for the RNA, it is common to detect electrophoretic migration patterns of the 28S, 18S, 5.8S, and 5S ribosomal subunit. These values represent the RNA integrity number (RIN) [67]. Pure samples of DNA should

be stored in solution because their dehydration leads to denaturation. In contrast, the RNA is best preserved dehydrated [59].

Concerning epilepsy and regardless of the level and the techniques used, the study of patients with this disorder is focused on the search for the "epilepsy gene." Unfortunately this approach is utopian because it is impossible to attribute the cause of epilepsy to a single gene since it represents a multifactorial disorder from a physiological point of view [68]. Also, the molecular analysis of genes has to be associated with different factors, such as type of epilepsy, the epileptic focus, the treatments administered prior to surgical resection, whether or not it is drug resistant, and other clinical variables.

Despite the plurality in studies, when a search in PubMed (NCBI) is performed with the words "epilepsy and genes in humans," they found only 6285 items, in which research ranges from the characterization and analysis of gene expression (the gene encoding the $GABA_A$ receptor subunit δ [69]) to variants associations (atypical variants, p.G257R, p.R323Q, p.I389V in $GABA_A$ receptor associated with Rolandic epilepsy [70]), polymorphisms and mutations (the SNP c.3435C > T in the MDR1 gene encoding the P-glycoprotein transporter [71]), identification and global study of microRNAs (miRNAs such as miR-204 and miR-218 that are downregulated in patients with TLE [72]), identification of biomarkers (TGFβ1 that is increased in CSF of patients with drug-resistant epilepsy [73]), epigenomic analysis (hypermethylation-associated genes observed in neurotransmission and synaptic transmission in patients with TLE [74]), and inheritance (the deletion of 18q21.32 relatives inherited from a family with cases of idiopathic epilepsies [75]). Thanks to all the studies using tissue of patients with epilepsy, many genetic aspects of the disease have been identified. However, there is still a long way to go in understanding the mechanisms. An undeniable fact is that studies of gene expression have opened the door to a new era where the final goal is the optimization and customization of treatments, thus improving the quality of life of patients.

5 Autopsy and Control Human Tissue

A good research design should always consider a control condition to eliminate variables that could lead to the wrong conclusion. The evaluation of human brain tissue of patients with epilepsy implies the analysis of control tissue obtained from neurologically healthy subjects. However, this situation is difficult to obtain.

Appropriate controls represent a crucial situation that must be addressed when human tissue is used for electrophysiology recordings [76]. It is clear that normal tissue samples cannot be used for comparison for obvious ethical causes. Resected samples of non-epileptic patients due to removal of deeply laying tumors have been

Table 2
Factors that affect the quality of the brain samples for molecular studies in epilepsy

Antemortem	Postmortem
Diseases	Postmortem interval (PMI)
Premedication	Structural alterations of the pathophysiology of epilepsy (hippocampal sclerosis and cortical dysplasia)
Fever	pH of the brain or cerebrospinal fluid
Hypoxia-ischemia	Ambient temperature in the postmortem period
Acidosis	Harvesting procedures
Agonal conditions	Storage temperature
	Accidental or systematic thawing and refreezing

used as "control tissue" [65]. Nevertheless, this tissue is not adequate for comparison because it comes from patients with a different pathology.

Many studies include the evaluation of autopsy samples obtained from subjects who died of different causes and without evidence of neurological illness. However, it is important to consider various antemortem and postmortem factors that can directly impact on the quality and therefore the validity of the results obtained from autopsy samples. For example, the evaluation of nucleic acids from autopsies is particularly difficult (Table 2) [76, 77]. Indeed, there are controversial studies indicating that the RNA of the brain tissue is stable and preserves integrity even several hours after death [78, 79], while other studies suggest a rapid and definitive RNA degradation as the postmortem interval progresses [76, 80].

6 Concluding Remarks

Certain epilepsy animal models have received great acceptance as they exhibit similar development of spontaneous seizures and a spectrum of histological changes like those of showed by patients with epilepsy [81]. However, the study of brain tissue obtained from patients with pharmacoresistant epilepsy provides valuable information about human epilepsy pathophysiology [22] and represents an excellent tool for individual diagnosis and specialized treatment. Indeed, correlations between the results obtained and different clinic data from the patients with pharmacoresistant epilepsy represent a valuable procedure to identify the possible influence of relevant conditions that can be manipulated to modify the expression of the disease.

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Chapter 12

The Blood–Brain Barrier and the Design of New Antiepileptic Drugs

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Abstract

The vertebrate's brain is vascularized during its development to provide oxygen and essential nutrients to newly born neurons. This brain vasculature is anatomically distinct from that of other organs. It comprises, in addition to endothelial cells, pericytes and astrocytes, which collectively form the neurovascular unit, structure that underlies the blood–brain barrier and regulates blood flow to match brain activity. The main features of the vertebrate blood–brain barrier are closed cell–cell junctions, a low rate of transcytosis, and the expression of various adenosine triphosphate-binding cassette transporters. The blood–brain barrier function is impaired in several neurological diseases including epilepsy. This chapter will provide a brief overview on the regulation of blood–brain barrier properties by the neurovascular unit and the relation between blood–brain barrier, seizures, and epilepsy. We will also summarize new therapies that aim to control seizure activity.

Key words Neurovascular unit, Blood–brain barrier, Seizures, Epilepsy, Antiepileptic drugs

1 Introduction

The brain constitutes only 2% of the body weight and can be considered the most complex and energy-demanding organ since it consumes approximately 20% of the human body's metabolic reserves. However, it has a limited capacity to store energy, and thus the maintenance of its optimal function depends on the proper delivery of oxygen, glucose, and other nutrients via the anatomically distinct brain vasculature. There is therefore a tight relation between the local microscopic cerebral blood flow and the changes in neuronal activity of specific brain regions. This regulating mechanism is named hyperemia and is activated in physiological situations such as reading or it can also be triggered in pathological situations such as seizures [1–3].

The complex interplay between changes in blood supply according to increased or decreased neuronal demand is accomplished by a group of cells of both vascular and neural origin, called

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the neurovascular unit [4]. Harder et al. [5] defined it as a synchronized interactive structure formed by neurons, interneurons, astrocytes, microglia, pericytes, endothelial cells, and extracellular matrix components, which establishes an anatomical and functional whole. One of the distinctive functional characteristics of the neurovascular unit is the blood–brain barrier, first described by Paul Ehrlich in 1885. However, it was Edwin Goldman who showed that the water-soluble dye trypan blue injected into cerebrospinal fluid readily stained central nervous tissue blue, contradicting Ehrlich's conclusion of a lower binding affinity of the central nervous system for these dyes and supporting the hypothesis of limited permeation from the circulatory system into the brain [6, 7]. More than a century later, we know that the blood–brain barrier serves as a functional and anatomical barrier able to control the flow of solutes in and out of the brain to maintain an optimal environment for neuronal functioning. Moreover, the blood–brain barrier is the site where drug entry into the brain is regulated by multidrug transporter molecules [8]. The knowledge about this structure is fundamental to understand some aspects of brain physiology, as well as the pathogenesis of several neurodegenerative diseases.

This chapter focuses on the cellular components of the neurovascular unit, the blood–brain barrier characteristics and its involvement in the generation of seizures and epilepsy, and the new therapies that aim to optimize drug delivery in the epileptic brain.

2 The Neurovascular Unit Components

Under physiological conditions, each component of the neurovascular unit (Fig. 1) plays an active and particular role, which results in a highly efficient regulatory system of the cerebral blood flow [9, 10]. However, the specific role of each component is not yet fully established [11].

2.1 Endothelial Cells

The endothelial cells that line the luminal side of blood vessels enter the brain parenchyma early during neural tube development [12]. It is currently known that endothelial cells produce trophic and vasoactive factors which are extremely important for the vascular tone control; some of these factors are dilatory as nitric oxide, and some others are vasoconstrictors as endothelin and thromboxane [13, 14].

Their morphological, biochemical, and functional properties are unique and distinguishable from all other endothelial cells in the body [15]. Specifically, the brain endothelial cells have longer tight junctions, sparse pinocytic vesicular transport systems, no fenestrations in their cytoplasm, a high amount of mitochondria, and a negative surface charge that repulses negatively charged

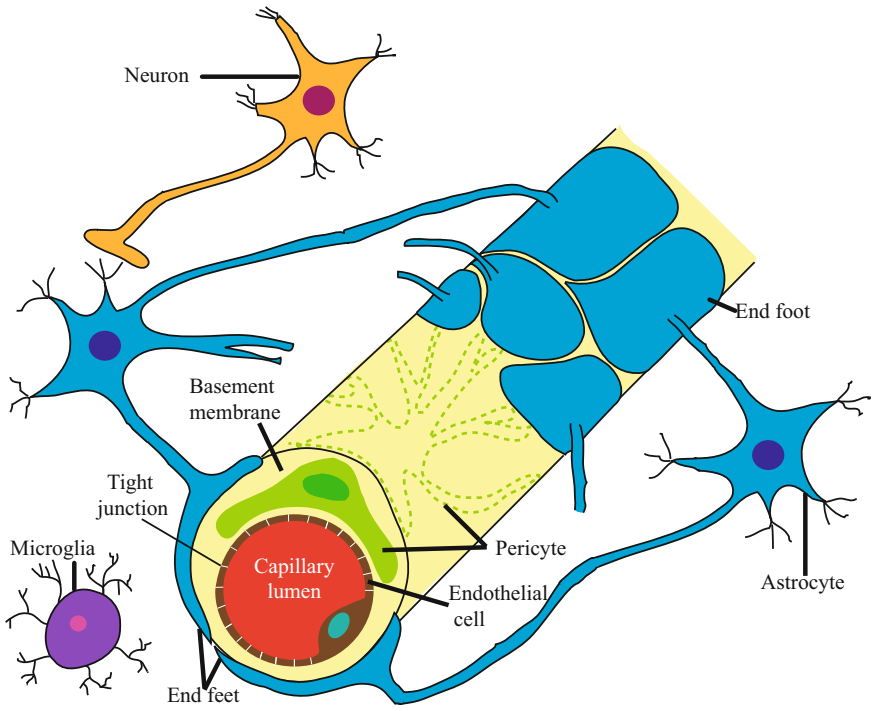

Fig. 1 Constitution of the neurovascular unit. Schematic representation showing the tight junctions between the endothelial cells surrounding a blood–brain capillary and ensheathed by the basement membrane. Pericytes are distributed discontinuously along the length of the cerebral capillaries. Cerebral endothelial cells and pericytes are enclosed by the basement membrane. Astrocytic end-feet processes conform a complex network surrounding the capillaries. Astrocytes provide links to neurons and microglia are central nervous system-resident immune cells

compounds. The diffusion of hydrophilic solutes is severely restricted since the capillary endothelium in the brain is 50–100 times tighter than peripheral microvessels [16]. However, these cells are set up with specific transport systems and receptors that will help to uptake nutrients and hormones required for brain function [17].

2.2 Vascular Smooth Muscle Cells and Pericytes

Vascular smooth muscle cells and pericytes, collectively known as mural cells, enter the brain parenchyma together with endothelial cells during angiogenesis [18]. The vascular smooth muscle cells surround small arteries, arterioles, and venules. It is believed that the blood flow of brain vasculature is controlled at the level of arterioles and thus involves vessel constriction and relaxation controlled by neurons and astrocytes that are mediated by vascular smooth muscle cells [19].

The pericytes are perivascular cells that tend to be aligned with the vessel axis, surrounding the endothelium around microvessels and capillaries in the brain, which provides support to endothelial cells. Pericytes share the basement membrane and are in direct

communication with astrocytes, neurons, and endothelial cells, exchanging information through tight junctions, gap junctions, adhesion plaques, and soluble factors [3, 20–23]. The contractile proteins expressed in pericytes, such as alpha-SM actin, play a fundamental role in the regulation of capillary blood flow since they provide vascular stability by modulating the vessel diameter [24]. Indeed, pericyte loss leads to locally reduce cerebral blood flow and breakdown of the blood–brain barrier [18].

2.3 Astrocytes

The astrocytes are glial cells characterized by their numerous processes containing the cytoskeleton intermediate filament, the glial fibrillary acidic protein [17]. Certain astrocytes cover the entire abluminal side of blood vessels with specialized processes called astrocyte end-feet [16, 25]. These astrocytic end-feet are slender processes, enriched with water and potassium channels, anchored to the basement membrane of blood vessels via the dystroglycan complex [26]. Due to the anatomic proximity to endothelial cells, the astrocytes play a central role in the restricted permeability and in the integrity of the blood–brain barrier. Basically, they enhance the formation of tight junctions between endothelial cells and reduce its gap junctional area, regulate the brain water and electrolyte metabolism, modulate the expression and polarization of transporters, and promote specialized enzyme systems [27–29].

Besides the relevance of the interplay between astrocytes and endothelial cells for blood–brain barrier properties, astrocytes are also crucial for proper neuronal function and, thus, for a functional neurovascular unit. In fact, astrocytes can signal vascular smooth muscle cells of blood vessels in order to regulate the cerebral blood flow in response to moment-to-moment changes in local neuronal activity [17]. Astrocytes also contribute to a variety of neuronal functions such as synapse formation and plasticity, energetic and redox metabolism, and synaptic homeostasis of neurotransmitters and ions [30].

2.4 Perivascular Macrophages

The perivascular macrophages are blood-derived phagocytic cells that reside in the perivascular spaces, which surround penetrating arterioles and are continuous with the subarachnoid space. These perivascular cells perform phagocytic functions and regulate the immune response and lymphocyte entry into brain parenchyma [31–33].

2.5 Microglia

The microglial cells invest the brain very early during development just before the endothelial cells invade and vascularize the tissue [34]. Since microglia are located in perivascular space, it is conceivable that their interactions with the endothelial cells may influence the blood–brain barrier properties. However, this is still an unclear issue that has led to contradictory findings in the literature.

Microglia plays a very important role in immune responses of the central nervous system. In fact, these cells present themselves in two forms: resting (under physiological conditions) and activated (under pathological conditions) microglia. When resting, cells have small bodies and long thin processes, known as ramified morphology; in contrast, activated microglia assume a phagocytic morphology by shifting from long to short processes [28].

2.6 Neurons and Interneurons

Neurons are characterized by a cell body, an axon, and numerous dendrites. It has been estimated that almost every neuron in the human brain has its own capillary, which shows the importance of the close neuronal–vascular relationship for normal brain functioning [28]. In fact, the neurons have been called the pacemaker of the neurovascular unit [35]. They are able to detect small variations in its supply of nutrients and oxygen and transform these signals into electrical and chemical messages to adjacent interneurons or astrocytes [36]. In some cases, neurons communicate with the vessels via the astrocytes, influencing the vascular tone and the blood supply to the area surrounding it [14, 37]. Furthermore, there is some evidence that neurons can regulate the function of blood vessels in response to metabolic requirements by inducing expression of enzymes unique for brain microvascular endothelial cells [25]. On the other hand, mature endothelium has a reciprocal function in inducing a stable brain microenvironment that enables proper neuronal activity [38].

2.7 Basement Membrane

The basement membrane is an important regulator of all epithelial cells and is crucial for the integrity and mechanical stability of brain vasculature. It surrounds the brain microvascular endothelial cells and pericytes and presents a duplicature that separates the pericyte from the endothelial cells and the astrocyte end-feet [39, 40].

The basement membrane does not act as a significant barrier to the diffusion of small molecules, but its anchoring function plays an important role in the integrity of cerebral microvasculature and, thus, on blood–brain barrier stability, contributing to tissue/cell organization and differentiation [25, 41]. The main basement membrane components are structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin), and proteoglycans (perlecan), organized in three apposed layers [26, 42]. In addition, the basement membrane also includes matrix adhesion receptors, known as cell adhesion molecules, as well as signaling proteins, which form an extensive and complex matrix [40]. The basement membrane is also essential for endothelial cell polarization and lumen formation. Proper endothelial polarization is needed for the correct localization of influx and efflux transporters [43].

3 Blood–Brain Barrier Characteristics

The blood–brain barrier is a collective term referring to a complex system of metabolic, physical, and transport filters or barriers that control access of blood-borne chemicals to the brain but, at the same time, ensure the delivery of essential nutrients and selected biomolecules. Altogether, these barriers maintain an optimal and stable physicochemical environment within which the central nervous system can operate [44].

3.1 Cell–Cell Junctions

Cell–cell junctions in the brain are closed and they even restrict the passage of ions, contributing to maintain endothelial polarization and the correct localization of blood–brain barrier transport proteins [45]. Two different types of endothelial cell–cell junctions are crucial for keeping the correct blood–brain barrier properties, the tight and the adherens junctions.

The tight junctions are elaborate structures located on the luminal region of the endothelial cells (Fig. 1). It is generally accepted that these junctions seal the interendothelial cleft forming a continuous blood vessel. They significantly reduce permeation of ions and other small hydrophilic solutes through the intercellular cleft (paracellular pathway) but cannot prevent that small highly lipophilic substances such as oxygen and carbon dioxide diffuse passively into the brain parenchyma along their concentration gradient [46–48]. Tight junctions primarily confer low paracellular permeability and high endothelial electrical resistance [49]. The tight junctions are composed of claudins (claudin-3, claudin-5, claudin-12), occludins, junction adhesion molecules (JAM-A, JAM-B, and JAM-C), and several transmembrane proteins [50].

The adherens junctions are typically found intermingled with the tight junctions [28]. They hold the cells together giving structural support and participate in the regulation of the paracellular permeability and the initiation of cell polarity [51]. Adherens junctions are composed of vascular endothelial cadherin and catenin [52].

3.2 Influx and Efflux Transporters

The tight junction-controlled paracellular impermeability of the brain capillary endothelium implies that the hydrophilic molecules must cross the endothelial wall by transcellular pathways to reach their neuronal targets or leave the brain [53]. Influx and efflux transporters carry nutrients, ions, and macromolecules into the brain and remove toxic and unwanted molecules into the circulatory system. These transporters are expressed in a polarized manner which allows the effective exchange of molecules and ions between blood and the brain parenchyma [47, 53].

The fact that the blood–brain barrier has a high density of mitochondria reflects high energy demands for active adenosine triphosphate-dependent transporters. The sodium pumps (Na^+, K^+-ATPase)

and the sodium-potassium-two chloride cotransporters are examples of ion transporters [54].

The solute carriers are transporters embedded in the luminal and abluminal membranes. This carrier-mediated transport allows the efflux of waste products and the influx of ions, nutrients, and molecules essential for the central nervous system metabolism such as glucose, amino acids, nucleosides, and neurotransmitters [48, 55].

The brain endothelium also expresses many different adenosine triphosphate-driven efflux pumps that are localized on the luminal side of the endothelium. The main blood–brain barrier efflux transporters are P-glycoprotein, multidrug resistance-associated proteins, breast cancer-related protein, members of the organic anion transporting polypeptide family, and members of the organic anion transporter family. These transporters have the potential to work together to reduce penetration of many drugs into the brain and to increase extrusion from the brain. Weak substrates of the blood–brain barrier efflux transporters generally pass the blood–brain barrier to a certain extent and exert central nervous system effects, showing that they are lipophilic enough to diffuse through the blood–brain barrier. However, their effectiveness may be affected by expression rates and the functional state of the relevant efflux transporter. In contrast, strong substrates of the blood–brain barrier efflux transporters do not pass the blood–brain barrier to a relevant extent, and their pharmacodynamic effects are restricted to the periphery [56–58].

3.3 Transcytosis

Transcytosis is the vesicular mechanism by which large molecules such as proteins and peptides can move across the brain endothelium. This mechanism can be specific (receptor-mediated transcytosis) or nonspecific (adsorptive-mediated transcytosis system) [47, 59]. In both cases, to achieve transcytosis of an intact protein or peptide, the lysosomal compartment within the cell needs to be avoided by routing the primary sorting endosome and its contents away from the degradative compartment. Routing away from the lysosome appears not to occur in many peripheral endothelia but it seems to be a specialized feature of the blood–brain barrier [60].

Large proteins such as insulin, transferrin, leptin, lipoproteins, and immunoglobulin G use the receptor-mediated transcytosis to cross the blood–brain barrier. This mechanism involves the binding of macromolecular ligands to specific receptors on the cell surface triggering an endocytic event. The first step of this event occurs when the receptor and its bound ligand cluster together and a caveolus is formed which pinches off into a vesicle. Then, both ligand and receptor are internalized into the endothelial cell and routed across the cytoplasm to be exocytosed at the opposite pole of the cell. Finally, the dissociation of the ligand and the receptor presumably occurs during cellular transit or during the exocytotic event [47]. The receptor-mediated transcytosis has been used for

drug delivery into the brain via a strategy known as Trojan horses. For example, different growth factors or anti-sense agents that normally do not cross the blood–brain barrier, or immunoliposomes carrying naked DNA, can be conjugated to monoclonal antibodies against one of the blood–brain barrier receptors (e.g., insulin and transferrin). Therefore, the monoclonal antibodies act as surrogate ligands and can be used to carry conjugated neurotherapeutics across the blood–brain barrier [61].

The adsorptive-mediated transcytosis system is a mechanism that requires an excess positive charge on the molecule, which renders it cationic. The posterior interaction with the cell surface binding sites will induce endocytosis and subsequent transcytosis [62]. Molecules as histone and albumin use this mechanism to access the blood–brain barrier [55].

4 Blood–Brain Barrier, Seizures, and Epilepsy

A wide range of neurological conditions such as Alzheimer disease, Parkinson disease, multiple sclerosis, trauma, brain tumors, stroke, and epilepsy are associated with perturbations in the normal blood–brain barrier that contribute to their pathology.

In order to study whether blood–brain barrier disruption can cause epilepsy, several studies have focused on the changes of the blood–brain barrier permeability during epileptogenesis. Their results show that a remarkable blood–brain barrier leakage is detected during the acute and latent phases (shortly after *status epilepticus*), when spontaneous seizures are still absent. This indicates that the blood–brain barrier disruption does not induce seizures immediately but is more likely to play a role in epileptogenesis [63–67]. Similarly, while extensive blood–brain barrier leakage is observed directly after traumatic brain injury in humans, seizure activity develops only at later time points [68–70].

On the other hand, a few attempts have been made to investigate the role of a single seizure on blood–brain barrier permeability. van Vliet et al. [64] were able to show that blood–brain barrier leakage occurs also during the chronic epileptic phase in rats as a result of a spontaneous seizure. These data suggest that a spontaneous seizure is associated with focal blood–brain barrier leakage, which probably may help to sustain the epileptic condition. In conclusion, although it seems that acute seizures can induce blood–brain barrier damage, it is not easy to determine whether disruption of the blood–brain barrier during the chronic epileptic phase is just a consequence of seizure activity or that it can also contribute to the occurrence of seizures.

One of the most obvious changes after blood–brain barrier disruption is the presence of serum proteins in the brain tissue. Previous studies showed that albumin is present in the parenchyma

but can also be taken up or bound to neurons, astrocytes, and microglial cells, particularly shortly after traumatic brain injury and *status epilepticus* [63, 64, 66, 67, 71–77]. Ivens et al. [72] described that albumin can be taken up by astrocytes via transforming growth factor β receptors. This is followed by a downregulation of inward-rectifying potassium (Kir 4.1) channels and water (aquaporin 4) channels in these astrocytes. As a result, the buffering of extracellular potassium is reduced, which facilitates *N*-methyl-d-aspartate-mediated neuronal hyperexcitability and eventually epileptiform activity. However, Frigerio et al. [76] have suggested that albumin is not neurotoxic by itself and that the features of cellular damage in albumin-positive neurons in epileptic tissue are determined by the underlying neuropathology. For example, considering that the albumin uptake in neurons can increase the synthesis and release of glutamate [78], this will in consequence enhance neuronal excitability. Therefore, the role of albumin accumulation in neurons and its contribution to epileptogenesis needs further study.

Moreover, albumin can also induce brain inflammation via the upregulation of cytokines and the activation of the transforming growth factor β receptor pathway [79, 80]. This brain inflammation can directly affect synaptic signaling and plasticity, reduce inhibitory transmission, affect blood–brain barrier permeability, and thereby increase the seizure susceptibility and promote epileptogenesis [81–84].

5 Design of New Antiepileptic Drugs

Epilepsy is one of the most prevalent neurological disorders affecting 65 million people worldwide [85]. Preclinical research has enabled the discovery of valuable drugs for the symptomatic suppression of seizures in epileptic patients. However, although more than 20 available antiepileptic drugs include compounds with a broad range of mechanisms of action, this symptomatic treatment is not successful in a third of all people with epilepsy, especially those with temporal lobe epilepsy [86].

The fact that the efforts in antiepileptic drug development have not solved the issue of pharmacoresistance has encouraged experimental and clinical research to focus on drug refractory mechanisms. In this sense, the multidrug transporter hypothesis has emerged as a mechanistic explanation of drug resistance. Based on data obtained from expression analyses in tissue dissected during epilepsy surgery as well as from rodent seizure and epilepsy models, this hypothesis suggests an increased expression of transporters in the luminal membrane of brain capillary endothelial cells which results in enhanced efflux transport into the capillary lumen, thereby limiting brain access of antiepileptic drugs [57]. Considering the broad substrate spectrum of the involved transporter molecules, it is not surprising

that blood–brain barrier efflux constitutes a major hurdle in central nervous system drug development [87]. Therefore, brain penetration of high-affinity substrates can be efficaciously limited, preventing any therapeutic effect in the central nervous system [88].

Studies in rodents have indicated that coadministration of P-glycoprotein modulators can enhance brain penetration of antiepileptic drugs. Local administration of verapamil increased brain penetration of different antiepileptic drugs in microdialysis experiments [89, 90]. In an epilepsy model, administration of tariquidar (a third-generation modulator) promoted brain penetration of phenytoin in those brain regions with overexpressed P-glycoprotein [91]. In addition, some case reports have been published describing add-on treatment with verapamil in patients [92, 93]. However, any future planning needs to consider the fact that P-glycoprotein serves an important physiological function protecting sensitive tissues and hematopoietic cells from harmful xenobiotics and regulating the distribution of endogenous compounds. Considering the physiological relevance of blood–brain barrier efflux transporters, preventing transporter upregulation might offer considerable advantages over modulating transporter function once an induction has occurred. Therefore, knowledge about the main signaling factors contributing to seizure-associated induction of efflux transporters is essential to identify key signaling factors in the cascade regulating their expression [58, 94, 95].

An alternate approach, which avoids compromising the protective function of efflux transporters, is to bypass transporter molecules. Different strategies are followed in this regard including nanoparticle encapsulation of transporter substrates [96]. Nanosized carrier systems including polymers, emulsions, micelles, liposomes, and nanoparticles may deliver their content into the brain by passive targeting [97]. However, the penetration and distribution rate into the brain is often limited since these carrier systems have to face very diverse physicochemical features and physiological conditions. In general, it is possible to achieve much higher concentrations of drug in the brain by local administration procedures [98]. Although implantable controlled release systems are already clinically used for treatment of brain tumors, these systems also face the fact that local penetration of the drug is limited due to the restriction of diffusion by the brain parenchyma [99]. Alternative approaches for direct delivery include gene therapy involving viral, lipid, polymer-based, and cell-based delivery strategies. For details, interested readers are referred to reviews that focus on these techniques [97, 98].

In order to identify new mechanisms involved in epileptogenesis and develop novel treatments, it is fundamental to know its pathways. In this sense, the blood–brain barrier leakage may be a candidate biomarker [100]. Several promising new therapeutic strategies that aim to restore blood–brain barrier function have been investigated in

experimental epilepsy models. These include inhibition of transforming growth factor β receptors [72, 79, 101], leukocyte–vascular interactions [102], interleukin-1 receptors [83, 103], and the mammalian target of rapamycin (mTOR) pathway [104–106]. Recently, the mTOR inhibitor everolimus improved the seizure control in the majority of tuberous sclerosis complex patients with drug-resistant epilepsy [107]. Similarly, in epileptic rats, a mTOR inhibitor reduced epileptogenesis and decreased blood–brain barrier leakage during the chronic epileptic phase [106].

6 Conclusion and Future Perspectives

The neurovascular unit components and their multifactorial interactions are extremely sophisticated and complex. The knowledge of each of the components and their respective pathways are crucial to understand various neurological disorders such as epilepsy. The blood–brain barrier is now considered as a potential therapeutic target. A better understanding of all these players can lead to novel treatment strategies and thereby prevent the development of pharmacoresistance.

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Chapter 13

Virtual Screening Applications in the Search of Novel Antiepileptic Drug Candidates

Alan Talevi and Luis E. Bruno-Blanch

Abstract

Virtual screening (VS) encompasses a wide spectrum of computational approaches oriented to prioritize which compounds from chemical libraries or repositories will be subjected to experimental in vitro and in vivo testing. VS can be broadly classified into structure- and ligand-based approaches. Which VS methodology is preferable is highly dependent on the targeted system and the availability of experimental data and also on background considerations such as existing technical and economic resources. To the moment, VS has been underexplored for the discovery of new antiepileptic agents. In this chapter we discuss details on the general ligand-based procedures to undertake VS campaigns, with emphasis on some particular considerations for the case of antiepileptic drug development.

Key words Epilepsy, In silico screening, Ligand-based approximations, Virtual screening

1 Introduction

The universe of accessible chemical compounds has been growing exponentially during the last 50 years: in 2015, the Chemical Abstract Service, which roughly reflects the chemical entities known by mankind, achieved its 100 millionth entry. Judging from the number of entries in PubChem, around half of those substances are small drug-like molecules. This vast and expanding population of available chemicals makes it probable to find novel therapeutic agents with virtually any desired pharmacological profile. Nevertheless, the systematic, massive screening of such molecular diversity remains challenging. Even automated and miniaturized approaches like high-throughput screening are technologically demanding and prohibitive for small academic centers or pharmaceutical companies.

The term virtual screening (VS) or in silico screening refers to the application of a diversity of computational approaches to rank digital chemical repositories or libraries in order to establish which drug candidates are more likely to obtain favorable results when

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experimentally tested through in vitro and/or in vivo models. Since they are meant to reduce the volume of experimental testing and optimize the results, VS techniques possess several advantages in terms of cost-efficiency, bioethics, and environmental impact. We may also mention that, since many computational applications and chemical databases are publicly available online and several of them run smoothly in any current personal computer, the technology gap between developed and emerging countries is considerably low for VS technologies compared with other screening approaches.

VS approaches can be essentially classified within two categories: structure-based (or direct) and ligand-based (or indirect) approximations. A persistent and major obstacle for the implementation of structure-based VS approaches comes from the fact that most validated targets for antiepileptic drugs are ligand- or voltage-operated ion channels whose structure has not been solved experimentally yet. In order to predict protein folding for unsolved structures, a drug designer can turn to homology modeling, which uses a known protein similar to the protein of interest (e.g., a homolog from other species or another member of the same protein family from the same species) as a template to predict the secondary and tertiary structure of the target protein [1]. These models can be in turn be used for design or VS campaigns. For instance, some attempts of homology models of epilepsy-relevant targets such as GABA transporters, GABA transaminase, and SV2A have recently been reported [2–4]. A remarkable exception is carbonic anhydrase, a putative AED target whose human isoforms have already been solved and are being actively used in the field of drug discovery [5–7].

Alternatively, ligand-based approaches can be applied whenever a model of the target structure is not available or to complement structure-based approximations. Concisely, ligand-based approaches can be classified into similarity searches, machine learning approaches, and superposition approximations [1, 8]. These techniques differ in a number of factors, from their requisites to their enrichment metrics or scaffold hopping. Similarity search uses molecular fingerprints derived from 2D or 3D molecular representations, comparing database compounds with one or more reference molecules in a pairwise manner. Notably, only one reference molecule (e.g., the physiologic ligand of a target protein) is required to implement similarity-based VS campaigns. Usually, similarity searches are the only alternative to explore the chemical universe for active compounds in the absence of experimental knowledge of the target protein or related proteins and when the number of known ligands is scarce. Machine learning approaches operate by building models from example inputs to make data-driven predictions on the database compounds. Machine learning approximations require several learning or calibration examples.

Finally, superimposition techniques are conformation-dependent methods that analyze how well a compound superposes onto a reference compound or fit a geometrical, fuzzy model (pharmacophore) in which functional groups are stripped off their exact chemical nature to become generic chemical properties (e.g., hydrophobic point, H-bond donor, etc.); such process is facilitated if the modeler counts on an active rigid analog with limited conformational freedom. As a general rule, the more complex approximations take the lead in terms of scaffold hopping, whereas simpler approaches are computationally less demanding while achieving good active enrichment metrics [9]; the efficacy of a given technique is, however, highly dependent on the chosen molecular target, and frequently different techniques are complementary in nature [10].

Since, as it has been mentioned, most of the human proteins validated as molecular targets for antiepileptic drugs have not been solved experimentally, this chapter will focus on ligand-based VS protocols. Similarity search protocols are basically simple: their sole complexity lies in a number of decisions that the user has to make regarding what similarity metric will be used, what fingerprint system, and the size of the molecular features (molecular substructures) considered. Thus, we will aim our attention at more complex ligand-based approaches that rely on inferring a model from a set of instances or examples, in particular, machine learning approaches.

2 Building a Model

2.1 Dataset Compilation

Naturally, the first step when one tries to infer a model (a generalization on some structure–property relationship with a variable degree of abstraction) from a number of examples is to compile such training or calibration instances. We will refer this as dataset compilation. It is often heard that models are as good as the biological and chemical data from which they are derived [11, 12]. Therefore, the dataset compilation is one of the most important steps when developing a model for VS purposes. When intending to build a model for VS applications, some specific considerations must be taken into account: (a) compile a dataset as diverse as possible; (b) ideally, the dependent variable (the modeled property, i.e., the biological activity) should span at least two or three orders of magnitude [13–16], from the least to the most active compound in the series; (c) the available biological data on the training set compounds should, preferably, be uniformly distributed across the range of activity or at least follow some defined statistical distribution, usually normal distribution – the same principle applies to the model's independent variables [13]; and (d) the biological activities of all the training instances should be of comparable quality – ideally, they should have been determined in the

same laboratory using the same conditions [14, 15], so that variability in the measured biological activity only reflects treatment variability (this is rarely the case, though).

