Author/s:
Maljevic, S;Reid, CA;Petrou, S

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DR. SNEZANA MALJEVIC (Orcid ID : 0000-0003-1876-5872)

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Models for discovery of targeted therapy in genetic epileptic encephalopathies

Snezana Maljevic[1], Christopher A. Reid[1], Steven Petrou[1,2,#]

[1]The Florey Institute of Neuroscience and Mental Health, and

[2]University of Melbourne, Melbourne, Australia

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#Corresponding author:

Prof. Steven Petrou

The Florey Institute of Neuroscience and Mental Health,

Kenneth Myer Building, 30 Royal Parade, 3052 Parkville, VIC

Email: steven.petrou@florey.edu.au

Abstract

Epileptic encephalopathies are severe disorders emerging in the first days to years of life that commonly include refractory seizures, various types of movement disorders, and different levels of developmental delay. In recent years, many *de novo* occurring variants have been identified in individuals with these devastating disorders. To unravel disease mechanisms the functional impact of detected variants associated with epileptic encephalopathies is investigated in a range of cellular and animal models. This review addresses efforts to advance and use such models to identify specific molecular and cellular targets for the development of novel therapies. We focus on ion channels as the best-studied group of epilepsy genes. Given the clinical and genetic heterogeneity of epileptic encephalopathy disorders, experimental models that can reflect this complexity are critical for the development of disease mechanisms based targeted therapy. The convergence of technological advances in gene sequencing, stem cell biology, genome editing, high throughput functional screening together with massive unmet clinical needs provides unprecedented opportunities and imperatives for precision medicine in epileptic encephalopathies.

Keywords: epilepsy, ion channels, disease models, induced pluripotent stem cells, therapy

Abbreviations

AED – antiepileptic drugs

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASO – antisense oligonucleotide

BFNS – benign familial neonatal seizures

BFNIS – benign familial neonatal-infantile seizures

BFIS – benign familial infantile seizures

CBD – cannabidiol

CHO – Chinese hamster ovary cells

EEG – electroencephalography

EIMFS – epilepsy of infancy with migrating focal seizures

GABA – γ-amino butyric acid

GEFS+ – genetic epilepsy with febrile seizures plus

HEK – human embryonic kidney cells

iPSC – induced pluripotent stem cell

MEA – multi-electrode arrays

MGE –medial ganglion eminence

MTH – molecular Trojan horses

NMDA – N-Methyl-D-aspartic acid

SMA – spinal muscular atrophy

SMN – surviving motor neuron

SUDEP –sudden unexplained death in epilepsy

RMT – receptor-mediated transfer

Epileptic Encephalopathy

Epileptic encephalopathies present as a heterogeneous group of early onset and childhood disorders characterized by severe, intractable seizures, specific electroencephalographic (EEG) signatures and different levels of developmental delay or regression, often with a poor prognosis. Initially, the term was used for disorders in which the severe epileptic activity results in developmental slowing or regression. Recent classification introduced the term "developmental and/or epileptic encephalopathy" recognizing that developmental impairment can occur independently of epileptic activity (Scheffer *et al.* 2017).

Clinical features of epileptic encephalopathies have been extensively reviewed elsewhere (McTague *et al.* 2016; Noh *et al.* 2012; Shbarou and Mikati 2016), and we will only introduce a few examples of well-established syndromes, such as Ohtahara, West, Lennox-Gastaut, and Dravet syndrome. Ohtahara syndrome, also known as early infantile epileptic encephalopathy, starts in the first three months of life and is characterized by tonic or focal seizures, or infantile spasms, severe to profound delay and limited responsiveness to therapy. As with many other epileptic encephalopathies, the interictal (between the seizures) EEG in patients with Othahara syndrome shows a typical pattern of alternating high voltage activity and little or no activity (known as burst suppression). In more than 70 % of cases, Ohtahara syndrome will evolve into West syndrome, which starts at about six months of life and encompasses infantile spasms, an interictal EEG pattern with chaotic activity including high irregular spikes and waves in the background, named hypsarrhythmia, and developmental delay. It can sometimes progress to Lennox-Gastaut syndrome, which mostly arises between 3 and 5 years of age, and includes numerous seizure types, mostly drug resistant, slow background activity in the interictal EEG and cognitive impairment. Interestingly, developmental delay precedes the onset of epilepsy in about half of the patients (Shbarou and Mikati 2016; McTague *et al.* 2016). In Dravet syndrome, starting at 5 to 8 months of age, the development is normal in the first year but slows and deteriorates with episodes of status epilepticus, which may be very frequent in some of the patients. Febrile seizures, photosensitivity, and drug resistance are other features of this syndrome. It was also among the first epileptic encephalopathies with a defined genetic cause: mutations in the *SCN1A* gene, encoding Nav1.1 sodium channel, have been detected in about 90 % of Dravet cases. Other epileptic encephalopathy syndromes include early myoclonic epilepsy, epilepsy of infancy with migrating focal seizures, epilepsy with myoclonic-atonic seizures and epilepsy aphasia spectrum (Harkin *et al.* 2007; Noh *et al.* 2012; McTague *et al.* 2016). The clinical syndromes can often show overlapping features and cases of unclassified (atypical) epileptic encephalopathy are also commonly described in the literature.

Only ten years ago, the etiology of the majority epileptic encephalopathy disorders was mostly unknown. With the development of next generation sequencing techniques, this knowledge gap has been filled with many novel genes and variants detected in the affected children, often

defining novel clinical entities. Interestingly, the vast majority of identified variants occur *de novo*, while some are inherited from healthy or mildly affected mosaic parents. Cases of recessive inheritance have also been reported (McTague *et al.* 2016). Many of the detected *de novo* variants have been found in known epilepsy genes, expanding their phenotypic spectrum to include both mild and severe epilepsy. Moreover, variants in ion channel genes are amongst the most prevalent causes of epileptic encephalopathy as shown in a host of genetic studies (Johannesen *et al.* 2016; Shen *et al.* 2016; Syrbe *et al.* 2015; Lemke *et al.* 2013; Epi4K Consortium *et al.* 2013; Weckhuysen *et al.* 2012; Veeramah *et al.* 2012; Epi4K Consortium 2016; Howell *et al.* 2015; EuroEPINOMICS-RES Consortium *et al.* 2014; Møller *et al.* 2017; Nava *et al.* 2014). Defining the genetic architecture of these rare disorders has significantly improved diagnosis and is revealing new opportunities for the development of targeted therapies.

The involvement of ion channels in genetic epilepsy has been extensively studied at the molecular, cellular and neuronal network level. This review will, therefore, provide an overview of mechanisms and related strategies for specific treatments using the example of ion channel genes associated with epileptic encephalopathy. We will further highlight the need for and the availability of newly developed experimental models, including those based on human induced pluripotent stem cells (iPSCs). Finally, we will assess the utility of some recent methodological advances, such as genome editing, generation of neuronal organoids, or *in vivo* transplantation and their potential for delivery of precision medicine in the treatment of epileptic encephalopathies.

Genes

Epilepsy presents a disease of neuronal hyperexcitability, which is probably why a significant proportion of identified epilepsy genes encode ion channels. Table 1 summarizes ion channel genes linked to various forms of epileptic encephalopathy. The list includes voltage-gated sodium, potassium or calcium channel genes as well as those encoding ligand-gated GABA and glutamate receptors.

Voltage-gated sodium channels

SCN1A, *SCN2A*, *SCN3A*, and *SCN8A* encode major brain sodium channel subunits Nav1.1, Nav1.2, Nav1.3, and Nav1.6, respectively. These pore-forming sodium channel α subunits are highly enriched at the axon initial segments (AIS) in neurons and play a crucial role in generation and propagation of neuronal firing. They show a specific pattern of expression during development, with the embryonic Nav1.3 sodium channel remitting in parallel to the rise in expression of the adult Nav1.1 (Cheah *et al.* 2013). Similarly, early expressed Nav1.2 channels are partially replaced by Nav1.6 channels, which emerge later in development, at the distal part of the AIS (Liao *et al.* 2010b). Brain sodium channels also show cell-specific expression, as Nav1.1 is mainly found in inhibitory and Nav1.2 and Nav1.6 predominantly in excitatory neurons (Reid *et al.* 2009).

