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Developmental and epileptic encephalopathies: from genetic heterogeneity to phenotypic continuum

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1 **Developmental and epileptic encephalopathies: from genetic**
2 **heterogeneity to phenotypic *continuum***

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17

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19

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25 **Table of Contents**

26	I. INTRODUCTION TO THE CONCEPT AND PATHOPHYSIOLOGY OF DEVELOPMENTAL AND EPILEPTIC	
27	ENCEPHALOPATHY (DEE).....	4
28	II. GENERAL CONCEPTS OF EPILEPTOGENESIS AND EPILEPSY	13
29	III. MAIN (KNOWN) DETERMINANTS OF NORMAL AND ABNORMAL BRAIN DEVELOPMENT THAT INFLUENCE	
30	EPILEPTOGENESIS	17
31	IV. NON-GENETIC DETERMINANTS OF THE EPILEPTOGENIC RISK.....	22
32	V. GENETIC DETERMINANTS OF DEE: EXPERIMENTAL MODELS	23
33	VI. DYSFUNCTIONS IN NEURAL CELL MIGRATION, PROLIFERATION, AND SYNAPTOGENESIS	25
34	VII. DYSFUNCTIONS IN INTRINSIC EXCITABILITY	29
35	VIII. DYSFUNCTIONS IN SYNAPTIC TRANSMISSION AND PLASTICITY	51
36	IX. DYSFUNCTIONS IN NEURONAL HOUSEKEEPING: MTOR AND AUTOPHAGY	67
37	X. THE SEARCH FOR PERSONALIZED TREATMENT APPROACHES	73
38	XI. CONCLUSIONS	79
39		

40 **ABSTRACT**

41 Developmental and epileptic encephalopathies are a heterogeneous group of disorders
42 characterized by early-onset, often severe epileptic seizures, EEG abnormalities, on a background
43 of developmental impairment that tends to worsen as a consequence of epilepsy. DEEs may result
44 from both non-genetic and genetic etiologies. Genetic DEEs have been associated with mutations
45 in many genes involved in different functions including cell migration, proliferation, and
46 organization, neuronal excitability, and synapse transmission and plasticity. Functional studies
47 performed in different animal models and clinical trials on patients have contributed to elucidate
48 pathophysiological mechanisms underlying many DEEs and explored the efficacy of different
49 treatments. Here, we provide an extensive review of the phenotypic spectrum included in the
50 DEEs, of the genetic determinants and pathophysiological mechanisms underlying these
51 conditions. We also provide a brief overview of the most effective treatment now available and of
52 the emerging therapeutic approaches.

53

54 **Keywords:** developmental and epileptic encephalopathies; epileptogenesis; channelopathies;
55 synaptopathies; personalized treatment approaches.

56

57 **CLINICAL HIGHLIGHTS**

58 Epilepsy is the third leading contributor to the global burden of neurological disorders and affects
59 65 million people worldwide. Based on the clinical and EEG features, etiologies, and comorbidities,
60 different categories of epilepsy types and syndromes are recognized. Among them, developmental
61 and epileptic encephalopathies (DEEs) represent the most severe end of the spectrum. This review
62 will help Physiological Reviews' readership to increase their knowledge about the different types
63 of DEEs, their etiologies and pathophysiological mechanisms, and available and emerging
64 treatments.