Let us discuss point by point. Regarding the chemical diversity of the training set, while for computer-aided drug design the use of homologous series of chemicals might be advantageous, in the case of VS, we are expecting to apply the model for the screening of vast collections of chemicals (typically, thousands to millions). As we will comment later, the predictions of the models will only be reliable within the chemical space covered by the training set: thus, if we want to cover a wide chemical space, the training set should ensure the most possible chemical diversity. On the other hand, guaranteeing a wide coverage of the activity space allows the model to capture not only essential molecular features needed to elicit the desired activity, but features that diminish activity as well. Data distribution should be studied in order to avoid poorly populated regions within the studied chemical space as well as highly populated narrow intervals [13]. It is often heard that modelers should avoid data extrapolation; however, interpolation could also be dangerous if too sparse regions exist within the data. Histograms can be of great help to visualize the distribution of both the model dependent and the independent variables; still, analysis of the multivariate space can reveal empty or scarcely populated data regions that separate analysis of the independent variables may not [17]. The second issue related to inadequate data distribution is the existence of *leverage points* (outliers) among the data points. We will resort to the terminology adopted by Cruz-Monteagudo and collaborators to discuss the subject of exceptional data points [18], though their nomenclature is not universal. An outlier is a type of data exception represented by extreme values in the descriptor or property (response) space that cannot be attributed to mislabeling due to annotation or measurement errors (Fig. 1). Outliers exert great influence on the model calibration (hence they are called leverage points) especially when a quantitative model with a continuous response/output (regression model) is used. It is likely that some degree of overfitting will appear when outliers are present among the training examples. Even if no

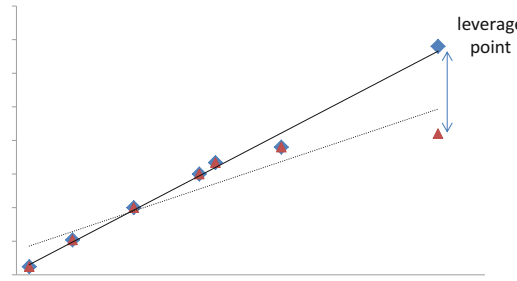

Fig. 1 Example of a leverage point. Note how much the isolated point on the extreme right influences the fit of the regression line

systematic error in the experimental measurement of the modeled property exists, random error will have greater impact on the model in the case of outliers. There are a huge number of methods to detect outlier behavior (and, consequently, remove atypical points from the calibration samples) [19]. The very simple approach by Roy et al. [20] can be used to detect outliers provided that the data is normally distributed, which is relatively frequent in our field (and the stand-alone application is publicly available online!). However, as always when using parametric methods (which assume that the dataset fits a known distribution or probability model), it may be a good idea to run statistical discordance tests to check if the assumed distribution is optimal or close to optimal. Finally, since most of the machine learning applications are based on datasets that have been directly or indirectly compiled from literature, the last requisite on the dataset (homogeneous data quality) is seldom accomplished. Compiling the dataset from literature could also give raise to another type of data exceptions: noise. Noisy data points emerge from large experimental errors or from wrong annotations. To mitigate this issue, conscientiously curate your dataset: read your data sources carefully and dispose of those training examples extracted from inadequate or dubious experimental protocols. At present, there are several databases that compile experimental data for small molecules (e.g., ChEMBL); though such resources are manually curated from primary scientific literature, it will not hurt to review the experimental procedures from which biological data is extracted. When possible, ChEMBL tries to normalize bioactivities into a uniform set of endpoints and units. Remarkably, they currently flag activity values that are outside a range typical for that activity type, potentially missing data and suspected or confirmed author errors. Finally, classification models can be used to alleviate the influence of data heterogeneity, as will be discussed later. See *Note number 1* for an extensive additional discussion on the topic of dataset compilation in relation to antiepileptic drug discovery.

2.2 Partitioning the Dataset into Training and Test Sets

Once the dataset has been compiled, it is typically split into a training (or calibration) set (from which the model will be inferred) and an independent test set which will be used to assess the predictive power of the model. Partitioning the dataset is not a trivial task. The general objective of this procedure is to attain representative subsets of the whole dataset. Often, such subsets are obtained through random sampling (a randomly chosen subset of the dataset is tagged as test set) or activity range algorithms (the dataset is divided in groups according to activity values, and test set compounds are chosen from each group to cover the activity range uniformly). While these approaches are appropriate when training and test sets are comparable in size [21, 22], better results are obtained with more rational partitioning procedures such as sphere exclusion algorithms when test sets are small (but larger than five compounds)

in comparison with the corresponding training sets [21]. Note that typically only 10–20% of the dataset is selected for external validation [12]. It should also be considered that ideally, the number of active and inactive compounds in the training set should be balanced in order to avoid potential bias toward the prediction of the overrepresented category of training examples [12].

2.3 Choosing and Calculating Molecular Descriptors

Briefly, molecular descriptors are numerical variables that reflect chemical information encoded within a symbolic representation of a chemical compound, e.g., a molecular formula, a chemical graph, and a geometric molecular model. There is a wide diversity of molecular descriptors available to characterize relevant aspects of a molecule, from simple functional group counts to time-demanding quantum descriptors. Once the dataset has been compiled and divided into appropriate training and test sets, the nature of the molecular descriptors that will be allowed into the model should be decided. Some studies suggest that the choice of descriptors plays a more important role than the choice of a modeling technique [23, 24]. Two fundamental aspects are considered at this stage of the modeling process: first, the throughput speed associated with different types of descriptors and, second, the interpretability of each type of descriptor. We should keep in mind that this chapter is focused in models which will eventually be used to screen large collections of chemicals. Thus, the selected molecular descriptors should ideally be easy to compute and readily interpretable. Unfortunately, these two aspects are usually inversely related: in general, the more interpretable a model is, the more it is computationally demanding. 3D QSAR models often provide a graphical output which is easy to interpret in familiar chemical terms. Most 3D QSAR methods, however, implement the pharmacophore concept and are conformation and sometimes alignment dependent. What conformation should be used to compute the correspondent 3D descriptors? An ideal solution to account for the conformation dependency would be to determine the bound (also called bioactive) conformation [25, 26]. Defining the bound conformation is often an enormously difficult and time-consuming task. Remember that the focus of this chapter is VS applications; consistently, any conformational analysis should eventually be applied not only to the training and test set compounds but also to the screened database molecules, which could well include millions of compounds. Bound conformations of ligands can be obtained experimentally by NMR or X-ray crystallography. However, as has been pointed out in the introduction of the chapter, most of the targets for antiepileptic drugs have not been solved yet. Furthermore, crystal structures also have limitations, from data acquisition and data refinement errors to the potential inadequacy of crystal structures to represent the conformational ensemble in solution [26]. Valuable hints on the active conformation might be obtained when

rigid ligands with restricted conformational freedom are available. For instance, some antiepileptic agents like phenytoin and carbamazepine have very few rotatable bonds and have been used to propose pharmacophore models [27]. When no hints on the bioactive conformation can be inferred from experimental data or rigid ligands, the modeler has no alternative, but to sample the potential energy surface of the ligands. A number of methods (all of them computationally demanding) are available for such purpose, including systematic search, stochastic approaches, and molecular dynamics. Note that very frequently very rough approximations are performed in this stage, from using the presumed global energy minimum or a local energy minimum (which is not representative of the bioactive conformation) to energy minimization procedures in vacuum, which ignore solvent effects. If bound conformations can be defined, eventually the conformational energy (energy difference between the bound and unbound conformation, sometimes called strain energy) should be calculated for each of the chemicals in the database subjected to VS, retaining those molecules with calculated strain energy below a user-defined threshold, which is typically below 10 kcal/mol. On the other hand, when alignment-dependent methods are used, defining alignment criteria for structurally diverse compounds can be tough (if not impossible). On the basis of all the previous limitations of 3D QSAR methods, we believe that conformation-independent QSAR methods (2D QSAR) are more easily automated and adapted to the task of VS since they neither require conformational search nor structural analysis [28]. Judging from the success of such approaches, simple representations of the molecular descriptors such as chemical graphs seem to implicitly contain a large amount of biologically relevant molecular information. They are naturally inappropriate, though, to differentiate geometrical isomers. Moreover, they are usually less transparent to interpretation; many 2D models behave like a black box: effective but inscrutable. In any case, 2D approaches could be used as a first screening approach to reduce the number of potential drug candidates, which can be later complemented with more computationally demanding techniques.

Once the molecular descriptors that will be used have been chosen, it is time to curate chemical structures. The required degree of curation depends on the descriptors that are to be used. Do not underestimate the importance of this step: it has been shown that, in average, there are two structural errors per medicinal chemistry publication [12] and a variable rate of errors in compounds indexed in chemical databases which may be as high as 8% [29]. Even slight errors in chemical structures may lead to significant loss of prediction accuracy in the subsequent model [29]. The first step for cleaning chemical records is to remove those data points that are usually not handled by conventional cheminformatics techniques: inorganic and some organometallic compounds,

counterions, salts, and mixtures [12]. Some additional details on this subject are provided in *Note number 2*. Duplicates should also be removed; otherwise the incidence of a single compound on the model would be exaggerated. Note that different compounds might act as duplicates depending on the chosen molecular descriptors: e.g., stereoisomers are distinct but act as duplicates if 2D descriptors are used. Some chemical functions and moieties that can be represented in multiple ways should be standardized, e.g., aromatic rings, nitro groups, etc. [30]. Finally, tautomeric groups should also be curated. In order to decide which tautomer should be kept, the mechanism of action of the compounds could be considered [29] or, alternatively, the dominant tautomer at physiologically relevant conditions should be used. Many of the previous steps can be performed in an automated manner by specialized software applications (e.g., ChemAxon's Standardizer); still, it is advisable to manually verify a randomly picked subset of the compounds to ensure everything has gone well. Also note that many software tools for molecular descriptor calculation impose restrictions to the molecular representations (e.g., explicit or implicit hydrogens, aromatic rings, etc.).

2.4 Building a Model

So far we have explained the cautions needed to compile an adequate training set, curate the correspondent molecular structures, and compute a set of molecular descriptors. At this point, the subset of descriptors that best correlates with the property of interest should be selected (variable or feature selection step), and the dependent variable should be mapped with those preselected descriptors (i.e., the incidence of each descriptor on the dependent variable should be weighted). There is a plethora of techniques to execute these tasks [31–33], and their analysis is out of the scope of this chapter. However, we would like to discuss two particular aspects of model building that are generally pertinent no matter which modeling technique is considered. The first relates to the principle of parsimony and the problem of overfitting. The second relates to the convenience of choosing between regression and classification approaches.

Overfitting means gaining explanatory power on the training examples at the expense of generalizability (predictive power). As in any learning task, memorizing is discouraged since the goal is to extract a generalization from learning examples that can be later applied to other cases/situations. The principle of parsimony affirms that we should use the simplest method that provides the desired performance level. This includes avoiding the use of excessively flexible approaches if they are not required (e.g., avoid using nonlinear methods if linear methods can provide an appropriate solution) and also avoiding the inclusion of more parameters/features than needed [34]. In our personal experience, the models that explain too well the training data fail at predicting external

data, while models with moderately good performance on the training examples tend to behave similarly on external instances. Overfitting can be avoided retrospectively (once a model has already been built, using adequate validation procedures as discussed in the next subsection) or prospectively (during the model-building stage). Prospective avoidance of overffiting usually involves a rule of thumb regarding the ratio of learning examples to predictors (molecular descriptors) included in the model. Though a ratio equal or greater than five is often suggested for multivariate regression approaches [13, 35–37], in our opinion at least ten training compounds per independent variable are safer. Some methods such as partial least squares allow a higher number of descriptors. A final point that should be regarded is the influence of the total number of variables screened for possible correlation with the modeled activity (i.e., the size of the descriptor pool) on the statistical significance of the obtained correlation [38]: the larger the descriptor pool, the greater the probability of arriving to spurious, chance correlations. Software for molecular descriptor calculation can typically compute hundreds to thousands of descriptors. In our experience, we have found that the use of small random subsets of descriptors is a useful strategy to mitigate the chance of spurious correlations (note that this issue is intimately related to the problem of overfitting, since the general idea still is avoiding inclusion of independent variables that reflect meaningless particularities of the training examples; thus, the strategies that have been already discussed to minimize the risk of overfitting are also suitable to reduce the probability of chance correlations). Fisher's randomization test, which is discussed in the next section, is also a valuable tool to assess the probability of random correlations. Some additional hints on the matter of variable selection are presented in *Note number 3*.

With regard to the selection of a classification or regression model, classification models might be more adequate when biological data has been obtained in different labs and it is subjected to large interlaboratory variability, possibly introducing noise to the model [39]; this approach might be valuable to mitigate the influence of leverage data points. Note that, typically, biological data from cellular and animal models is prone to present high variability; as we discuss in the *Notes*, many modeling efforts directed to predict anticonvulsant activity are based on in vivo data obtained from seizure models; even when models of seizure are naturally more controlled than models of epilepsy, which often require sub-chronic administration of pro-convulsant stimuli, high interlab variability is to be expected. Classification models are quantitative models based on relationships between independent variables (in this case molecular descriptors) and a categorical response variable of integer numerical values that represents the class of the corresponding sample. Here, the term "quantitative" is

referred to the numerical value of the independent variables needed to classify the chemicals in the qualitative classes (a categorical response); such variables specify the quantitative meaning of a QSAR-based classification process [36]. As clearly stated by Polanski et al. [40], extensive data independence implies qualitative and not quantitative solutions; classification models might be considered somehow in the middle, because they provide a qualitative response (yes or no, active or inactive, etc.) while retaining quantitative analysis through the numerical independent variables. Those same authors point out that the combination of different data handling schemes seems particularly effective to provide robust solutions. When using classification models, the sources of noise are majorly restricted to those points that lie in the frontier between the categories of objects under consideration (e.g., those whose activity value is near the activity threshold that has been defined to differentiate active from inactive compounds). Also note that scientific literature is generally biased toward the report of highly active compounds, resulting in a general relative abundance of active compounds in comparison to inactive ones. Classification methods can ameliorate this issue since the modeler might include putative inactive compounds within the inactive class: though this is a potential source of error (the inactive nature of such presumed inactive examples has not been verified), it can be assumed that this error will not be significant if the dataset is sufficiently large.

2.5 Model Validation

Model validation implies the *quantitative* assessment of the model robustness and predictive power [41, 42], which serves to detect the occurrence of overfitting and chance correlations. In the context of signal processing applications (within which we can enclose QSAR modeling), robustness refers to *approaches that are not degraded significantly when the assumptions that were invoked in defining the processing algorithm are no longer valid* [40]. Validation techniques can be divided in internal and external validation. In the internal validation approaches, the training set itself is used to assess the model stability and predictive power; in external validation, a holdout sample absolutely independent from the training set is used to test the predictive ability of the model. Though there is a diversity of techniques that can be used for internal validation purposes, the most frequent are cross validation and Y-randomization.

In cross validation, groups of training examples are iteratively held out from the training set used for model development; the model is thus regenerated without the removed chemicals, and the regenerated model is used to predict the dependent variable for the held-out compounds [43]. The process is typically repeated until every training compound has been removed from the training set at least once (Fig. 2). When only one compound is held out in each

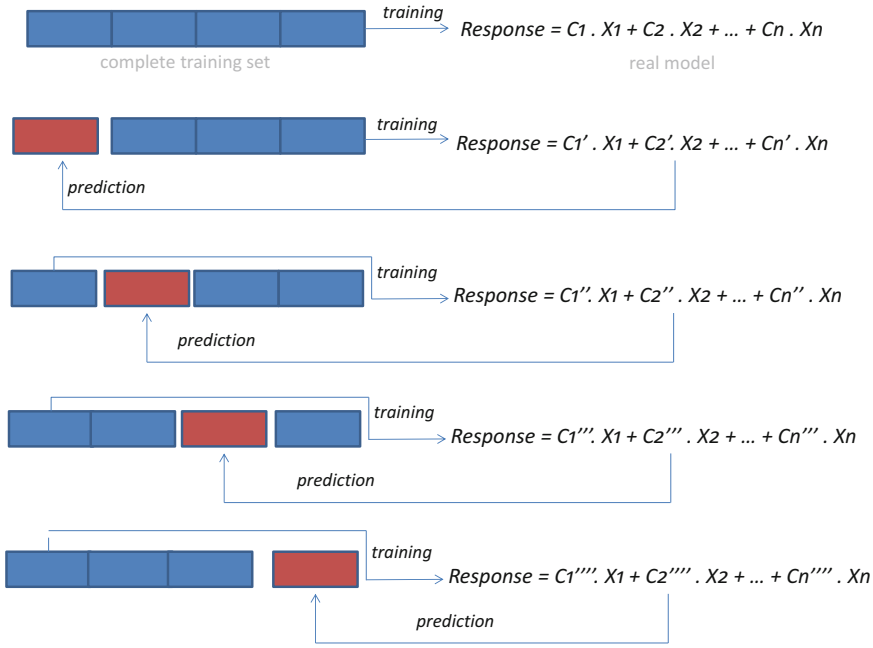

Fig. 2 A fourfold cross validation is schematized as an example. In each cross validation round, 25 % of the training compounds (shown in *red*) are randomly removed from the training set and used as internal test sample, while the remaining compounds (in *blue*) are used for training purposes. Note that in the example the same model parameters that are included in the original model are present in the new models, but the regression coefficients change reflecting the variability introduced to the training sample. Here, the process has been repeated until every instance in the original training data has been removed once

cross validation cycle, we will speak of leave-one-out cross validation. If larger subsets of training examples are removed in each round, we will speak of multifold, leave-some-out, leave-group-out, or leave-many-out cross validation. Naturally, the more compounds removed per cycle, the more challenging the cross validation test. Cross validation in general and leave-one-out cross validation in particular tend to be overoptimistic [41, 43, 44]: good cross validation metrics are a necessary but not sufficient condition to prove the predictive power of a model. When leave-many-out cross validation is used, the results for each (held-out) fold or subsample can be averaged or otherwise combined to produce a single estimation. See *Note number 4* for some additional discussion on this subject.

Y-randomization (Fig. 3) involves scrambling the value of the experimental/observed dependent variable across the training instances, thus abolishing the relationship between the response and the molecular structure. Naturally, since the response is now randomly assigned to the training examples, no correlation is expected to be found if the model is regenerated from the scrambled data.

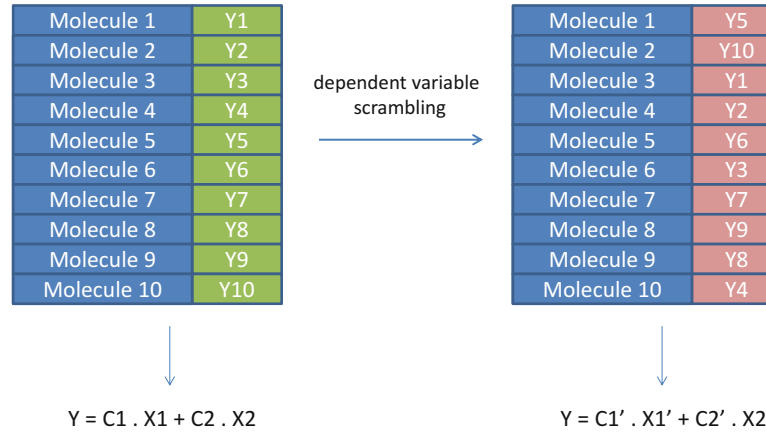

Fig. 3 In randomization test, the Y-response is scrambled among the training samples, and the relationship between the molecular structure and the response is thus abolished, hopefully leading to poorly performing randomized models. Note that process randomization has been illustrated here, as can be deducted from the different parameters included in the randomization models and the real one

External validation, i.e., using an independent test set to establish the model predictive power, has been regarded as the most significant validation step [41, 43], though one condition should be observed for the results to be reliable: the test sample should be representative of the training sample; at least 20 holdout examples are advised when the test set is randomly chosen from the dataset and, if possible, at least 50. Some authors have pointed out, however, that dividing the dataset into training and test sets may result in loss of valuable chemical information which otherwise could have been used for model building [34, 42, 45], suggesting that only internal validation is advised for small (<50 instances) datasets. In that situation, leave-group-out using folds comprising 30% of the training set gives robust results across several small datasets [43].

3 Virtual Screening

3.1 Pilot VS Campaign

Once the model has been built and properly validated and before it is applied in a real VS campaign, it is convenient to assess the model performance in a pilot VS campaign. Note that the active and inactive compounds are generally balanced in the dataset that was used to build the model, but in real VS applications, the inactive compounds greatly outnumber the active ones. This constitutes an intrinsic limitation of the VS approach, determining that even models with very good performance will tend to have low positive predictive value (PPV, i.e., activity probability of a given predicted hit) in VS, since, as follows, such probability is influenced not only by intrinsic features of the model (specificity, Sp,

and sensitivity; Se) but also by the yield of active Ya of the screened database [46]:

$$\mathrm{PPV} = (\mathrm{Se} \times \mathrm{Ya}) / \left[\mathrm{Se} \times \mathrm{Ya} + (1 - \mathrm{Sp})(1 - \mathrm{Ya})\right] \quad (1)$$

While Ya is uncertain in real VS applications, it is unequivocally low. Thus, it is desirable to perform a pilot VS campaign in the first place, seeding a relatively low number (below 5 %) of known actives among a large number of observed and presumed inactive compounds. As mentioned previously, finding reported inactive compounds in the literature is hard due to the bias to report positive results, which makes it difficult to accomplish the precedent condition. Therefore, one will often resort to putative inactive compounds as decoys. A good decoy should share certain physicochemical features with the active compounds in order to pose a more rigorous challenge to the model [47]. The enhanced directory of useful decoys (DUD-E) is an online public resource that automatically generates valuable matched decoys for user-supplied ligands (such decoys are matched by a number of physicochemical properties but are topologically dissimilar to the actual active compounds) [48]. Receiver operating characteristic (ROC) curves in which the model Se (true positive rate) is plotted versus 1 minus Sp (true negative rate) for a diversity of score thresholds are a valuable tool to test the performance of a given VS model/method and also for benchmarking purposes [46]. They are also used to optimize the score threshold that will define if a given compound from the screened database will be considered a predicted active or inactive, allowing selecting an adequate Se/Sp balance and thus optimizing the PPV. A scheme describing how ROC curves are built is presented in Fig. 4. With the help of ROC curves, different VS approaches can be statistically compared between themselves and to random behavior; the area under the ROC curves is usually used for those purposes. Though owing to the saturation effect, the total area under the ROC curve is not a suitable metric to assess the VS approaches in relation to the early recognition (i.e., the ability of a VS method to rank actives early in the ordered list) [49], the partial area under the ROC curve is well suited for this purpose. pROC is an excellent free open-source package for R which can be used for partial and total ROC curve comparison [50]. Some additional remarks on this subject are included in *Note number 5*.

3.2 Virtual Screening and Applicability Domain Estimation

After making sure that the model is suitable for real VS applications and selecting an appropriate cutoff score value, one can proceed to the real VS campaign. There exist numerous publicly available databases containing drug-like molecules, such as ZINC [51], DrugBank [52], Sweetlead [53], or the Universal Natural Product Database [54]. The election of the screened database depends on

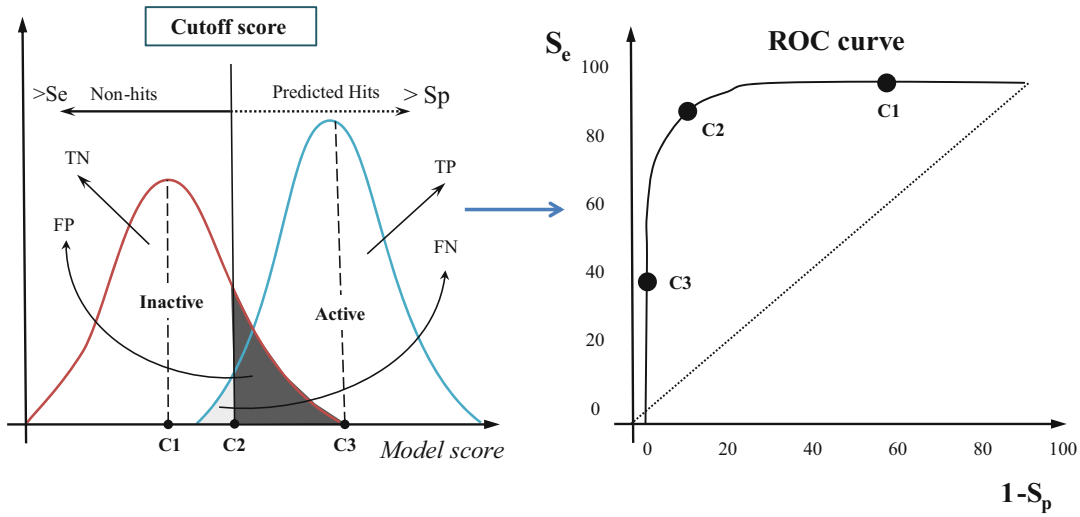

Fig. 4 Schematic representation of ROC curve construction

which compounds the researcher is focused on. For instance, DrugBank and Sweetlead are the collections of choice when the VS is oriented to drug repurposing, since they compile approved and investigational drugs from the FDA and other regulatory agencies. The Universal Natural Product Database, as its name implies, compiles (more than 200,000) compounds from natural sources. It must be checked if every screened compound belongs to the applicability domain of the model (in order to determine whether a given prediction is or is not reliable); for this, there are a number of methods available, including distance-based, parametric, and nonparametric approaches, among others [17]. Most of these approximations can be easily implemented with most statistical software packages; if the molecular descriptors included in the training set follow a normal distribution, the parametric approach discussed by Roy et al. might be applied [20], and it is readily available online. Note, however, that it has been observed that applicability domain assessment often limits the chemical space coverage of the resulting (reliable) predictions [23]; the same authors have observed that consensus scoring (combining the scores or ranks from different VS approximations) might reduce the necessity of applicability domain estimation while retaining a wider coverage. Remarkably, consensus scoring could also mitigate the influence of noisy data achieving robust solutions [40].

3.3 ADME Filters

The pharmaceutically relevant processes of absorption, distribution, metabolism, and excretion (ADME) determine the pharmacokinetics of a given drug and therefore the extent of drug action. Including ADME filters as secondary criteria to select drug candidates is particularly important when implementing VS applications

to discover novel AEDs, whose pharmacology is often critically influenced by biodistribution and metabolism issues. Besides the frequently used Lipinski rules (or other similar rules, e.g., Veber's rules) to filter out compounds with likely unfavorable oral absorption, having in mind the role of the blood–brain barrier regulating the influx of chemicals into the brain, specific rules or algorithms to predict central nervous system bioavailability should be considered. For example, the "rule of 2" states that a log octanol–water partition (log *P*) coefficient of 2 is optimal to assure brain bioavailability for those compounds that enter the brain through passive diffusion [55]. Other more complex (yet simple) filters such as the central nervous system desirability score proposed by Wager et al. could also be included [56]. If the VS campaign is focused on novel treatments for refractory epilepsy, it could be a good idea to include in silico filters to predict affinity for ABC transporters (some models on these antitargets are discussed in other chapters of this volume). Whenever using a model built by other developers, ensure that such model's predictions include applicability domain assessment. At last, many known AEDs are involved in CYP induction or suffer extensive CYP metabolism; accordingly, filters to predict affinity for the main CYP isoforms (e.g., CYP3A4, CYP2D6, CYP2C9) or nuclear receptors might also result useful.

4 Notes

1. *Dataset compilation*. Traditionally, the requirements on the training set examples were even more stringent than the ones listed in Sect. 2.1. For instance, it is usually affirmed that, especially in the case of 3D QSAR, all the training examples should share the same mechanism of action (and the same binding mode) and all the inactive compounds in the training set should be truly (and not putative) inactive [14–16, 57]. It is argued that all 3D QSAR methods were conceived to describe only one interaction step in the lifetime of ligands [14], a statement which is partially supported by the fact that many 3D QSAR methods are highly alignment dependent (the results depend on the position and orientation of the molecular representation in space). Furthermore, it is also said that only in vitro biological data should be considered, since in vivo data reflects a number of parallel processes (e.g., transport, metabolism, binding to multiple targets), while by definition it is not possible to reach equilibrium in an in vivo system [14, 15]. It is true that in vitro data is cleaner than in vivo data, in the sense that interpretation of the test results is more straightforward and less affected by confounding factors; all the other systems undergo significant time-dependent changes. Personally, however, we believe that such excessive reductionist approach could be

dangerous when dealing with complex disorders such as epilepsy. There are many good reasons to take dogmatic principles with caution. First, very frequently biological data emerging from phenotypic models (e.g., in vivo or cellular models) are used to obtain QSAR models, and in spite of this, the models achieve considerable explicatory and predictive ability (see, e.g., [58–62]). A common mechanism could be presumed when compounds of the same chemical series are being considered, but the modeler cannot be truly sure regarding the specific action mechanism explaining the phenotypic observation or the number or identity of the pharmacologically active chemical species. The complex nature of the biological response makes it impossible to describe, a priori, a well-defined action mechanism or to discriminate the influence of other processes on the modeled activity (transport processes, bioactivation, etc.). And yet, the so obtained models work. Sometimes one will not have a satisfactory explanation to an observation, but facts should not be ignored in favor of a preestablished principle. Second, it is now understood that multifactor, complex disorders (e.g., mood disorders, neurodegenerative disorders, or epilepsy) are usually better addressed with drugs with complex pharmacology (i.e., multi-target drugs) [63–65]; in fact, once and again it is mentioned that the "one target, one drug" paradigm has been disappointing in terms of innovative treatments [66–68]. Most currently approved antiepileptic drugs are in fact multi-target agents [64]. Therefore, it is possible that we should resort to tailored multi-target drug discovery and/or return to phenotypic-based drug discovery to identify new pharmacological solutions to epilepsy. Under this perspective, going against the doctrine and using biological data obtained from animal models might be in fact a better approach toward VS for novel antiepileptic agents. Note that many successful QSAR and VS applications focused on antiepileptic drugs have indeed used in vivo biological data for modeling purposes [27, 28, 69–72], including reports by leading experts in the QSAR field [28]. Third, not all available molecular descriptors are conformation and/or alignment dependent, and some of them are capable of describing more general properties than those relevant for a single binding event. For instance, molecular complexity and molecular size have been directly and inversely correlated with drug promiscuity [73]; these molecular features can be captured by descriptors such as information indices or molecular weight, respectively. Fourth, QSAR theory has greatly evolved in the last years; multitasking QSAR models are suitable to predict multiple features and complex behaviors, exploiting latent commonalities across tasks [74, 75]. Finally, as it has already been discussed, classificatory models might be able to mitigate potential noise linked to experimental error or

simultaneous incidence of multiple parallel processes on the bioactivity data. All in all, there is no reason to exclude a model where the mechanism is not known or if there are multiple mechanisms [35]. A final remark must be done in relation to activity cliffs: while continuous structure–activity relationships where gradual change in the molecular structure results in moderate changes in the biological response are benign to modeling efforts, sometimes small modifications in the molecules introduce huge changes in biological activity [17]. Whereas highly informative (they provide valuable information on molecular features essential to the activity) activity cliffs can be really problematic for modeling purposes; some algorithms have been developed to identify these "instances that should be misclassified" [76].

2. *Curation of chemical structures.* While the properties of salts can be very different from those of the corresponding neutral molecules, before excluding salts from the QSAR analysis, some questions should be (if possible) answered. The first question would be if the descriptors that will be used are sensitive to charge. If they are insensitive to charge, just neutralize the cations or anions that are left when removing counterions. If at least some of the descriptors that you will use are sensitive to charge, it might be a good idea to go through the original publications from which biological data of your dataset have been extracted and see if the pH of the tested solutions and the test system are known or can be figured out from the media composition. In any case, the (experimental or at least theoretical) pKa/s of the dataset compounds will be needed to assign their protonation states. The pH of the drug solution is more likely to affect in vivo than in vitro data. In vitro assays usually require buffered systems, thus limiting the influence of the drug solution pH; in general, the protonation state in in vitro models would be thus determined by the test media pH. In contrast, in vivo absorption and thus pharmacokinetics can be greatly influenced by the pH of the drug solution. If the administered dose is known and assumed to be bioavailable, one might choose to assign the protonation state depending on the pH of the target organ; e.g., in the case of antiepileptic drugs, the pH of the brain is around 7 [77]. Always remember to analyze your set of chemicals at those physiologically relevant conditions for the administration route and therapeutic goal; also keep in mind that some pharmaceutically/pharmacologically relevant conditions may vary due to a physiopathological process. If data to afford the previous analysis is not available, neutralizing ions is acceptable [30].
3. *Excluding redundant variables.* When building the model, it is often advised that simultaneous inclusion of highly correlated (redundant) independent variables should be avoided.

Correlated independent variables lead to multicollinearity, which can increase the standard errors associated with the regression coefficients and cause problems in interpreting the results of a regression equation. Orthogonalization procedures can be applied to obtain orthogonal descriptors [78]. Redundant variables can be avoided prospectively by setting a high tolerance value (or by defining a threshold value for its inverse, the variance inflation factor, VIF). Descriptor calculation software usually includes some option to exclude descriptors correlated above a programmer- or user-defined threshold. However, it must be taken into account that even highly correlated descriptor pairs might be included in the model without losing statistical significance ([79] *and references therein*): it has been suggested that the VIF should be compared to the model inflation factor (MIF) and only if VIF > MIF, one of the seemingly redundant descriptors should be excluded.

4. *Validation*. In our opinion, averaging the predictive performance on the folds used for the multifold cross validation is more useful, since it allows computing a confidence interval (or an estimate of the standard deviation) to evaluate, for instance, if the statistical behavior of the original model (the one built using the entire training set) significantly differs from the behavior of the cross validation models on the held-out samples. It is advisable to perform stratified multifold cross validation, in which the folds are selected so that the mean response value is approximately equal in all the folds. For instance, if you are validating a classification model, the folds might comprise an equal number of examples from each class. In the case of Y-randomization, the confidence interval of the metric used to characterize the performance of the randomized model should not contain the value of the performance metric for the original, nonrandomized model (although other ways of analyzing the randomization results have been proposed [13]).

   Finally, note that internal validation procedures can be performed at varying confidence levels. One can study the whole process from the variable selection step, or, alternatively, one can validate the influence of the experimental design on the weighting scheme used to measure the contribution of an independent variable to the response. The former is, of course, a more strict challenge to the model robustness, since one is leaving aside any influence of the original training data on the variable selection. The importance of applying cross-validation at the variable selection step has been signaled in the specialized literature [42, 43]. It has also been highlighted that the randomization technique can be of two types: *process randomization*, in which the values of the dependent variable are scrambled and variable selection is done freshly using the whole descriptor pool, and *model randomization*, in which case the response is scrambled but the new QSAR models so

obtained include the same set of independent variables as present in the nonrandom model [80] which is of course a less strict validation approach. In conclusion, use validation techniques to validate the whole modeling procedure, including variable selection, for more reliable results.

5. *ROC curve analysis.* The adequate balance between the true positive rate and the true negative rate is context dependent and not a statistical matter. Sp and Se evolve in an opposite way and therefore they cannot be optimized simultaneously. If the budget for experimental testing is limited, Sp can be prioritized to reduce the false positive rate (predicted hits that will yield negative results) at the expense of sacrifying some true positives; if, in contrast, chemical novelty of the hits is the priority and funding abounds, Sp may be relaxed in favor of Se.

5 Final Remarks

We have presented an overview of the most relevant considerations that must be made in order to develop QSAR models and apply them in VS campaigns. Regarding particular considerations for the case of antiepileptic drug discovery, the complex nature of the disease and the multi-target nature of most of the existing antiepileptic drugs suggest that modeling in vivo data (i.e., phenotypic-based drug discovery) might lead to more efficacious drug candidates. As discussed in separate chapters, the classical "the more potent the better" paradigm might not apply to the particular task of antiepileptic drug discovery. Finally, considering the critical role of pharmacokinetics in antiepileptic drug pharmacology, it is advised to include in silico ADME filters during the screening process to predict the brain bioavailability and potential biodistribution and metabolism issues of the selected drug candidates, along with potential drug interactions.