Mutations in *SCN1A* have been linked to a spectrum of epilepsy phenotypes, including febrile and non-febrile seizures in genetic epilepsy with febrile seizures plus, GEFS+ (Escayg *et al.* 2000; Wallace *et al.* 2001b), and Dravet syndrome, also known as severe myoclonic epilepsy of infancy (SMEI) (Claes *et al.* 2001). The majority of associated mutations occur *de novo* and are nonsense or splicing alterations yielding truncated proteins, but missense mutations have also been reported (reviewed in (Reid *et al.* 2009; Escayg and Goldin 2010)).

SCN2A and *SCN8A* have been linked with both benign forms of early onset epilepsy, benign familial neonatal-infantile seizures (BFNIS) and benign familial infantile seizures (BFIS), respectively, and the severe epileptic encephalopathies and intellectual disability (Heron *et al.* 2002; Reid *et al.* 2009; Howell *et al.* 2015; Gardella *et al.* 2016; Veeramah *et al.* 2012; Blanchard *et al.* 2015; Larsen *et al.* 2015). *SCN2A,* in particular, has emerged as one of the dominant genes for epileptic encephalopathies with the clinical spectrum including Ohtahara syndrome, epilepsy of infancy with migrating focal seizures (EIMFS), infantile spasms (West syndrome), Lennox-Gastaut syndrome, Dravet-like syndrome as well as unclassified epileptic encephalopathy (Howell *et al.* 2015; Wolff *et al.* 2017). Furthermore, it presents a prominent gene in the autism spectrum disorders, and mutations have also been identified in cases of schizophrenia (Ben-Shalom *et al.* 2017; Carroll *et al.* 2016; Wang *et al.* 2016). *SCN8A*-related

epileptic encephalopathy typically has a poor prognosis and includes refractory seizures, developmental delay, and movement disorders with ataxia (Blanchard *et al.* 2015; Larsen *et al.* 2015; Veeramah *et al.* 2012).

Voltage-gated potassium channels

About 40 genes encode voltage-gated potassium channel alpha subunits in humans. Unlike the sodium channels alpha subunits, which contain four homologous repeated domains that form the ion-conducting pore, potassium channel pore-forming subunits assemble into tetramers to form functional channels. Different expression stoichiometries and subunit-specific biophysical characteristics yield a bewildering array of channel properties to support a diverse spectrum of physiological roles. Thus, some potassium channels regulate the repolarization of an action potential while others open only in response to prolonged firing or determine the resting membrane potential. Despite a significant number of different potassium channels and the fact that they play a critical role in the regulation of neuronal excitability, only a few genes have been linked to epileptic encephalopathies and epilepsy in general, including *KCNA2*, *KCNB1*, *KCNQ2*, *KCNQ3,* and *KCNT1*.

KCNA2 encodes Kv1.2 channel subunits expressed abundantly in the brain. *De novo* mutations in this gene were found in patients with epileptic encephalopathy including both febrile and afebrile, as well as focal and generalized seizures. Furthermore, mild to moderate intellectual disability, delayed speech development, and severe ataxia have also been described (Syrbe *et al.* 2015). Several *de novo* mutations causing epileptic encephalopathy have been identified in a related Kv2.1 potassium channel, encoded by *KCNB1* (Torkamani *et al.* 2014; Thiffault *et al.* 2015; Saitsu *et al.* 2015). While Kv1.2 is localized to axons and involved in the transmission of electrical signals, Kv2.1 is localized to soma and dendrites and contributes to the integration of the incoming signals.

KCNQ2 and *KCNQ3* code for the voltage-gated potassium channels Kv7.2 and Kv7.3 responsible for the slow and non-inactivating potassium current that can be suppressed by muscarinic receptor agonists thus called the M-current (Brown and Adams 1980). The M-current regulates membrane potential at the subthreshold range and suppresses repetitive neuronal firing.

KCNQ2 and *KCNQ3* variants can be inherited and cause a mild self-limited form of epilepsy - benign familial neonatal seizures (BFNS) (Charlier *et al.* 1998; Biervert *et al.* 1998; Singh *et al.* 1998; Maljevic and Lerche 2014). *De novo* variation in *KCNQ2* and *KCNQ3* is also seen and typically associated with severe early onset epileptic encephalopathy (Weckhuysen *et al.* 2012; Weckhuysen *et al.* 2013; Maljevic and Lerche 2014; Miceli *et al.* 2015a; Miceli *et al.* 2015b).

KCNT1 encodes a sodium-activated potassium channel thought to be involved in modulation of action potential waveforms (Markham *et al.* 2013). Mutations in *KCNT1* have been identified in inherited and sporadic cases of the rare autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) with occasional psychiatric abnormalities. *KCNT1* variation accounts for about 40% of cases with EIMFS (Barcia *et al.* 2012; Heron *et al.* 2012). Other epileptic encephalopathies, such as Ohtahara syndrome, have also been linked to this gene ((Møller *et al.* 2015); reviewed in (Lim *et al.* 2016)).

$GABA_A$ receptors

$GABA_A$ receptors belong to the family of pentameric ligand-gated channels that open in response to the binding of the neurotransmitter, gamma-amino butyric acid (GABA), to conduct chloride and bicarbonate anions. In the adult brain, this leads to a membrane hyperpolarization and thus inhibits neuronal firing. Six different subunit classes of $GABA_A$ receptor subunits are encoded by 19 genes. Functional receptors usually contain two α, two β and a subunit from one of the other classes. The most abundant receptor conformation is probably $2\alpha_1 2\beta_2 1\gamma_2$ (Sieghart *et al.* 1999; Pirker *et al.* 2000).

Variation in the *GABRA1*, *GABRB3* and *GABRG2* genes encoding the α_1, β_3, and γ_2 subunits of $GABA_A$ receptors, have been described in a broad spectrum of epilepsy phenotypes, from febrile seizures, GEFS+, absence seizures, juvenile myoclonic epilepsy (Macdonald *et al.* 2012; Kananura *et al.* 2002; Baulac *et al.* 2001; Cossette *et al.* 2002; Wallace *et al.* 2001a; Harkin *et al.* 2002; Maljevic *et al.* 2006) to epileptic encephalopathy, including Dravet syndrome (Møller *et al.* 2017; Carvill *et al.* 2014; Johannesen *et al.* 2016; Kang and Macdonald 2016; Shen *et al.* 2016; Janve *et al.* 2016; Papandreou *et al.* 2016). Furthermore, β_3 was defined as one of the

leading causes of epilepsy with myoclonic-atonic seizures (MAE), and correlation with the West syndrome and other unspecific types of epilepsy has also been suggested (Møller *et al.* 2017).

Glutamate NMDA receptor

Different classes of ionotropic receptors that mediate excitatory actions of glutamate include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-Methyl-D-aspartic acid (NMDA) receptors, named by their specific agonists. These channels open in response to glutamate binding to allow nonselective passage of cations. NMDA receptors are formed by three different subunits (GluN1-3), encoded by *GRIN1*, *GRIN2A*/*B*/*C*/*D*, and *GRIN3A/B*, respectively. The functional heterotetrameric channels comprise two GluN1 assembled with two GluN2 and/or GluN3 subunits. Both glutamate binding and the prolonged membrane depolarization that removes the Mg^{2+} block from the pore are required for the activation of NMDA receptors, suggesting that they do not underlie fast synaptic transmission, but rather regulate different neuronal plasticity processes via activation of calcium-dependent signaling pathways.

Heterozygous *GRIN1* mutations have been linked to a spectrum of severe epileptic encephalopathies, which include seizures, developmental, intellectual and speech disability, cortical visual impairment, oculomotor and movement disorders (Lemke *et al.* 2016). Two missense homozygous mutations have been detected in consanguineous families with intellectual disability and autism spectrum disorder (Lemke *et al.* 2016; Rossi *et al.* 2017), while a homozygous truncation was found to cause a severe neonatal epileptic encephalopathy (Lemke *et al.* 2016). *GRIN2A* has been associated with idiopathic focal epilepsies with centrotemporal spikes (Lemke *et al.* 2013), whereas *de novo* mutations in *GRIN2B* were found in individuals with West syndrome, focal epilepsy and developmental delay (Lemke *et al.* 2014; Smigiel et al., 2016). One *GRIN2D* mutation was also detected in two unrelated patients with epileptic encephalopathy (Li *et al.* 2016).