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Chapter 14

Discovering New Antiepileptic Drugs Addressing the Transporter Hypothesis of Refractory Epilepsy: Ligand-Based Approximations

Manuel Couyoupetrou, Mauricio Di Ianni, Melisa Gantner, Guido Pesce, Roxana Peroni, Alan Talevi, and Luis E. Bruno-Blanch

Abstract

About one third of the epileptic patients cannot control their symptoms with antiepileptic drugs, despite the introduction of more than 15 novel therapeutic agents to the market since 1990. The most studied hypothesis to explain the phenomenon of drug resistance in epilepsy maintains that it might be related to regional overactivity of efflux transporters from the ATP-binding cassette (ABC) superfamily at the blood–brain barrier and/or the epileptic foci. Here, we review scientific evidence supporting the transporter hypothesis along with its limitations. We also cover some technical aspects of computational and experimental approaches used for the early detection of substrates of such efflux systems.

Key words ABC transporters, Breast cancer resistance protein, CETA, Ligand-based approaches, Meta-classifiers, MDCK–MDR1, P-glycoprotein, Refractory epilepsy, Transport assays

1 Introduction

1.1 Refractory Epilepsy: Current Explanations

About 30 % of the epileptic patients suffer from refractory or intractable epilepsy [1], i.e., they fail to achieve seizure freedom through adequate trials of two tolerated appropriately chosen antiepileptic drug (AED) schedules [2]. A clear and universal definition of refractory epilepsy is fundamental to understand the limitations of the neurobiological explanations to refractory epilepsy, which are discussed later in the present chapter. This scenario has not changed substantially despite the introduction of more than 15 AEDs from 1990 to the present [3]. Biological mechanisms underlying drug-resistant epilepsy have not been fully elucidated [4], though there are several hypotheses that explain this phenomenon; among them, the more prominent are the transporter hypothesis [5, 6], the target hypothesis [6, 7], the neural network hypothesis [8], the gene variant hypothesis [9], and the intrinsic severity hypothesis [10].

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The transporter and target hypotheses have been proposed earlier and have thus been more extensively studied. The former states that drug resistance in epilepsy may be a consequence of the local overactivity of ATP-binding cassette (ABC) transporters at the blood–brain barrier (BBB) and/or the epileptic foci. An overview on the evidence and limitations of this hypothesis is provided under the next subsection. The target hypothesis proposes that the reduced sensitivity to AEDs might be linked to acquired modifications in the structure and/or functionality of AED targets. While some years back constitutive alterations of drug transporters or targets were also included within the scope of the transporter and target hypothesis [7, 11], leading experts in the field now categorize intrinsic alterations of drug targets within the gene variant hypothesis [4]. The latter, however, also covers other possible genetic causes of drug resistance, such as polymorphic variants of CYP biotransformation enzymes. The nature of the pharmacokinetic or pharmacodynamic alteration is not trivial since it could have a profound impact on the clinical approach to the drug resistance issue. While genetic causes of pharmacoresistance might currently be detected through simple diagnostic tests even before starting the treatment, acquired modifications linked to the pathophysiology of the disease are more difficult to prove and nowadays still require more invasive procedures (e.g., surgery resection). Recently, the possible role of epigenetics in drug-resistant epilepsy has also been underlined [12].

It has been noted that none of the hypotheses provides a full or universal explanation to nonresponsive patients with epilepsy: a certain hypothesis might be applicable to a particular subgroup of patients, or alternatively, some patients could require multiple hypotheses to explain their refractoriness [4, 11, 13]. It is worth highlighting that the best treatment approach should be highly dependent on the underlying drug resistance mechanisms observed in a given patient.

1.2 Strong and Weak Points of the Transporter Hypothesis

In eukaryotes, ABC transporters are transmembrane efflux transporters characterized by wide substrate specificity [14, 15]. They are highly expressed in barrier tissues (e.g., blood–brain barrier) and elimination organs (such as the liver and kidneys), restricting the bioavailability of xenobiotics and being consequently involved in multidrug resistance phenomena. They also play a role in the traffic of physiologic compounds (e.g., cholesterol or amyloid beta) and the pathogenesis of diverse disorders [15–17]. Most of the research on ABC transporters has focused on P-glycoprotein (Pgp), the first historically identified member of the superfamily, although the attention of the scientific community now also extends to other members, namely, multidrug resistance proteins (MRPs or ABCCs) and breast cancer resistance protein (BCRP or ABCG2).

Validation of the transporter hypothesis for drug-resistant epilepsy has been achieved at the preclinical level, where drug resistance in animal models of seizure and epilepsy has been reverted by coadministration of ABC transporter inhibitors. In 2001, Potschka and coworkers proved that the levels of carbamazepine in the extracellular fluid of the cerebral cortex could be enhanced through perfusion of the Pgp inhibitor verapamil and the MRP1/2/5 inhibitor probenecid [18]. Next, the same group proved that coadministration of probenecid and phenytoin increased phenytoin anticonvulsant effect in kindled rats [19]. Neither 50 mg/kg probenecid nor 6.25 mg/kg phenytoin exerted significant anticonvulsant effect when given alone. It was argued that such raise in the seizure threshold was unlikely to result from additive effects of the chosen sub-anticonvulsant doses. Similar results were later obtained in the focal pilocarpine model of limbic seizures [20]. These pioneering works had two important limitations: (a) they used weak, unspecific modulators of ABC transporters; and (b) experiments were performed without discriminating between responder and nonresponder animals. The first issue was solved with tariquidar, a third-generation and more specific inhibitor of Pgp [21]. Inhibition of Pgp by tariquidar increased the phenytoin brain-to-plasma ratio. Definitive preclinical proof of concept of the transporter hypothesis was obtained by coadministration of tariquidar to drug-resistant animals with Pgp overactivity [22]. Similar results were observed in the 3-mercaptopropionic acid model of refractory seizures, which is associated with Pgp upregulation at the blood–brain barrier, neurons, and astrocytes [23]. Remarkably, verapamil add-on therapy did not improve seizure control in a study on phenobarbital-resistant dogs, and some animals showed a worsening of seizure control [24], which highlights the potential influence of interspecies variability and the necessity to validate the transporter hypothesis at clinical trials.

Concerning clinical data, substantial evidence shows high expression levels of ABC transporters at the neurovascular unit of nonresponders [25–32]. Most of these studies compare samples from patients with intractable epilepsy subjected to surgical removal of the epileptic focus with samples of human brain from people with no history of seizures. While brain tissue from drug-responsive epileptic patients would be a more adequate control, such samples are unavailable since the invasive procedure to obtain them is unacceptable in responders. This limitation has recently been overcome using positron emission tomography [33, 34] which showed that the plasma-to-brain transport rate constants K1 for [11C]verapamil and (R)-[11C]verapamil tend to be reduced in different brain regions of drug-resistant epileptic patients in comparison with both healthy individuals and seizure-free patients.

Reversal of drug resistance after blocking ABC transporters would constitute definite proof of the transporter hypothesis.

Anecdotal cases of refractory patients who have shown improvement when AEDs were coadministered with verapamil have been reported [35–38], but it is unclear if the observed results could be a consequence of the intrinsic anticonvulsant effects of verapamil and/or other drug interactions of pharmacokinetic nature. More recently, a study was conducted on seven children with drug-resistant epilepsy [39]. They received verapamil as add-on therapy to baseline AED. Three subjects with genetically determined Dravet syndrome showed a partial response to adjunctive verapamil; another patient with the same syndrome but no known mutation showed partial seizure control during 13 months followed by seizure worsening. Two subjects with structural epilepsy and one with Lennox–Gastaut syndrome showed no improvement. In spite of the limited number of patients participating in the study, the results seem in agreement with the idea that some therapeutic interventions might be more effective in certain subgroups of refractory patients. A double-blind, randomized, single-center trial (initial sample size = 22) showed mild benefits of verapamil in comparison to placebo as add-on therapy for refractory epilepsy for a subset of the participants [40]. Randomized controlled multicentered trials and studies addressing the effect of selective inhibitors of Pgp with no intrinsic activity are still needed to obtain definitive clinical evidence for the transporter hypothesis. Regarding a possible association between genetic variants of ABC transporters and drug-resistant epilepsy, the available studies are controversial and sometimes inconclusive; while former meta-analysis failed to establish an association between ABCB1 variants and refractory epilepsy [41], subgroup analysis in more recent ones suggests associations in Caucasian and Asian subjects [42–44].

The main argument against the transporter hypothesis is that not all AEDs are Pgp substrates. Seemingly contradictory evidence exists regarding which AEDs are substrates and which are not [45, 46], but it should be kept in mind that results are highly dependent on the experimental setting, including the type of assay (in vivo, ex vivo, or in vitro, human versus animal models, concentration equilibrium transport assay, or nonequilibrium conditions). Still, it seems safe to say that some AEDs are unlike Pgp substrates. A number of points should be considered to reach a conclusion regarding the assigned category (substrate or non-substrate). Possible interspecies variability in substrate specificity should not be excluded. Bidirectional transport assays in the presence and absence of a selective Pgp inhibitor might lack sensitivity since directional transport might be masked by the contribution of passive diffusion; this is especially true when high concentrations of the test drug are used. The magnitude of this effect depends on the expression levels of the transporter in the cell culture, the substrate-tested concentrations, the drug affinity for the transporter, and the physicochemical features of the test drug, among others

[47]. Starting the assay with equal drug concentrations on both sides of the cell monolayer (concentration equilibrium transport assay, CETA) removes the concentration gradient, avoiding net diffusion and enhancing sensitivity [48].

Even if some available AEDs are not Pgp substrates, does this entirely preclude the validity of the transporter hypothesis? Not really. First, Pgp is one among many other efflux transporters possibly involved in drug-resistant epilepsy. Most of the studies determining the directional transport of AEDs have focused on Pgp; however, some of the AEDs are transported by other members of the ABC superfamily. The role of BCRP in the drug resistance phenomena might have been overlooked: while previous work seemed to suggest that AEDs were not transported by BCRP [49], more recent studies using double-knockout Mdr1a/1b(−/−)/Bcrp(−/−) mice and the CETA model suggest otherwise [50, 51]. Interestingly, proteomic studies have revealed ABCG2 as the transporter with the highest expression levels at the BBB of healthy subjects [52]. Moreover, due to the partial overlapping of the substrate specificity of different ABC transporters (together with reported co-expression and co-localization patterns that point to a cooperative role in the disposition of common substrates) [50–52], the role of a certain ABC transporter might be obscured owing to the function of others, their concerted function, and possible compensatory regulation, thus requiring complex models to study the phenomena. The difficulties to quantify the levels of expression of a given transporter in different regions of the brain of patients who have not been subjected to surgical procedures and the uncertainties regarding the ability of experimental models to reflect the absolute and relative expression levels of the different ABC efflux transporters at the epileptic foci and the BBB contribute to the difficulties to study the influence of a given transporter in the regional AED bioavailability in the brain.

The current definition of refractory epilepsy itself suggests that the transporter hypothesis may hold even if some of the known AEDs are not recognized by ABC transporters. Since the definition indicates that a patient should be considered unresponsive after failure of two well-tolerated and appropriately chosen and used AED trials, the key to the preceding reasoning lies in what is considered an appropriate drug choice. The definition of drug-resistant epilepsy weakens the transporter hypothesis if and only if one of the two appropriate therapeutic interventions was in fact a non-substrate for ABC transporters. At present, in the absence of definitive clinical proof of the transporter hypothesis, clinical guidelines for the management of epilepsy do not recommend to try at least one non-substrate AED; thus, the quality of substrate or non-substrate is presently unrelated to the appropriateness of the intervention. If the transporter hypothesis was validated, then a method for patient selection capable of identifying patients that

may benefit from therapeutic strategies targeting efflux transport will be required; what is more, patient selection should also be considered when designing clinical trials to study the clinical relevance of the transporter-associated resistance [53], excluding other sources of drug resistance as possible confounders.

1.3 Therapeutic Approaches to Transporter-Mediated Refractory Epilepsy

There are a number of possible therapeutic solutions that are being explored in relation to the transporter hypothesis. Inhibition of ABC transporters by adding on transporter blockers has already been studied as a possible therapeutic solution to multidrug resistance in cancer, though clinical trials have so far been disappointing ([13, 15, 53] and references therein) due to safety issues. The reader should bear in mind the physiologic role of ABC transporters as a general detoxification mechanism and their involvement in the traffic of endogenous substrates, which discourages the use of add-on inhibitors in the context of long-term therapeutic interventions (such as the ones used in epilepsy). The potential effects of such inhibitors in the pharmacokinetics of other drugs should also be considered in a polymedication scenario owing to the high probability of adverse drug interactions. Moderate or weak inhibitors of ABC transporters emerge as possible solutions; so do therapeutic agents directed to the signaling cascade that regulates the transporter expression [53]. A deep review on such approaches can be found in the excellent articles by Potschka and Luna-Munguia [53, 54].

The use of a "Trojan horse" approach to deliver therapeutic levels of the ABC transporter substrates to the epileptic focus, avoiding the recognition of the efflux pumps, could also be mentioned. This type of intervention encompasses prodrug design and particulate delivery systems (mainly, pharmaceutical nanocarriers) ([55, 56]; the reader may also refer to the special chapter on this subject, in this same volume). Finally, the design of novel AEDs which are not recognized by ABC transporters constitutes an interesting but presently overlooked alternative solution. In the following sections, we will describe some protocols directed to the early in silico and in vitro identification of substrates of ABC transporters in the frame of AED discovery programs.

2 In Silico Identification of Substrates for ABC Transporters

The general procedure to build ligand-based computational models has already been discussed by Talevi and Bruno-Blanch in another chapter of this volume. Similarly, the use of target-based approximations for the early recognition of Pgp substrates through homology modeling and subsequent virtual screening has been discussed by Palestro and Gavernet. Thus, we will only discuss under this

section particular aspects of ligand-based approximations aimed to in silico early recognition of ABC transporter substrates, with emphasis on the suitability of meta-classifiers for this purpose. Naturally, the general objective here is to discard substrates of ABC transporters; that is, ABC efflux pumps will be regarded as antitargets, i.e., *a biological target that causes undesirable effects when interacting with a drug*. Note that unlike common screening campaigns, where the compounds that interact with a given biological target are retained for further studies, the compounds interacting with an antitarget will not progress to additional testing.

We suggest starting any protocol to identify potential new treatments for efflux transporter-associated refractory epilepsy with high-throughput cost-efficient in silico screening tools and then gradually advance to computationally demanding models with lower throughput, reserving in vitro and in vivo models to the last stages of the screening. This cascade "in silico and in vitro first, in vivo later" approximation is not only cost-efficient (experimental tests are always more expensive than in silico experiments) but is also in good agreement with the 3R's bioethical principle, since it helps replacing and reducing animal testing as much as possible.

The reader may choose between developing its own in-house in silico model and resorting to any of the (many) models reported previously (see, for example, the models reviewed in references [57–59]). Some model developers offer their models online or in software packages, either freely or commercially. See, for example, Biozyne (http://pgp.biozyne.com/, last assessed January 2016) and Althotas Virtual Laboratory (http://pgp.althotas.com/, last assessed January 2016) [60, 61]. Some models can be reproduced from literature reports provided that you can access the required software tools. If you decide to use models developed by someone else for your predictions, it might be a good idea to examine the original papers in which such models are described, in order to assess the suitability of the procedures that have been used for model building and possible limitations of the approach. Do not rely blindly in computational models whose details have not been disclosed. Note that very frequently reported models related to ABC transporters are based on unbalanced training sets in which substrates significantly outnumber non-substrates, resulting in possible bias toward the prediction of the dominant category. On the other hand, many of the reported models have been derived from congeneric series of molecules, severely restricting their chemical space coverage. Finally, models to predict interaction with ABC transporters are seldom complemented by experimental validation of the results. As a general rule, when using models related to ABC transporters developed by other modelers, you should try posing the following questions: Have the models been derived from a balanced training set? Have the models been

inferred from a chemically diverse training set? Is it possible for you to estimate whether a predicted compound belongs to the applicability domain of the model? Have the models been appropriately validated using at least external validation procedures? If your answer to any of this questions is no or if you do not know the answer, avoid using the correspondent model(s). As a final advice on this matter, try to review the quality of the biological data used for training purposes. Section 3 will discuss some advice for in vitro permeability assays that could be used as reference. As discussed in the chapter by Talevi and Bruno-Blanch in this same volume, there are some robust modeling approaches (namely, classification models and ensemble learning) that can help mitigating noise related to dubious or heterogeneous experimental data. Finally, note that accuracy metrics reported in the literature for a given model often correspond to a single score cutoff value. If you are able to build a receiving operating characteristic (ROC) curve, you may even optimize the score threshold value according to your own scenario, opting for a cutoff value that favors specificity or sensitivity depending on your particular needs [62]. From the work by Truchon and Bayly, it is suggested to study the behavior of a given virtual screening/ranking method by seeding a relatively small number of known hits among a large number of decoys [63]. Through analytical work and proper statistical simulation methods, these authors demonstrated that the standard deviations in different metrics used to assess ranking methods tend to converge in such conditions, simultaneously removing a possible "saturation effect." In brief, do not excessively rely in conclusions regarding ranking metrics when they have been drawn from a limited-size test sample; instead, conduct your own evaluation through a pilot screening campaign dispersing a small sample of known hits among a large number of decoys: you might find the directory of useful decoys valuable for this purpose [64].

If you choose to build your own model(s) to predict affinity for ABC transporters, you must assume similar considerations. The key point here is that, owing to the polyspecificity that characterizes ABC transporters and the high interlab variability associated with experimental data, predicting whether a substance is or is not transported by a given member of the ABC superfamily is particularly challenging. Back in 2007, based on the high variability of Pgp experimental affinity data, Zhang and colleagues estimated the upper bound of accuracy for Pgp models in 85% [65], which is quite low compared with the accuracy achieved in the frame of other modeling problems. With some exceptions that overcome that theoretic upper bound (see, for instance, [66]), most of the reported models on ABC transporters display an overall accuracy similar to 80%. Typically, modeling efforts rely on biological data and chemical datasets compiled from literature: classification models can be used to alleviate the noise associated with such heterogeneous experimental data and large interlaboratory variability [67]; as Polanski et al.

affirm, extensive data independence implies qualitative rather than quantitative solutions [68].

The intrinsic difficulty of predicting affinity for ABC transporters has led many researches in the field to contemplate more flexible techniques such as nonlinear models [59] and more robust approximations such as ensemble learning/consensus QSAR or locally weighted methods [58, 69–75]. Also note that there is some evidence that ensemble learning could reduce the necessity of applicability domain estimation, assuring wider coverage of the chemical space [76]. Despite a very large number of models and algorithms for the computer-aided recognition of substrates for ABC transporters have been reported, very few have been applied in the specific field of drug discovery for refractory epilepsy, including the models by Di Ianni et al. [74] which have been used either alone or combined with docking protocols, as described by Palestro and Gavernet in this same volume.

Finally, when compiling training data, interspecies differences and other sources of variability (e.g., expression systems, genetic variants) must be considered.

3 In Vitro Permeability Assay to Identify ABC Transporter Substrates

In vitro models for the prediction of drug transport across biological barriers include cell cultures that reproduce physiological characteristics of a variety of barriers, such as the intestine and the blood–brain barrier. As previously insinuated, one of the main limitations of such systems is the high variability in permeability estimations, which makes difficult the comparison and combination of data from different laboratories and demands careful validation and continuous suitability demonstration: proper standardization of preexperimental, experimental, and post-experimental factors helps in reducing intra- and interlaboratory variability [77–79]. In the conclusion of their recent and remarkable study assessing the influence of a diversity of factors (mainly, days between seeding and experiment, passage number, coating, and data analysis approach) on variability and permeability, Oltra-Noguera and coworkers conclude that a similar study should be undertaken in each laboratory to evaluate the influence of protocol variables on the cell monolayer properties, in order to standardize the conditions and set acceptance criteria [77]. Caco-2, MDCK, and MDCK Pgp-transfected clone (MDCK–MDR1) cell lines are the most frequently used to determine in vitro permeability values and characterize drug transport mechanisms. Among them, we are currently using MDCK–MDR1 cells to study if AED candidates are or are not Pgp substrates. MDCK–MDR1 constitute a fast maturation model; note that MDCK–MDR1 lines with low values of transepithelial resistance (TEER) have also been used as blood–

brain barrier model [80, 81]. Back to the study of Oltra-Noguera, MDCK–MDR1 showed constant CV among passages, protocols, and experimental conditions, but permeability values were affected by all the studied conditions mentioned previously, indicating that for this cell line, standardization of experimental conditions is particularly critical to obtain comparable results between different laboratories [77].

Since the more relevant transporters possibly contributing to drug-resistant epilepsy are preferentially expressed in the apical membrane of the cells, the efflux (or transport) ratio can be generally used to define whether a given drug is or is not a substrate from an ABC transporter. Briefly, using a permeability chamber in which a donor and a receiver compartment are separated by the cell monolayer growing on a polycarbonate membrane, apparent permeability coefficients (Papp) are calculated in both apical-to-basolateral and basolateral-to-apical directions; the efflux ratio is defined as the apparent permeability obtained in the basolateral-to-apical direction divided by those obtained in the apical-to-basolateral direction. If the only drug transport mechanism is diffusion, then no significant difference between both permeability coefficients is expected, and the efflux ratio will be similar to 1; in contrast, the movement of a substrate for an efflux transporter preferentially expressed in the apical membrane will be restricted in the apical-to-basolateral direction, and the efflux ratio will tend to be larger than 1. Assuming sink conditions (negligible drug concentration in acceptor versus donor compartment, i.e., acceptor concentration <10% of donor concentrations), Fick's first law may be used to compute the correspondent Papp as follows:

$$\frac{\mathrm{d}Q}{\mathrm{d}t} = \mathrm{Papp} \times S \times \mathrm{Co}$$

where dQ/dt is the appearance rate of drug in the receiver side, calculated using linear regression of amounts in the receiver chamber versus time, S is the surface area of the monolayer, and Co is the initial drug concentration in the donor compartment. Since after starting the experiment the concentration in the donor compartment is not really Co but constantly changes with time, the precedent equation can be corrected by replacing Co by Cd, the concentration in the donor compartment at each sample time (which can be simply calculated from the difference between the initial drug amount in the donor compartment and the drug amount in the receiver compartment at each sample time, assuming biotransformation within the cells can be neglected) [82]. If the sink condition is not verified, one should resort to non-sink equations, which are valid both in sink and non-sink conditions [77]. Note that concentrations should be corrected to consider the dilution effect related to media replenishment. Ideally, the mass balance should be checked at the end of the experiment by determining the amount of drug in cell membranes and inside the cells,

though the percentage of compound retained in the cell compartment is often negligible [77]. Note that a given cell line will express more than one drug transporter. Thus, the efflux ratio should be repeated in the presence of a specific inhibitor of the transporter being studied. Provided that the test drug is actually a substrate of the (anti)targeted transporter, such experiment should remove the influence of the (anti)targeted transporter returning the efflux ratio to around 1.

According to the results of Oltra-Noguera and collaborators, the best conditions to study compounds subjected to active efflux include using an intermediate or late passage number, polycarbonate filter without collagen coating, and sink corrected equations [77]. Within our group the following experimental conditions are observed: MDCK II–MDR1 cells are grown in 25 cm^2 culture flasks using DMEM with 10% fetal bovine serum, 1% l-glutamine, 1% nonessential amino acids, and penicillin and streptomycin at 37 °C in 5% atmosphere. Cells are split twice a week at 70–80% confluence in a ratio of 1:20 or 1:30 using trypsin–EDTA solution (0.25%). All transport assays were done with cells from passages 19 to 43. Cells were kept at 37 °C in 5% CO_2. The cells are seeded in 6-well Costar Snapwell plates with polycarbonate membrane inserts at a density of 50,000 cells per insert (1.12 cm^2) and grown for 4 days in culture medium. The medium is replaced every day. The apical media volume is 0.5 ml, and the basal volume is 2 ml. Integrity of the cell monolayers is determined by measuring the TEER using an epithelial voltammeter (Millicell-ERS, Millipore Corporation); normal TEER in MDRK and MDCK II–MDR1 cells is about 190 Ω cm^2 [83]. In addition, the integrity is also checked using atenolol (which is transported by the paracellular pathway). The Papp of atenolol across MDCK II–MDR1 cell monolayers in these conditions is typically $1–5 \times 10^{-7}$ cm/s. The expression of Pgp is checked by Western blot analysis and by transport assay with trimethoprim, a substrate for Pgp [84]. On the day of the experiment, culture medium is removed, and cells are washed three times with media transport (HBSS, Hanks' balance salt solution, pH 7.4, Gibco-BRL). The filter inserts containing the cell monolayers are placed in an Ussing chamber and kept at 37 °C and under constant gassing with carbogen. Test compounds are added to the donor side (4 ml for the apical and basal chamber). At 20, 40, 60, 80, 100, and 120 min, samples (400 μl) are taken from the acceptor compartment followed by the addition of 400 μl of transport media. For the inhibition experiments, cell monolayers are incubated with amiodarone chlorhydrate (50 μM) [85] for 1 h in apical and basolateral compartments before adding the test compound. A schematic representation of the device is shown in Fig. 1.

A critical point in transport assays is the AED concentrations used in these studies [48]; the role of drug transporters may be concealed by the contribution of passive diffusion, especially when

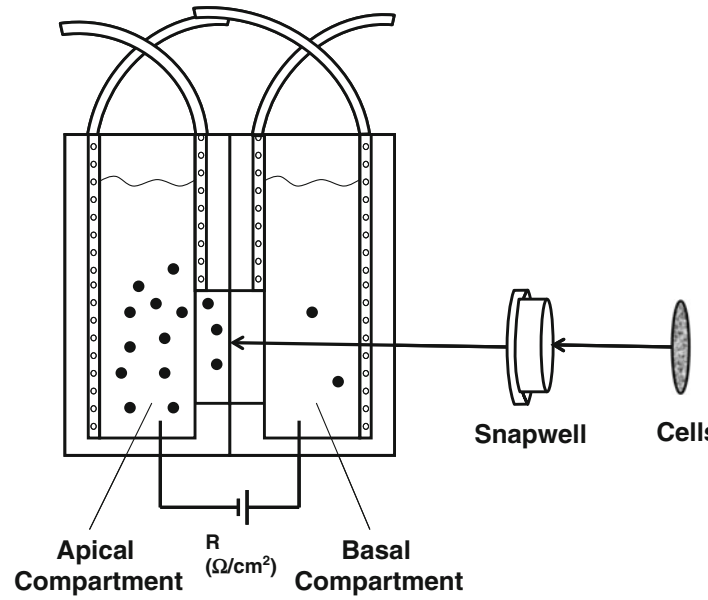

Fig. 1 Diffusion chamber used in MDCK II–MDR1 permeability studies

highly permeable compounds (which is the case for most AEDs) and/or weak substrates are assayed. This effect could be more pronounced at high concentrations, since the concentration gradient is the driving force for passive diffusion. It is advised to test therapeutically relevant drug concentrations [48]. Note that free drug levels in plasma seldom exceed micromolar concentrations and brain levels are usually even smaller. Thus, the use of as low concentrations as possible is suggested. However, the lower limit of the possible concentration range to be used depends on the sensitivity of the analytical detection method used. HPLC MS/MS methods usually fulfill the requirements. Naturally, the analytical methods should be properly validated. Pay close attention to the fact that some ABC transporter substrates such as verapamil display a biphasic behavior depending on the concentration [48, 86]; thus, using a concentration range covering from low nanomolar to micromolar concentrations is highly preferred to using a single concentration, when possible. If due to sensitivity issues of the available analytical method the lowest concentrations of that range are precluded, try to use at least therapeutically relevant concentrations; the use of as low concentrations as possible could help reduce the impact of passive diffusion on the study results. Take into consideration that in general expression levels of the transporter at the monolayer will not represent local expression levels at epileptogenic regions in the brain and that ABC transporters display stereospecificity for some substrates.

Alternatively, using identical initial concentrations at both sides of the monolayer (apical and basolateral compartment) is an

elegant solution to remove the passive diffusion component, increasing the assay sensitivity (i.e., CETA) [48]. In such case, only the net direction of the transport should be analyzed. In the absence of mediated transport, no net drug movement should be registered. In contrast, if the drug acts as a substrate for an efflux transporter expressed at the apical membrane, a net movement in the basolateral-to-apical direction should take place.

4 Ex Vivo Permeability Assay to Identify ABC Transporter Substrates: Evert Gut Sac Model

The in vitro everted gut sac model was first introduced in 1954 by Wilson and Wiseman [87]. Since then modifications and improvements have been made to the model to increase the viability of tissue and to maintain intact mucosal epithelium that mimic the in vivo conditions [88, 89]. The everted sac model has been explored to carry out pharmacokinetic investigations of the impact of efflux transport modulators on the absorption of drugs [90–94]. The main advantage of this technique with respect to in vitro assays is that frequently results from the everted intestinal sac model have been in agreement with in vivo findings [92, 95–98]. On the other hand, the cell culture assays conducted in either human cancer lines expressing ABC tansporters or transfected cell lines with high density of transporter molecules, which is required for their function to be predominant over membrane permeation and passive diffusion processes, have high negative predictive value, but often give false positives [99]. Indeed, there are evidences that the absorption rate of test compounds is not always similar in the everted gut sac than in the Caco-2 monolayer [100]. For oral absorption studies, Caco-2 cell line experiments were approved by the Food and Drug Administration, but these cell lines are not always the right substitute for the ex vivo everted gut sac model [101]. For that reason, new drug candidates screened for interactions in silico and in vitro culture assays during early stages of drug development should be tested in a subsequent step by ex vivo assays such as everted gut sac, the isolated intestinal perfusion, or Ussing chamber system [102].

The advantages of this everted gut sac model as an ex vivo tool to study the mechanisms and kinetics of drug absorption are a relatively large surface area available for absorption and the presence of a mucus layer [103]. On the other hand, the tissue viability is one of the main limiting parameters having a maximum of approximately 2 h [88].

Different animals have been chosen for everted gut sac experiments, but the everted rat intestinal sac is the most commonly used [87, 88, 90, 104]. The reproducibility of the rat everted gut sac

suggests that this ex vivo model is a useful tool for studying transport of substrates and modulators of ABC transporters [93].

4.1 Localization of ABC Transporters along the Intestinal Tract

One of the keys for the success of the analysis of the participation of ABC transporters in drug absorption by the everted gut sac technique is to select the correct intestinal segment. A cellular and subcellular localization description of the most frequently studied ABC transporters in human and in rats is briefly described below. For a full scan of the intestinal expression of Pgp (MDR1/ABCB1), BCRP, and MRP2 (ABCC2) in male and female rats, consult McLean et al. [105].

The expression of Pgp increases from proximal to distal portions in the apical membrane of the intestinal crypts in human, resulting in the highest expression levels in the colon [106, 107]. In the same way, it shows an increase from proximal to distal regions in the rat [105].

In the rat and human intestine, MRP2/ABCC2 mRNA expression is highest in the apical membrane of the enterocytes in the duodenum and subsequently decreases in direction to the terminal ileum and colon where it is only minimal [103, 108]. Protein levels of MRP2 in rats decrease along the intestinal axis from proximal to distal parts [105].

Transcription of BCRP in the human jejunum is higher than that of MDR1 and comparable with that of MRP2 [109]. BCRP/ABCG2 mRNA is also expressed with apical localization in the epithelium of the small intestine of the rat showing duodenum and jejunum levels similar to those found for MRP2 and then decreasing slightly to the more distal portions [109]. In the large intestine, a continuous decrease of the BCRP mRNA toward the distal portions is observed finding in the rectum a half of the levels found in the small intestine [110]. However, protein levels of BCRP show an arcuate pattern with the highest expression toward the end of the small intestine in the rat [90, 105].

On the other hand, MRP3/ABCC3 localizes to the basolateral membrane of enterocytes in rats. It is expressed in low levels in the duodenum and jejunum but markedly increases in the ileum and colon [108].

There are not sex differences in the mRNA and protein expression levels of ABC transporters in the entire intestine of the rat [105].

As described above, the localization of ABC transporters not always matches thorough the intestine between rat and human beings. Moreover, differences exist between the mRNA expression and the subsequent translation to protein because of post-transcriptional modifications. Furthermore, it is important to note that expression levels of transporters can be modified under various physiopathological conditions, in the presence of polymorphisms, and by administration of drugs. For example, we

observed overexpression of BCRP in the ileum after chronic oral administration with the antiretroviral efavirenz that reduces its own intestinal penetration [90]. Yumoto et al. demonstrated that in situ intestinal absorption of methotrexate, a substrate of Mrp2, is decreased by oral treatment with ursodeoxycholic acid that causes Mrp2 upregulation [92]. Moreover, the metabolic syndrome generated by enhanced fructose intake in rats decreases the protein expression and activity of ileal Pgp, thus increasing the bioavailability of rhodamine [93]. Changes in expression level, subcellular localization, and functional properties can all be involved in interindividual differences in drug pharmacokinetics. For that reason, the expression levels of the ABC transporter of interest need to be checked in experimental models before carrying out the everted gut sac technique to achieve optimal experimental conditions and to avoid false results.

4.2 Everted Gut Sac Preparation

Under anesthesia with urethane (1.2 g/kg body weight), rapidly remove the jejunum or duodenum or ileum of the intestine and wash each segment with an ice-cold oxygenated Krebs solution (pH 6.5) containing 7 g/l sodium chloride, 0.34 g/l potassium chloride, 1.8 g/l glucose, 0.251 g/l disodium hydrogen phosphate, 0.207 g/l sodium dihydrogen phosphate, and 46.8 mg/l magnesium chloride. Gently evert the washed intestine over a glass rod and divide into segments (5–6 cm each). Clamp one end of the everted intestine and tie with a silk braided suture and then place a short intravenous cannula at the other end and tie the intestine around it using a second braided silk suture making sure it does not break to hang it so that this end is available for the administration of fluids. It is very important to prevent the intestinal wall to enter the cannula being damaged because the fluid inside the bag was lost by these sites giving erroneous results. Previously, enter a stainless thin wire in the cannula cap that acts as a hook at the other end so as to be able to hang the everted sac in the container where it will conduct the test. Hang the everted filled sac in the incubation tube containing drug in 5–10 ml oxygenated Krebs solution at 37 °C alone or in the presence of a fixed dose of ABC inhibitors preventing the sac to touch the walls. Fill the sac with 600–800 μl Krebs solution containing the drug to be tested using a 1 ml syringe. Sampling could be performed at different intervals considering that the same amount of medium as the sample is taken must be replaced. Depending on the transport direction to be analyzed, e.g., serosal to mucosal or the opposite, drugs to be tested could be added in the incubation container or into the sac [90, 93, 103].