Non-ion-channel genes

We will only mention here a few examples of non-ion-channel genes that have been detected in patients with epileptic encephalopathy. Mutations in *SLC2A1* coding for GLUT1 glucose transporter expressed at the blood–brain barrier cause a severe epileptic encephalopathy known as GLUT1 deficiency syndrome (De Vivo et al., 1991). The X-chromosomal gene *PCDH19*, encoding protocadherin involved in interneuronal connections in early development is one of the most common causes of epileptic encephalopathy in females (Dibbens *et al.* 2008; Duszyc *et al.* 2015), while another X-chromosomal gene *CDKL5* coding for serine/threonine kinase cyclin-dependent kinase-like 5 causes severe epileptic encephalopathy sharing some features with Rett syndrome (Kilstrup-Nielsen *et al.* 2012). Mutations have also been found in *CHD2,* coding for chromodomain helicase DNA binding protein 2 that regulates chromatin structure and gene expression (Suls *et al.* 2013), in addition to mitochondrial and many other genes (McTague *et al.* 2016). Several genes encoding the components of the synaptic transmission pathway have also been associated with epileptic encephalopathy. Among them are *DNM1* coding for dynamin, a GTPase binding protein involved in endocytosis (EuroEPINOMICS-RES Consortium *et al.* 2014), *STXBP1* (Saitsu *et al.* 2008) and *STX1B* (Schubert *et al.* 2014) genes that encode presynaptic proteins and *SYNGAP1* that codes for a postsynaptic protein (Epi4K Consortium *et al.* 2013; Mignot *et al.* 2016). An ever increasing number of genes and variants will continue to be uncovered before the full genetic landscape of the epileptic encephalopathies is revealed.

Summary

The prodigious detection of genetic variants associated with epileptic encephalopathies has led to a new era in epilepsy research. We have learned that the genes initially associated with familial epilepsy syndromes also occur with *de novo* variation in a host of clinically severe epileptic encephalopathy syndromes. Knowledge of the causative gene has led to a 'gene-centric' view of epileptic encephalopathies, which are now more often described in relation to the gene rather than to the clinical syndrome. For example, following genetic ascertainment, a case of West syndrome could be referred to as *SCN2A* epileptic encephalopathy. Precision

medicine is arising as a result of this gene-centric view where the focus turns to gene-specific mechanisms rather than emergent pathologies.

Experimental models of EE

Translating genetic findings into targeted therapy requires the use of different experimental paradigms, from single cells to *in vivo* models (Figure 1). While we describe them here concentrating on commonly affected and well-studied ion channel genes, these concepts can also be applied in the analysis of other biological pathways involved in epileptic encephalopathies. Especially studies of genes directly involved in the regulation of neuronal excitability, such as synaptic transmission components, will share many similarities with the presented experimental approaches.

Heterologous expression in non-neuronal cell lines is used as the primary assay to investigate the protein dysfunction caused by a genetic mutation. It can help confirm that a detected variant is disease-causing by identifying a functional change or a dominant-negative effect the mutated protein exerts on the available wild type counterparts. A more sophisticated neuronal *in vitro* models (rodent primary neuronal cultures or human stem cell-derived two-dimensional and three-dimensional models) can provide information about the impact of protein dysfunction on the neuronal and network properties. Finally, animal models, from *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish, rodents and non-human primates, engineered with the gene variation of interest, enable different approaches for accessing the pathomechanisms that emerge on various temporal and spatial scales. While each of these models can help discern the disease mechanism at different levels, they all find utility in the specific phases of preclinical research, with their scalability and complexity often being inversely correlated (Table 2). We will focus here on the modeling cascade that we see as the most useful in our quest for the development of precision medicine strategies for epileptic encephalopathies and potentially epilepsy in general.

Heterologous expression systems

The initial experimental step to study the effects of detected genetic variants is their expression, in parallel to the wild type, in cells that do not typically contain the gene product. For ion channels, commonly used approaches are two microelectrode voltage clamp on *Xenopus laevis* oocytes and patch clamp electrophysiological analysis in various mammalian cell lines, like the human embryonic kidney (HEK293), or Chinese hamster ovary (CHO) cells. The procedures include engineering of the variants into the coding sequence of the affected gene and their transfer into the selected cells, for electrophysiological or biochemical and expression analysis using antibodies.

There are many examples of heterologously expressed epileptic encephalopathy mutations. We can best illustrate their advantages and disadvantages using the examples of sodium and potassium channels. Sodium channel mutations have often been analyzed in HEK293 and CHO cells lines using patch clamp recordings. The observed biophysical effects of mutations include shifts in activation and inactivation curves, altered persistent (steady-state, non-inactivating) sodium current and kinetics of inactivation, and changes of overall current amplitudes (Reid *et al.* 2009). These studies commonly revealed a loss of function of the detected missense mutations in *SCN1A* and a gain of function of the *SCN2A* mutations. Having in mind the expression pattern of the affected channels, these effects could be intuitively translated into reduced firing of inhibitory neurons (*SCN1A*) or increased excitability of principal neurons (*SCN2A*), and thus explain the occurrence of seizures. However, some *SCN2A* mutations have also shown loss of function (Reid *et al.* 2009; Oliva *et al.* 2012; Wolff *et al.* 2017), with recent studies suggesting that these are correlated with milder epileptic encephalopathy phenotypes (Wolff *et al.* 2017) or autism (Ben-Shalom *et al.* 2017). Explaining how and why a loss of sodium channels in excitatory neurons could result in either of these phenotypes is not feasible using the analysis in HEK cells and should be addressed in more complex expression systems. Still, important information that can be obtained from expression in non-neuronal cells could help clinicians decide whether a sodium channel blocker antiepileptic drugs (AEDs) should be used in these patients, as in the case of *SCN2A* dysfunction it may not be effective and even show negative impact on seizure activity (Wolff *et al.* 2017).

Expression and analysis of potassium and ligand-gated channels has often been performed in *Xenopus laevis* oocytes. Apart from their large size, relative ease of handling and robust levels of exogenous protein expression, one of the major advantages of this system is the use of microinjection to deliver the DNA or RNA of interest into the cell. This reduces the cell to cell variability seen in transfections and provides better control of protein ratios in the cases of co-expression of different subunits. One example of heterologous expression in *Xenopus* oocytes is the *KCNT1* channel. Expression of *KCNT1* disease variants reveals increased potassium currents compared to the wild type and the observed gain of function seems to correlate well with the phenotype severity. Moreover, use of quinidine as a potential blocker of these currents has also been successfully tested in oocytes, revealing differential responsiveness, hence providing important information for its potential clinical use (Milligan *et al.* 2014).

Overall, the heterologous expression is a scalable, relatively cheap, fast and efficient approach that can detect changes in biophysical and molecular properties of the mutated channels to confirm the genetic findings, establish genotype-phenotype correlation and single out variants that should undergo further more complex studies. Furthermore, the responsiveness of the detected variants to specific channel openers and blockers is potentially informative for precision therapy. The perceived disadvantage of the heterologous approach is that analyzed channels may behave differently or not reveal their disease relevant pathology when expressed in non-native cellular environments. While many studies in the past few decades showed that the biophysical behavior in both heterologous systems and neuronal tissue is comparable, it is the posttranslational regulation, cellular and protein trafficking processes, interactions with other molecules and temperature dependence (e.g. experiments in *Xenopus* oocytes are performed at 14-17^{o}C) that require use of more refined experimental models.

Neuronal *in vitro* models

To assess the effects of mutations on intrinsic neuronal properties, synaptic and neuronal networks function *in vitro*, they can be expressed in the primary rodent cultures (Harrill *et al.* 2015; Giordano and Costa 2011), or neuronal cultures derived from iPSCs obtained from patients can be analyzed (Parent and Anderson 2015). While these approaches provide

neuronal context, there is insufficient evidence that the randomly generated *in vitro* networks possess sufficient complexity or the appropriate topology required to predict clinically relevant disease mechanisms and therapeutic outcomes reliably. Nevertheless, the ease with which neuronal properties can be investigated and the scalability to meet the demands of drug screening make them interesting disease and therapy models.