There are several factors that need to be taken into account due to the fact that they impact on the outcome and conclusion of everted gut sac studies, e.g., animal factors (age, sex, species, diet, disease state, chronic treatment, and toxicity), intestinal segment (ileum, jejunum, duodenum, and colon), and experimental factors

(e.g., pH, aeration, temperature, concentration of substances). Those factors have been extensively revised by Alam et al. [111].

Finally, to achieve sensitivity and specificity, it is important to select the best quantification method for each compound as spectrophotometry [93, 112], liquid chromatography [92, 93, 96], and radioactivity [106] and to perform the analysis in the presence of highly specific pairs of substrate inhibitors [112].

5 Final Remarks

Despite the transporter hypothesis of refractory epilepsy has long been studied (with convincing preclinical evidence and encouraging yet limited clinical evidence), such hypothesis is still underexplored in the drug discovery field to assist the search of new treatments for refractory epilepsy. Although a considerable number of computational models related to the early identification of substrates for ABC transporters have indeed been developed, few of them have been applied specifically in the field of AED discovery.

ABC transporter polyspecificity and interlaboratory variability of experimental data on their substrates and non-substrates constitute major challenges to the development of accurate computational models oriented to the prediction of affinity for efflux pumps. We have reviewed some general criteria to circumvent or at least minimize the impact of such limitations when building computational models for that purpose, prominently, the use of classifiers and meta-classifiers. Whenever applying external (i.e., non-in-house models), estimation of the model's coverage/applicability domain is essential.

Regarding experimental substrate assessment, some relevant issues include measuring interaction with not one but a number of ABC transporters with (partially) overlapping substrate specificity (which often act in a concerted manner and can be subjected to compensatory regulation) and testing a wide range of therapeutically relevant concentrations (from nM to μM) in order to discard possible biphasic responses. The use of small concentrations helps in minimizing the influence of passive diffusion on the study outcome; alternatively, the CETA is a sensitive model to measure the affinity for ABC transporters of highly permeable drugs and/or weak substrates. Experimental variables should be carefully study to enhance the reproducibility of the obtained results [113].

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Chapter 15

Discovering New Antiepileptic Drugs Addressing the Transporter Hypothesis of Refractory Epilepsy: Structure-Based Approximations

Pablo Palestro and Luciana Gavernet

Abstract

Drug resistance represents a major obstacle to the success of epilepsy treatments, therefore intense investigations have been carried out to explain its origins. One of the most experimentally corroborated theories is the transporter hypothesis. It proposes that, at least for a subset of patients, the failure of the anticonvulsant drugs is caused by their inability to reach the molecular targets due to the regional overexpression or activation of efflux transporters. Among them, P-glycoprotein (P-gp) showed overactivity in drug-resistant patients as well as proved interactions with known anticonvulsant drugs. In this chapter we summarized the structure-based approximations employed to identify substrates/inhibitors of the glycoprotein, with special attention in describing the structural data available of the target- and the docking-based simulations. We also pointed out our results regarding the identification of new anticonvulsant candidates that avoid P-gp interactions by means of a sequential ligand-based and target-based screening, along with practical details related to this protocol.

Key words Refractory epilepsy, Docking, P-Glycoprotein, Virtual screening

1 The Transporter Hypothesis of Refractory Epilepsy

Despite the successful discovery of new antiepileptic drugs (AEDs) with better absorption, distribution, metabolism, excretion, and toxicity (ADME/TOX) profiles (or, in some examples, with novel mechanisms of action), the efficacy of drug treatment of epilepsy has not substantially improved over the years. According to the international consensus, refractory epilepsy is described as the failure to achieve seizure freedom with two or more well-tolerated AEDs, given in an appropriate manner [1]. About 30% of the patients fit to this definition, since they respond inadequately to known drug therapies. This fact represents a main concern for the experts in the field, who elaborated several hypotheses to explain the origins of the drug-resistant epilepsy.

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Abundant evidence acquired from epidemiological analysis, studies in animal models, and human epileptic tissues of patients undergoing surgical resection indicates that the refractory epilepsy is a multifactorial phenomenon. It might be associated with the development of tolerance during prolonged administration of the AEDs [2], the etiology and severity of the seizures [3], changes of the drug targets [3–6], alterations in neuronal connectivity [7, 8], blood–brain barrier dysfunctions [9], and genetic variants of the proteins involved in the biodistribution, metabolism, and mechanism of action of the drugs [10]. In addition to these causes, the *transporter hypothesis* claims that the failure of AEDs in some refractory patients is originated by their inability to reach the molecular targets, due to the overexpression or activation of efflux transporters in brain tissues [11]. This mechanism of drug resistance is highly supported by experimental data [11–25] and provides a possible explanation about one important characteristic of refractory epilepsy: patients that fail to control the seizures with one AED have a small chance (lower than 10%) to control them by other AEDs, even with drugs that act through the interaction with different molecular targets [10, 11, 26].

The most-studied efflux transporter is P-glycoprotein (P-gp). It is a member of the ATP-binding cassette (ABC) transporter family, and it is expressed in brain cells (neural cells, glial cells, and capillary endothelium at the blood–brain barrier) as well as in other barriers and excretory tissues, with the function of detoxifying them by preventing exogenous compounds from entering susceptible organs [27, 28]. In contrast to this beneficial effect, the regional overactivity of P-gp in drug-resistant patients limits the access of AEDs into the brain targets, rendering them ineffective [13, 21, 22, 29, 30]. Moreover, studies about the interaction between P-gp and AEDs confirmed that phenytoin, phenobarbital, topiramate, lamotrigine, levetiracetam, oxcarbazepine, and eslicarbazepine acetate (and some of their metabolites) are substrates or inhibitors of the P-gp [31–35]. In this context, it became advisable to include in the early phases of AED discovery the analysis of the interactions of the newly designed anticonvulsants with P-gp.

According to the characteristics of the binding, compounds can be categorized as substrates, inhibitors, or modulators of P-gp. Substrates are actively transported by P-gp, whereas inhibitors affect the transporting function. Modulators interact with active sites distinct from the substrates, reducing the strength of its interactions by a negative allosteric binding [36]. Since both inhibitors and modulators impair the transport function, they are frequently named equally as inhibitors [37]. Additionally, as modulators, inhibitors, and substrates interact with P-gp in one way or another, here we will refer them together as *binders*.

There are several biological assays developed to test the ability of compounds to interact with P-gp [38, 39]. Among them, in vitro transport experiments are recommended by the US Food and Drug Administration as the preferred data to decide if a drug

is a P-gp binder. They suggested a bidirectional transport assay using cultured cells as the initial test, followed by the validation that the efflux is inhibited by the presence of one or more inhibitors [40]. The in vitro methods with Caco-2 cells are the most frequently employed, followed by the MDCKII-MDR1 cells (multidrug resistance protein-1-transfected Madin–Darby canine kidney cells) [41]. However, any experimental screening to evaluate P-gp interactions with new active compounds is expensive and time demanding, so computational models provide a valuable and complementary tool for the virtual recognition of P-gp binders.

2 In Silico Approaches to Predict P-gp Binders

The impact of P-gp on the drug resistance to treat not only epilepsy but also other diseases (such as cancer, Alzheimer, and HIV [42–45]) has promoted the development of in silico studies to identify P-gp binders. It is known that P-gp has the capability to interact with unrelated structures, and this poli-specificity makes it difficult to find a common pattern for the recognition of binders. However, numerous ligand-based approaches were developed to find the requirements shared by small molecules to be P-gp substrates or inhibitors. The initial pharmacophoric patterns proposed the importance of aromatic rings and lipophilic centers, basic nitrogen atoms, and hydrogen-bonding interactions [46–48]. After that, other ligand-based approaches were developed to predict P-gp binders, which include new pharmacophoric patterns, machine-learning algorithms, and quantitative structure–activity relationship (QSAR) studies among others [47, 49–60]. Details of the ligand-based models are widely explained in a previous chapter of this book. Some of them present a high prediction accuracy for given classes of drugs or drug candidates, with the additional advantage of being less computationally demanding than target-based approaches.

Structure-based methods deal with the high computational cost (particularly for virtual screening campaigns with large datasets), but they are able to provide atomic details on the protein–ligand interactions. Therefore, these approaches allow the prediction of the binding modes between new (or known) compounds and the biological targets as well as the structural optimization of the ligands (to improve/avoid their interactions with the macromolecules). Of course, the application of structure-based approximations implies the knowledge of the target at atomic level.

2.1 Tridimensional Structure of P-gp

The elucidation of the 3D structure of membrane proteins from crystallography still represents a challenge in structural biology. Regarding crystals of transporters in complex with some substrates, the experiments get even more problematical because the low binding affinities increase the difficulty to solve their positions.

In 2009 Aller and coworkers elucidated the first X-ray structure of the eukaryotic (*Mus musculus*) P-gp, improving enormously the knowledge of the protein architecture at atomic level [61]. Before this finding, the 3D structure of P-gp was modeled by comparative analysis (homology-modeling techniques) with templates that share low sequence identity to the human protein, such as the bacterial ABC transporter MsbA [62–64]. Conversely, the sequence of mouse P-gp has more than 80% sequence identity to human P-gp and similar size [65], suggesting a high level of conservation of the 3D structure.

Additionally, Aller and coworkers crystallized two protein–ligand complexes providing the first structural details about how P-gp interacts with their binders [61]. The reported apo and drug-bound structures showed inward-facing conformations of P-gp, which correspond to the initial stage of the transport cycle. Subsequently, other experimental models were also elucidated and deposited in the protein data bank [66] improving the information about the overall 3D structure of P-gp and the architecture of the binding sites (Table 1).

As other ABC proteins, P-gp employs the hydrolysis of ATP to efflux out of the cell substrates across the biological membrane. It comprises two pseudosymmetric halves. Each one has six transmembrane helices (TMs) connected with loops and short helices to one cytosolic nucleotide-binding domain (NBD) (Fig. 1).

Table 1

Experimental 3D models of P-gp deposited in the protein data bank after the mouse structures elucidated by Aller in 2009 [61]

Date	Authors	Related PDB codes (resolution)	Organism
2009	Aller et al. [61]	3G5U (3.80 Å)	*Mus musculus*
		3G60 (4.40 Å)	
		3G61 (4.35 Å)	
2012	Jin et al. [67]	4F4C (3.40 Å)	*Caenorhabditis elegans*
2013	Li et al. [68]	4M1M (3.80 Å)	*Mus musculus*
		4M2S (4.40 Å)	
		4M2T (4.35 Å)	
2013	Ward et al. [69]	4KSB (3.80 Å)	*Mus musculus*
		4KSC (4.00 Å)	
		4KSD (4.10 Å)	
2015	Szewczyk et al. [70]	4Q9H (3.40 Å)	*Mus musculus*
		4Q9I (3.78 Å)	
		4Q9J (3.60 Å)	
		4Q9K (3.80 Å)	
		4Q9L (3.80 Å)	

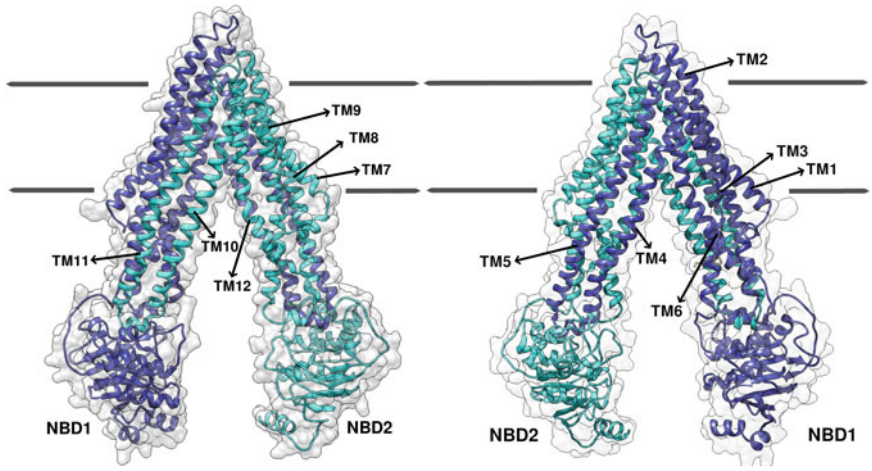

Fig. 1 Overview of the crystal structure of mouse P-gp (PDB code 4Q9H). Transmembrane domains are labeled (TM). *Horizontal lines* approximate the region of the lipid bilayer

Regarding the binding sites for substrates and inhibitors, previous analyses have proposed up to seven binding regions located into the internal cavity of the protein, which is generated by the two sets of TMs (TMs 1–3,6,10,11 and TMs 4,5,7–9,12) [71, 72]. The existence of multiple binding sites (some of them overlapped) in the P-gp internal cavity is consistent with the poli-specificity observed for the protein. Moreover, this cavity has a volume around 6000 Å^3, which is big enough to accommodate more than one binder simultaneously in different subsites [61] (Fig. 2). Recently, another potential binding site has been detected by X-ray crystallography [70]. It is placed on the exterior of the P-gp structure but close to the region proposed as a potential intramembranous access of the substrates to the cavity [73, 74]. The authors suggested that there is an initial weak interaction of the substrates with this site before achieving their final position into the internal cavity [70] (Fig. 2).

2.2 In Silico Predictions of P-gp Binders Through Target-Based Methods

The structural information of P-gp provides the starting point for the application of target-based methods of drug design. Among them, molecular docking protocols were employed to locate P-gp putative binding sites and to predict the binding affinities of small molecules. Note that the results of a docking protocol are highly dependent on the experimental data available, the predefined scoring functions, and the conformational search algorithms provided by the software. Consistently, it is always convenient to test a diversity of docking alternatives and appropriately validate the suitability of the protocol to reproduce the experimental conformation of ligand–target complexes, if they are available. In the same line, it is opportune to test their ability to discriminate known binders from non-binders through their docking score. It is expected that they predict better affinities (i.e., lower calculated binding energies) for binders.

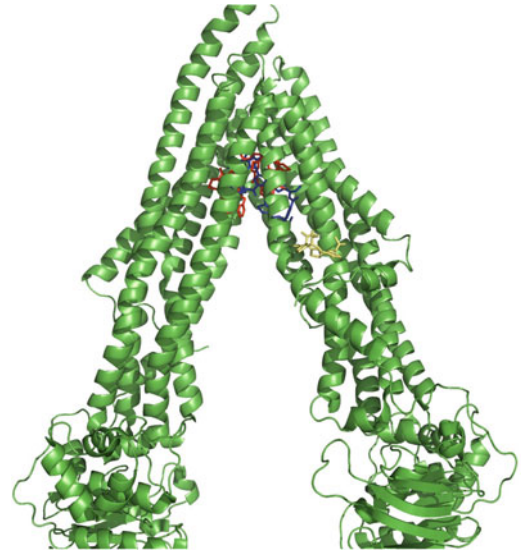

Fig. 2 Overview of P-gp co-crystallized with valine–cyclopeptide structure (PDB code 4Q9J). Two molecules of the ligand are located in the internal cavity (highlighted in *blue*), whereas another is placed on the exterior of the structure (highlighted in *yellow*). The position of the other two ligands (phenylalanine cyclopeptide, co-crystallized in the PDB complex 4Q9L) was also included in *red*, to show the versatility of the active site

Becker and collaborators constructed a P-gp homology model based on the 3D structures of the transporters Sav1866 and MsbA to dock the ligands verapamil, rhodamine B, colchicine, and vinblastine into the binding cavity [64]. The results showed good correlation with the experimental data available since the docking poses interact with residues previously identified in the active site. Pajeva et al. employed the structure of mouse P-gp (PDB code: 3G61) as template to construct a P-gp human 3D model, and they docked a small set of quinazolinone inhibitors to support the results obtained from pharmacophoric patterns about the common structural features of P-gp binders [75]. Later on, other authors evaluated the ability of docking programs to discriminate known binders from non-binders [49, 60, 76, 77]. They constructed bigger test sets than Becker and Pajeva with different chemical backbones to consider the promiscuity of the P-gp binding sites. The final proposition was to employ docking algorithms to predict the P-gp binding affinities of untested molecules.

Chen et al. evaluated the prediction capability of molecular docking by using the two drug-bound P-gp structures provided by Aller et al. (PDB codes: 3G60 and 3G61, Table 1). They docked 157 substrates and 88 non-substrates with Glide docking software (http://www.schrodinger.com/Glide) and scored them by the two Glide modes: SP (standard precision) and XP (extra

precision). The authors concluded that the docking protocols were unable to clearly discriminate substrates from no substrates by using the best score criterion [49].

Dolghih and coworkers applied docking protocols to analyze their capacity to differentiate 30 binders (26 drugs and four metabolites) from 98 presumed non-binders (metabolites). They employed the target structure provided by Aller (PDB code: 3G60) and run rigid and flexible docking (with induced fit algorithms). The force fields were provided by Glide SP, Glide XP, and MM-GB/SA scoring functions [76]. Additionally, they tested 13 binders and 34 non-binders selected from a dataset of FDA-approved drugs. The active compounds of the set were positive in two experimental assays (monolayer efflux and calcein-AM inhibition assays) whereas the non-binders were negative for both experiments. Finally they benchmarked the docking conditions with a blind test on a series of peptidic cysteine protease inhibitors. The authors concluded that flexible receptor models have the ability to differentiate known binders from non-binders. They proposed that the better results in flexible docking might be associated with the mobility of the binding site residues but also with the low resolution of the target structure. They also suggested that P-gp substrates could bind deeper in the P-gp cavity than the ligands in the crystallized complex [76].

Dolghih et al. also docked rhodamine B, a known P-gp substrate with experimental binding data available [78, 79], to validate if docking can predict accurately the geometry of experimental complexes. In the same line, they strip and docked the ligand (a cyclic peptide) back into the crystal structure 3G60 (named QZ59-RRR). Again, the flexible receptor poses reproduced better the experimental binding interactions. Interestingly, one of the best docking conditions found in this investigation was then applied by the authors to estimate the efflux ratio of the molecules (the ratio of their two transport rates in opposite directions: basal to apical and apical to basal across a single layer of cells) as a measure of their brain penetration [80].

On the other hand, Bikardi et al. combined ligand-based and target-based methods to find a cost-effective protocol to predict potential P-gp substrates and their molecular interactions [50]. The identification of possible P-gp substrates was in charge of a support vector machine method (with a training dataset of 197 known P-gp substrates and non-substrates), and the docking calculations were employed only to predict the interactions at atomic level of the selected compounds. The authors validated the docking protocol (Autodock Vina software) by re-docking the QZ59RRR ligand to the mouse X-ray P-gp structure. They found an acceptable agreement between the experimental and predicted ligand conformations (RMSD value of 1.27). However, the binding predictions were performed on a human P-gp model as the target. The authors constructed a 3D homology model (using mouse P-gp as template) and tested it by docking rhodamine B.

The results of this docking calculation were consistent with experimental data [78, 79]. The algorithms are available in a Web server, which enables the users to predict if a compound is a P-gp substrate, as well as its binding conformation into the P-gp active sites.

Bikardi et al. did not use docking for classifying P-gp binders from non-binders. Conversely, Klepsch and collaborators compiled a large set of 1076 inhibitors and 532 non-inhibitors to test the capacity of the scoring functions implemented in GOLD package to differentiate them [60]. They employed a model of the human P-gp as target, which was constructed from murine P-gp (PDB code 3G5U). Initially, the ligands were docked in two protonation states with ChemScore or GoldScore functions. Then, the resulting docking poses were rescored with five scoring functions: ChemScore, GoldScore, Astex Statistical Potential (ASP), Piecewise Linear Potential (ChemPLP), and XScore [60]. In summary, 20 final models were obtained and their prediction capabilities were investigated. The best one was based on ChemScore, and it was able to predict 76% of P-gp inhibitors and 73% of non-inhibitors. Additionally, the authors combined the results obtained from ChemScore-based docking with the log P values of the compounds, which relates to the ability of the compounds to cross the membranes by diffusion. It caused a slight improvement in the prediction of true inhibitors, with values in the confusion matrix of 0.81 for sensitivity (i.e., 81% of the inhibitors predicted), at expenses of a decrease in the detection of non-inhibitors (0.69 of specificity) [60]. Note that this approach is philosophically consistent with the enhanced sensitivity of the CETA assay [35] to identify lipophilic weak substrates of P-gp by removing the diffusion component from the permeability assay.

It is worth mentioning that docking protocols are unable to provide a complete explanation about the ligand interactions during efflux cycle in all the models mentioned before. More than a few doubts remained unsolved about the binding sites' specific locations for substrates and/or inhibitors. Moreover, during the course of the efflux cycle, P-gp undergoes large-scale conformational changes to pump the drugs out of the cellular membranes, and the mechanisms of these conformational transitions are still unclear. In that direction, numerous attempts have been performed by means of molecular dynamic simulations to better understand the mechanisms of drug uptake and binding in a flexible protein like P-gp [81–89]. The studies confirm the significant conformational change that facilitates the extrusion of the molecules and the importance of the TM12 and TM6 regions for the flexibility of the macrostructure.

2.3 Structure-Based Design of New Anticonvulsants to Treat Refractory Epilepsy

The early recognition of potent anticonvulsant candidates with no interaction with P-gp represents a useful strategy to design anticonvulsant drugs. On the other hand, the virtual identification of compounds with strong interactions with P-gp could be implemented for developing inhibitors for adjuvant therapies.

This last strategy has serious limitations since it implies to cancel the physiological role of the glycoprotein (a first line of defense in several barriers), and it involves the development of very selective and specific inhibitors to minimize side effects.

To the extent to our knowledge, the docking-based protocols mentioned before in this chapter have not been employed to find new anticonvulsants that avoid interactions with P-gp. On the contrary, we and other authors have run a virtual screening campaign to identify anticonvulsants for the treatment of P-gp-mediated drug-resistant epilepsy [77]. Initially we applied four ligand-based models on the ZINC 5 [90] and DrugBank [91–93] databases to propose new possible non-substrates of P-gp with anticonvulsant properties. Briefly, a topological discriminant function was employed to identify active compounds against the maximal electroshock-induced seizure (MES) test, and a three-model ensemble of 2D classifiers was used to differentiate P-gp substrates from non-substrates.

From the ligand-based screening, 380 candidates were selected for a second round of analysis by docking simulations. We first analyze the capacity of different docking software and conditions to discriminate known binders from non-binders of the test set, which was constructed after a thorough analysis of the bibliographic data. Special attention was given to the selection of the compounds of the test set, since there is some controversy in the literature regarding the classification of substrates/inhibitors and non-substrates/non-inhibitors. For example, Polli et al. classifies verapamil as non-substrate according to the monolayer efflux experiment in MDCK cells [94], whereas Feng et al. consider it as substrate in the same assay [95]. Doan et al. [96] reports fluoxetin as a non-substrate (monolayer efflux experiment in MDCKII-MDR1 cells), but in the assay of calcein-AM (CAM) inhibition (same cellular line), it behaves as an inhibitor [97]. In order to construct a representative and diverse set, the biological results from multiple publications were considered and priority was given to in vitro assays over in vivo tests [65]. After a thorough analysis, we included in the test set those substrates/inhibitors that were detected in two or more publications in different assays (if possible more than three). Conversely, we considered non-binders those compounds found as non-substrates preferably in two or more different assays and not reported as substrate/inhibitors (or reported in only one test). The compounds reported with some controversy in the results (e.g., one test where the compound was considered substrate/inhibitor and another where it behave as non-substrate) were discarded.

We benchmarked Glide, Autodock, and Autodock Vina software [65]. The compounds were docked into a homology model of human P-gp based on the mouse P-gp structure as template (PDB code: 3G61). The target was considered either rigid or flexible. Two different sets of amino acids were allowed to move in the

flexible simulations. In one model (model A) we allowed the movement of the amino acids that interact with the experimental ligands in the mouse complexes (Phe-335, Phe-343, Phe-728, Phe-732; PDB codes 3G60 and 3G61). For model B, we examined the conformation of the flexible residues in model A after the docking simulations. We found that Phe-343 and Phe-978 showed different conformations depending on the ligand, whereas Phe335, Phe732, and Phe728 adopted practically the same conformation in all the tested compounds. Therefore we choose another set of flexible residues that includes Phe-343, Phe-978, and other amino acids (Tyr-307, Tyr-953) that interact with the compounds of the test set according to the docking results with model A. The results were analyzed by means of the receiver-operating characteristic (ROC) curves for each docking system [98] to decide the most favorable docking conditions and to choose the limiting docking score (best threshold value) that can be used to discern between binders and non-binders classes.

The ROC curves graph the relation between the false positive rate (on the X axis) and the true positive rate (on the Y axis) for all possible threshold levels (see as example Fig. 3). These two values are defined as follows:

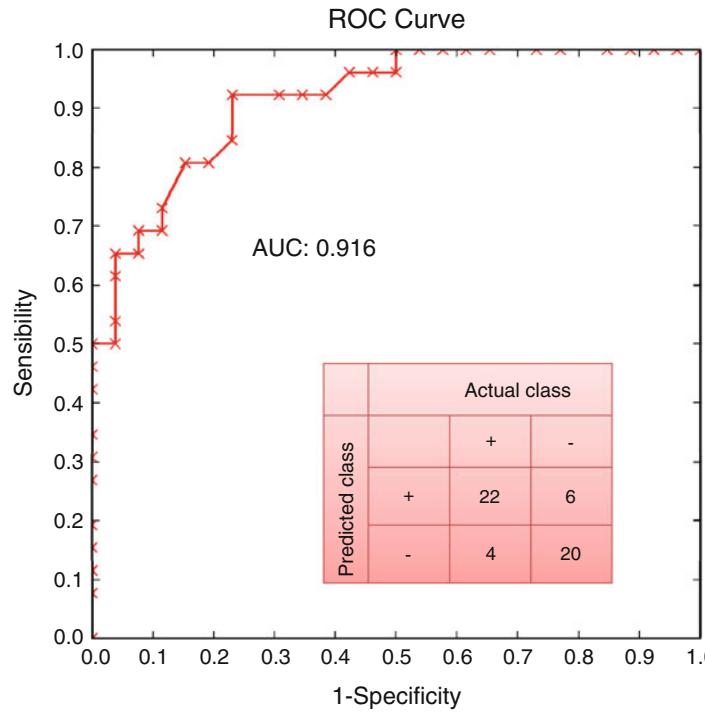

Fig. 3 ROC-type curves obtained for the best simulation. The confusion matrix with threshold −7.4 shows the number of well-categorized binders (22 of 26) and non-binders (20 of 26)

False positive rate = FP/(TN + FP)

True positive rate = TP/(TP + FN)

where TP means true positives, FN false negatives, FP false positives, and TN true negatives. The true positive rate is also defined as sensitivity and the false positive rate is also named as 1-specificity, since specificity is the rate of true negatives: TN/(TN + FP).

The ROC plots provide an objective picture of the methods' performance through the area under the curve (AUC) [98]. A random classification would give an AUC of 0.5, whereas a perfect classification would give an AUC of 1. Therefore, the AUC of the "real" models ranges between these two values. The analyses of the curves are useful to compare different methods and to have an overview of their capacity to discriminate known active from known inactive compounds.

As mentioned before, to construct the ROC curve we need to determine the selectivity and specificity values. Continuous data (as docking scores) requires a threshold value that divides the predicted active and inactive compounds. Depending on this cutoff, the selectivity and specificity will vary, and each pair of values represents a point in the curve.

On the other hand, for virtual screening purposes, we need to select the best threshold value that allows deciding which new compounds are good candidates to experimental analysis. If this cutoff is selected so that the sensitivity of the model increases (high rate of true positives), the specificity will decrease and we will expect a high rate of false positive compounds. On the contrary, if specificity is favored over sensitivity, more true positives will be classified as inactive.

There are several criterions to balance both sensitivity and specificity, and the threshold values are selected according the given system. For example, the highest G-mean value (square root of the product between selectivity and specificity) could be used to select the best cutoff for virtual screening. Another practical criterion is based on the accessibility of the experimental assays. As the resources involved in the experimental test are limited, it would be useful to minimize the evaluation of false positive candidates. This criterion prioritizes specificity over sensitivity, at expenses of losing active compounds.

In our investigation the best ROC curve was achieved by means of Autodock Vina with flexible receptor (model B) and ligands at physiological pH. The resulting simulation is shown in Fig. 3, with an area under the ROC curve of 0.916 (best threshold of docking score: −7.4). This protocol is able to predict the 85 % of the binders (sensitivity value of 0.85) and the 77 % of non-binders (specificity value of 0.77) with a global accuracy value of 0.81 [65].

Finally we performed the docking simulation to the 380 compounds selected by the ligand-based virtual screening. According to the ROC curves, we considered as P-gp binders the structures that showed a docking score lower than −7.4 kcal/mol. With this

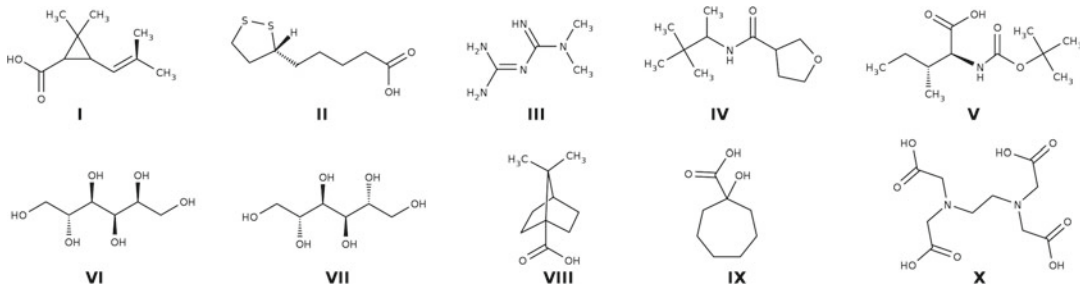

Fig. 4 Compounds selected by sequential virtual screening that showed anticonvulsant activity against MES test. I, chrisantemic acid; II, 7,7-dimethyl-1-norbornane carboxylic acid; III, thioctic acid; IV, metformin; V, mannitol; VI, sorbitol; VII, *N*-(*tert*-butoxycarbonyl)-l-isoleucine; VIII, 1-hydroxycycloheptanecarboxylic acid; IX, *N*-(3,3-dimethylbutane-2-yl)-2-methylfuran-3-carboxamide; X, EDTA [65]

threshold, 275 structures passed the docking filter as non-binders, evidencing a high level of consensus between the ligand-based and target-based protocols (more than 72 % of the initial structures were recovered by docking). From them, ten diverse molecules were selected for acquisition and subsequent pharmacological evaluation (Fig. 4). All showed anticonvulsant properties in animal models of acute seizures (MES test), proving the accuracy of our predictions in relation to the protective effects of the candidates [77].

3 Conclusions

P-glycoprotein is probably one of the most studied antitargets (together with hERG channel and CYP3A4 enzyme) due to its participation in drug resistance and drug interactions.

Particularly, the P-gp awareness for the design of new anticonvulsants represents an interesting strategy to treat P-gp-mediated refractory epilepsy. In this context, target-based methods offer a virtual alternative to ligand-based protocols, since they might discern possible binders from non-binders using, for example, the values of the docking scores.

Docking on P-gp deals with the intrinsic limitations of the software (characterized by specific-search algorithms and force fields) to correctly quantify the binding energies of the complexes. Additionally, human P-gp is a very flexible membrane protein and its experimental structure is not currently available. Therefore, the simulations have to be run on experimental structures of mouse P-gp (which show low resolution in the position of the amino acid side chains) or by comparative models of the human target. Despite these difficulties, the docking protocols mentioned in this chapter showed, in general, an acceptable performance. Additionally they were able to reproduce the binding modes of known binders and could provide atomic details on the new protein–ligand complexes.

The final step in the virtual screening campaigns is to test the candidates in adequate experimental models. The implementation of models of drug-resistant epilepsy, taking into consideration overexpression of P-gp, would optimize the process of selection of compounds. They could serve to identify new chemical entities that control resistant patients and to better understand the mechanisms of drug resistance.

In vitro cell assays include for example the use of isolated brain capillaries, primary or immortalized brain endothelial cells, and immortalized cells from peripheral tissues (some of them mentioned before in this chapter) [99, 100]. On the other hand, in vivo models of drug-resistant seizures associated with overexpression of P-gp, such as the model of seizure induced by 3-mercaptopropionic acid, are available [100].

We believe that the sequential screening of large databases with ligand-based filters coupled by target-based protocols represents an opportunity for the discovery of new anticonvulsants that evade P-gp interactions in a timely and cost-efficient manner. Moreover, the prediction of the P-gp-binding modes might serve to optimize the new leaders without losing their anticonvulsant action. These investigations, in conjunction with suitable biological models, could afford new effective drugs in patients with P-gp-mediated refractory epilepsy.

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Chapter 16

Nanoformulations of Antiepileptic Drugs: In Vitro and In Vivo Studies

María E. Ruiz and Guillermo R. Castro

Abstract

Epilepsy is a chronic neurological disorder that represents a unique therapeutic challenge displaying high population incidence with more than 60 million people worldwide. Nearly 70 % of people are provisionally responding to the treatment, but 20–60 % of patients become resistant to current antiepileptic drugs (AEDs) (WHO 2015, Epilepsy Fact Sheet No. 999). Also, there is a negative social impact of the pathology since patients and their families suffer stigma and discrimination in many parts of the world. Furthermore, patients who positively respond to the anticonvulsant treatment are subjected to high systemic concentrations of drugs to achieve therapeutically effective levels at the site of action in the central nervous system (CNS), which results in undesirable side effects that threaten their quality of life and their adherence to the treatment.

A complete assessment of the described situation is far beyond the possibilities of the present book chapter, and the sanitary problem of epilepsy (as well as other CNS diseases treated with anticonvulsant drugs) has already been described in the previous chapters. Hence, we will focus in the pharmacokinetic aspects of the problem of epilepsy treatment, including the low brain bioavailability of AEDs due to the restrictions imposed by the blood–brain barrier (BBB) and the high efflux rate of drugs from the CNS caused by the overexpression of ABC transporters at the BBB. In any case, the development of nanoformulations of AEDs seems like a promising strategy to improve their pharmacokinetic profile by increasing the fraction of drug that reaches (and stays in) the CNS, as well as by optimizing the drug's distribution/metabolism and elimination profile.

Despite the abundance of recent works in the field of pharmaceutical nanoformulations, there is not much to be found in the particular case of epilepsy, especially if one looks for nanosystems with proven in vivo effectiveness. Therefore, this chapter begins with a short overview of the possibilities offered by the pharmaceutical nanotechnology to improve the antiepileptic therapy to continue with a detailed analysis of the methods and the results of the in vitro and in vivo evaluation of nanoformulations of AEDs reported so far.