Primary neuronal cultures

Dissociated neuronal cultures are most commonly obtained from hippocampi or cortices of embryos at late stages of *in utero* development, or from pups on postnatal days P0-P3, and kept in culture for about four weeks. One approach is to introduce genes of interest into wild type neuronal cultures using a variety of delivery methods, including lipofection, electroporation or viral infection. Limitations of such overexpression models include: (i) isolation of specific ionic currents carried by the channel of interest from other, similar currents present in neurons, (ii) presence of endogenously expressed channel proteins that may reduce the impact of the exogenously added channel and (iii) overexpression of added channels due to the use of strong non-neuronal exogenous promoters. Furthermore, only mutations with a dominant impact can be analyzed in this way, as the effect of loss of function variants may be indiscernible in the context of a full wild type background. Examples of the successful application of this approach are given below.

A de novo, recurrent epileptic encephalopathy mutation in *GRIN2D* encoding NMDA receptor subunit GluN2 showed a gain of function in *Xenopus* oocytes. Interestingly, when transfected in primary neurons, it caused dendritic swelling and neuronal cell death, which fits well with the excitotoxicity due to increased NMDA receptor function (Li *et al.* 2016). Another study showed that a *KCNB1* mutation affecting the voltage sensing domain reduced voltage sensitivity of the Kv2.1 channel, while a pore domain mutant yielded no currents and exerted a dominant-negative effect when expressed in transfected pyramidal neurons. As a result, the neuronal firing of the transfected neurons was reduced due to incomplete repolarization after an action potential. It was, therefore, suggested that the insufficient neuronal firing might affect the

development of neurons and neuronal circuits leading to an epileptic encephalopathy (Saitsu *et al.* 2015).

The fact that rodent neurons develop functional networks within one to two weeks in culture makes them even more attractive for epilepsy modeling as well as drug screening. Similarly, although on a longer time scale, iPSC-derived neurons can generate network activity (see below). Extracellular recordings of neuronal electrical activity within these networks can be performed using multielectrode arrays (MEA), cell culture dishes with embedded electrodes. MEA recordings can provide numerous parameters reflecting firing and synchronicity of these cultured networks (Chiappalone *et al.* 2006; Schock *et al.* 2012; Illes *et al.* 2009). For instance, hippocampal neurons obtained from an *SCN1A* epilepsy mouse model showed increased firing activity and higher network synchronicity (Hedrich *et al.* 2014). Thus, a scalable approach to test novel or established compounds on neurons expressing epileptic encephalopathy variants is readily available.

Human stem cell-based models

Human cellular models have become increasingly useful in the past ten years with the development of iPSCs' approaches that enable studies of patient-derived neuronal populations (Parent and Anderson 2015). Biochemical, morphological, electrophysiological or other properties of these neurons can then be compared to those of neurons derived from unaffected individuals of the same age and sex, or more powerfully, to the cells in which the particular mutation has been corrected using genome editing techniques.

The first reprogramming of skin fibroblasts into iPSCs was performed by Yamanaka and colleagues using retroviral transduction of the four factors Oct4, Klf4, Sox2 and c-Myc (Takahashi *et al.* 2007). In the meantime, other somatic cell types, obtained from wisdom teeth, hair roots, blood, and urine have been successfully used as source tissues. Considering the young age of individuals with epileptic encephalopathies, this is a relevant issue, since the invasiveness of the procedure and the amount of available tissue may present a challenge. Therefore, the increasing use of hematopoietic cells for reprogramming is potentially a more favorable option than fibroblasts that require biopsy. Studies suggest that iPSCs are affected by

the epigenetic properties of the source tissue and the age of the donor, but prolonged culturing can reduce this epigenetic imprinting (Raab *et al.* 2014; Lo Sardo *et al.* 2017). "Older" cells also show a higher number of genetic abnormalities (Lo Sardo *et al.* 2017), which is important for the selection of proper population control cell lines. Reprogramming protocols have also evolved to avoid the use of retroviral gene transfer that can have oncogenic and other effects on the targeted genome due to the vector integration. Instead, nonintegrating episomal or Sendai virus vectors have become more standard (Parent and Anderson 2015).

Advances in stem cell studies have paralleled the developments in the field of genome editing. Three major approaches for genome editing include; Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (Gaj *et al.* 2013). These methods enable generation of isogenic control iPSC lines by reverting the disease mutation to the reference gene sequence. Gene correction can be performed either in iPSCs or somatic cells in parallel with the reprogramming (Howden *et al.* 2015). Same procedures can also be used to introduce a disease mutation of interest in a control stem cell line, providing a possibility to study its impact, even when it is not possible to obtain cells from the patient. Similarly, different fluorescent markers, reporters or effectors can be integrated into the genome of the studied line (Mirzapour Delavar *et al.* 2016).

Neural differentiation protocols

The available differentiation protocols are aimed at producing different types of neurons that can potentially reveal the disease phenotype. The presence of seizures, EEG abnormalities, and cognitive impairment suggests that modeling epileptic encephalopathies requires the generation of cortical inhibitory and excitatory neurons. Unlike reprogramming into iPSCs, which is becoming an increasingly streamlined process, neuronal differentiation protocols are still very slow, variable, inefficient, and not sufficiently characterized or reproducible. Three commonly used strategies for generation of cortical neurons include (i) generation of embryoid bodies and isolation of neural rosette cells, (ii) application of small molecules or (iii) forced viral overexpression of transcription factors. Each of these approaches attempts to recapitulate in a

certain way the neuronal development processes and they are all still being optimized regarding the type and maturity of generated neurons, and time needed to observe the functional neurons and networks (Kim *et al.* 2014; Zhang *et al.* 2013). A commonly used small molecule strategy exploits two inhibitors of SMAD signaling, Noggin and SB431542, to induce rapid conversion of stem cells grown under adherent culture conditions into the early neurectoderm, which can be passaged into rosettes or turned into different types of neuronal cells via specific patterning factors (Chambers *et al.* 2009; Kim *et al.* 2014). To circumvent the long period needed to obtain functional neurons using this protocol, which can reach between three to six months, a forced viral expression of *Ngn2* is used to effectively generate active neurons within two to three weeks after infection. The function of neuronal networks generated in this way is further assissted by co-culturing astrocytes (Zhang *et al.* 2013). Selection of a differentiation procedure will depend on the gene under study and related phenotype. For instance, the investigation of an *SCN1A* disorder will require the generation of a population of cells containing GABAergic neurons, whereas abundantly expressed channels like *KCNQ2*, *SCNA2* or *KCNA2* can be analyzed in more homogenous populations of excitatory neurons. However, for the optimal *in vitro* analysis, mixed populations containing different types of neurons are probably the best option.

Dravet syndrome is the first epileptic encephalopathy to be modeled using human iPSCs and provides an example of how the field moved rapidly to develop modeling paradigms that recapitulated accepted pathomechanism findings in mouse models while offering the advantages of human cellular context and *in vitro* scalability. In the first study using iPSC modeling of Dravet syndrome, loss of function of derived inhibitory neurons was found (Higurashi *et al.* 2013). Subsequent studies did not support this view and showed *increased* neuronal activity in excitatory (Jiao *et al.* 2013) or both excitatory and inhibitory neurons (Liu *et al.* 2013). With methodological improvements including the use of CRISPR/Cas9 to generate isogenic control cells (Liu *et al.* 2016) or specific fluorescent reporters of excitatory and inhibitory neurons (Liu *et al.* 2016; Sun *et al.* 2016) the reduced firing of inhibitory neurons became more evident and the accepted view. Thus, even though the iPSC approach proved useful in identifying the Dravet syndrome pathomechanism, having other cellular and animal

models that support these findings is still necessary. Further optimization of the iPSC approach, especially concerning significant variability between the generated iPSC lines and reproducibility of the detected neuronal phenotype is still needed to provide a valid platform for drug testing and therapy development in the context of human cell biology.