Key words Antiepileptic drugs, Bioavailability, Biopolymers, Blood–brain barrier, Central nervous system, Drug delivery systems, Epilepsy, Intranasal route of administration, Nanoformulations, Nanoparticles, Nanosystems, Nanotechnology, Solid lipid nanoparticles

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1 Introduction

The currently available pharmacological treatment for epilepsy is symptomatic: seizures are inhibited by the periodic administration of one or more antiepileptic drugs (AEDs). However, it is not possible to achieve the control of convulsions in all the patients, mainly due to the lack of an adequate therapeutic response (refractory patients) or because of the high attrition rates observed in anticonvulsant therapy, where long-term therapies are needed with drugs that cause several unwanted side effects [1, 2]. Conventional dosage forms are characterized by the lack of specificity in its distribution: to achieve therapeutic levels at the site of action, other tissues are unnecessarily subjected to high levels of the drug.

From a therapeutic point of view, the epilepsy treatment implies, similar to all central nervous system (CNS) disorders, additional challenges due to the presence of the blood–brain barrier (BBB) that limits the access of drugs to their molecular target in the brain [3]. The BBB includes not only particularly tight junctions that limit the passage of drugs through the paracellular pathway but also high expression levels of polyspecific efflux pumps of the ABC superfamily (e.g., P-gp, BCRP, MRPs, among others), which in turn restrict the transfer of their substrates through the transcellular pathway [4].

One of the classic strategies to address these difficulties in order to improve the anticonvulsant therapy has been the search for new drugs (or prodrugs) with more potency and/or higher bioavailability (BA) [5]. However, although the number of new AEDs has significantly increased over the last 20 years, this has not been followed by a decrease in the rate of treatment failure: about one third of patients remain resistant to drug therapy [6], and the results of clinical studies available so far are not conclusive in suggesting a therapeutic improvement or a better tolerability of the new AEDs [7]. Moreover, the excessive cost of these new anticonvulsant drugs makes them inaccessible for many of the epileptic patients, since about 80% of the population affected by epilepsy live in low- and middle-income countries [8].

Considering the role of the efflux transporters at the BBB, blocking their function, downregulating their activity, or inhibiting the upregulation associated with the disease seem at first sight as a logical strategy to enhance the localization of the therapeutic agents in the brain [9, 10]. However, the associated risks are high: long-term decline of the function of ABC transporters has been associated with xenobiotic toxicity and enhanced predisposition to neurodegenerative diseases such as Parkinson's and Alzheimer's [11, 12].

Therefore, other methods have been proposed to bypass the BBB and deliver drugs directly into the CNS, like the reversible disruption of the BBB by the infusion of hyperosmotic solutions or

the administration of vasoactive compounds to temporarily open the tight junctions. Moreover, invasive methods like the intracerebral (or intraparenchymal) administration of solutions or implants and the convection-enhanced delivery (CED) of drugs have also been proposed [3]. Many are the drawbacks of these approaches, specially the elevated risks of brain damage, infection and/or toxicity, and a possible lack of control of the release kinetics.

There is, however, a less invasive alternative approach to bypass the BBB: direct nose-to-brain delivery of drugs. The intranasal administration of drugs represents a promising route for CNS drug delivery; its characteristics are discussed in Sect. 4.

For all the aforementioned issues, it is imperative to develop novel strategies to achieve a proper control of epileptic seizures in all the patients through innovative treatments and novel technologies. In this regard, pharmaceutical nanotechnology will most probably play a key role to substantially improve the treatment of epilepsy, as it has been the case for other diseases like cancer [13].

The encapsulation of anticonvulsant drugs using nanoscale systems may provide, among other advantages, an optimized drug distribution profile, with controlled and higher levels at the target site and lower efflux of the drugs from the CNS by the ABC transporters present at the BBB [14]. A formulation with such characteristics would have seemed idealistic a few years ago. However, today advances in nanotechnology and nanobiotechnology are allowing us to approach and develop novel vehicles and strategies showing very interesting prospects for the treatment of CNS diseases [15].

2 Nanoformulations in Epilepsy Treatment

As discussed so far, it is clear that many are the difficulties that a drug must overcome to achieve their therapeutic effect at the CNS. In addition to the undesirable side effects and the resistance mechanisms by nonspecific efflux transporters already mentioned in the previous section, there are other factors that threaten or endanger the success of the therapy, such as metabolic reactions that can cause the drug inactivation and/or the production of active metabolites with unfavorable pharmacokinetic properties, as well as the low aqueous solubility exhibited by many of the already known active ingredients or by the ones currently in the development pipeline. In an excellent review published in 2013, Vlieghe and Khrestchatisky affirmed that most small molecules and virtually all macromolecules do not cross the BBB by passive diffusion due to their hydrophilicity and/or their high molecular weight [3]. Therefore, by encapsulating a drug into a nanosystem, it is expected to attain a global improvement of both the release and the biodistribution profiles of the drug.

A drug incorporated into a nanosystem of about 100–200 nm size could be hidden from the body clearance mechanisms [16], which in turn will result in more drug reaching the target site. In other words, the nanoformulation is expected to efficiently cross the blood capillary endothelial cells at the BBB producing greater drug concentrations at the brain parenchyma [17].

In general, a drug encapsulated or entrapped into a nanoparticle (NP) could enter the CNS by adsorptive-mediated transport (AMT) or alternatively by receptor-mediated transport (RMT) if the NP possesses a suitable ligand to a BBB receptor. In any case, the efflux transporters at the BBB are avoided, which is especially relevant since it has been demonstrated that many currently used AEDs are substrates of those carriers, including phenobarbital (PBT), phenytoin (PHT), lamotrigine (LTG), and oxcarbazepine (OxCBZ), among others [18].

Besides a diminished efflux from the CNS, nanosystems are expected to provide additional advantages over the classic dosage forms. As mentioned before, epilepsy treatment with AEDs is characterized by several undesirable side effects that this group of drugs produces to the patients, partly due to its high systemic levels and unspecific distribution [19]. Moreover, cell/organ toxicity could be increased by the metabolites produced from the biotransformation of the drug under physiological conditions.

Generally, AEDs are well absorbed (with oral BA ranging from 70% to higher percentages), highly bound to plasma proteins, and also highly metabolized by the hepatic enzymes that convert the AEDs into one or more metabolites some of which also possess antiepileptic activity [19]. Carbamazepine (CBZ), for example, is not a P-gp substrate but is metabolized (in a percentage variable between 10 and 80%) to its 10,11-epoxi-CBZ (E-CBZ) derivate, a compound of similar antiepileptic activity to that of the parent drug but with much lower plasmatic half-life (near 6 ± 1 h, meanwhile the CBZ half-life is around 26 ± 5 h [19]) and susceptible to P-gp-mediated transport [20]. In addition, due to the elevated plasma protein binding ability of almost all AEDs (near 50% for PBT and CBZ-E, 75% for CBZ and even 90% for PHT), there is a small fraction of drug circulating in its free form, and it is precisely this fraction the one that could enter the CNS through passive diffusion [21].

Finally, and compared with other strategies to address the problem (like the search for new drugs or prodrugs), the development of nanoformulations of AEDs could provide the additional advantage of rapidly reaching clinical development, since they can involve already approved drugs. The screening and development of novel drugs usually starts from about 10,000 or more prospective molecules to finally get a couple good candidates, a process that requires between 12 and 15 years to reach the market and a huge amount of money [22].

Regarding the inactive substances of the formulation, namely, excipients, their choice depends mainly on the type of nanosystem intended to be developed. The classification and the advantages and drawbacks of each class of nanosystem have been extensively discussed in other texts (see, *e.g.*, [23, 24] and references therein). Despite that virtually all kinds of nanosystems could be used for the delivery of AEDs, the preference for the use of excipients already approved by the FDA for their use in drug products [25] makes some of them more suitable than others, like lipid-based over polymeric-based nanosystems.

The use of this kind of excipients also avoids safety issues if the formulation does not exceed the maximum limits established for each component (which could also depend on the administration route). Other characteristics of the nanosystems, as the drug-loading capacity, physical and chemical stability, cost of the production method, sterilization possibilities, and scale-up properties, must also be taken into account.

3 Proposed Nanosystems for the Administration of AEDs

This section is dedicated to the review and discussion, with special emphasis on the in vitro/in vivo evaluations performed, of some of the articles published so far on the field of antiepileptic therapy that meet the following inclusion criteria: (1) related to formulations of drugs with proven anticonvulsant effect, (2) administered as nano (but not micro)-sized carriers, and (3) published in the last 5 years (with the exception of three articles prior to 2010, included to illustrate some key concepts of the section). The articles selected, as well as their main features, are listed in Table 1.

It is worth highlighting that all the results presented in this section were reported at different preclinical stages. There is so far no approved nanoformulation for epilepsy treatment.

Though many other interesting works can be found on the topic, we limited our analysis to the ones presented in Table 1 to illustrate the potential of pharmaceutical nanocarriers applied to the particular case of epilepsy.

3.1 In Vivo Pharmacokinetic/Pharmacodynamic (PK/PD) Studies of Nanoformulations of AEDs

Back in 2000, Friese et al. assayed polymeric nanoparticles for the delivery of a novel noncompetitive NMDA receptor antagonist (MRZ 2/576) [26]. When administered as a solution, the free drug demonstrated to be very potent in vivo (maximal electroshock seizure—MES test ED50 7.7 mg/kg), but with a very short duration of action, the maximum effect was reached around 5 min after intravenous (IV) administration due to its extrusion from the CNS by the carboxylic acid transporters. Therefore, the authors tested the effect of a pretreatment with probenecid (PBC, 200 mg/kg intraperitoneal (IP) injection as an aqueous suspension 30 min

Table 1
Summary of the nanosystems discussed in this section

Drug	Type of nanosystem	Studies included				Authors and reference	Year
		In vitro drug release	In vitro permeability	In vivo (PK)	In vivo (PD)		
MRZ 2/576	PBCA NP	–	–	–	MES	Friese et al. [26]	2000
Valproic acid (VA)	PBCA NP coated with PS 80	–	–	Yes	–	Darius et al. [27]	2000
Diazepam (DZ)	SLN	Yes	–	–	–	Abdelbary and Fahmy [51]	2009
Valproic acid (VA)	NLC	Yes	–	–	–	Varshosaz et al. [76]	2010
Valproic acid (VA)	Chitosan NP	Yes	–	–	–	Hamidi et al. [77]	2011
Ethosuximide (ETS)	Chitosan NC	Yes	–	–	Yes[a]	Hsiao et al. [35]	2012
Carbamazepine (CBZ)	Chitosan SLN	Yes	–	–	MES	Nair et al. [36]	2012
Clonazepam (CZ)	SLN	–	BBB model (hCMEC/D3 cells)	–	PTZ	Leyva-Gómez et al. [37]	2014
Lamotrigine (LTG)	Pluronic 123 micelles	Yes	–	Yes	–	Liu et al. [30]	2014
Gabapentin (GPT)	Albumin NP coated with PS 80	Yes	–	Yes	MES and PTZ	Wilson et al. [33]	2014
Phenytoin (PHT)	Angiopep-2-conjelectro-responsive hydrogel NP	Yes	BBB model (bEnd3 cells)	Yes	AK	Ying et al. [34]	2014
Oxcarbazepine (OxCBZ)	PLGA-NP	Yes	BBB (hCMEC/D3) and human placenta (BeWo cells) models	–	–	Lopalco et al. [78]	2015

[a]The anticonvulsant effect was evaluated in epileptic rats
PK pharmacokinetic, *PD* pharmacodynamics, *PBCA* poly(butylcyanoacrylate), *NP* nanoparticles, *PS* polysorbate, *NLC* nanostructured lipid carriers, *SLN* solid lipid nanoparticles, *PLGA* poly(lactide-coglycolide), *BBB* blood brain barrier, *VA* valproic acid, *DZ* diazepam, *ETS* etosuximide, *CBZ* carbamazepine, *LTG* lamotrigine, *GPT* gabapentine, *PHT* phenytoin, *OxCBZ* oxcarbazepine, *MES* maximal electroshock seizure test, *PTZ* pentylenetetrazol test, *AK* amygdala kindling test

before the drug administration), an uricosuric agent that competitively inhibits the aforementioned transporters, and a significant prolongation of the drug action time (up to 150 min) was observed possibly due to the simultaneous effect of PBC at the BBB and the kidneys. PBC however has its own pharmacological activity and hence a combined administration of MRZ and PBC could not be considered as a therapeutically viable option.

Consequently, the authors tested the IV administration of the drug in poly(butylcyanoacrylate) NP (140–335 nm) finding that the NP were able to prolong the MRZ anticonvulsant activity up to 60 min (or even 210 min, if the NP were coated with polysorbate 80) when mice were subjected to the MES test (100 Hz, 20 mA, 0.5 s shock duration applied by corneal electrodes).

Despite the lack of important complementary studies of the nanosystem, such as in vitro drug release, toxicity, and/or biodistribution studies, the work of Friese et al. is still a good example of how nanotechnology can help to overcome pharmacokinetic problems of promising drug candidates that would be otherwise dismissed.

In the same year, the effect over the PK profile of valproic acid (VA) administered in a NP formulation was reported by Darius et al. [27]. The preparation technique included the polymerization of 1% butylcyanoacrylate in the presence of dextran 70,000 or Tween 85 as stabilizers, followed by the lyophilization of the produced NPs, resuspension in buffer PBS, and adsorption of VA (30 mg/ml) onto the NP surface. A third formulation was also prepared by coating the dextran 70,000 NPs with polysorbate 80 (PS 80).

The three formulations and the control (VA solution of the same concentration) were IV administered (0.1 ml/10 g of body weight, *i.e.*, 300 mg/kg of VA) to four groups of mice (three mice per time point), and VA was quantified in plasma and brain by gas chromatography at 5, 20, and 60 min post-dose. Interestingly, none of the three formulations displayed differences compared to the control group in terms of plasma and brain exposures. The work also included the quantification of several VA metabolites, some of which (including a neurotoxic one) showed significantly lower serum levels when administered as NP.

From the presented results, it seems that the nanoformulation did not modify the influx and efflux of VA from the CNS (processes known for being asymmetric [28, 29]). Unfortunately, the in vitro drug release profile and the encapsulation efficiency were not reported, which would have allowed having a better description of the system, especially interesting in this case in which the drug is adsorbed onto, and not encapsulated into, the NP.

When preclinical studies are carried out with a drug adsorbed or encapsulated into a nanosystem, it is essential to also perform a complete in vitro characterization of the system by means of the determination of the total drug content, the percentage encapsulated, the stability of the system over the time (under the proposed storage

conditions), and the in vitro release profile of the drug, both at time zero and after the estimated period of use. This information allows a better interpretation of the results of the in vivo studies, usually performed with the suspension resulting from the NP synthesis (without a prior separation step) and not necessarily immediately after it.

Nevertheless, the most relevant conclusion of the Darius et al. work is the possibility of the modification of a drug's metabolic clearance by a nanocarrier. As in the case of Friese et al., this is another nanotechnology-based strategy to develop an optimization of the PK profile of a drug.

On the contrary, Liu et al. found significant differences in brain exposure when the same dose of LTG (10 mg/kg) was IV administered as a solution or incorporated into pluronic 123 (P123) polymeric micelles with an average size of 20 nm [30]. Despite that the authors attributed these results to the P123 ability of modulating the P-gp activity at the BBB, they did not find differences in brain exposure of LTG-P123 micelles when the animals were pretreated with the P-gp inhibitor verapamil.

To elucidate the mechanisms underlying the improvement in brain BD, an in vitro bidirectional permeability study could be performed (e.g., in Caco 2 or MDCK cells transfected with P-gp) to comparatively assess the permeability of the free drug, the encapsulated drug, and the drug plus the inactive components of the NP formulation (excipients). Such experiment, performed both with and without specific inhibitors of the transporter, would help to prove the authors' hypothesis about a potential effect of P123 over P-gp.

Furthermore, due to the specialized transport mechanisms that exist in each epithelium or endothelium, it is important to also assess, if possible, the permeability in adequate in vitro specific cellular systems (*e.g.*, brain microvascular endothelial cells).

The developed micelles are promising in terms of the high BA achieved by the nanoformulation, and the results allow to speculate about the presence of several mechanisms involved in that outcome, like the effect of the nanosystem over other efflux transporters, since LTG is a P-gp substrate [18] and also a BCRP substrate [31], a transporter not inhibited by verapamil [32].

As in the case of Liu et al., the work of Wilson et al. also achieved a better biodistribution for the AED gabapentin (GPT) administered in a nanosystem (compared to a solution of the drug), but in this case the authors also assessed the anticonvulsant activity of the formulation in vivo to confirm that the better PK properties actually translate into better PD properties [33].

In the aforementioned work, GPT-loaded albumin NPs were prepared by pH-coacervation method, freeze-dried, and then coated with PS 80 showing an average size of 140 nm. The drug released from the NPs in the brain was almost twofold higher than the control made by GPT solution. Meanwhile, the coated NPs achieved brain levels more than threefold higher compared with

the control. In addition, when the NPs were tested in vivo, the coated formulation significantly reduced the duration of all phases of convulsion compared to the control, both in MES and PTZ test.

The last of the selected studies about drug biodistribution is the one of Ying et al., who reported PHT-loaded electro-responsive hydrogel NP (ERHNP) brain targeted by means of its conjugation with angiopep-2 (ANG-ERHNP), with an average size of 140 nm and a %EE near 80% [34]. The main results of this very comprehensive work could be summarized in the following points: (1) conjugation with the brain-targeting peptide significantly increased the cell permeability of ANG-ERHNP in bEnd3 cells (derived from brain microvascular endothelial mouse cells as an in vitro BBB model); (2) ANG-PHT-ERHNP exerted the same protection as the control PHT solution in the in vivo amygdala-kindling (AK) epilepsy model; for a given dose (50 mg/kg), the free drug only displayed protective effects for 2 h, while the nanoformulation still showed protection (measured as reached seizure stage and after discharge duration) 8 h post-dose; and (3) the maximum drug concentration in the brain was reached 0.5–1 h post-dose but remained high (relative to the free drug) during the following 4 h of the study, which is consistent with the PD data.

Finally, Hsiao et al. [35], Nair et al. [36], and Leyva-Gómez et al. [37] described nanosystems for the delivery of ethosuximide (ETS), CBZ, and clonazepam (CZ), respectively, the first two with the common feature of using chitosan-based systems. Chitosan is a highly bioadhesive and biodegradable cationic polysaccharide, suitable for the synthesis of biocompatible and nontoxic drug delivery systems.

Hsiao et al. obtained chitosan nanocapsules of ETS with thermo-gelling properties that could be potentially used as an injectable depot for drug delivery. The in vivo therapeutic effect was evaluated by the subcutaneous (SC) injection of 1 ml of the thermo-gelling solution (that forms, at body temperature, a SC gel implant with 10 mg/ml of ETS) into five Long–Evans rats with spontaneous spike-wave discharges (SWDs), suggested to be an absence-like seizure model that is known to be suppressed by ETS [38]). The treatment reduced the number of recorded SWDs to about 50% on days 1–3 after injection, and also the amplitude of the SWDs that occurred during the other days of the study were monitored by electrodes surgically implanted over the frontal cortex of the rats. From day 4 the number of SWDs increased, which coincided with a gel volume reduction greater than 50%. The work did not include a control group treated with a solution of ETS, what could have revealed the typical differences between immediate and prolonged drug release kinetics.

In 2012, Nair et al. reported a new chitosan–solid lipid nanoparticle (SLN) system for the administration of CBZ [36]. Orally administered chitosan–SLN–CBZ formulation exerted

shorter durations of all epileptic phases in MES test in vivo with respect to the control group (CBZ solution) and significantly delayed the onset of convulsions in the isoniazid induced convulsion model. From the reported results, however, it is not possible to establish neither the dose actually administered to the groups of mice nor the mechanisms underlying the improved activity. As stated previously in this section, unfortunately the lack of a complete in vitro characterization of a nanosystem limits the conclusions of in vivo experiments.

Probably, the final effect is the resultant of various combined effects, like an improved oral BA, as in the case of Leyva-Gómez (below), and improved metabolic/distribution profiles of the drug. CBZ is not a P-gp substrate [18], but, as mentioned before, near 10–80% of the dose is metabolized to its 10,11-epoxide derivative, a compound with activity similar to CBZ [39] that presents a shorter plasma half-life and is a P-gp substrate [20]. Therefore, the overall effect would be a higher anticonvulsant effect due to a higher brain exposure to the drug if the nanoformulation is capable of reducing the CBZ metabolism as in the previously reviewed work by Darius et al. [26].

Leyva-Gómez et al. synthesized and studied the in vitro permeability and in vivo activity of SLN containing the drug CZ [37]. Permeability studies were performed in hCMEC/D3 cells (human brain endothelial cell line) and showed a significantly higher permeability of the drug encapsulated in SLNs compared to the drug alone, even though CZ permeability is already high across the in vitro BBB. The anticonvulsant effect was assessed by the pentylenetetrazol (PTZ) seizure test, and the authors found that when administered orally to mice, the SLN-CZ significantly delayed the onset of the first convulsive event (respect to the control group that received a CZ solution); the same effect was not observed when the administration was IP. These findings are in line with the results reported by Nair et al. about the possibility of an improved oral BA due to some components of the SLN [36], a hypothesis that could be tested by studying the in vitro permeability of the loaded SLN, in comparison with the free drug and a solution of free drug plus SLN constituents, in adequate models of intestinal permeability (*e.g.*, Caco 2 cells).

Regardless of the positive results, benzodiazepines are not suitable for chronic therapies due to tolerance development. They are, however, a first choice for acute seizures, a situation in which the intranasal administration could be a more suitable drug delivery route, as we will see in Sect. 4.

As a last remark on the present section, it would be of great importance to also perform the in vivo preclinical studies (both PK and PD) in animal models of resistant epilepsy, in which biochemical alterations may be present, like the overexpression of the efflux transporters at the BBB [40].

3.2 In Vitro Drug Release Studies

Drug dissolution tests have near a century of development. However, in recent decades they have attracted more interest, especially due to their application to the study of solid drug dosage forms which somehow predicts the drug BA. To be absorbed, an active ingredient should be previously dissolved; to cross a biological membrane, a compound must be solubilized in the fluids in contact with the membrane [41]. Additionally, if the active ingredient is included in a dosage form, it must be released to be dissolved, a common feature of all the administration routes of drugs, both enteral and parenteral.

Therefore, the study of release and dissolution kinetics becomes the in vitro assay of choice for a first predictive assessment of a drug's in vivo performance, and even in some cases it is enough to subrogate the drug of in vivo BA or bioequivalence (BE) studies (biowaiver) [42]. The biorelevance of the test, i.e., its ability to adequately predict what will happen in vivo, is thus essential: the test should be able to detect those differences between formulations which would lead to a different in vivo behavior and at the same time should not be over-discriminating to differences that would be irrelevant at a physiological level.

The aforementioned highlights a key aspect of these in vitro studies: their comparative nature, either against a reference formulation (as in the regulatory case) or between different alternative formulations, as well as between a formulation and the pure drug, comparisons that can only be done if certain experimental parameters are standardized. While strict protocols are followed to perform an in vitro dissolution test during a pharmacopeial quality control [43], several variables should be modified to assess the release performance of a drug in a nanoformulation, but some general guidelines should also be followed to yield comparatively useful results.

The use of **biorelevant media**. In its simplest form, these are buffer solutions of a pH coincident with some portion of the gastrointestinal (GI) tract, *i.e.*, ranging from 1.2 to 6.8, as proposed by the WHO [44]. However, more complex simulated biological fluids have been proposed for oral dosage forms, which release the active ingredient to be absorbed in the GI tract: fasted-state (FaSSIF) and fed-state (FeSSIF) simulated intestinal fluid [45, 46], simulated gastric fluid also in the fasted or fed state (FaSSGF and FeSSGF, respectively), and colonic fluid in both states (FaSSCoF and FeSSCoF) [47]. However, a medium to simulate the dissolution conditions at the CNS has not been developed yet, which is natural if one bears in mind that conventional dosage forms are not able to reach the BBB to release their contents into the CNS, which is now possible with nanometric formulations.

It should be emphasized that, since nanoencapsulated drugs may cross biological barriers as such, dissolution testing should

always be complemented with permeability studies in appropriate models.

Working under **sink conditions**. The United States Pharmacopeia (USP) establishes that these conditions are met if the concentration of saturation of the drug in the medium is at least three times the working concentration [48]. In the case of poorly soluble drugs (class II and IV of the BCS), that condition may require volumes incompatible with the experimental possibilities (conventional dissolution equipment allows for a maximum of 1 l per vessel). To overcome these limitations, several alternatives are available (use of cosolvents, surfactants, biphasic systems, medium removal strategies, among others), but of them only the use of certain surfactants has been officially accepted [49].

Finally, another interesting aspect that tends to be underestimated is **the influence of hydrodynamics** during dissolution tests. The type and intensity of agitation, closely related to the type of dosage form and equipment used [50], is a major factor to ensure biorelevant results, since little or inefficiently agitated systems are likely to be over-discriminant, while the opposite happens if the agitation is excessive.

Taking into account these considerations, Table 2 summarizes the conditions used to study the in vitro release kinetic of the nanoformulations of AEDS described in the previous section of this chapter.

Despite not having any in vivo results, the work of Abdelbary and Fahmy was analyzed because it is one of the most biorelevant in vitro test reported [51]. The authors prepared SLN loaded with diazepam (DZ) by modified high-shear homogenization and ultrasound techniques and assessed the in vitro drug release by using a buffer solution of biorelevant pH as dissolution media and the USP apparatus 1 (basket), which allows for the system to be correctly agitated at 37 °C. On the other hand, and since the dose of DZ used for the test is declared, it is possible to affirm that the test was performed under *sink* conditions (the volume used is more than three times the solubility of DZ in the medium). This is especially relevant since a common failure found in the experimental design of many of the reviewed articles is the lack of specification of the amount of drug placed in the dissolution media.

Finally, despite that the agitation speed could seem low for the apparatus used (in general, rotation speeds of 100 rpm are used for in vitro dissolution tests in the USP apparatus 1 [42]), it should be kept in mind that these conditions are trying to simulate the flow of the nanosystem through the systemic circulation and not peristaltic movements.

If an oral route of administration is proposed, the nanosystem could release the drug in the GI tract or be absorbed as such. In the first case, a classical drug release profile under the accepted biorelevant conditions for orally administered dosage forms should

Table 2
Description of the experimental conditions used to evaluate the in vitro release performance of the nanosystems of AEDs reviewed in this section

Amount/dose	Experimental array	Medium, agitation, and temperature	Sampling time and quantification method	% Dissolved/time
DZ-SLN [51] An amount of SLN dispersion equivalent to 2 mg of DZ	Dispersion was placed in a glass cylinder fitted at its lower end with a cellulose membrane (MWCO: 12–14 kDa) and suspended in a USP dissolution apparatus 1 (basket) vessel	400 ml of phosphate buffer (pH 7.4) was added to each flask and allowed to rotate (25 rpm) at 37 °C	Sampling time: 8 h. Aliquots were withdrawn and replaced with fresh buffer and the drug content was determined spectrophotometrically	Near 90 % of DZ is dissolved in 8 h (for the two selected SLN formulations)
VA-NLC [76] 3 mL of aqueous dispersion	Dispersion was sealed into dialysis bags (MWCO: 12.4 kDa) with a constant length of 10 cm and placed in a glass test tube	100 mL of pH 6 PBS with 0.1 % Tween 80. 200 rpm magnetic stirrer agitation. Temperature is not disclosed	Sampling time: 21 days. Aliquots were withdrawn and replaced with fresh medium. Drug content was determined by HPLC	Less than 75 % is dissolved in 8 days (for the selected NLC formulation)
VA-hydrogel NP [77] The nano-dispersion was divided into 1 mL portions	Each portion was placed in 1.5 mL polypropylene micro tubes, placed in a vertically shaking incubator designed and assembled in-house	The samples were shaken gently (15 rpm) while being incubated in 37 °C	Sampling time: 7 days. At each time point, one of the aliquots was harvested and the total and free drug were quantified by HPLC	Almost 60 % of VA is released in the first 24 h, reaching 90 % in 7 days
ETS-chitosan NC [35] Portions of 2 ml of Ethosuximide nanogels (0.1 g/10 ml)	2 ml portions (prepared with the crosslinking agent genipin to prevent gel erosion during the test) were prepared in 15 ml falcon tubes	In each tube, 2 ml of pH 7.4 simulated body fluid (SBF) were added[a]. The test was carried out at room temperature. No agitation described	Sampling time: 20 days. Aliquots were withdrawn and replaced with fresh buffer and the drug content was determined spectrophotometrically	Near 75 % of ESM is dissolved in 21 days (more than 55 % in the first 6 h)
CBZ-SLN [36] 2 ml of the SLN dispersion	The dispersion was placed in the donor compartment of a Franz diffusion cell fitted with a dialysis membrane (MWCO: 3.5 kDa)	20 ml of PBS was placed in the receptor compartment, and the system was kept at 37 °C. No agitation described	Sampling time: 24 h Aliquots were withdrawn and the drug content was determined spectrophotometrically	One of the three SLN formulations reach the 65 % of CBZ dissolved in 24 h

(continued)

Table 2
(continued)

Amount/dose	Experimental array	Medium, agitation, and temperature	Sampling time and quantification method	% Dissolved/time
LTG-polymeric micelles [30] 1 ml of LTG-incorporated micellar solution	The solution was introduced into a pre-swollen dialysis bag (MWCO: 3.5 kDa)	The bag was immersed into 50 mL of PBS (pH = 7.4) with 0.1% Tween 80, and kept at 37 °C, with stirring at 100 rpm	Sampling time: 24 h Aliquots were withdrawn and replaced with fresh medium. The drug content was determined spectrophotometrically	75% of LTG is dissolved in 6 h and remained constant until the last sampling time
GPT-albumin NP [33] NP equivalent to 5 mg of Gabapentin	NPs were suspended in 2 ml of pH 7.4 phosphate buffer and the suspension was placed in a dialysis bag (MWCO: 5 kDa)	The end-sealed bag was dialyzed against 50 ml of pH 7.4 phosphate buffer, at 37 °C, with stirring at 100 rpm	Sampling time: 24 h. Aliquots were withdrawn and replaced with fresh medium. The drug content was determined spectrophotometrically	Near the 45% of the drug is released in 24 h
PHT-electro-responsive NP [34] 2 mL of the NP equivalent to 1 mg of PHT were used[b]	The solution was sealed into a dialysis bag (MWCO: 8–14 kDa). Additionally, two parallel Pt electrodes were inset in the bag and DC voltage was applied to the solution with a current of 100 or 200 μA for 1 min	The bag was immersed into 50 mL of PBS (pH = 7.4) buffer and kept at 37 °C in a thermal bath with continuous stirring	Sampling time: 24 h Aliquots were withdrawn and the drug content was determined by HPLC	At 4 h, the release increased from 35 to 60–90% when currents of 100–200 μA were applied, respectively
OxCBZ-polymeric NP [78] NP suspensions were diluted 1:20 (v/v) in the medium	Volumes used are not disclosed, but the suspension were directly added and mixed with the dissolution media in vials placed on an orbital shaker	Dissolution medium was pH 7.4 PBS, orbitally shaked at 50 rpm in a hot box at 37 °C	Sampling time: 24 h. Aliquots were withdrawn and the drug content was determined by HPLC	Among 60–70% released in the first 3 h, and remain almost constant until 24 h

[a]SBF composition: 140 mmol/ml Na; 5 mmol/ml K; 1.5 mmol/ml Mg; 2.5 mmol/ml Ca; 147.8 mmol/ml Cl; 4.2 mmol/ml HCO_3; 1.0 mmol/ml HPO_4 ; 0.5 mmol/ml SO_4
[b]Control PHT solution (500 μg/ml) was used to remove the retardation effect of the PHT molecule via the membrane of the dialysis bag

be expected [44]. In the second case, however, the situation is more complex: under GI simulated conditions, the formulation should remain stable long enough to be absorbed without a significant loss of its drug content. How to experimentally measure this has not been addressed yet, but it is not illogical to think that larger release times under GI-simulating conditions (pH 1.2–6.8, agitation at 75–100 rpm) would be favorable if the drug is expected to be absorbed in the encapsulated form. However, under no circumstances the test should be performed without any agitation and/or accurate temperature control.

A similar analysis can be done regarding to the composition of the dissolution media. For parenteral administration, it is correct to select a pH 7.4 buffer, despite that for GI administration the same drugs may require more acidic media, *e.g.*, the evaluation of DZ, LTG, and GBP release from solid oral dosage forms is recommended in HCl 0.01–0.1 N [43]. The use of additives should be more carefully addressed: as it was mentioned before, if the release is expected to happen in the GI tract, tensioactive substances may be considered, although some commonly used compounds (*e.g.*, Tween 80, pluronic 123, PEG) have been shown to negatively modulate P-gp activity [52, 53] and could therefore lead to the results' misinterpretation. For example, both VA capsules and CBZ tablets' USP dissolution tests are performed with a small amount of sodium lauryl sulfate (*i.e.*, SDS) in the medium [43]. The biorelevance of the use of Tween 80 to simulate plasmatic conditions should be further investigated.

A last point to consider is the logical need of some adaptations to perform the in vitro release test of nanoformulations. The use of a dialysis bag, for example, is one of the most commonly found. Despite that with a good agitation system a significant alteration of the release profile is not expected, it is always convenient to assess whether there is a retardation effect (for the diffusion through the membrane) in order to correct the results. Of all the articles reviewed, this verification was found only in one of them [34]. As a general rule, one could think that every alteration introduced to the classical system should be previously validated to ensure that it would not hamper the biorelevance of the test. As a classical system, we understand a vessel with enough fresh dissolution medium to achieve sink conditions at a biorelevant pH, correctly agitated and kept at 37 °C.

4 Nose-to-Brain Delivery of AEDs

Intranasal (IN) drug administration has always been regarded as an alternative administration route especially useful for those drugs that are extensively metabolized or that result unstable on the GI tract conditions [54].

Nasal mucosa is highly vascularized and of easy access for drugs, and its epithelium is mainly formed by ciliated cells, together with goblet cells and seromucous glands, which are responsible of producing secretions for the endonasal mucus [55]. It is a highly porous, nonkeratinized epithelium that lies over a thin basal membrane and that acts as a barrier for the absorption of drugs and xenobiotics [56].

Historically, the IN administration of drugs has been restricted to local uses since the systemic absorption achieved by the route is usually low and erratic. Furthermore, it shows a great interindividual variability since many are the physiological factors involved in the absorption process, like the dimensions of the nasal cavity (which in turn determines the surface available for the absorption), the blood flow, and the metabolic capacity of the mucosa as well as the composition and volume of nasal secretions. Additionally, pathologic factors associated with some allergic or infectious diseases may modify the secretion levels and/or the blood flow of the tissue.

Nevertheless, this administration route has been proposed as a noninvasive alternative to deliver drugs to the brain since it possesses a unique anatomic characteristic: the nasal epithelium is innervated by the trigeminal and olfactory nerves. Once in the olfactory epithelium, a drug is absorbed and enters the olfactory bulb through the dendritic processes of the olfactory neurons that are directly exposed in the upper nasal passage. Therefore, as it can be seen in Fig. 1, the drug is capable of diffusing through the olfactory and trigeminal nerve, effectively distributing throughout the brain [57].