Organoids

Complex neuronal disorders including epilepsies are challenging to model *in vitro,* using dissociated cells or even two-dimensional neuronal networks. Therefore, attempts are being made to generate three-dimensional cultures that will more faithfully recapitulate organization, development, and maturation of neuronal networks. These three-dimensional cultures can be referred to as organoids, mini-brains, brainoids, or corticoids. As with two-dimensional cultures, neuronal differentiation protocols can rely on the application of external patterning factors, which will drive the developing organoid towards a particular brain region. However, intrinsic patterning can also yield semi-organized organoids containing cells from different parts of the brain (Quadrato *et al.* 2016). Remarkably, intrinsic patterning was used to generate the first iPSC-derived cerebral organoids that showed primitive cortical organization, a dorsal forebrain-like progenitor zone, and marker specification of hippocampus and forebrain. Moreover, organoids obtained from a patient with primary microcephaly presented morphological and cellular defects, proving that these structures can reveal a disease-related structural phenotype (Lancaster *et al.* 2013).

Irrespective of the protocol used, there are significant challenges posed, including the size of the generated organoids, the increased apoptosis within the organoids due to hypoxia,, the time needed to achieve neuronal maturity, which can reach six to twelve months, and reproducibility. However, existing studies have highlighted the extraordinary opportunities these mini brain structures offer, especially for the analysis of neuronal migration and interconnections or for the replication of specific brain regions (Qian *et al.* 2016; Quadrato *et al.* 2017). As pointed above, these processes are probably critical in at least some epileptic encephalopathies, and difficult to investigate in neuronal cultures or mouse models at early developmental stages.

Genetic mouse models

Epileptogenic mechanisms are by far the most studied in rodent models, including those with spontaneously occurring mutations resulting in an epileptic phenotype, those carrying disease mutations detected in patients, and models of chronic epilepsy or acute seizures induced by different stimuli (Kandratavicius *et al.* 2014; Guerrini *et al.* 2014). For studies of genetic epileptic encephalopathy, the most interesting are mouse models harboring mutations corresponding to the ones detected in patients, as we will illustrate here using examples of sodium channel disorders.

Two knock-in mouse models of genetic epileptic encephalopathy caused by *SCN1A* truncating mutations found in Dravet patients were critical for revealing disease mechanisms (Ogiwara *et al.* 2007; Yu *et al.* 2006). Both mouse lines presented with similar phenotypes including spontaneous seizures, decreased threshold for thermally-induced seizures, interictal EEG discharges, reduced weight, and premature death. Seizures were only seen in C57/Black 6 and not in 129 background, and all symptoms were more pronounced in the homozygous mice. Probably the most significant finding of the two studies was that Nav1.1 presents the main sodium channel subunit in inhibitory neurons, as revealed both by immunohistochemical studies using Nav1.1-specific antibodies (Ogiwara *et al.* 2007) and electrophysiological recordings in dissociated cultured neurons with interneuron-specific bipolar morphology (Yu *et al.* 2006). The recorded interneurons from mutant mice showed significantly reduced firing rates compared to wild type due to the reduced presence of functional Nav1.1 channels, indicating disinhibition as the major pathomechanism related to the Dravet syndrome. Indeed, studies using a conditional mouse model, have shown that the deletion of Nav1.1 in inhibitory neurons is sufficient to cause severe epileptic phenotype and premature death. Interestingly, additional deletion of *Scn1a* in excitatory neurons rescued the disease phenotype in this model, extending the potential role of Nav1.1 beyond the inhibitory neurons (Ogiwara *et al.* 2013).

A well-established mouse model of epilepsy related to the *SCN2A* is a transgenic line *Scn2a*^{Q54}, expressing a non-disease related Nav1.2 mutation with increased persistent current (Kearney *et al.* 2001). These mice show spontaneous seizures and behavioral arrest, focal seizure activity on

EEG, morphological changes in the hippocampus, and shortened lifespan. Ensuing studies revealed strain-dependence as well as the influence of genetic modifiers on the phenotype (Thompson *et al.* 2017; Calhoun *et al.* 2016). A knock-in mouse line, modeled on a human *SCN2A* BFNIS mutation that showed enhanced persistent current in HEK cells (Liao *et al.* 2010a), presented with frequent seizures and increased mortality. At the cellular level, current clamp recordings revealed increased excitability in hippocampal pyramidal neurons. The heterozygous mutation was also shown to exacerbate neurodegeneration in a mouse model of multiple sclerosis (Schattling *et al.* 2016).

Many of the analyzed *SCN8A* epileptic encephalopathy mutations show increased function in heterologous systems, which can lead to higher neuronal activity and seizures. However, some mutations reduce the channel function. It has been suggested that the gain of function causes a more severe epileptic encephalopathy phenotype, and the loss of function a milder phenotype, including intellectual disability, often without seizures (Blanchard *et al.* 2015). This finding corresponds well with the available data from mouse models. Both spontaneous seizures and sudden unexplained death in epilepsy (SUDEP) have been described in a knock-in mouse model with an *SCN8A* epileptic encephalopathy mutation (Wagnon *et al.* 2015). In contrast, *Scn8a* knock-out mouse shows a protective effect when crossed with genetic models of epilepsy (Martin *et al.* 2007) and also against chemically and thermally-induced seizures (Makinson *et al.* 2014).

Attempts to model human loss of function mutations with knock-out mouse models have only been moderately successful. Possible reasons include homeostatic plasticity or failure to capture the dominant-negative effect of a human mutated protein with a complete knock-out of the mouse orthologous gene. For instance, heterozygous *Kcnq2* knock-out does not present with spontaneous seizures (Watanabe *et al.* 2000) unlike a mouse line carrying an epilepsy loss of function mutation in this gene (Otto *et al.* 2009; Singh *et al.* 2008). Similarly, comparison of the *Gabrg2*$^{+/-}$ with the *Gabrg2*$^{+/R43Q}$ heterozygous state of the R43Q knock-in mouse model of absence epilepsy (Tan *et al.* 2007) revealed that only mice carrying the mutation show thermally-induced seizures in a non-strain dependent manner, while absence seizures occurred in both models and depended on the strain background (Reid *et al.* 2013). Comparing the

Gabrg2$^{+/-}$ to a heterozygous mouse carrying truncating mutation associated with epileptic encephalopathy, *Gabrg2*^{Q390X}, revealed mild epilepsy and impaired biogenesis of remaining wild type subunits in the former, and severe seizures, behavioral changes and reduced overall expression of γ_2 subunits in the latter mouse model (Warner *et al.* 2016). This suggests that missense and truncating mutations that exert a dominant-negative effect on the remaining wild type subunits may be responsible for the more severe phenotype, but also easier to model than a mere haploinsufficiency.

Even though the procedures for generation of the genetic mouse models have significantly improved in recent years as novel genome-editing techniques became available, the number of reported epileptic encephalopathy mouse lines in literature is still small, suggesting that both costs and time strains of generating and studying such models are still a limiting factor. However, the possibilities of studying the disease mechanism both *in vivo* (behavior, EEG, imaging), *ex vivo* (brain slices electrophysiology and imaging) or *in vitro* (primary neuronal cultures) offered by such models make us believe this is about to change.

Apart from the opportunities they offer, the examples above showed that genetic mouse models do have several disadvantages. Firstly, the genetic background of the inbred mouse strains can largely affect the epileptic phenotype, with some of them showing more susceptibility to seizures; this yields experimental and breeding complexity and creates studies that are long and expensive (Schauwecker 2011). Secondly, severe epileptic encephalopathy mutations may not reveal phenotypes in heterozygous mice yet lead to early developmental lethality in homozygous mice, making the analysis of the disease mechanisms practically impossible. Lastly, the early onset epileptic phenotypes are difficult to assess in newborn pups, and the temporal correlation with the disease onset in humans is not always clear. To circumvent some of these challenges, one useful strategy is the generation of conditional mouse lines that restrict expression to a particular cell type or a specific period of development. As an example, this approach was used to study a dominant-negative *KCNQ2* mutation and detect the impact of its expression during development (Peters *et al.* 2005).