Despite that the drugs absorbed by this route effectively bypass the restrictions imposed by the BBB, the amount of drug reaching the brain from the nasal cavity is still very low, near the 0.1 % of the administered dose [54]. The major obstacle for the absorption process is the mucus layer that covers all the epithelium and reduces the contact time of the substances with the absorptive surface. In a process called mucociliary clearance, the mucus layer (and all the particles and compounds associated to it) is transported by the cilia movement toward the nasopharynx, where it is either swallowed or expelled via coughing.

Therefore, IN dosage forms of AEDs could be a suitable option to ensure a direct delivery of the drugs into the CNS, with less systemic drug losses, faster onset of action, noninvasiveness, easy application, and circumvention of the BBB. A proof of this is that, despite the aforementioned drawbacks, this administration route has been successfully applied for the delivery of anticonvulsant drugs like CBZ [58, 59], CBZ microspheres [60], and LTG [61]. Furthermore, IN delivery of benzodiazepines (midazolam, diazepam, lorazepam) is especially useful in acute crisis management, since it allows for the reduction of the time between drug administration and cessation of seizures [62].

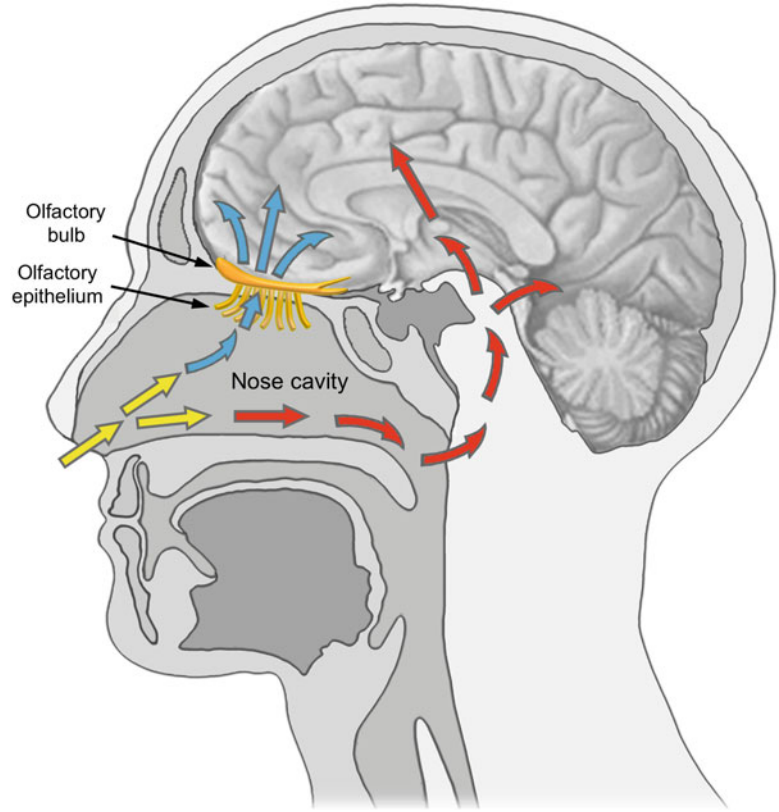

Fig. 1 Intranasal delivery pathways. Intranasally applied drugs are rapidly transported into the brain tissue both by the peripheral olfactory route (shown in *blue*) and by the peripheral trigeminal route (shown in *red*). *Reprinted with permission from Goldsmith* et al. *Precision nanomedicine in neurodegenerative diseases. ACS Nano 8(3): 1958–65. © 2014 American Chemical Society*

In the next sections, we will discuss the articles about nanosystems for IN administration of AEDs that have so far been published.

The use of this administration route for the delivery of biotherapeutics will not be discussed here, but it is a very interesting option to deliver macromolecules that are usually potent enough to achieve therapeutically active levels in the brain in spite of the small fraction that is absorbed through the nasal mucosa. The nasal route offers several advantages for this kind of molecules that are too large and too unstable to be administered by other routes [56]. The work series of Kubek et al. represents a good example of how the IN route could be applied to the administration of NP-encapsulated biomolecules for epilepsy treatment [63–65].

4.1 Nanoparticulated IN Delivery Systems

In nasal formulations, the site of drug deposition depends on several variables such as the diameter and size distribution of the particles, some matrix properties, and, in the case of aerosols, the speed of the particles. Therefore, formulating a drug into a nanoparticulated

system for IN administration would provide, besides all the classic and already mentioned advantages of nanocarriers, the achievement of a better control over the deposition place, and thus over the drug absorption, while simultaneously protecting the drug from the degrading environment in the nasal cavity (*e.g.*, lytic enzymes and pH present in nasal secretions).

It is very important to use biocompatible materials to reduce the risk of irritation of the mucosa during the application. Despite that sporadic administration is not likely to produce damage to the epithelium and/or the cilia, chronic administration could produce severe irritation and toxicity, either by the drug or the ingredients of the formulations (*i.e.*, preservatives, salts, etc.), which could lead to serious damage of the cilia, compromising this natural defense mechanism.

There are several articles within the scientific literature that present nanoparticle formulations for IN administration, but only three of them involved AEDs: two based on nanostructured lipid carriers (NLC) and the other one using polymeric nanoparticles.

Back in 2011, Eskandari et al. developed NLC-containing VA for IN administration to achieve sustained protection against the MES model of seizure with lower drug concentrations, thus reducing systemic side effects of the drug. The main features of their work are presented in Table 3. Intranasally administered, the nanosystems showed a protective effect, measured as the decrease in the extension/flexion (E/F) ratio, equal or greater than the one obtained with the VA solution given by the same route but with much lower doses (4 mg/kg of the VA-NLC vs. 30 mg/kg of the VA solution). Furthermore, the intranasally given VA-NLC (4 mg/kg) protected in the same way as the IP VA solution of 150 mg/kg. As it can be expected from the preceding results, the VA quantification revealed a brain/plasma (B/P) ratio of 8.4, 6.77, and 0.42 for the intranasal VA-NLC, VA nasal solution, and VA IP solution, respectively [66].

On the other hand, the group of Alam et al. developed and optimized NLC for the IN administration of LTG. The work included an extensive in vitro characterization of the nanosystem, a biodistribution study of the ^{99m}Tc-labeled nanosystem by γ-scintigraphy, and an in vivo PD study of its activity in the MES test, measured as the percentage of protected animals and as the latency and duration of the tonic hind limb extension (THLE) experienced by the unprotected animals. Other experimental details are listed in Table 3.

Latency times were as follows: nasal NLC > nasal solution > oral solution; all of them were statistically greater than the control (no treatment). However, only the group that received the nanoformulation was protected against seizures (100% and 75% of protection at 1 h and 24 h post-dose, respectively). As in the case of latency times, brain concentration of LTG was greater for the

Table 3
Summary of the experimental protocols used to test the anticonvulsant effect of nanoformulations developed for nose-to-brain delivery of AEDs

AED and nanosystem	Groups	Dose (route)	Intranasal administration	Anticonvulsant animal model	Biodistribution studies
Nanoparticles					
VA-NLC [66]	Control (NLC)[a]	0 mg/kg (IN)	100 µl in each nostril with a syringe tubing	Rats were given shock (110 mA, 0.2 s,	VA in the blood and brain by GC
Drug loading:	Control (NLC)[a]	0 mg/kg (IP)	Animals held in supine	100 Hz) 15, 30, 60,	Rats were sacrificed at
47 ± 0.8%	VA-NLC[b]	4 mg/kg (IN)	position under light	90, and 120 min	maximum protection
Particle size:	VA-NLC[b]	20 mg/kg (IP)	ether anesthesia	post-dose. The E:F	time
154 ± 16 nm	VA solution[b]	30 mg/kg (IN)		ratio was recorded	
Zeta potential:	VA solution[b]	150 mg/kg (IP)			
−10 ± 0.5 mV	($N = 6 \times 6 = 36$)				
LTG-NLC [67]	Control	Without any treatment	100 µl in each nostril	Rats were given shock (150 mA, 0.2 s, 60 Hz) 1 and 24 h post-dose	LTG in the blood and brain by HPLC
Drug loading:	LTG solution[c]	24 mg/kg (oral)	Animals held in slanted	Latency and THLE	Rats were sacrificed at
96.6 ± 4.3%	LTG solution[c]	4.8 mg/kg (IN)	position under light	duration were	24 h post-dose
Particle size:	LTG-NLC[d]	4.8 mg/kg (IN)	ether anesthesia	recorded	
151.6 ± 7.6 nm	($N = 4 \times 4 = 16$)				
Zeta potential:					
−11.7 ± 3.0 mV					

(continued)

Table 3
(continued)

AED and nanosystem	Groups	Dose (route)	Intranasal administration	Anticonvulsant animal model	Biodistribution studies
Micro- and nanoemulsions					
CBZ-Mucoadhesive nanoemulgel [71]	Control	Sham operated	100 µl in each nostril with a syringe tubing	Mice were injected with 100 mg/kg of PTZ (IP) 5 min post-dose	Not performed
0.1% w/w CBZ-loaded nanoemulgel with 0.1% xanthan gum as anionic mucoadhesive polymer	CBZ-MNEG (Both of $N=10$)	25 mg/kg (IN)	Animals were then allowed to rest for 5 min to recover from the anesthesia completely and to allow time for the drug to reach the brain	Onset of clonic convulsions and death times were recorded Mice were given shock (99 mA, 11 s, 95 Hz) 5 min post-dose The tolerated number of shocks were recorded	
Globule size: 45–146 nm	Control CBZ solution (Both of $N=5$)	ST vehicle(IV) 25 mg/kg (IV)			
	Control CBZ-MNEG (Both of $N=10$)	Sham operated 25 mg/kg (IN)			
	Control CBZ solution (Both of $N=5$)	ST vehicle(IV) 25 mg/kg (IV)			

CBZ microemulsion [73]	Control	Without any treatment	*ca.* 88 µl in each nostril with a micropipette attached with a	Rats were given shock (150 mA, 0.2 s, 60 Hz) 1 h post-dose	CBZ in the brain and plasma by HPLC
40% w/v CBZ Globule size: 51.3 ± 2.5 nm pH = 5.78	CBZ solution[e] CBZ solution[e] CBZ-ME CBZ-ME ($N = 5 \times 6 = 30$)	8.2 mg/kg (IP) 8.2 mg/kg (IN) 8.2 mg/kg (oral) 8.2 mg/kg (IN)	0.1 mm id of LDPE tubing	THLE duration and time for complete recovery were recorded	Rats were sacrificed 15 min post-dose
PHT microemulsion [72]	Control	Without any treatment	ca. 88 µl in each nostril with a micropipette attached with a	Rats were given shock (150 mA, 0.2 s, 60 Hz) 1 h post-dose	PHT in the brain by HPLC
40% w/v PHT Globule size: 16 nm pH = 6.0	PHT solution[e] PHT-ME PHT-ME ($N = 4 \times 6 = 24$)	8.2 mg/kg (IP) 8.2 mg/kg (oral) 8.2 mg/kg (IN)	0.1 mm id of LDPE tubing	Duration of the THLE was recorded	Rats were sacrificed 15 and 30 min post-dose

[a]Prepared in the same way as the VA-NLC but without the drug
[b]Pre-dialyzed against purified water (24 h) to separate NP from free drug
[c]In DMSO 50% v/v
[d]Medium for resuspension of lyophilized particles is not specified
[e]In 60% PEG 400

NLC, followed by the IN solution, while plasmatic LTG concentrations were IN solution > oral solution > nasal NLC [67].

Unfortunately, the authors did not include an excipient control group (with the same constituents as the NLC but without the drug) in the report. Also, the medium where the LTG-NLC were suspended for the in vivo studies is not clear, which is a very important issue when dealing with absorptive processes that may be influenced by cosolvents (like the DMSO present in the solutions) or pH.

The third work related to the use of NP for nose-to-brain drug delivery of AEDs is the one of Sharma et al., who formulated lorazepam-loaded PLGA nanoparticles (LZ-PLGA-NP) intended for IN administration. The formulation was optimized in terms of particle size and Z-potential (167–318 nm, −18.4 mV), and the amount of drug encapsulated (ca. 90%) resulted stable for at least 90 days and showed no appreciable toxicity in a cell viability assay. Despite that we could not include this work in Table 3 for its lack of in vivo PD studies, the NP showed promising brain-preferential biodistribution: when a fixed dose of LZ was administered to rats as IV solution, IN solution, and IN PLGA-NP, the brain concentration was higher for the latter in the whole time interval (0–8 h). At maximum concentration time (2 h), the B/P ratios were 0.59, 0.76, and 0.87, respectively [68]. It would be interesting to analyze the PLGA's degradation products, considering that the lactic acid ($pKa \approx 3.86$) released from the NPs is considered a strong acid and the brain pH is about 7.2, with the potential toxic consequences.

4.2 Micro- and Nanoemulsions for IN Administration of AEDs

Particle size is not the only factor that determines the IN absorption of a drug, since despite that smaller particles are capable of reaching the superior nasal epithelium (with the greater permeability), they are also cleared faster by the mucociliary system. The use of bioadhesive materials delays this clearance mechanism and extends the time that particles are in contact with the epithelium, thus promoting the absorption of the drug. For example, Alam et al. postulated that the greater brain exposure found with their LTG-NLC could be due to the mucoadhesive effect of the poloxamer 188 used, which proceeds via an immediate adhesion of the polyoxyethylene chains to mucus through secondary forces, followed by their diffusion into mucus where they undergo molecular interactions [69].

Vyas et al. prepared two CZ microemulsions, one of them with polycarbophil 0.5% for mucoadhesive properties, and compared their biodistribution after IN administration to rats by γ-scintigraphy. As in the previously reviewed articles, they found greater brain/plasma ratios for the IN route compared with an IV administration, with greater brain accumulation of the mucoadhesive formulation [70].

Samia et al. developed a CBZ-loaded mucoadhesive o/w nanoemulgel (0.1 % w/w CBZ-MNEG), based on a nanoemulsion of oleic acid in water, with Labrasol® (caprylocaproyl polyoxylglycerides) as surfactant and xanthan gum as mucoadhesive polymer. Along with the PD studies (both in the PTZ and MES models, see details of the experimental protocol in Table 3), the authors evaluated the bioadhesion strength of the formulation by measuring the force required to detach the gel from a bovine nasal mucosa.

Briefly, the CBZ-MNEG was stable during a 12-month period, showed strong bioadhesive properties, and protected animals in both models of seizures. In the PTZ model, the formulation prolonged the onset time for convulsion and death (the obtained values were higher than the IV ones for the same dose and significantly greater than the ones of the control group). For electrically convulsive mice, CBZ-MNEG prevented the convulsions in the first shock, while all control animals died within the first shock. Expressed as number of tolerated shocks, CBZ-MNEG resulted in 2.0 ± 0.1 and IV solution in 1.5 ± 0.2 [71].

Lastly, the group of Acharya et al. developed microemulsions (ME) for nose-to-brain delivery of PHT [72] and CBZ [73] and evaluated their in vivo protective effect in the MES model (details in Table 3). In both cases, the ME caused higher brain concentration levels than the IP solution of the same dose, but showed similar anticonvulsant effect. It would have been interesting to assess the B/P ratio at 60 min, the time employed for the MES test, since these results seem somehow contradictory: in the case of CBZ, for example, the ME showed a B/P ratio 10–15-fold higher than the IP solution of the same dose, but the same anticonvulsant effect was attained with both formulations.

It is worth highlighting that Acharya et al. presented nasal ciliotoxicity studies, very important for this administration route and not included in any of the other reviewed articles. The studies were performed in vivo in rats (CBZ-ME) or ex vivo in sheep nasal mucosa (PHT-ME), followed by the microscopic examination of the nasal mucosa to evaluate the tissue damage. Both formulations were considered nontoxic, as no change could be noticed in the gross morphology and histology of the nasal mucosa, supporting what was already found by other authors for the same excipients [74].

Although the clinical potential of the IN delivery route still remains controversial, the interest in continuing the exploration of the nose-to-brain delivery of drugs is understandable. As seen in the works reviewed in this section, there are many alternatives (type of nanovector, particle size, mucoadhesive materials, and penetration enhancers, among others) that can be tailored to improve brain BA through IN administration. If this route allows the drugs to reach the CNS in a sufficient amount, it could generate interest in previously abandoned drugs and enable a novel approach to CNS drug delivery [75].

5 Conclusions

Conventional administration of AEDs has been proved unsatisfactory in many ways, from insufficient drug levels at the biophase, high toxicity, drug bioconversions and degradability, improper administration routes, and lack of targeting. Nanotechnology is an emerging field that will offer novel drug formulations through the development of novel carriers which could provide an improved pharmacokinetic profile, extended drug release, higher BA, and better patient compliance among other properties. A better understanding of the pathology and characteristics of the patient will allow targeting specific cells avoiding the capture of the nanodevice by nontargeted organs (*e.g.*, liver, kidneys). In this sense, the nanocarrier morphology, shape, size, and surface topography could be used to determine the drug fate. Possibilities are huge, since many are the molecules available in the market.

However, nanomedicine is still in its infancy, and thus we still need to develop and design novel and standardized assays, including the toxicological ones and preclinical and clinical trials for the matrix components and the nanodevices, that allow for the study of their interactions with the drugs and the body. The combination of AEDs and tailored nanodevices will provide novel and efficient platforms to improve the therapeutic treatment of epilepsy and consequently the life quality of patients.

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Part III

Potential Contributions of Network Pharmacology and Drug Repurposing in Antiepileptic Drug Discovery

Chapter 17

Side Effects of Antiepileptic Drugs

Hana Kubova

Abstract

Adverse effects of antiepileptic drugs (AEDs) are common and result in treatment discontinuation in up to 25% of patients. The profile of adverse effects varies greatly among AEDs and markedly affects drug selection for individual patients. The most common adverse effects like cognitive impairment, coordination difficulties, and other CNS-related adverse effects are predictable, dose dependent, and reversible. They are of particular concern in patients who work or study. Idiosyncratic adverse reactions are unexpected events that cannot be explained by known mechanisms of action. Typically, they are not related to dose and they are associated with high risk of morbidity or even mortality. Some of them, like weight gain, can negatively affect treatment adherence. Many of AEDs increase the risk of congenital malformations or reproductive problems. New AEDs are usually better tolerated and some of them have no effect on hepatic drug-metabolizing enzymes which results in lower potential for drug interactions. Comparative, well-designed, and long-term trials are however needed to confirm better tolerability of the new AEDs and to assess their effect on quality of life, tolerability, and teratogenic potential.

Key words Epilepsy, Antiepileptic drugs, Side effects

1 Side Effects of Antiepileptic Drugs

Approximately 65 million people worldwide have epilepsy, making it the most common neurological disorder after stroke. Epilepsy is a multifactorial disorder that encompasses many seizure types and syndromes with different prognoses and sensitivities to available treatment. Treatment is symptomatic and limited to seizure suppression. Thus, current antiepileptic drugs (AEDs) are more accurately called "anti-seizure drugs" because they do not prevent or reverse pathological changes underlying development or progression of epilepsy and epilepsy-related comorbidities.

Adverse effects represent a leading cause of treatment failure, are a major impediment to optimal dosing for seizure control, and result in early treatment discontinuation in nearly 25% of patients. Adverse effects also negatively affect patient adherence to AEDs [1]. Furthermore, adverse effects are a major source of disability and mortality in patients with epilepsy and substantially contribute to the use

Alan Talevi and Luisa Rocha (eds.), *Antiepileptic Drug Discovery: Novel Approaches*, Methods in Pharmacology and Toxicology,
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and costs of healthcare systems. Recently, many new AEDs have been introduced into clinical practice. They have brought new therapeutic options, but their efficacy is not greater than that of old AEDs, and their use does not reduce the frequency of drug-resistant epilepsy in at-risk patients. Approximately 20–30% of patients continue to be pharmacoresistant with ongoing seizures, high risk of adverse effects, and considerable psychiatric comorbidities. In some patients, remission is achieved only at the expenses of serious side effects. In patients with epilepsy, drug treatment is usually necessary for several years, but it can last a lifetime. During such a long duration, various adverse effects of AEDs may appear and negatively affect the patient's quality of life. Systematic research and new methods of assessment of toxic effects may result in more effective strategies to detect and tackle the adverse effects of AEDs and to improve the quality of life for epileptic patients.

The frequency at which adverse effects are reported and, to a certain extent, their patterns are highly dependent on the method of their assessment. Particularly, studies on CNS effects often generate controversial data. Outcomes of these studies are affected by many variables; among them criteria used for selection of patients, length of therapy, and domains of measured effects play critical roles. The use of standardized and validated screening methods also helps to obtain comparable data and allows identification of populations at high risk for developing adverse effects.

It should also be stressed that an adverse effect is an unpleasant experience arising during drug exposure that is not necessarily caused by the drug. Thus, establishing causality can be challenging in uncontrolled studies and case reports, particularly when relevant information concerning dose, other treatments, or reversibility after discontinuation of therapy is missing (for a review, see reference [2]).

2 Definitions and Classification of Adverse Effects

The WHO's definition of an adverse drug reaction, which has been used since 1972, is *A response to a drug which is noxious and unintended and which occurs at doses normally used in man for the prophylaxis*, *diagnosis*, *or therapy of disease or for the modifications of physiological function*. The term *adverse effect* encompasses all unwanted effects and is preferable to other terms such as *toxic effect* or *side effect*. A toxic effect is always dose related and occurs as an exaggerated therapeutic effect. On the other hand, a side effect is not associated with therapeutic effect and it may or may not be dose related [3]. Thus, the term "adverse effect" makes no assumption about mechanisms and avoids the risk of misclassification.

The term "adverse effect" must be distinguished from "adverse event." An adverse effect is an untoward experience that can be attributed to some action of a drug; an adverse event is an adverse outcome arising while the patient is taking a drug, but the event is not necessarily attributed to the drug [3].

Adverse drug effects can be classified by symptoms, severity, frequency, underlying mechanisms, or other parameters. According to the WHO, adverse drug reactions were originally divided into two basic groups, type A and type B. Type A is defined as "expected exaggerations of the drug's known effect that is usually dose dependent." Type B is "an idiosyncratic and unpredictable reaction, usually unrelated to the drug's known pharmacology." For the demands of modern pharmacology, two other categories were added to this classification, labeled type C (reaction related to the dose and time) and type D (delayed reaction). The latter classification was later split into withdrawal reaction (type E) and unexpected failure of therapy (type F) (for details, see reference [3]). Indeed, this classification, like others, has certain limitations because an adverse drug reaction can be difficult to classify into one of these categories. This classification was modified recently by Perucca and Gilliam [2] for AEDs (*see* Table 1).

Table 1

Modified classification of adverse effects according to Perucca and Gilliam (reference [2])

Type	Features	Example	Management
Type A	Related to known mechanisms of drug actions	CNS-related adverse effects (dizziness, somnolence, agitation)	Dose reduction or drug withdrawal, dosage modification
	Dose related		
	Common or very common, predictable		
	Low mortality		
Type B	Related to individual vulnerability (immunological, genetical)	Skin rash, hepatotoxic effects, pancreatitis, agranulocytosis, aplastic anemia	Immediate withdrawal, drug avoidance in future
	Not related to mechanism of action of the drug		Slow dose titration
	Not dose related		
	Unexpected, uncommon		
	High mortality		
Type C	Related to cumulative dose, occur after some time after the use of drug	Decreased bone density, endocrine effects, sexual and reproductive dysfunction, folate and vitamin D deficiency	Dose reduction or withdrawal, vitamin supplementation
	Time related		
	Uncommon, chronic, usually reversible		

(continued)

Table 1
(continued)

Type	Features	Example	Management
Type D	Teratogenic and carcinogenic effects Usually dose related Irreversible, delayed	Birth defects	Avoid drugs with known teratogenic effects in patients in risk (women with childbirth potential)
Type E	Adverse drug interactions Common, predictable	Increased risk of CNS neurotoxicity in combination of Na^{2+} blockers, decreased efficacy of warfarin in combination with carbamazepine, interaction between hormonal contraception and lamotrigine (reduced efficacy)	Avoid necessary polytherapy
Type F	Withdrawal related Usually related to abrupt withdrawal Uncommon	Status epilepticus, insomnia, agitation, psychiatric withdrawal symptoms	Slow withdrawal

3 Type A Effects

Type A adverse effects are defined as common, predictable, and related to a known pharmacological action of the drug. They are dose dependent, and they usually appear at the beginning of therapy or after dose escalation. The CNS effects are the most frequently reported type A adverse effects of AEDs and typically include fatigue, drowsiness, dizziness, coordination problems, concentration difficulties, memory problems, and irritability. Although the profile of CNS adverse effects varies from one drug to another, nearly all AEDs induce sedation and coordination disturbances to some extent (for a review, see reference [2]). Cognitive adverse effects observed as a result of AEDs are usually fairly modest, but can nevertheless be clinically significant because patients with even subtle adverse CNS symptoms display significant worsening of perceived quality of life [4]. Furthermore, cognitive and behavioral deficits are highly individualized and must be considered independently in every patient. Polytherapy or elevated plasma levels of AEDs increase the risk of CNS adverse effects.

In addition to AED effects, seizure etiology, and frequency, age of epilepsy onset and cerebral lesions also contribute to

cognitive functions in patients with epilepsy. It should be emphasized that epilepsy patients, as a group, have been shown to perform more poorly on a variety of cognitive measures compared to healthy controls.

As documented by many reports, AEDs exhibit a whole spectrum of CNS effects, not all of which are only negative with unfavorable impact on quality of patient's life. Some AEDs have beneficial effects on mood and behavior, and many AEDs are also used in psychiatry in non-epileptic patients. Available data from patients with epilepsy, however, demonstrate substantial differences in the CNS response to individual AEDs. For example, beneficial behavioral and mood-stabilizing effects of lamotrigine have been documented in many studies, whereas other studies report the development of psychosis in patients treated with lamotrigine. Such controversial data suggest that the behavioral response to AEDs in individual patients cannot be simply predicted only on the basis of a known mechanism of drug action.

In 1999, Ketter and collaborators [5] divided AEDs on the basis of their predominant psychotropic profiles into two global categories. One group of AEDs, acting predominantly through potentiation of inhibitory gamma-aminobutyric acid (GABA) neurotransmission, is assumed to have "sedating" effects in association with fatigue, cognitive slowing, and possible anxiolytic and antimanic effects. This group involves drugs such as barbiturates, benzodiazepines, valproate, gabapentin, tiagabine, and vigabatrin. The other group involves felbamate and lamotrigine and attenuates excitatory glutamate neurotransmission. These drugs are expected to be "activating," possibly with anxiogenic and antidepressant effects. AEDs with mixed GABAergic and antiglutamatergic actions may have "mixed" profiles. More recent analysis of available literature, however, did not bring conclusive evidence for this hypothesis (for a review, see reference [6]). Thus, in addition to mechanisms of action (*see* Table 2), there are other variables that play a role in the behavioral and mood-modifying effects of AEDs. Among these variables, age, the presence or absence of brain lesions, and the baseline mood state have to be considered in particular. Additionally, the extreme *age groups*, children and elderly patients, appear to be particularly sensitive to the cognitive or mood effects of AEDs. Immaturity of the nervous system in children may be responsible for the paradoxical responses described after administration of some AEDs, and several reports have indicated some differences in the mood effects of phenobarbital, carbamazepine, phenytoin, and valproate in pediatric populations. In particular, children on phenobarbital demonstrated poorer performance than children treated with valproate. Unlike in adults, administration of both phenobarbital and benzodiazepines in children often produces an acute reaction that includes hyperactivity, irritability, or aggression. Additionally, animal studies

Table 2

Main mechanisms of action of AEDs

Drug	Main mechanism of action
Phenobarbital	Enhancement of GABA-meditated inhibition
Phenytoin	Na^+ channel blocker
Primidone	Enhancement of GABA-meditated inhibition
Ethosuximide	Blockade of T-type of Ca^{2+} channel
Benzodiazepine	Enhancement of GABA-meditated inhibition
Carbamazepine	Na^+ channel blocker
Valproate	Multiple (enhancement of GABA-meditated inhibition, glutamate (NMDA) inhibition, Na^+ channel, and T-type calcium channel blockade)
Vigabatrin	Enhancement of GABA-meditated inhibition
Lamotrigine	Na^+ channel blocker, attenuation of excitatory transmission
Oxcarbazepine	Na^+ channel blocker
Gabapentin	Blockade of α2δ subunit of Ca^{2+} channel, effects on GABA turnover
Tiagabine	Enhancement of GABA-meditated inhibition, inhibition of glial GAT-1
Topiramate	Multiple (GABA potentiation, AMPA inhibition, Na^+ and Ca^{2+} channel blockade)
Levetiracetam	SV2A modulation
Zonisamide	Na^+ and T-type Ca^{2+} channel blocker, carbonic anhydrase inhibition
Stiripentol	Enhancement of GABA-meditated inhibition, Na^+ channel blocker
Pregabalin	Blockade of α2δ subunit of Ca^{2+} channel
Rufinamide	Na^+ channel blocker
Lacosamide	Enhancement of slow inactivation of voltage-gated Na^+ channel
Eslicarbazepine acetate	Na^+ channel blocker
Perampanel	Glutamate (AMPA-Rp) antagonist

This table summarizes the predominant mechanisms of action for each AED. It is, however, important to realize that only a limited number of AEDs (ethosuximide) are characterized by a single mechanism of action. In fact most of AEDs have multiple mechanisms of action

have suggested that children may be at increased risk for enduring cognitive impairment because of the possible interference of the AEDs with normal development. In elderly patients, the increased risk of adverse cognitive effects is usually associated with age-related changes in pharmacokinetics.

Mood and anxiety disorders represent the most frequently reported psychiatric problems in patients with epilepsy, and both

biological and psychological reasons were identified [7]. Biological contributors involve neuroanatomical factors, including involvement of temporal lobe structures, severity and distribution of brain lesions, and various epigenetic changes at molecular, cellular, and structural levels. Such changes may both lead to rebuilding of the brain circuitry and participate in functional alterations occurring in epilepsy patients. In addition, epilepsy is a chronic, stigmatizing disorder that brings various social limitations that can contribute to development of psychiatric problems. Seizure reduction with related improvement in quality of life can positively affect the patient's mood. On the other hand, in people with learning disabilities associated with ongoing seizures, sudden seizure control can result in "release phenomenon" because the patient did not have chance to develop proper skills in how to use their new-found abilities. Similarly, patients who are not able to express their concerns about adverse effects in words can instead react aggressively. Such behavioral disturbances are not direct effects of the drug, but they have yet to be well identified in order to be eliminated (for a review, see reference [8]).

Apparently, proper assessment of both beneficial and adverse CNS effects is of high clinical relevance, and the selection of optimal AEDs can help not only to manage seizures but also to reduce psychiatric problems accompanying epilepsy. However, improper selection of AEDs or their combinations can cause mood-related problems and result in severe behavioral disturbances. The following part of this review therefore not only summarizes the adverse effects of AEDs but also presents brief insight into possible beneficial CNS effects of these drugs.

4 Old Antiepileptic Drugs

Phenytoin, primidone, phenobarbital, carbamazepine, valproate, ethosuximide, and benzodiazepines have been used in monotherapy or polytherapy for many years, and their adverse effects are relatively well established. Mood effects of these drugs are usually moderate and can be clinically significant. Typically, they include sedative effects and coordination disturbances such as vertigo, imbalance, ataxia, nystagmus, or diplopia. For *phenytoin*, agitation, increased anxiety, and alterations of emotional state have been reported, however, only after high doses [9, 10]. *Phenobarbital*, like other barbiturates and *benzodiazepines*, has sedative effects in adults, whereas it often induces hyperactivity in children with epilepsy. Both *phenobarbital* and *benzodiazepines* have the most detrimental effects on cognition.

Consistent with the structurally similar tricyclic antidepressants, *carbamazepine* is used in psychiatry for the treatment of mood disorders, primarily mania and rapid cycling bipolar disorders, depression, and dysphoria and with some limitation for

bipolar and unipolar depression and dysphoria (for a review, see reference [11]). Carbamazepine may help control agitated or disruptive behaviors, viewed dimensionally across a spectrum of psychiatric disorders, including attention deficit/hyperactivity, intermittent explosive behavior, post-traumatic stress, and personality disorders, as well as in mental retardation, dementia, and alcohol and possibly benzodiazepine withdrawal. Fenwick [12] characterized carbamazepine as a mood-stabilizing drug. However, data regarding carbamazepine therapy and psychosis in patients with epilepsy are controversial. A double-blind study with adjunctive carbamazepine revealed improvement in chronically psychotic psychiatric patients with temporal lobe epilepsy [13]. In contrast, no studies have documented serious mood effects. There are studies reporting occasional development of acute psychosis in some epilepsy patients receiving carbamazepine, but these studies are usually case reports. The effects of carbamazepine on cognition are relatively mild, and only a few cognitive differences between carbamazepine, phenytoin, phenobarbital, or primidone were reported in a large group of new-onset epilepsy patients who were followed for at least 36 months [14]. In a more recent study, Keene et al. [15] did not find any differences in cognitive side effects between add-on therapies with carbamazepine, phenytoin, and clobazam in children with refractory epilepsy.

Valproate, a drug with multiple mechanisms of action, may relieve mood symptoms and agitation in epilepsy patients [16]. Epilepsy patients with concomitant neurological problems (abnormal EEG, head injury) had better rates of affective improvement than patients without neurological abnormalities [16]. Valproate rarely causes somnolence, fatigue, and mild cognitive impairment [17].

Contradictory effects of valproate on mood function have been reported in pediatric patients. In some studies, no behavioral effects were found [18], whereas in children with behavioral problems, mental retardation and hyperactivity were reported [19, 20]. However, Herranz and collaborators [21] reported behavioral effects of valproate in 65 % of children on valproate monotherapy. In a minority of these children, sedating effects were observed, while most showed increased irritability or hyperactivity. Similarly, like in adults, valproate seems to be more beneficial in children with a "mood-activated" profile, while valproate may lead to behavioral disturbances in children without baseline problems [6].

There is limited information concerning the mood and behavioral effects of *ethosuximide*. Dizziness, fatigue, and somnolence represent the most frequently reported CNS adverse effects observed in patients treated with ethosuximide (for a review, see reference [22]). A controlled study did not find any mood effects of ethosuximide or its association with cognitive impairment [23].

5 New Antiepileptic Drugs

As mentioned above, the new AEDs are expected to be better tolerated and to affect cognitive functions to a lesser extent than the old AEDs. Although most of the previous studies dealt with the putative advantages of newer over older AEDs, some serious adverse CNS effects are observed after their administration.

Similarly, sedative effects and coordination disturbances of old AEDs represent the most commonly reported CNS-related adverse effects of new AEDs although sedative effects are more frequent and severe with the old AEDs: benzodiazepines, phenobarbital and primidone [2]; however, coordination problems were documented in all third-generation AEDs (gabapentin, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, retigabine, tiagabine, topiramate, zonisamide) [24, 25].