Another experimental approach is the use of *in utero* electroporation to genetically manipulate neural precursor cells at different brain locations by microinjection and electroporation of plasmid DNA into the brain of embryonic mice while they still develop within the uterus (Briz *et al.* 2017). Using this method, it is possible to obtain a large number of newborn pups that express the transfected plasmid. Co-transfection of fluorescent cellular markers enables studies of neurodevelopmental processes, including migration and morphology, as well as of the function of transfected neurons. For instance, it was shown that *in utero* transfection of a loss of function *KCNQ2* mutation into cortical layer 2/3 pyramidal neurons causes their hyperexcitability (Niday *et al.* 2016). Similar information can also be obtained using transplantation of human stem cell-derived neuronal precursor into the rodent brains (Upadhya *et al.* 2016; Imaizumi and Okano 2014), see below. To conclude, generation of novel rodent models is not only valuable for our understanding of disease mechanisms but also critical for the development of novel therapies as these models present currently irreplaceable preclinical tools.

Therapeutic modalities

Standard AEDs have shown poor efficacy in the treatment of epileptic encephalopathies. The phenotypic specificity of these disorders demands the development of novel therapeutic approaches, which should focus not only on seizure control but the quality of life-destroying co-morbidities. We will illustrate here the available and emerging concepts that may provide more successful therapeutic outcomes by addressing the underlying disease mechanisms, paving the way for the introduction of precision medicine for the treatment of these devastating disorders.

Genetic and functional studies have already had significant impact on clinical practice. For instance, one of the initial observations showing that the loss of Nav1.1 function may be involved in the generation of seizures was the seizure-exacerbating effect of sodium channel blockers observed in some Dravet patients. This soon became a clinically actionable observation underscoring the importance of genetic testing and, in the presence of *SCN1A* mutation, refraining from sodium channel blocking AEDs (Wirrell 2016). Similar observations have now been made for *SCN2A* and *SCN8A* suggesting enhanced adoption of theranostic approaches into

clinical practice. A recent study showed that early onset syndromes, caused by the gain of function *SCN2A* mutations show a relatively good response to sodium channel blockers, in contrast to the syndromes starting after the first trimester caused by loss of function variants (Wolff *et al.* 2017). Not surprisingly, it has also been observed that Nav1.6 gain of function mutation carriers respond relatively well to sodium channel blockers (Boerma *et al.* 2016). These examples nicely illustrate the concept of the therapeutic targeting of ion channel epilepsy genes. Importantly, randomized clinical studies are still required to measure the effect of sodium channel blockers in epileptic encephalopathy patients carrying mutations in sodium channels.

As we have seen, epilepsy mutations cause either gain or loss of function, which can lead to the generation of seizures and developmental delay. While the precise pathophysiological sequelae of biophysical changes may remain unknown, targeting the identified gain or loss of function of the affected protein is a promising approach for precision medicine that can be attained by a myriad of different treatment strategies.

Drug repurposing

Identification of genetic causes and underlying mechanism of severe epilepsy enables the potential to reuse already marketed drugs in the treatment of these diseases. The approach avoids the very long, expensive and uncertain drug development process and promises to deliver patient benefits in short time frames. Furthermore, drug development for severe, but rare syndromes is not attractive for the pharmaceutical sector with small market sizes and challenging preclinical and clinical development hurdles further deterring interest.

Various examples of drug repurposing for the treatment of epileptic encephalopathy have already been tested in preclinical and small clinical trials. One example is quinidine, a known antiarrhythmic drug, shown to block KCNT1 channels as well as some of its gain of function mutations, thus revealing a potential for treatment of KCNT1-related refractory epilepsy (Milligan *et al.* 2014). Several studies reported differential effect in patients, with those treated at very young age showing a more positive response in contrast to (young) adults receiving the treatment (Mikati *et al.* 2015; Chong *et al.* 2016; Fukuoka *et al.* 2017). This suggests that there

is a clear need to define a therapeutic window for this kind of pharmacological intervention, but also to explore other repurposing opportunities using drugs with more appropriate efficacy and pharmacokinetic profiles.

Another example is the use of FDA-approved NMDA receptor antagonists in cases where the gain of function of these receptors was detected. This approach was tested in the *GRIN2D* mutation carriers. As an example, administration of oral memantine, NMDA receptor blocker used in the treatment of Alzheimer's disease, resulted in mild to moderate improvement. When one of the probands developed refractory status epilepticus, treatment with anesthetic ketamine and magnesium, as the NMDA receptor blocker, proved helpful (Li *et al.* 2016). As noted above, these anecdotal observations in individual patients need to be confirmed in randomized controlled clinical trials.

Small molecules

Many bioactive molecule libraries comprising potential antiepileptic or neuroprotective compounds are available for high and medium throughput screening, which require platforms that can make use of different cellular and animal models. Small molecule programs also use identified genetic and molecular targets such as gain or loss of function of various ion channels to develop specific drugs, i.e. channel openers or blockers, on traditional expression platforms. The complexity of experimental models is inversely correlated with the scalability, suggesting simpler animals such as *Drosophila melanogaster*, *Caenorhabditis elegans*, or zebrafish may be more suitable for these screens than mouse models. Their primary advantage is the relatively simple genetic modification and possibilities of the high throughput screening. However, the utility of these models is yet to be confirmed, especially regarding modeling of co-morbidities such as intellectual disability.

When the initial screen yields a proof of principle compound, it is tested in other, more complex preclinical models for efficacy, specificity, toxicity and potential mechanism of action including identification or confirmation of pharmacological targets. Further modifications to its chemical structure can be made to improve effectiveness.

An interesting example is the development of retigabine, a specific opener of KCNQ2 and KCNQ3 channels (Rundfeldt and Netzer 2000). The drug has been initially approved for the treatment of pharmacoresistant partial epilepsies, but the side effects seen in some adults have led to its removal from the market (Tompson *et al.* 2016). The fact that retigabine counteracts M-current loss of function seen in *KCNQ2/3* variants led to its use in the treatment of epileptic encephalopathy patients, with studies revealing significant improvement in some of them (Weckhuysen *et al.* 2013; Millichap *et al.* 2016). Therefore, new compounds using a similar mode of action and reduced side-effects, are currently being developed.

Natural products

Similarly to small molecules, natural products can also be screened for the development of novel therapies. An NIH - curated list (https://nccih.nih.gov/grants/naturalproducts/libraries) includes companies and institutes that provide compounds sourced from microorganisms, plants, and animals.

One of the natural products that has drawn a broad interest in the recent years due to the anecdotal evidence that it could ameliorate seizures and co-morbidities in children with severe epileptic encephalopathies, such as Dravet syndrome, is cannabidiol (CBD), a major non-psychoactive component of cannabis (Devinsky *et al.* 2014). The mechanism of CBD action is not completely understood, but it affects a large number of receptors and shows predominantly anticonvulsive effects in animal models. Also, CBD shows anti-inflammatory and neuroprotective action. Randomized controlled trial in children and young adults with treatment-resistant epilepsy revealed that CBD may reduce seizure frequency. This study also reported that CBD has a satisfactory safety profile (Devinsky *et al.* 2016). A recent trial in patients with Dravet syndrome showed that CBD reduced the frequency of convulsive seizures per month significantly more than a placebo, but was also associated with increased rates of adverse effects (Devinsky *et al.* 2017). Several companies are currently developing different formulations and further clinical trials are underway.

Other natural products with potential anticonvulsive effects can be found in venoms from spiders, scorpions, and snakes. These toxins serve to elicit pain, discomfort or paralysis in their

prey. To do so, venom components specifically affect ion channels and receptors regulating these processes. A recent study reported tarantula toxins that can activate Nav1.1 sodium channels (Osteen *et al.* 2016). This mechanism can potentially be used in the treatment of the Dravet syndrome.

Diet therapies

The most common diet intervention in epilepsy and epileptic encephalopathy is the ketogenic diet, which is based on the removal of carbohydrates and increased use of fats in the diet. The potentially typical ketogenic diet uses ratios of 4:1 and 3:1 of fats towards other macronutrient groups, which is an effective treatment for seizures, but with compliance issues. Therefore, other, less stringent forms of the diet, including the addition of medium chain triglycerides (especially the decanoic fatty acids) or the modified Atkins diet have also been used (Sampaio 2016). The increased fat and reduced carbohydrate content results in the generation of ketone bodies, which in the absence of glucose, present the primary source of energy in the brain. While this process specifically targets the condition in GLUT1 deficiency syndrome by providing an energy source for the brain independent of the defective glucose transporter, the marked improvement in other epileptic syndromes is still being explored. The suggested mechanism is either the stabilization of the energy resources in the brain, the direct effect on AMPA receptors, or changes in DNA methylation (Boison 2017). In addition to seizure control, the ketogenic diet, particularly when started early, can lead to cognitive improvement especially in GLUT1 deficient patients (Weber *et al.* 2008). Outside of GLUT1 disorders, the ketogenic diet is useful for some epileptic encephalopathy patients, and its efficacy shows no particular correlation with underlying genetic cause (Schoeler *et al.* 2013).

Antibodies

Recombinant monoclonal antibodies are commonly used in biological studies due to their specific binding to selected molecular targets. These proteins have also been successfully utilized for targeted treatment of different diseases, especially cancer. An antibody molecule comprises two heavy and two light chains connected via disulfide bridges, both containing a

variable domain responsible for recognition of an antigen. Antibodies used for targeted treatment always include the variable domain, with different portions of the remaining structure (reviewed in (Neves *et al.* 2016)). The use of antibodies in the treatment of brain disorders is limited by the permeability of the brain-blood-barrier. Different strategies have been employed to provide delivery of molecules into the brain. For larger molecules, molecular Trojan horses (MTH), exploiting the endogenous receptor-mediated transfer (RMT) of proteins such as transferrin, insulin, low-density lipoprotein, etc. have been developed. The resulting therapeutic antibody will thus contain a region responsible for RMT and a part for recognition of the targeted molecule.

Defining mechanisms underlining severe epilepsy can identify potential targets for therapeutic antibodies. It is very likely that gain of function variants could be addressed by specific inactivating antibodies more readily than the loss of function variants by activating antibodies. Targeting ion channels can be challenging as their extracellular regions are often short or covered by other interacting molecules. In this regard, the use of nanobodies could be favorable. Nanobodies are a single-chain antigen-binding camelid antibody fragments that show many advantages compared to other recombinant antibodies, including cell-penetrating properties (Hassanzadeh-Ghassabeh *et al.* 2013). A recent study revealed that the P2X7 receptor could be successfully blocked by nanobodies, which resulted in reduced inflammation markers in mice and in human blood (Danquah *et al.* 2016).

Gene therapies

Genetic tools for precision treatment of epileptic encephalopathy can include interventions at different levels to provide either correction of a disease mutation in the genomic DNA or modify expression levels of mutated or wild type alleles. Since the development of genome editing CRISPR/Cas9 technique, *in vivo* clinical use of genome editing has become an ever growing possibility. However, many challenges remain both from an ethical and methodological point of view. The major methodological problems include delivery, the efficiency of correction, off-target editing, selection of target cell types and determination of therapeutic windows. While molecular tools are still in development, ongoing studies in animal models are also

aiming to deliver CRISPR/Cas9 molecules into neurons, using ribonucleoproteins or viral constructs. Thus far, deletions of specific genes and transgenic expression of fluorescent or other regulatory proteins have been successfully performed in rodents showing the possibilities of genome editing in non-dividing cells (Staahl *et al.* 2017).

At the RNA level, the focus for many years has been on the regulation of the levels of mRNA which can then translate into the amount of available protein. The gain of function mutations could be counteracted for instance using morpholinos, siRNA, or antisense oligonucleotides (ASOs). Furthermore, naturally occurring antisense RNA transcripts regulate the expression levels of certain genes. Interference with these transcripts can be utilized for the loss of function mutations as it could lead to an increased expression of the non-mutated allele. This approach was exploited in the Dravet syndrome, with the detection of a natural antisense noncoding RNA that was successfully blocked by specific oligonucleotides leading to a targeted upregulation of *SCN1A* expression in different *in vitro* and *in vivo* Dravet models and non-human primates (Hsiao *et al.* 2016). These concepts are also well illustrated in the treatment development for the spinal muscular atrophy (SMA), a severe disease arising from the lack of survival motor neuron protein encoded by two genes, *SMN1* and *SMN2*. The disease is caused by a recessive mutation in *SMN1*, while a variable copy number of the homologous *SMN2* gene is present. However, *SMN2* contains a splice enhancer nucleotide polymorphism leading to the generation of nonfunctional proteins lacking exon 7. The use of splice switching oligonucleotides that enhance the expression of the full *SMN2* mRNA by including the skipped exon has just been approved as a treatment for SMA (Morrow 2017). Furthermore, a long noncoding RNA that binds to and inhibits the promoter of *SMA2* was identified and could efficiently be blocked by an ASO to increase the expression of this gene, suggesting a combination of two ASOs may be another useful strategy in this disease (d Ydewalle *et al.* 2017).

Cell therapies

Stem-cell-based therapeutic approaches have been reported in different neurological diseases including stroke, multiple sclerosis, and Parkinson's disease, but have also been explored as a

potential treatment for epilepsy (Rao *et al.* 2017). Many studies focused on repairing and enhancing the GABAergic system by transplantation of GABAergic progenitor cells into epileptic brain regions (Shetty and Upadhya 2016). For instance, grafting of neural stem cells into the hippocampus of a rat model of status epilepticus-induced chronic epilepsy was shown to reduce seizures via long-term survival of the transplanted cells and their differentiation into GABAergic neurons (Hattiangady *et al.* 2008). Other studies used progenitor cells from the medial ganglionic eminence (MGE), the primary source of GABAergic neurons in the early embryonic development, to reduce seizures in different experimental models of temporal lobe epilepsy (reviewed in (Shetty and Upadhya 2016)). Human cortical interneurons derived from an embryonic stem cell line and transplanted into the mouse cortex reproduced migratory behaviors and electrophysiological activity of cortical interneurons (Maroof *et al.* 2013). However, differentiation of MGE precursors, derived from human embryonic stem cells and human iPSCs, into the physiologically mature GABAergic interneurons took about seven months both *in vitro* and when transplanted into the rodent cortex, corresponding to the developmental timeline in human (Nicholas *et al.* 2013). Notably, grafting of iPSC-derived maturing GABAergic interneurons into the brain of the temporal lobe epilepsy mouse model revealed that their functional integration results in seizure suppression and improvement of behavioral and cognitive phenotype (Cunningham *et al.* 2014). Therefore, the possibility to generate and differentiate human iPSCs enables development of autologous cell replacement therapy also increasing its attractiveness as a treatment of epilepsy.

Filling in the Gap

Genetic research has already made a giant leap to identify causes of epileptic encephalopathy syndromes. We have now stepped into an exciting era of precision medicine, with a hope to both discern the mechanisms of these devastating syndromes and develop effective therapies tailored to specific needs of individual patients. Since epileptic encephalopathy disorders are

often regarded as the model disease for epilepsy, these therapeutic approaches are likely to provide novel treatment options for patients with more common forms of epilepsy.

The available studies and models have increased our understanding of pathological mechanisms, but also raised many questions. For instance, how can both gain and loss of function of the same channel cause similar disease, as seen for *KCNQ2* epileptic encephalopathy mutations, or why do different phenotypes emerge at different points of development as recognized for *SCN2A*-related disorders. The time dependence of the initial seizure occurrence is another unknown, as are the causes for the observed structural changes in the brain found in some epileptic encephalopathy cases.

So far, animal models have been most useful in tackling critical neurobiological and neurophysiological processes, including those related to the disease. But differences in gross brain structure, regional organization and gene expression cannot be ignored. Furthermore, even with the advancement of genome editing tools, generation and characterization of an animal model may take months if not years. Lastly, the most obvious use of such models for phenotypic, morphological, optical and electrophysiological studies should, in the case of epileptic encephalopathy, be performed at a particular point in the early development. This task is both methodologically challenging as it is difficult to define such a window in animal models that has translational relevance in humans.