Because of fatal adverse hematological and hepatological effects, *felbamate* is currently used only in severe refractory forms of epilepsy such as the Lennox-Gastaut syndrome. Felbamate is an NMDA receptor antagonist, and at the same time, it potentiates GABA-mediated events. In children, somnolence, insomnia, and anorexia are the most frequently reported CNS effects. A similar profile including diplopia and headache was also reported in adults [26]. Adverse psychiatric effects are consistent with the activating profile of felbamate and include anxiety, manic depression, irritability, aggression, mania, and psychosis [27, 28]. Patients with previous psychiatric problems may or may not be at increased risk of aggravation of psychiatric problems. Favorable behavioral effects of felbamate were reported in patients with Lennox-Gastaut syndrome. Gay et al. [29] documented significant improvement in social, intellectual, and motor functioning and improvement of attention, concentration, and memory.

Mechanism of action for *gabapentin* is not fully understood, but it binds to the Ca^{2+} channel α2δ subunit. It also affects GABA turnover. Clinical studies documented rather positive mood effects of gabapentin, including decreased anxiety and depression or mood stabilization [30]. In epilepsy patients, gabapentin is generally well tolerated despite some sedative effects [31]. In adult patients, development of psychosis or depression was reported only sporadically [32]. Reports from pediatric patients are controversial. Some studies have documented behavioral disturbances including hyperactivity, irritability, and agitation in children with add-on gabapentin [33, 34]. The risk of developing these behavioral disturbances was higher in children with preexisting attention deficits and other behavioral problems. In contrast, Besag [35] did not report frequent or serious behavioral disturbances in children or teenagers with learning difficulties after gabapentin was added to their current therapy.

The positive mood effects of gabapentin were also reported in patients with epilepsy. Almost 50% of patients reported improvement in general well-being compared to those with placebo [36]. Effects of gabapentin on cognitive performance are less adverse than those of carbamazepine [37]. Additionally, the risk of developing depression is low (for a review, see reference [38]).

There are many reports on the beneficial behavioral effects of *lamotrigine* in patients with epilepsy, in particular on bipolar mood disorders and depression (for a review, see reference [8]). Lamotrigine is a Na^+ channel blocker, but it was also found to attenuate glutamate release. Lamotrigine-treated patients not only performed better in 48% of neuropsychological measures compared with those on carbamazepine but also scored better on measures of memory, attention, and a number of quality-of-life measures [39]. As documented by a multicenter international trial, a majority of children and adolescents on lamotrigine improved on global assessments [40], and significant behavioral improvement was also observed. In a double-blind, placebo-controlled study, *lamotrigine* was not associated with any cognitive impairment [41], and multiple studies comparing lamotrigine with other AEDs reported fewer adverse cognitive effects. Although adjunctive lamotrigine induces more somnolence than placebo, sedative effects are less common than with carbamazepine [42]. Placebo-controlled trials have shown low rates of psychosis and depression in patients taking lamotrigine (>1%; for a review, see reference [38]). Mood improvements have been reported in several open clinical studies in both adults and children taking lamotrigine [42–44]. There are only a few case reports of psychosis attributed to lamotrigine treatment. The development of psychosis was documented in both adult and pediatric patients receiving lamotrigine as add-on therapy (for a review, see reference [8]).

Levetiracetam possesses a unique pharmacological activity profile; it binds selectively to the synaptic vesicle protein, SV2A, which is involved in the exocytosis of neurotransmitters [45]. The most commonly reported CNS adverse effects of levetiracetam include somnolence, dizziness, irritability, and behavioral disturbances, but their incidence is relatively low [31]. The most severe adverse effects, such as psychosis, are most common in patients with a previous history of psychotic diseases [46]. Similar to felbamate and tiagabine, levetiracetam presents an intermediate risk of depression (4%). A more recent study with add-on levetiracetam in children and young adults [47] is in line with previously published data confirming the safety of this drug.

Topiramate is a drug with multiple mechanisms of action that involve GABA potentiation, AMPA inhibition, and Na^+- and Ca^{2+}-channel blockade, suggesting that it might have beneficial effects in some patients and detrimental effects in others. In older literature, the use of topiramate was associated with a relatively high risk

of developing neurocognitive and behavioral problems compared to newer AEDs (lamotrigine, gabapentin, vigabatrin) [48]. An audit of topiramate, carried out by Crawford [32], determined a risk of psychotic syndromes at 12%. A slow titration schedule, however, is associated with lower prevalence of psychiatric adverse effects. Another relevant risk factor is having a history of psychiatric disorders [49]. Beneficial behavioral effects of topiramate in patients with mood disorders were reported by Marcotte [50], who found improvement in 52% percent of patients with bipolar affective disorders.

Tiagabine inhibits reuptake of GABA. This drug is associated with a relatively high risk of coordination problems, somnolence, and dizziness, as reported in up to 22% of patients [51]. Incidence of serious psychiatric adverse events such as psychosis is not significantly higher than those with placebo [52]. According to individual studies, risk of depressed mood ranges between 1% [53] and 5% [54]. Some studies suggested beneficial effects of tiagabine in patients with mania, but data are limited (for a review, see reference [8]).

Because peripheral vision constriction occurs in up to 40% of patients [55], the use of *vigabatrin* is limited only to situations when it is absolutely necessary. Major CNS adverse effects of vigabatrin, which acts as a suicide inhibitor of GABA transaminase, include dizziness, fatigue, and drowsiness. Its administration is associated with increased risk of behavioral disturbances, ranging from irritation to psychosis. Patients with a previous history of psychosis are at increased risk of psychotic symptoms [56]. It is of interest that vigabatrin seems to be better tolerated in pediatric patients, and psychotic reactions have rarely been reported in children [57]. Review of data from double-blind, placebo-controlled studies revealed increased incidence of depression in patients treated with vigabatrin [58]. Treatment with this drug did not produce significant cognitive deterioration and had no effects on the quality-of-life measures [59].

The most common CNS adverse effects of *oxcarbazepine* are similar to those of carbamazepine. Behavioral disturbances are not usually reported. Oxcarbazepine is generally well tolerated and rather beneficial compared with the adverse CNS effects that have been reported (for a review, see reference [8]).

Zonisamide acts through multiple mechanisms of action. It blocks sodium and T-type calcium channels and inhibits carbonic anhydrase. The most frequently reported CNS adverse events are agitation and irritability, with higher incidence compared to placebo (data from manufacturer). Frequency of psychosis with zonisamide was similar to that reported with other AEDs. Incidence of depression was higher than in placebo, but this effect is likely dose dependent, and its risk increases with used dose. Compared to placebo, somnolence, anorexia, and ataxia were slightly more common with zonisamide treatment [60–62].

Data concerning adverse CNS effects of the newest AEDs are still limited, but in general, adverse CNS effects were reported in all of them. Meta-analysis of double-blind studies on neurological adverse events of new-generation sodium blockers, *eslicarbazepine acetate*, *lacosamide*, and *oxcarbazepine*, demonstrates increased risk of dizziness, diplopia, vertigo, or coordination problems compared to placebo. These effects were more frequently observed after oxcarbazepine than after eslicarbazepine acetate or lacosamide [63]. Similar to gabapentin, *pregabalin* interacts with calcium ion channels and increases the level of GABA in neuronal tissue. It is well tolerated, causing dizziness, somnolence, and ataxia of only mild to moderate intensity [64]. Similar adverse CNS effects were reported in patients treated with *perampanel*, a new AMPA glutamate receptor antagonist, and *retigabine*, acting primarily through the opening of voltage-gated KCNQ2/3 potassium channels. Both drugs have also demonstrated the ability to induce neuropsychiatric symptoms [65].

The renal adverse effects of AEDs are dependent on the mechanism of action. The risk of kidney stones is associated with the AED's potential to inhibit carbonic anhydrase. The development of calcium phosphate kidney stones was reported in approximately 1% of patients treated with *topiramate*, *zonisamide*, and *acetazolamide*. The risk increases with dose and treatment duration [66–68]. Due to its direct effects on the smooth muscles of the bladder, administration of *retigabine* in higher doses is associated with approximately 10% risk of urinary retention [69].

6 Type B Effects

Type B or idiosyncratic adverse reactions refer to unexpected events that cannot be explained by known mechanisms of action and occur unpredictably in susceptible individuals. Typically, they are not related to dose. Individual susceptibility can be due to immunologic, genetic, or other mechanisms. Type B adverse events are less common than type A effects. They usually arise shortly after the therapy onset, and they can be reversed after drug withdrawal. Type B effects are associated with high risk of morbidity or even mortality.

The most common types of idiosyncratic reaction to AEDs include cutaneous, hematological, hepatic, and pancreatic responses (for a review, see reference [70]). Skin rash occurs in approximately 5–17% of patients on *lamotrigine*, *phenytoin*, *carbamazepine*, and *phenobarbital* (for more details, see reference [71]). Stevens-Johnson syndrome or toxic epidermal necrolysis affects 1–10 of 10,000 new users of these AEDs [72]. Known risk factors for cutaneous adverse reactions are high starting dose and rapid dose escalation, history of immune system disorders, and age. For example, lamotrigine-induced serious and benign skin rashes occur more frequently in children than in adults, probably because of

age-related differences in drug metabolism [70]. Additionally, pharmacogenetics may be helpful in selecting patients with increased risk of serious dermatologic hypersensitivity reaction. In Asia, the presence of human leukocyte antigen HLA-B*1502 is highly associated with Stevens-Johnson syndrome induced with carbamazepine and probably also with phenytoin, lamotrigine, and oxcarbazepine [73]. Among others, HLA genes and the antigen HLA-A*3101 are associated with carbamazepine-induced cutaneous reactions. However, this association was found in more diverse ethnic groups (for a review, see reference [71]).

AEDs are among the drugs that most frequently cause liver necrosis leading to transplantation, and hepatotoxic effects of AEDs can occur in isolation or as a part of DRESS (drug rash with eosinophilia and systemic symptoms). *Valproate*, *phenytoin*, and *felbamate* carry the highest risk of hepatic failure. Transient elevation in liver function tests appears in 15–30% of patients with valproate [74]. Valproate and phenytoin were responsible for drug-induced acute hepatic failure leading to liver transplant in 7.3% of patients. Pediatric patients are at greater risk and valproate-induced hepatotoxicity occurs in one in 500–800 cases in young children under the age of 2 years receiving valproate polypharmacy [75]. For felbamate, the risk for hepatic failure is estimated at one per 18,500–25,000 exposures (for a review, see reference [76]).

Several AEDs can induce life-threatening hematological adverse effects. In general, exposure to AEDs excluding patients on felbamate is associated with a nine times higher risk of developing aplastic anemia [77]. *Felbamate* is associated with the greatest risk of fatal aplastic anemia with an incidence of 127 per one million [78]. Risk of developing aplastic anemia in patients with felbamate is up to 20 times higher than in those with carbamazepine. Predictors of felbamate-induced aplastic anemia include immune diseases, especially lupus erythematosus, allergy, and prior cytopenia (for a review, see reference [76]). *Carbamazepine* has the highest potential for causing agranulocytosis [79].

7 Type C Effects

Chronic adverse effects are those that manifest after a prolonged period of exposure lasting from months to years. They progress slowly and although some of these effects are reversible, others can be irreversible. Most serious adverse effects belonging to this group are associated with exposure to enzyme-inducing AEDs. Among the most important adverse effects, type C effects are reproductive disorders in man, abnormalities in bone health, cardiovascular problems, and weight gain.

Long-term treatment with AEDs can cause decreased bone mineral density and increased risk of fractures. A study by Pack

[80] revealed that epilepsy patients have a greater risk of fracture than the general population, and osteopenia or osteoporosis was detected in 38–60% people with epilepsy (for a review, see reference [81]). The risk is particularly high in enzyme-inducing drug exposure. Changes seen after exposure to these drugs are related to their hepatic enzyme inducting properties which are responsible for accelerated degradation of vitamin D. Negative effects on bone mineral density are associated primarily with administration of phenobarbital and phenytoin. Valproate and carbamazepine can also have negative impacts on bone health, but data are inconsistent (for a review, see reference [82]).

AED therapy is often associated with endocrine adverse effects. Sexual dysfunctions, reproductive disorders, and changes in hormone levels represent common problems among epileptic patients. The most robust effects on sexual hormone levels were reported in patients treated with enzyme-inducing AEDs primarily with *phenytoin* and *carbamazepine*. Enzyme-inducing AED exposure is associated with acceleration of the breakdown and production of sex hormone-binding globulin (SHBG). This results in increased SHBG and reduced levels of biologically active estrogen and androsterone. Additionally, reduced levels of serum dehydroepiandrosterone sulfate (DHAES) have been reported in men and women taking *phenytoin* and *carbamazepine* (for a review, see reference [83]). *Valproate* does not induce liver enzymes, but it does reduce serum gonadotropin levels, probably through direct central effects [84]. In addition, valproate, but not carbamazepine, was found to affect semen morphology and motility [85]. In women with epilepsy, administration of valproate is associated with the highest risk of endocrine and reproductive problems. These women have significantly more menstrual disorders than controls, and these were frequently associated with polycystic ovary syndrome and/or hyperandrogenism, which were detected in 70% of women receiving valproate, 20% of *carbamazepine*-treated women, and 19% of women acting as controls [86]. In addition, results of several studies in pubescent girls treated with valproate indicate that young ovaries are more susceptible to long-lasting endocrine changes (for a review, see reference [83]). *Lamotrigine* and *levetiracetam* were not found to cause endocrine disturbances or reproductive problems, but data on levetiracetam are still inconclusive [84]. There is growing evidence that *topiramate* administration can cause sexual dysfunction. Data concerning other newer AEDs are sparse and randomized studies are still lacking (for a review, see reference [83]).

In addition to effects on sexual hormones, some AEDs can also affect thyroid function. In spite of their clinical importance, these adverse effects are only rarely mentioned. Significant alteration of thyroid functions has been reported in patients treated long-term with *carbamazepine oxcarbazepine* and *phenytoin*, but not with valproate [87, 88].

Changes in bodyweight represent another typical type C effect of AEDs. Weight gain is typically reported in patients treated with valproate, gabapentin, pregabalin, vigabatrin, retigabine, and, to a lesser extent, carbamazepine. Weight gain poses a serious health hazard and carries an increased risk of hypertension, type 2 diabetes mellitus, and dyslipidemia [89]. It also increases risk of non-adherence or discontinuation of treatment. In contrast, topiramate, zonisamide, felbamate, stiripentol, and rufinamide can cause weight loss, which is potentially beneficial in overweight or obese individuals [90].

Vigabatrin-induced restriction of the bilateral visual field is another serious type C adverse effect of AEDs. Loss of the visual field is irreversible and was reported in 44% of adult and 34% of pediatric patients. Risk factors involve cumulative dose and increasing age (for a review, see reference [91]).

8 Type D Effects

Through clinical experience and pregnancy registries, it is known that children of women with epilepsy are at increased risk of congenital defects and neurodevelopmental deficits. While a variety of problems can contribute to neurodevelopmental problems in children of women with epilepsy, AEDs appear to play a major role. Possible teratogenic effects of AEDs are a major concern in women of childbearing potential treated for epilepsy. In addition to increased risk of serious birth defects, the risk for mental retardation related to intrauterine growth retardation, reduced head circumference, and other factors was also reported (for a review, see reference [92]). A relatively high risk of congenital malformations and neurodevelopmental problems are associated with exposure to old, enzyme-inducing AEDs. Their teratogenic potential is determined by the chemical attributes of the AEDs and also by the genetic attributes of the patient. Drug metabolism plays a critical role in teratogenesis because not only maternal compounds but also intermediate, active metabolites can be responsible for teratogenic effects. For example, drugs containing an aromatic ring, such as carbamazepine or lamotrigine, are converted to a reactive epoxide or arene oxide that can interact with macromolecules to produce toxicity (for a review, see reference [93]).

Risk of congenital malformations varies across AEDs, with *valproate* and *phenytoin* carrying a higher risk than carbamazepine, phenobarbital, and lamotrigine. According to the most recent data from the Australian Pregnancy register [94], valproate monotherapy is associated with a five- or sixfold risk of fetal malformation and carbamazepine monotherapy with a little over twofold increase relative to that in pregnancy not exposed to AED. Exposure to lamotrigine in monotherapy was not associated with a significantly higher risk of malformations than other newer AEDs taken as monotherapy. Polytherapy carries increased risk of birth defects especially when valproate is used.

In addition to increased risk of fetal malformations, many studies also report neurodevelopmental deficits in children of mothers with epilepsy suggesting behavioral teratogenicity of AEDs (for a review, see reference [92]). Most frequently, cognitive impairment, learning difficulties or behavioral impairment are mentioned, but methodological differences and variability in patient populations are probably responsible for inconsistencies among the data. Additionally, there are many other variables that can influence the neurodevelopmental outcome of children born to epileptic mothers such as generalized tonic-clonic seizures or genetic factors (for a review, see reference [95]). However, it is apparent that not all AEDs are the same with regard to behavioral teratogenicity. The highest risk of unfavorable neurodevelopmental outcome is associated with fetal exposure to *valproate*. The prospective NEAD study demonstrated significantly lower IQ in children exposed to *valproate* in utero compared to those exposed to phenytoin, lamotrigine, or carbamazepine. Association between IQ and valproate was dose dependent [96–98]. Poor cognitive outcome was also reported in children exposed to *phenobarbital* and exposure involving the last trimester was the most detrimental [99]. Although risk appears to be lower than with valproate, cognitive deficits were also reported in children exposed to *phenytoin* [100]. Whereas the data are contradictory, exposure to carbamazepine or lamotrigine appears to confer only a low risk of neurodevelopmental problems.

9 Type E Effects

Adverse reactions due to drug interactions in epilepsy patients are relatively common and usually clinically important. Most AEDs affect the activity of drug-metabolizing enzymes or are substrates for the same enzymes; therefore, pharmacokinetic drug interactions are particularly important. Pharmacokinetics concern processes related to drug absorption, distribution, metabolism, and elimination from the body. Consequently, interactions can occur at all of these levels. If the drug has pharmacologically active metabolites, then interaction may involve both the parent drug and these metabolites.

Drug interactions associated with distribution closely relate to the degrees of their binding to blood albumins. Any interactions are likely to occur when at least 90% of drug is protein bound. *Phenytoin*, *valproate*, and *tiagabine* fulfill this criterion, whereas *gabapentin*, *pregabalin*, *vigabatrin*, and *ethosuximide* are not considerably protein bound [101, 102]. Displacement of the drug from the protein-bound state may lead to an increase in its free plasma levels. Because many AEDs have a narrow therapeutic index, increased levels of free drug can increase the risk of toxic effects.

Metabolic interactions most frequently occur at the level of cytochrome P-450 (CYP) or UDP-glucuronosyltransferase (UGT).

The CYP pathway is involved in the metabolism of first-generation AEDs such as *carbamazepine*, *phenobarbital*, *primidone*, and *phenytoin*. Among newer AEDs, the CYP pathway plays an important role in the metabolism of *felbamate*, *topiramate*, *tiagabine*, and *zonisamide*. The UGT pathway metabolizes *lamotrigine* and *valproate*. *Carbamazepine*, *phenobarbital*, *primidone*, and *phenytoin* are typical enzyme inductors, and they act as metabolic activators. They reduce the serum concentration and efficacy of a wide range of medications, including cardiovascular, psychotropic, antimicrobial, and antineoplastic drugs, as well as oral contraceptives or immunosuppressants. For example, carbamazepine enhances the metabolism of warfarin [103]. On the other hand, some drugs, such as *valproate*, act as CYP inhibitors. They may cause an increase in plasma levels of parent drugs or their toxic metabolites and consequently enhance the risk of toxic effects. Concomitant administration of valproate and carbamazepine increases serum level of carbamazepine-10,11-epoxide, an active carbamazepine metabolite, which is responsible for most of its serious toxic effects (for a review, see reference [103]).

With regard to the UGT pathway, *oxcarbazepine* is a UGT activator that accelerates metabolism of lamotrigine. On the other hand, *valproate* is a UGT inhibitor, and it may affect the metabolism of lamotrigine (for a review, see reference [103]). Although most of the newer AEDs have a significantly reduced potential for drug interactions, they are not free of type E adverse effects. Importantly, *oxcarbazepine*, *lamotrigine*, *felbamate*, *topiramate*, and *rufinamide* can reduce serum levels of oral contraceptives [104].

Interactions at the level of renal excretion are unlikely to happen among AEDs; however, interactions with other drugs excreted in the same way cannot be excluded (for a review, see reference [103]).

Pharmacodynamic interactions at the site of action have also to be considered in epilepsy treatment. In contrast to pharmacokinetic interactions, pharmacodynamics interactions are not associated with changes in the serum or brain levels of combined drugs. Pharmacodynamic interactions assume summation or even potentiation of drug receptor or non-receptor effects (for a review, see reference [102]). In particular, co-prescription of AEDs with the same mechanisms of action can lead to summation/potentiation of their neurotoxic effects (for a review, see reference [105]).

10 Type F Effects

Despite its benefits, stopping AEDs in seizure-free patients is associated with the increased risk of seizure recurrence for up to 2 years compared with continued treatment. Furthermore, the outcome of treating a seizure recurrence in patients who have been seizure-free for years may be surprisingly poor (for a review, see reference

[106]). However, patients chronically treated with AEDs are at risk of serious adverse effects, including possible teratogenic effects (for a review, see reference [107]).

Abrupt discontinuation of AEDs carries a particularly high risk of serious withdrawal reaction frequently involving frequent seizures, status epilepticus, and psychiatric withdrawal symptoms. Antiepileptic drug non-adherence is a common cause of SE across all ages, particularly in children and adolescents. Prompt and reliable recognition of non-adherence is imperative for correct management (Lie et al. [108]).

11 Conclusion

The long-term safety of AEDs primarily depends on their systemic and metabolic effects and on the genetic predisposition of the host. Adverse effects of AEDs are common, frequently contributing to treatment failure. They have also emerged as one of the strongest predictors of impaired quality of life, independent of seizures. However, the majority of adverse effects of AEDs are predictable. Patient education can therefore substantially decrease the risk of developing long-term, serious adverse events. Overtreatment should be avoided to minimize risk of dose-dependent adverse effects. Pharmacoepidemiological resources can help to identify the individual profiles of patients at high risk of specific adverse effects. Additionally, the successes in pharmacogenomics may help to select patients with increased risk of serious, life-threatening toxic effects such as Stevens-Johnson syndrome of toxic epidermal necrolysis. In addition, assessment of potential risks should include age, sex, childbearing potential, and presence of somatic and psychiatric comorbidities. Introduction of new AEDs with new mechanisms of action provides better opportunities for individually tailored pharmacotherapy for each patient to maximize efficacy and to minimize the risk of adverse events. New-generation AEDs are not free of adverse effects, although they typically have better tolerability and reduced potential for drug interactions.

Despite progress made in the safety of AEDs, new AEDs with fewer adverse effects and better efficacy than the currently available drugs are still needed. Treatment that would prevent or favorably modify processes leading to the development of epilepsy in patients with a known risk due to genetic predisposition or brain injury is an unmet need in epileptology.

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Chapter 18

Network Pharmacology and Epilepsy

Alan Talevi

Abstract

In contrast with the reductionist "one gene, one target, one drug" approach, network pharmacology proposes the use of multi-target therapies, a strategy that seems particularly suitable to treat disorders of complex etiology, among them epilepsy. As a matter of fact, most of the existing antiepileptic drugs are indeed multi-target unintended agents. Whereas a number of authors have recently advocated the use of network-based approximations in the antiepileptic drug discovery field, such strategy has so far not produced deliverables. Here, we review some practical considerations which could be used to assist in silico and wet screening for novel antiepileptic agents.

Key words Epilepsy, Network pharmacology, Systems biology, Multi-target drug, Tailored multi-target drug

1 Introduction

Some decades back, the pharmaceutical sector adopted a target-driven, reductionist approach to drug discovery. The idea was appealing: highly selective agents interacting with (and only with) a validated target would avoid off-target interactions, representing safer therapeutic solutions. Furthermore, the target-driven approach allowed the implementation of rational drug design campaigns and the bioethical (and cost-efficient) replacement of low-throughput animal models by high-throughput in vitro models as primary screening platform. Whereas such clean drugs seem well suited to find therapeutic agents to treat Mendelian disorders where a single gene is associated to the disease, they have generally shown limited efficacy for the treatment of complex disorders (e.g., cancer, psychiatric and neurological disorders). The former reductionist approach has once again been proposed as one of the possible explanations for the decline in productivity in the pharmaceutical sector [1–3], a reality that particularly hits the field of central nervous system (CNS) pharmacology [4, 5]. Curiously,

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the number of first-in-class small molecule drugs emerging from phenotypic screening seems to outnumber those from target-centered discovery [6].

From a systems biology viewpoint, living organisms are understood as robust entities, and disease can also be considered a robust state emerging from multiple and simultaneous perturbations of a resilient system [2]. Recently, a number of authors have discussed that epilepsy, being a multifactorial, polygenic, and dynamic disorder, could be particularly suited to be approached through network pharmacology [7–12]. Such belief is supported by the fact that, while it is currently thought that highly selective agents could be useful to treat specific syndromes [9], most of the existing antiepileptic drugs (AEDs) are actually fortuitous multi-target drugs which have emerged from phenotypic screening [8, 11]. On the other hand, a number of studies in animal models of seizure and epilepsy suggest that the combination of drugs associated with different mechanisms of action tends to enhance the efficacy of the treatment [9, 13–16]. At least, contrary to the general bioethical trend, in vivo models remain the primary screening assay to identify AEDs, which underlines the complex nature of the disorder. Whereas the majority of primary assays used within the pharmaceutical industry for the early drug discovery rely upon the creation of stable mammalian cell lines or upon the overexpression and purification of recombinant proteins to establish biochemical assays [17], the absence of immortalized cell lines mimicking the epileptic condition precludes this possibility. And while in vitro AED screening using animal or human ex vivo tissue (brain slice preparations) is also possible [18–21], such approach is limited by tissue availability, the rarity of spontaneous epileptiform activity in the tissue, and the viability of the tissue (a limitation that has lately been partially overcome thanks to the advances of organotypic culture techniques) [22].

Having said so, it is very surprising that the network pharmacology approach has yet not been fully embraced within the AED discovery field, which seems to have fallen a little behind in comparison with other complex disorders such as cancer or Alzheimer's disease. Stressing the previous statement, Fig. 1 displays the evolution over time (according to Scopus) of the number of scientific articles in periodicals that present the terms "multi-target" and "epilepsy" within their title, abstract, or keywords, in comparison with the coappearance of "multi-target" and "cancer" and "multi-target" and "Alzheimer." The previous observations are in agreement with the opinions of leading experts in the epilepsy field, who have stressed the urgent need of innovative approaches for AED discovery [9, 23]. While some recent articles have beautifully reviewed the experimental and theoretical basis that support adopting systemic, integrative approaches for AED discovery, here we will discuss some practical considerations for the implementation of this paradigm shift.

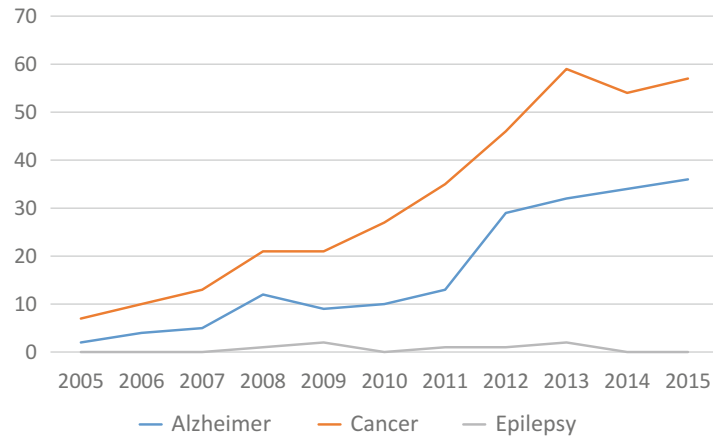

Fig. 1 Frequency of co-occurrence of the terms "multi-target" and "Alzheimer," "cancer," or "epilepsy" in scientific publications (2005–2015, Scopus). Whereas the relative frequencies could also reflect the comparative interest shown for each condition by the drug discovery sector, the epilepsy community seems to have embraced the network pharmacology paradigm more slowly

2 Tailored Multi-target Agents

Before target-focused drug discovery, new lead compounds emerged from serendipitous discovery, traditional medicine, and phenotypic/physiologic screening in cellular or animal models of disease. Though it was possible to find multi-target agents through such approaches, those targets were, hopefully, defined a posteriori, and the combination of targets attacked was unplanned and sometimes not fully known.

In the discussion on target- versus phenotypic-based strategies (or reductionist versus integrative approaches), tailored multi-target agents can be regarded as the dialectical synthesis that pick the best out of each paradigm. Multi-target therapies are an extension of the target-centered approach that incorporates the viewpoint of network pharmacology. Tailored (or designed) multifunctional agents are deliberately devised to selectively modulate a number of chosen targets, usually relying on computer-aided design and data analysis applications and simplifying the expensive target deconvolution. Theoretically, multi-target agents are equivalent to combined therapies with different single-target agents, but they are advantageous in terms of diminished chances of drug interactions, simpler pharmacokinetics, and improved patient compliance [24].

Multi-target agents could be a viable solution to deal with drug-resistant epilepsy linked to acquired or constitutive target modifications [25]. They could also be specifically designed to

address, in a simultaneous manner, the signs and symptoms and underlying causes of the disease. For example, it is suspected that seizures and inflammation take part in a complex interplay that results in a vicious circle [26, 27] where inflammation would be both cause and consequence of seizures. Clinical evidence shows that steroids and other anti-inflammatory treatments display anticonvulsant activity in some drug-resistant epilepsy syndromes, and some of the most widely used AEDs (e.g., valproate) have proven anti-inflammatory effects [28]. It could be guessed that a combination of anti-inflammatory and anticonvulsant properties in a single molecule could have a positive impact on epilepsy management. The preclinical evidence on the effects of anti-inflammatory agents in epilepsy is reviewed in a separate chapter of this same volume. Similarly, due to the high prevalence of comorbid mood disorders (mainly, anxiety and depression) in epileptic patients [29, 30], the simultaneous treatment of core and comorbid manifestations of epilepsy constitutes an additional potential application of multi-target agents in epilepsy management. Remarkably, many widely used antiepileptic drugs have shown efficacy against different psychiatric conditions, as clearly reviewed in Chap. 17 by Kubova.

2.1 Practical Considerations to Apply in the Design of Antiepileptic Multi-target Drugs

Etymologically, the word *pharmacophore* comes from the Greek and means "cure carrier" or "medicine carrier." Presently, it alludes to an abstract (geometrical) description of molecular features which are necessary for molecular recognition of a ligand by its molecular target. In other words, the pharmacophore is the molecular framework which is essential to elicit a biological response. A multi-target drug must either combine different pharmacophores in a single molecule (one for each specific recognition event intended) or present a common pharmacophore for different targets (which implies that the different targets display shared determinants of specificity). That is, different degrees of pharmacophore overlapping can be found in multi-target drugs [31] (Fig. 2).

Those multi-target drugs that use different sets of atoms (anchors) to interact with each target protein tend to violate drug-like criteria, presenting oral bioavailability issues [7, 32, 33]; understandably, the chance of violating drug-likeness rules and endangering bioavailability increases with the number of separate anchors. Thus, if designing multi-target drugs, watch carefully for violations of more than one of Lipinski's rules (no more than five H-bond donors, no more than ten H-bond acceptors, molecular mass below 500 g/Mol, and calculated Log*P* below 5) [34], and check if Veber's rules are accomplished (ten or fewer rotatable bonds and a polar surface area below 140 square Ångström) [35]. Note that, owing to the more challenging diffusion barrier posed by the blood–brain barrier, the physicochemical properties required to achieve brain bioavailability are even more stringent than those

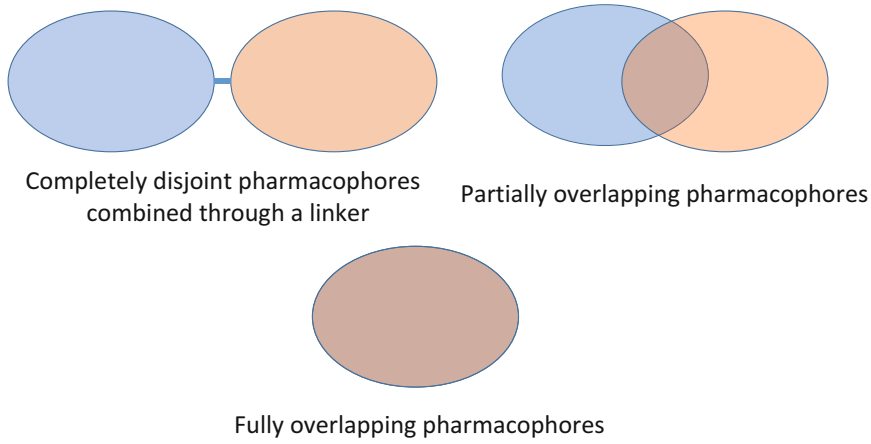

Fig. 2 Multi-target agents can display different degrees of merged pharmacophores

needed to attain oral bioavailability. Accordingly, the biopharmaceutical properties of multi-anchor ligands intended for the treatment of epilepsy or other central nervous system conditions must be specially watched. As discussed elsewhere in this volume, the very interesting work from Wager and coworkers presents a central nervous system multiparameter optimization approach that delivers a desirability score that can be especially useful to assist the design of multi-anchor multi-target AEDs on the basis of readily computable physicochemical properties [36]. The score is easily computed by calculating six theoretical properties, most of which are available in almost every modern chemoinformatics software package and also frequently provided by public chemical databases as PubChem or ZINC: calculated partition coefficient (clog*P*), calculated distribution coefficient at pH = 7.4 (clog*D*), molecular mass, number of H-bond donors, topological polar surface area, and the pKa of the most basic center. A direct relationship between Wager's desirability score and key in vitro attributes (absence of permeability issues, P-glycoprotein efflux, safety, metabolic stability) has been observed in marketed central nervous system drugs as well as Pfizer's candidate set. Remarkably, almost all the approved AEDs present high desirability scores (around 5).

Ligand efficiency metrics are another aspect that should not be disregarded during the design of multi-target agents [7, 33]. The empirical analysis by Juntz et al. revealed that, across a wide variety of ligand-macromolecule complexes, maximal contributions to binding free energy per ligand non-hydrogen atom are similar to −1.5 kcal/mol; the authors also observed a trend to a smaller free energy contribution per atom as the molecular mass of the ligand increases [37]. Ligand efficiency metrics have gained increasing acceptance within the drug discovery community, with retrospective analysis of recently marketed oral drugs showing that they usually have highly optimized ligand efficiency values [38]. In the case

of the bianchor agents, it might be speculated that efficiency metrics will tend to be low since only a fraction of the molecule participates in each independent binding event between ligand and each molecular target. Consequently, the density of efficient contacts between the drug and the targets is expected to be low. Merged pharmacophores may serve to solve the binding efficiency and bioavailability issues that characterize fragment-based approximations to multi-target drugs.