So which models and methodological approaches may lead to a more efficient development of targeted treatments? The models should faithfully represent human brain processes, including the presence of different neuronal and glial cell types, the formation of functional synapses and networks and a possibility to study developmental processes. Reproducibility, scalability, attainability for the functional analysis should also be included. Even though it is early days, we believe that the generation of stem cell-derived organoids in combination with genome editing, optogenetic and advanced imaging approaches, presents a potentially useful addition to other *in vitro* and *in vivo* models presented in this review. New technologies, such as high-throughput single cell RNA sequencing, may also offer answers to many questions about the expression of affected genes, their specific alleles (Quadrato *et al.* 2017), and gene networks (Johnson *et al.* 2015).

The existing and newly developed disease models will continue to expand our understanding of brain function and reveal disease mechanisms for specific genes and variants associated with epileptic encephalopathy. These experimental approaches will provide novel molecular targets driving clinical efforts that could include small molecule discovery programs, drug repurposing efforts, and application of molecular technologies such as antisense oligonucleotides, genome-editing or cellular therapy. Further, these disease models will have strong preclinical utility allowing us to test new therapeutic strategies in assays that are more predictive of human efficacy. Together, this will expedite the delivery of disease-based treatments to severely affected patients.

--Human subjects --
Involves human subjects: No
If yes: Informed consent & ethics approval achieved:
=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

ARRIVE guidelines have been followed:
Yes
=> if No or if it is a Review or Editorial, skip complete sentence => if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines." unless it is a Review or Editorial

Conflicts of interest: None
=> if 'none', insert "The authors have no conflict of interest to declare."
=> otherwise insert info unless it is already included

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Figure legend

Figure 1. Experimental models used in the analysis of genetic epileptic encephalopathies. Genetic variants detected in a patient can be studied using the heterologous expression in *Xenopus laevis* oocytes and mammalian cell lines (top left). *In vitro* neuronal cultures (top right), either extracted from the mouse brain or differentiated from iPSC lines derived from patients somatic cells, can reveal the impact of a mutation on the neuronal and network activity. Stem-cell-derived neurons can also grow in the form of organoids (bottom right), which may be used to study the neurodevelopmental processes, such as cell proliferation and migration, *in vitro*. Finally, genetic mouse models (bottom left) are used to analyze *in vivo* changes, including behavioral and EEG alterations, caused by mutations. Although a genetic variant can be analyzed in each of the models independently (red arrows), common strategies start with simpler and develop towards more complex models (gray arrows).

Table 1. Ion channel defects associated with epileptic encephalopathy

Gene	Protein	Epileptic encephalopathy syndrome	Mechanism	References	Possible targeting strategies
Voltage-gated channels					
Sodium channels					
SCN1A	Nav1.1	Dravet syndrome	loss of function	(Claes *et al.* 2001)	specific Nav1.1 openers; increased expression of the remaining allele
SCN2A	Nav1.2	Ohtahara syndrome, EIMFS, West syndrome, Lennox-Gastaut syndrome, Dravet-like syndrome, unclassified epileptic encephalopathy	gain and loss of function	(Shi *et al.* 2009; Nakamura *et al.* 2013; Ben-Shalom *et al.* 2017; Howell *et al.* 2015; Wolff *et al.* 2017)	gain of function: sodium channel blockers; gain/loss of function: regulation of expression of mutant or wild type allele
SCN8A	Nav1.6	epileptic encephalopathy with movement disorders and ataxia	gain and loss of function	(Larsen *et al.* 2015; Blanchard *et al.* 2015; Veeramah *et al.* 2012)	
SCN1B	β_1-subunit	Dravet syndrome, epileptic encephalopathy	recessive inheritance, loss of function	(Ogiwara *et al.* 2012; Ramadan *et al.* 2017)	replacement of the defective protein
Potassium channels					
KCNA2	Kv1.2	epileptic encephalopathy including febrile and afebrile, focal, but also	loss and gain of function	(Syrbe *et al.* 2015)	gain of function: potassium channel blockers loss of function:

		generalized seizures			openers or increased expression of the remaining allele
KCNB1	Kv2.1	epileptic encephalopathy developmental delay, severe infantile generalized seizures	loss of function; loss of ion channel selectivity	(Torkamani *et al.* 2014; Thiffault *et al.* 2015; Saitsu *et al.* 2015)	channel openers, block/removal of mutant subunits causing loss of ion selectivity
KCNQ2	Kv7.2	epileptic encephalopathy	loss and gain of function	(Millichap *et al.* 2016; Weckhuysen *et al.* 2013; Weckhuysen *et al.* 2012; Orhan *et al.* 2014; Miceli *et al.* 2015a)	loss of function: specific potassium channel openers (such as retigabine); gain of function: channel blockers, reducing expression of mutant proteins
KCNQ3	Kv7.3	epileptic encephalopathy	loss and gain of function	(Miceli *et al.* 2015b; Miceli *et al.* 2015a)	
KCNT1	Slack	EIMFS, Ohtahara syndrome	gain of function	(Barcia *et al.* 2012; Milligan *et al.* 2014; Lim *et al.* 2016; Mikati *et al.* 2015)	channel blockers (such as quinidine); reduced expression of the mutant protein
Calcium channels					
CACNA1A	Cav1.1	epileptic encephalopathy with progressive nerve atrophy (compound	loss of function	(Reinson *et al.* 2016; Epi4K Consortium 2016; Damaj *et al.* 2015)	channel openers and modulators

		heterozygous or other comorbidities (heterozygous)			
CACNA2D2	$\alpha_2\delta$-2	epileptic encephalopathy with dyskinesia, cerebellar atrophy, dysmorphic features	homozygous null or loss of function mutation	(Edvardson *et al.* 2013; Pippucci *et al.* 2013)	replacement of the missing protein
Hyperpolarization activated cyclic nucleotide gated channels					
HCN1	HCN1	fever-sensitive, intractable epileptic encephalopathy	gain and loss of function	(Nava *et al.* 2014)	gain of function: specific channel blockers; gain/loss of function: regulation of expression of mutant or wild type allele
Ligand-gated channels					
$GABA_A$ receptors					
GABRA1	α_1	Dravet syndrome, epileptic encephalopathy	loss of function	(Carvill *et al.* 2014; Johannesen *et al.* 2016)	replacement of missing protein, agonists, increased expression of other subunits, decreased expression of mutated/ truncated proteins
GABRB3	β_2	MAE, Dravet syndrome, West syndrome, unclassified epileptic encephalopathy	loss of function	(Epi4K Consortium *et al.* 2013; Papandreou *et al.* 2016; Møller *et al.* 2017)	
GABRG2	γ_2	Dravet syndrome, epileptic encephalopathy	loss of function	(Kang and Macdonald 2016; Shen *et al.* 2016)	

NMDA receptors					
GRIN1	GluN1	spectrum of severe epileptic encephalopathies, speech disability, movement disorders	loss of function	(Lemke *et al.* 2016)	loss of function: replacement of missing protein, agonists, increased expression of the second allele, decreased expression of mutated/ truncated proteins; gain of function: NMDA blockers negative modulators
GRIN2A	GluN2A	idiopathic focal epilepsies with centrotemporal spikes	loss and gain of function	(Lemke *et al.* 2013; Addis *et al.* 2017)	
GRIN2B	GluN2B	West syndrome, focal epilepsy and developmental delay	gain and loss of function	(Lemke *et al.* 2014; Smigiel et al. 2016)	
GRIN2D	GluN2D	epileptic encephalopathy	gain of function	(Li *et al.* 2016)	

Table 2. Properties and utility of different experimental models of epileptic encephalopathies for the development of targeted therapies.

Systems	Experimental Models	Initial Functional Screening	Disease Modeling	Economic value	Scalability	Preclinical Use
Heterologous expression	***Xenopus laevis* oocytes**	***	*	***	***	*

	mammalian cell lines (HEK293, CHO)	***	*	***	***	*
Neuronal *in vitro* models	**rodent neuronal cultures**	*	**	**	***	**
	iPSC-derived neuronal cultures/ brainoids	*	**	*	***	**
Animal models	***Caenorhabditis elegans***	*	**	**	**	*
	Drosophila melanogaster	*	*	**	***	*
	zebrafish	*	**	**	***	*
	rats	*	***	*	*	***
	mice – knock-out/ knock-in	*	***	*	*	***

* low, **moderate, ***high