A number of physicochemical molecular properties could be tuned to promote promiscuity. Several reports suggest that ligand promiscuity is inversely related to molecular weight [39–41], but others have failed to find a correlation or have even shown an opposite trend [42, 43] suggesting that the relationship between molecular mass and promiscuity might be context dependent [44]. Sturn et al. observed a class of multi-target compounds that they called "superpromiscuous," which could bind to nonhomologous targets and shared some of the atoms involved in direct interactions with each target [39]. Curiously, these superpromiscuous ligands tend to present either low or high complexity. Direct correlations have also been found between promiscuity and calculated log*P* [43, 44]. Bases and quaternary bases are markedly more promiscuous than acids, neutral compounds, or zwitterions [43]. The molecular topology can also influence promiscuity: the number of rings and the fraction of molecular framework (f_{MF}) have also shown to be directly correlated with promiscuity at least for large (above 0.65) molecular framework values [43, 44]. The f_{MF} is defined as the atom of heavy atoms in the molecular framework (MF) divided by the total number of heavy atoms in the molecule. In other words, a smaller molecular framework and more side-chain atoms will improve selectivity. A clear (and graphical) definition of the molecular framework can be found in the original work by Bernis and Murcko [45].

In the particular case of AEDs, our group, while looking for quantitative structure-activity relationships (QSAR) to identify anticonvulsants with activity in the maximal electroshock seizure (MES) model, observed that compounds with anti-MES effects tend to be small and display low complexity [46]. Figure 3 shows the distribution of molecular mass for AEDs approved between 1912 and 2012. Observe that the average molecular mass is 243.5, with a standard deviation of 70. If we compare these data with the average values of molecular mass for the compounds patented by 18 big pharmaceutical companies between 2000 and 2010, across the major drug-target classes [47], we will observe that the average molecular mass for approved AEDs is considerably lower (note that a definite trend toward higher molecular mass and clog*P* values, within Lipinski's rule limits, has been observed between 2000 and 2010, when the target-centered approaches still prevailed). Interestingly, Yang et al. [44] have studied the

interplay between promiscuity, log*P*, and molecular topology. They defined four different topological classes according to the number of terminal ring systems and the presence of a molecular bridge. The "one terminal ring system" (1TR) class includes molecules with only one ring system. The "two terminal ring systems" (2TR) contain molecules with two ring systems directly connected to each other. The 2TR + B class comprehends molecules with two terminal ring systems and a molecular bridge. Finally, the 3TR + B class contains molecules with three terminal ring systems and a molecular bridge. While, in general, promiscuity showed an uptrend from simpler (1TR) to more complex (2TR + B) motifs, the topology class did not influence promiscuity for compounds with clog*P* values ranging from 1 to 3. In the case of approved AEDs, Fig. 3 also shows that, for most AEDs, the f_{MF} tends to be high (with some exceptions, e.g., aliphatic compounds such as valproic acid or pregabalin). About 80% of the approved AEDs belong to the simpler topological classes (TR or 2TR), which a priori suggests a reduced tendency to promiscuity, but they also show, in most cases, clog*P* values in the range of 1–3 (Fig. 3), which is optimal for passive diffusion through the blood–brain barrier and where the degree of promiscuity seems to be indifferent to the topological class (with some remarkable exceptions like vigabatrin, tiagabine, or perampanel, which emerged in the context of the target-centered paradigm).

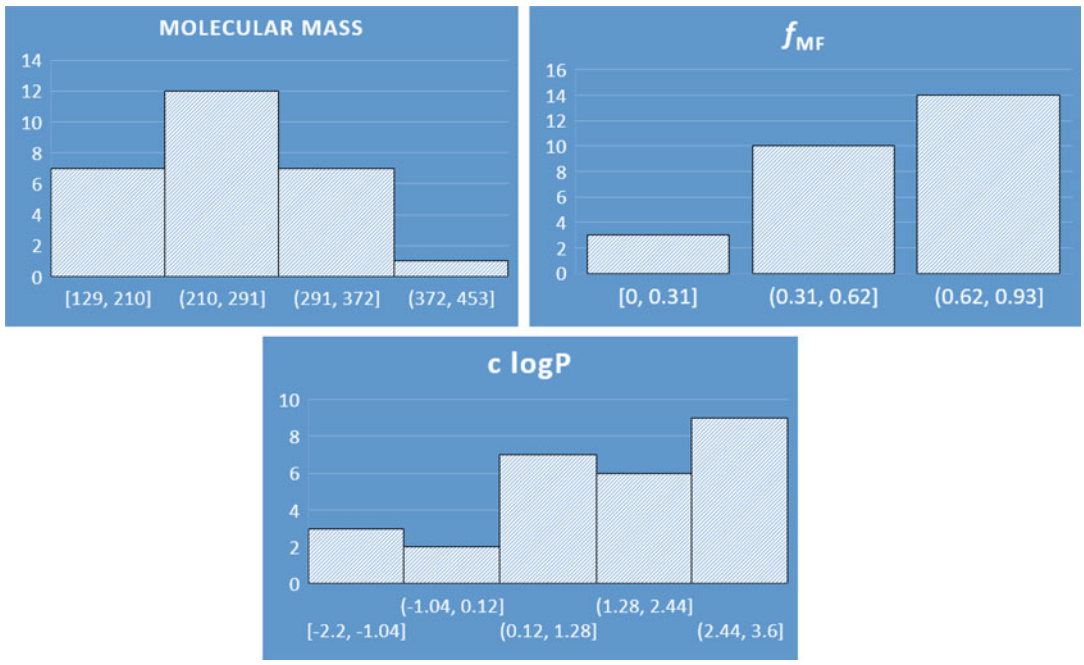

Fig. 3 Molecular mass, fraction of molecular framework and clogP distribution for AEDs approved between 1912 and 2012. The analyzed drugs have been extracted from ref [9]

The previous analysis confirms our observations and provides some keys to the proven promiscuity of most AEDs: low molecular mass and low complexity, high f_{MF}, and an adequate interplay between clog*P* and molecular topology. The analysis also suggests that novel scaffolds should simultaneously explore the 3TR+B topology and higher lipophilicity, while keeping high f_{MF} values (i.e., few side chains), which represents an unexplored and promising region of the chemical space for AEDs.

Regarding virtual screening campaigns focused on multi-target agents, one should remember that application of independent models to identify multi-target agents is expected to yield lower *positive predictive values* than virtual screening campaigns focused on single-target drugs [4, 32, 33, 48]. If it is assumed that being a ligand for one of the intended targets does not enhance or reduce the probability of being a ligand for another one (a situation that corresponds to nonoverlapping pharmacophores), each model applied in the virtual screening process works as a structural restriction that filters out all the molecules that do not accomplish the model requirements; subsequently, finding chemical compounds accomplishing all the model structural constraints becomes increasingly difficult as the number of target increases. Having this in mind, when optimizing the score thresholds, it is advised to gain sensitivity at the expense of specificity (be thus prepared to observe higher false-positive rates: such strategy results in an increment of experiment-related costs due to reduced active enrichment).

Also keep in mind that the pharmacophores correspondent to two targets could be mutually exclusive. Some pharmacophoric features are irreconcilable (they cannot coexist in the same point of the molecule, e.g., a charged moiety and a lipophilic one), while others are not (e.g., negative charge and H-bond acceptor). Choosing the pursued targets on the basis of empirical or theoretical evidence on common determinants of specificity (resulting in overlapping ligand specificity due to common pharmacophores) could be a good advice to expand the likelihood of success (remember that multi-target ligands with merged pharmacophores are preferred from the ligand efficiency and bioavailability perspectives). Bioinformatics tools capable of detecting protein coevolution [49] can be useful for detecting molecular targets with similar binding sites.

2.2 The Most Potent, the Better?

From a network pharmacology standpoint, attacking hubs (highly connected nodes in a biochemical network) may not be the best strategy, particularly if we are targeting sensitive organs like the brain. Designing low-affinity multi-target drugs to modulate multiple nonessential nodes nearby key nodes seems a more rational approach to restore the network to its normal functioning without serious toxic effects that could otherwise be expected when

blocking a key node [50]. As openly voiced by Bianchi and coworkers, "the complexity of neural processes underlying seizure activity may be more amenable to multiple small perturbations than a single dominant mechanism" [11]. At this point it may be worth highlighting the difference between potency and efficacy. Potency is related to the amount of drug that is needed to produce a given effect and is related to the affinity of the drug for its molecular target(s) and the number of units of the molecular target(s) available. In contrast, efficacy is linked to the maximum effect that a drug can produce, regardless of dose. From these definitions, it follows that *a drug could be more efficacious than others without being more potent*.

Memantine represents an outstanding example of the potential benefits of low-affinity multi-target ligands on CNS disorders [51, 52]. This drug is presently prescribed for the treatment of moderate to severe Alzheimer's disease and other dementias when acetylcholinesterase inhibitors are not well tolerated. In contrast, high-affinity uncompetitive inhibitor of the *N*-methyl-d-aspartate receptors (NMDARs) dizocilpine has not reached the market due to serious adverse reactions including Olney's lesions, cognitive disruption, and psychotic reactions. Memantine possesses low-affinity binding to NMDARs (in the high nM to low μM range), fast on/off kinetics, and almost no selectivity among subtypes [51], being consequently much better tolerated. Memantine also shows uncompetitive antagonism on other receptors, e.g., serotonin 5-HT3 [53] and dopamine D2 receptors [54], with similar affinity than for the NMDA receptors.

The old paradigm (the more potent, the better) still prevails in the primary screening for novel AEDs. The NIH's Anticonvulsant Screening Program considers the potency of drug candidates in acute seizure models as one of the criteria to select which drug candidates will advance to further testing [55]; this decision-making scheme may underestimate the efficacy of the drugs in on a long-term basis [56]. An equivalent principle is often applied in the context of some computer-aided screening campaigns (e.g., those based on docking and regression models), where the hits with higher predicted affinities are more likely to be selected for experimental validation. In the light of this paradigm shift, it could be appropriate to discard mere potency as selection criteria of AED candidates, preferring drugs with multiple (small) actions on different targets. Protein network analysis could prove useful to reveal weakly and moderately connected nodes as potential new targets for epilepsy therapies. In this line, it has been stressed that levetiracetam (a new generation AED that enjoys increasing attention within the epilepsy community and perhaps the third generation AED with the most innovative pharmacologic profile) exerts various mild modulatory actions on neurons [8].

2.3 What Response Should Be Modeled When Building Computational Models to Detect Novel AEDs

In the background of classical QSAR theory and more reasonably in the case of 3D QSAR, the compound dataset used to build and validate the model should present a common mode of action and even the same binding mode [57–59]. Alignment-dependent 3D QSAR methods have been conceived to describe one specific interaction step in the lifetime of ligands [57]. Obviously, the same principle applies for molecular docking and other structure-based approaches that explicitly predict the ligand-target interaction. Consequently, in vitro affinity data could be considered the gold standard for traditional QSAR modeling. However, considering the possible benefits of network pharmacology in the field of epilepsy and as an alternative to tailored multi-target agents, it is possible to detect novel antiepileptic drugs by modeling "dirty" responses obtained in phenotypic/physiologic models, instead of "clean" affinity data. Molecular descriptors reflecting more general structural patterns than those required for the specific ligand-recognition event by a certain target could be probably more suitable for this job. Many of the successful QSAR models and virtual screening applications for the discovery of AEDs have relied on in vivo biological data for modeling purposes [46, 60–70], and they include work by leading experts in the QSAR field [60].

Since most of the previously cited articles report models to predict the effect of a drug in seizure models (prominently, MES test), the current challenge is probably to face modeling campaigns based on biological data obtained from actual models of epilepsy (e.g., pilocarpine and kindling models), refractory epilepsy, and acute models so far understudied through the QSAR theory (e.g., 6 Hz test).

3 Comparing Gene-Wide Profiles

It is now known that drugs, particularly those administered in a repeated manner (e.g., AEDs), do not only provoke a given biological response in a direct way; they also indirectly elicit regulatory effects on gene expression profiles which could be even more relevant than the direct drug-target interaction.

Gene expression profiles offer a snapshot of globally measured transcript levels in a given cell, tissue, or organism at a specific point of time [71]; gene signatures are representative of specific conditions, i.e., exposure to a given xenobiotic or a disease state. Gene signatures are particularly relevant to characterize the phenotypic response to long-term exposure, shed light about the modes of action of a drug, and identify potential treatments for a certain disorder. The Broad Institute has pioneered such applications through its Connectivity Map, a publicly available resource designed to link disease and drugs through gene profiles [72]. This resource collects gene signatures derived from the exposure of human cells to a huge number of xenobiotics, including 1300

FDA-approved drugs. Query signatures can be compared to the stored ones through similarity matching algorithms: those at the top and bottom of the resulting ranking are thought to be related to the query state by shared or opposite expression changes. Compounds eliciting similar expression changes would exacerbate such condition; compounds displaying inverse signatures would function as therapeutic agents.

A diversity of microarray-based gene expression profiling studies have been conducted to elucidate the molecular changes underlying epilepsy and epileptogenesis [73–77]. They and other similar works could be a fair starting point to find novel drugs with disease-modifying properties by application of the inverse similarity idea proposed by the Connectivity Map.

4 Final Remarks

In spite of recent authoritative opinions on the potential contribution of network pharmacology to the development of more efficacious AEDs, such considerations have so far not been translated into new drug candidates, contrasting advances in other complex diseases such as neurodegenerative conditions or cancer.

We have presented four strategies to incorporate a network pharmacology perspective in the field of AED discovery: tailored multi-target agents, reexamining the validity of "the more potent, the better" paradigm, building QSAR models based on biological responses emerging from phenotypic models, and gene signature comparison.

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Chapter 19

The Importance of Drug Repurposing in the Field of Antiepileptic Drug Development

Alan Talevi

Abstract

Drug repurposing involves finding new therapeutic uses for existing drugs, including marketed, discontinued, shelved, and investigational drugs. The advantages of this strategy are many: reduced drug development timeline, best chances of surviving Phase II and Phase III clinical trials, simplified dosage form development, and, of course, added value to drugs belonging to a company or laboratory portfolio. The relatively high number of antiepileptic drugs which have been successfully repurposed and the fact that many drugs from other categories have proven anticonvulsant effects at the preclinical and even clinical level suggest that this strategy should be widely exploited in the antiepileptic drug discovery field. Here, we present an overview of the current approaches to drug repurposing, along with practical considerations to face a drug repurposing campaign to find new treatments for epilepsy and novel therapeutic uses for antiepileptic drugs.

Key words Computer-guided drug repurposing, Drug repositioning, Drug reprofiling, Drug repurposing, Drug rescue, Epilepsy, Indication expansion, Indication shift, *In silico* drug repurposing, Off-label use

1 Introduction

Drug repurposing (also known as drug reprofiling, drug repositioning, indication expansion, or indication shift) involves finding new therapeutic uses for existing drugs, including marketed, discontinued, shelved, and investigational drugs. Repurposed drugs present considerably higher probability of success (at preclinical and clinical level) than de novo drugs and a reduced development timeline [1, 2]. Repurposed candidates have already survived preclinical toxicological testing; they have verified tolerable safety and possess acceptable, previously characterized pharmacokinetics. When repurposed drugs have already been used in the clinical practice, manufacturing and stability issues have already been solved; furthermore, off-patent repurposed drugs may provide relatively low-cost solutions for new problems [3].

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Plenty approved antiepileptic drugs (AEDs) have also been approved for second or further medical uses and/or possess different off-label uses [4–8] (see Table 1 for a summary of other approved uses and the most frequent off-label uses). This suggests

Table 1
Other approved and off-label uses of AEDs

Antiepileptic drug	Other approved uses	Off-label uses
Phenobarbital	Sedative (Short-term) Hypnotic Preanesthetic	Crigler-Najjar syndrome (type II) Gilbert's syndrome Neonatal abstinence syndrome Cycling vomiting syndrome
Phenytoin	–	Antiarrhythmic Neuropathic pain Wound healing and ulcers
Primidone	–	Essential tremors
Diazepam	Anxiety Skeletal muscle spasms Acute alcohol withdrawal	Eclampsia
Carbamazepine	Trigeminal neuralgia Glossopharyngeal neuralgia	Bipolar disorder Antipsychotic (adjunctive) Abuse substances withdrawal
Valproic acid	Bipolar disorder Migraine	Impulse control disorders
Clonazepam	Panic disorder	Anxiety Sleep disorders Restless legs syndrome Spasticity
Lamotrigine	Bipolar disorder	Trigeminal neuralgia Neuropathic pain Attention deficit Migraine Schizoaffective disorders

(continued)

Table 1
(continued)

Antiepileptic drug	Other approved uses	Off-label uses
Gabapentin	Postherpetic neuralgia	Neuropathic pain
		Anxiety disorders
		Nystagmus
		Smoking cessation
Topiramate	Obesity	Abuse substances withdrawal
		Essential tremor
	Migraine prevention	Obsessive-compulsive disorder
Zonisamide	–	Obesity
		Parkinson's disease
		Migraine
		Neuropathic pain
Tiagabine	–	Anxiety (adjunctive)
		Neuropathic pain (adjunctive)
Pregabalin	Neuropathic pain	Anxiety disorders
	Fibromyalgia	
	Postherpetic neuralgia	
Lacosamide	Neuropathic pain	–
Eslicarbazepine acetate	–	Trigeminal myalgia
		Bipolar disorder

that approved AED as well as AED candidates in the pipeline should be systematically examined for possible second uses, a strategy which could imply a significant added value for laboratories' portfolios. Note that many AEDs are persistently used to treat neurological and psychiatric conditions (e.g., neuropathic pain, anxiety disorders, bipolar disorders), which suggests that drugs belonging to this therapeutic class could be more likely to be repurposed from certain therapeutic indications.

In the next section of the chapter, we will review different approaches for the systematic, rational drug repurposing of AEDs.

2 Different Approaches to Rational Drug Repositioning

There are several approaches that can be used to propose second medical uses of existing drugs. The first of them involves serendipitous clinical (or, why not, preclinical) observations regarding possible unexpected beneficial effects of a drug. Such observations may arise in the regular clinical practice or in the framework of a clinical trial (as it happened in the famous sildenafil anecdote). Epidemiological retrospective studies could be an adequate methodological approach to reveal hidden additional benefits of existing drugs [9]. A second (and probably more rational) option is knowledge-based drug repurposing, in which chemical and pharmacological information on the drugs and pathophysiological knowledge on the diseases are examined to guide an indication expansion [10]. Owing to the unprecedented rate at which scientific knowledge is generated nowadays, computational data analysis approaches can provide a valuable support to organize information and gain knowledge which can in turn be used to guide repurposing campaigns. In the following subsections we will discuss some general aspects of computer-aided drug repurposing. Some universal considerations to keep in mind when selecting candidates for repurposing are also presented in Sect. 3.

2.1 Bioinformatics-Based Drug Repurposing

One of the general principles that support computer-aided drug repositioning is that health disorders linked to the same or similar dysregulated or dysfunctional proteins may be treated with the same drugs (disease-centric approach). Bioinformatic tools, from sequence alignment to domain similarity identification tools, might be useful to reveal hidden protein-protein similarities. While experts in a given disease are naturally familiarized with target proteins associated with their specific subject of study, they might well ignore which other diseases are linked to the same or closely related targets. There are many online public resources which could be used to find curated information on gene-disease associations. For instance, DisGeNET [11] is a discovery platform whose current version contains more than 429,000 associations, between more than 17,000 genes and 14,000 diseases, disorders, and clinical or abnormal human phenotypes (including both Mendelian and complex diseases). Given the large number of compiled associations the platform features a score based on the supporting evidence to prioritize gene-disease associations. Other interesting resources include the Comparative Toxicogenomics Database (CTD) [12], which provides information about interactions between environmental chemicals and gene products, and their relationship to diseases; the Online Mendelian Inheritance in Man (OMIM) [13], an online catalog of human genes and genetic disorders; and PsyGeNET [14], which focuses on associations between genes and

psychiatric disorders. Briefly, if you pretend to repurpose an AED for other indication and the molecular target/s linked to antiepileptic activity are known, you might want to find out which homologs (proteins sharing a common evolutionary ancestor) of such protein/s are associated with other disease. The simpler way of inferring homology is probably through sequence similarity; remember, however, that homologous sequences do not always share significant sequence similarity but are clearly homologous based on statistically significant structural similarity or strong sequence similarity to an intermediate sequence [15]. The previous fact suggests that using different approaches to find homologs could be convenient for a wider coverage. For local sequence alignments, like those produced by BLAST, the expected distribution of similarity scores by chance is described by the extreme value distribution. The expectation value depends on the size of the searched database, which does not mean that sequences can be homologous in one context (a smaller search) but not in another (large databases). If the alignment is significant in a small search the sequences are homologous, but that homology may not be detected in a larger search. In other words, sequence similarity search tools minimize Type I errors (i.e., false positives) but do not make claims about false Type II errors (i.e., false negatives). Consequently, it is easier to identify distant homologs when exploring smaller databases. Also note that protein:protein comparisons are more sensitive and accurate than DNA:DNA ones. To obtain highly curated and annotated sequences (e.g., the sequences that will be used as queries) the Swissprot subset of the UniProt database is recommended; it provides comprehensive information on modified residues, active sites, variation, and mutation studies that allow more accurate functional prediction from homologous alignments. As insinuated previously, homology identification based on sequence alignment can be complemented by structural homology approaches based on manual or automated comparison of 3D x-ray or NMR protein structures. For instance, Vector Alignment Search Tool (VAST) [16] is a computer algorithm developed at the US National Center for Biotechnology Information that identifies similar protein 3D structures by purely geometric criteria and can detect distant homologs which are omitted by sequence comparison. While this application is useful to work with individual proteins, its extension, VAST+, presents structural similarity at the level of biological assemblies or macromolecular complexes [17], a very attractive approach in the field of epilepsy if we have in mind that several AEDs such as ligand-operated ion channels are in fact macromolecular complexes of different proteins. Once homologous with suspected similar functions and conserved determinants of specificity have been found, the previously discussed public resources compiling associations between gene/gene products and disease can be used to propose new medical uses.

In relation with the previous discussion, it is worth mentioning that connections between ligand promiscuity and binding-site and global structure similarity have been established by Haupt and coworkers [18]. For that purpose, 164 ligands co-crystallized with three or more nonredundant targets were extracted from the Protein Data Bank. Such ligands were present in 712 nonredundant protein targets. All pairs of binding sites for all promiscuous drugs were aligned. Those sites with consistent binding mode of the ligand were kept for subsequent analysis, finding a direct correlation ($r = 0.76$) between the global structure similarity and the degree of promiscuity (drug target count), and also between the square root of the number of similar binding sites and the degree of promiscuity ($r = 0.81$). These results suggest that the binding-site similarity and the global structure similarity can be used as criteria to guide drug repositioning initiatives. Interestingly, a diversity of algorithms has been developed to identify, in an automated manner, similarities between binding sites. See for instance reference [19].

Another bioinformatics approach which can prove helpful to guide drug repurposing involves comparison of genome-wide gene expression profiles, e.g., through the Connectivity Map. The approach has been briefly discussed in another chapter of this volume dedicated to network pharmacology and epilepsy and the discussion will not be repeated here. The reader may also visit the excellent review by Qu and Rajpal [20]. For instance, by application of this approach Zhuo et al. recently revealed that the AED valproic acid is able to reverse acquired Erlotinib resistance of lung cancer [21], which has been validated experimentally. Similarly, Dudley and coworkers found, through application of the Connectivity Map, that the anticonvulsant topiramate is a potential therapy for inflammatory bowel disease [22]. These authors validated their prediction in vivo using a trinitrobenzenesulfonic acid induced rodent model of the disorder.

2.2 Cheminformatics-Based Drug Repurposing

The most common cheminformatic-based drug repositioning approach is no other thing than a very particular type of virtual screening campaign in which the screened chemical repository/database is focused on approved, discontinued, abandoned, and/or investigational drugs. The methods used in cheminformatic-based drug repositioning are thus classified in the same way that for general virtual screening approaches [23] and have been discussed in other chapters of this volume. Essentially, such classification includes structure- and ligand-based approximations. Lately, serial and parallel combinations of these methods have been extensively applied [24, 25]. DrugBank and Sweetlead constitute excellent resources to carry out *in silico* drug repurposing campaigns, since they compile approved, discontinued and investigational drugs and other regulated chemicals from the FDA and other national regulatory agencies [26, 27]. Note that it was recently observed that the

importance of bioactivation might have been underestimated in the context of *in silico* screening-based campaigns oriented to drug repurposing [28, 29]. Since around 10% of the known "drugs" are in fact unintended or intended prodrugs [30], it is suggested to take into consideration possible active metabolites of the potentially repurposed compounds during the screening protocol.

A different but conceptually interesting approach has been presented by Wu and coworkers [31], in line with previous work by Keiser and collaborators [32, 33]. The general idea behind their work is that different therapeutic indications could be related if each of them includes chemically similar drugs. The anatomic therapeutic chemical (ATC) classification system provided by the WHO Collaborating Centre for Drug Statistics Methodology was used as international standard to define the therapeutic indication. The authors used a 1151 FDA-approved drugs database to train a Cytoscape network. First, the chemical similarity between two drugs belonging to two different therapeutic classes under comparison is calculated by using three independent molecular fingerprinting systems: CDK, MACCS, and Pubchem fingerprints. Tanimoto scores are calculated by the three methods, and later averaged. An evaluating score is obtained by summing the Tanimoto score of all drug pairs. For each anatomic therapeutic chemical class, a Z score between it and the remaining classes is then computed. A permutation test is applied to obtain the Z score of each pair of therapeutic classes, in order to remove the influence of the drug set size and to assess the significance of the similarity sum. The permutated drug sets (with the same number of drugs than the original ones) are generated for each therapeutic class through random selection from the 1151 drugs. The distribution of the permutated scores is determined and the corresponding normal distribution is indicated. The μ and σ of the normal distribution of the permutated evaluating score values are used to calculate the final Z score of ATC pairs. Because the permutated evaluated score follows a normal distribution, it is considered that if the Z score for a given pair of therapeutic classes is above 1.96, such classes display a statistically significant correlation. Note that the previously described method is conceptually in good agreement with our previous observation on the high number of AEDs that have consistently proven to have effects on other disorders such as anxiety or bipolar disorders. Again, the regular efficacy of drugs belonging to a given therapeutic class on other disorders seems to speak of a possible pattern of repurposing opportunities between pairs of therapeutic classes, which encourages the systematic (and possible reciprocal) screening of drugs from one therapeutic class for the other. The approach reported by Wu could provide a rational basis to decide which pairs of therapeutic classes are more favorable to explore possible cross-repurposing.

A current trend in computer-aided drug repurposing consists in integrating large volumes of very heterogeneous types of data (e.g., experimental and predicted, chemical similarity and protein similarity) into large-scale drug protein networks or even drug-protein-disease networks [34–36]. While initially network analysis only considered the connectivity between the nodes of the network, recently much attention has been also given to semantic networks [10, 37].

Recently, a combination of bioinformatic and cheminformatic approximations led us to detect the anticonvulsant effects of artificial sweeteners cyclamate and acesulfame [38, 39], which in turn allowed us to identify the anticonvulsant effects of natural sweeteners (steviol glycosides) in mice [40]. Cyclamate has later shown selective inhibitory effects on one carbonic anhydrase VII, which has been proposed as a novel AED target [41].

3 Notes

Let us assume the potential of a number of drugs for repurposing has already been validated at some experimental level (e.g., in vitro). What features should a promising candidate gather? What criteria can be used to decide which candidate deserves more attention?

The first thing you should bear in mind is that, to fully exploit the advantages of repurposing, *the dose needed for the pursued (new) indication should be compatible with the ones used for the original indication*. Remember that one of the alleged benefits of repurposing is that repurposed candidates have proven to be safe in humans, thus increasing its chances to survive clinical trials required to gain approval for the second medical use. We could speculate that, if the new indication requires doses below the maximum tolerated dose for the previous indication, safety will not be an issue. Naturally, the lower the doses, the better. In vitro assays provide a fair idea of the concentration of the drug necessary to induce a desirable effect (e.g., EC_{50}). It is advised to review available literature on the drug candidate and compile pharmacokinetic data. The steady-state concentration obtained after repeated administration for the original indication could be used as a reference value: if the in vitro effective concentration for the intended second use results one or more orders of magnitude above the plasma steady-state concentrations for the original therapeutic use, the drug is not a straightforward candidate for repurposing. As stated elsewhere in this volume, in the absence of perpetual cellular models of epilepsy, animal seizure models are often used as primary screening for AEDs. You might then need to extrapolate approximate human doses from animal doses; allometric conversions could be useful for that purpose. Conversion of animal doses to human equivalent doses based on body surface area is usually convenient. Converting mouse doses

(mg/kg) to *estimated* human equivalent doses requires dividing the animal dose by 12.3; in the case of rat, you should divide by 6.2. These values assume 60 kg humans; for weights outside the standard range, the following formula should be used:

$$\text{HED} = \text{animal dose in mg / kg} \times \left(\text{animal weight in kg / human weight in kg}\right)^{0.33}$$

Note that this conversion is often used to calculate the dose that will be used at Phase I clinical trials (initial clinical dose), in which case a safety factor of at least 10 is applied to protect subjects taking part in the trial; nevertheless, here we are only estimating the effective dose in humans in order to study the compatibility between the doses used for the original indication and the approximate doses that would be required for the new, pursued (repurposed) indication. Therefore, safety factor is not needed here. You could check references [41–43] for additional considerations on interspecies dose conversion. Note that some drugs are unlikely to be amenable to simple allometric conversion, including those that are highly protein-bound, undergo extensive metabolism and active transport, experience significant biliary excretion, those whose targets are subject to interspecies differences in expression, affinity and distribution, and drugs that undergo extensive renal secretion [43]. If working with concentrations, have in mind that plasma drug levels will in general not coincide with the concentrations in other tissues. In the case of AEDs, we are particularly interested in brain bioavailability. Unbound (free) brain concentration would determine the activation of a specific target receptor and thus the intensity of the pharmacological response [44, 45], though information on free drug concentrations is still difficult to find in the literature. You should also examine the literature for data on protein and tissue binding and affinity for efflux and influx transporters expressed at the blood-brain barrier. Binding to plasma proteins will tend to increase total plasma concentration in comparison to brain concentrations; the opposite will occur if the drug takes part in extensive unspecific interactions with brain tissue. Affinity for efflux transporters will tend to increase the blood free drug concentration compared with free brain concentrations; the opposite will be true for compounds with affinity for influx transporters at the blood-brain barrier. Elimination half-life could also provide valuable information. Figure 1 shows the evolution of drug plasma levels after administration of repeated doses of the drug at fixed time intervals. The most common drug therapy interventions consist in administering a given dose of the drug at regular intervals (usually 6, 8, 12, or 24 h to help treatment adherence). Although it falls beyond the scope of this chapter, following the superposition principle and coupling multiple single dose equations, it can be easily demonstrated that, after a number of doses,

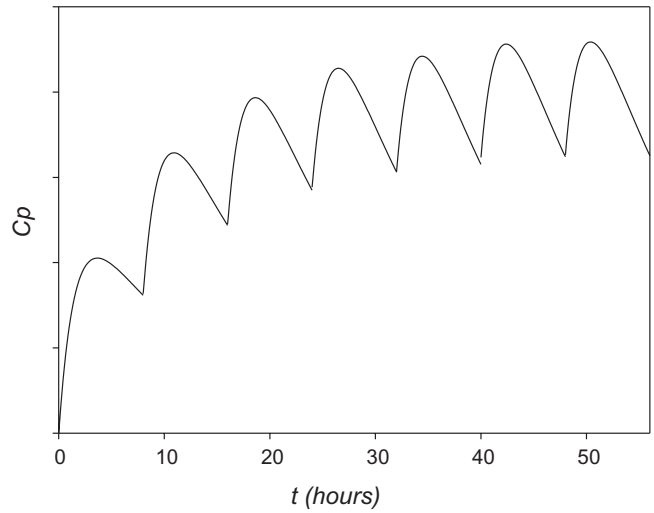

Fig. 1 Concentration-time profile for extravascular (e.g., oral) multiple doses delivered at regular intervals (in the example, doses are administered at regular intervals of 8 h)

drug plasma levels build up and a steady state is reached. During steady state, plasma drug levels will fluctuate between approximately fixed steady-state maximum (Cpmax,ss) and minimum (Cpmin,ss) concentrations. Four to five half-lives are required to achieve steady-state levels.

It can be inferred from the previous discussion on the importance of dose compatibility that the repurposed candidates linked to the larger doses for the original indication are more likely to be repurposed at lower doses. Naturally, drugs used exclusively as topical treatments are not good candidates for repurposing as systemic medications, since most of the advantages of the repurposing approach will be lost [29].

Once the compatibility between the doses used for the original indication and the ones that would be needed for the second indication (in our case, epilepsy) have been established, available data on the repurposed candidate should be scrutinized. It is important to detect possible contraindications or additional beneficial effects for the new indication [46]. For example, owing to the high prevalence of depression and anxiety in epileptic patients, repurposing a treatment for anxiety disorders or depression as AED could provide interesting additional benefits for the people suffering from epilepsy. This could eventually provide arguments to gain approval if multiple endpoints are later considered when designing the clinical trials. On the contrary, drugs displaying suicidal behaviors or depression as possible side effects would not constitute attractive candidates as AEDs, since they could exacerbate comorbid conditions associated with epilepsy.

Finally, one would probably disregard repurposed candidates linked to frequent and severe adverse reactions in favor of those candidates displaying a better safety profile.

4 Final Remarks

AEDs constitute, undoubtedly, a therapeutic category that possesses great potential for drug repurposing opportunities, which could be related with the multi-target nature and complex pharmacology of most of the approved antiepileptic agents, a feature that has opportunely been discussed in the chapter dedicated to network pharmacology. So far, from the knowledge on the AEDs pharmacology and the preclinical and clinical data available, the therapeutic indications for which AEDs could be repurposed seem to be neuropathic pain, mood disorders, and arrhythmias. However, hidden opportunities could be detected in a systematic manner though some of the computational approaches discussed in this chapter.

Regarding which therapeutic categories could be more interesting for screening as novel AEDs, we may mention anxiolytics, antidepressants, and anti-inflammatory agents. First, because it is possible to find reciprocal relationships between two therapeutic classes and, as mentioned already, existing AEDs frequently have effects on mood disorders. Second, because comorbid mood disorders (anxiety, depression) have high prevalence in epilepsy. In the case of inflammation, it seems to be involved in the disease onset and progression, and anti-inflammatory drugs with anticonvulsant properties could be useful as antiepileptogenic agents.

At last, we have discussed some general considerations to select which drug candidates are more attractive for repurposing. We advise to carefully examine the pharmacokinetic, toxicologic, and pharmacological available data on the candidate for repurposing. Drugs which are well tolerated at high doses for the original indication are more likely to be successful for repurposing. Drugs whose side effects might exacerbate the symptoms or etiology of the second disease (or some comorbid condition) should be disregarded. In contrast, drugs which could display additional benefits should be especially considered.

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