65

66 **I. INTRODUCTION TO THE CONCEPT AND PATHOPHYSIOLOGY OF DEVELOPMENTAL AND**
67 **EPILEPTIC ENCEPHALOPATHY (DEE)**

68 Epilepsy is the third leading contributor to the global burden of neurological disorders, and affects
69 65 million people worldwide (1). Based on the clinical and EEG features, etiologies, and
70 comorbidities, different categories of epilepsy types and syndromes are recognized (2).
71 Developmental encephalopathies (DEs) are a group of severe disorders with early onset of signs of
72 developmental impairment, associated with other neurological symptoms such as autonomic
73 dysfunction, behavioral disorders, and motor impairment (3). In DEs, developmental
74 delay/impairment is a prominent feature, whilst the epileptic activity (seizures and EEG
75 abnormalities) does not appear to be causally associated with developmental delay, stagnation, or
76 regression. Epileptic encephalopathies (EE) comprise a large, heterogeneous group of severe
77 epilepsy syndromes characterized by several seizure types, frequent epileptiform activity on EEG,
78 and developmental delay or regression (4). In EE, no pre-existing developmental delay is observed,
79 and the cause of delay is attributed to an interference that epilepsy has on physiological brain
80 processes. However, when severe epilepsy has a very early onset, it is often impossible to know
81 whether the underlying cause of the epileptic encephalopathy would not in itself have caused
82 developmental delay, even in the absence of epilepsy. For this reason, recent definitions refer to
83 'developmental and epileptic encephalopathies (DEEs)' to designate a heterogeneous group of
84 disorders characterized by early-onset, often severe epileptic seizures, EEG abnormalities, on a
85 background of developmental impairment that tends to worsen as a consequence of epilepsy (2).
86 This generates a complex clinical picture in which both developmental abnormalities and severe
87 epilepsy/EEG discharges contribute to the observed impairment, each to an extent that it is hard
88 to measure. The spectrum of DEEs by age of onset is described in **TABLE 1**, adopting the latest
89 proposal for the classification of epilepsy syndromes by the International League Against Epilepsy

90 (ILAE) Task Force on Nosology and Definitions (5–7). In certain DEEs, specific gene defects may
91 create recognizable etiology-specific syndromes, whereas, in others, a variety of genetic variants
92 may be associated with the same epilepsy syndrome. Such genetic heterogeneity is characteristic
93 of the infantile spasms syndrome (ISS) and Lennox-Gastaut syndromes (LGS), where genetic
94 variants affecting distinct molecular or signaling pathways may lead to similar electroclinical
95 syndromes (8–12). Moreover, DEEs may also result from nongenetic etiologies, including
96 structural, toxic/metabolic, infectious, or immune, which may appear either independent of or in
97 the setting of certain genetic etiologies (**TABLE 1**). Despite the advances in diagnostic tools, the
98 underlying etiology may remain unknown in a significant portion of patients with specific
99 syndromes. For example, in ISS, a third of the patients have unknown etiology, ~24% have genetic
100 or genetic-structural, and almost half have structural/metabolic or other acquired etiologies (13).
101 Similar distributions of etiologies are also encountered in Lennox-Gastaut syndrome, although, as
102 discussed above, increasing numbers of genetic variants and associations have emerged, as use of
103 genetic tests becomes more widespread (14).

104 In DEEs, the co-occurrence of epilepsy and intellectual disability (ID) can involve at least two non-
105 exclusive mechanisms. These mechanisms include uncontrolled, frequent or prolonged seizures
106 that can interfere with brain developing programs, resulting into inadequate construction of
107 cortical networks and poor cognitive outcomes (15), as well as genetic mutations or adverse
108 environmental factors that can induce both seizures and cognitive impairment. For example,
109 genetic mutations inducing specific synaptic defects might cause seizures because of aberrant
110 connectivity, as well as intellectual disability because of altered synaptic plasticity (16). In many
111 DEEs, epilepsy co-exists with other comorbidities, both neurological and extra-neurological.
112 Neurological comorbidities differ in type and severity, and span from subtle learning difficulties to

113 psychiatric features, such as autism spectrum disorder (ASD) or depression to psychosocial
114 concerns (2).

115 In spite of their apparent phenotypic *continuum*, DEEs include a large collection of specific
116 neurogenetic disorders. Several studies have been performed over the last two decades to identify
117 molecular determinants and characterize pathophysiological mechanisms underlying DEEs,
118 contributing to a greater understanding of their neurobiological and clinical aspects.

119 Next generation molecular testing has boosted gene discovery for many human disorders.
120 According to the Online Mendelian Inheritance in Man catalogue (OMIM, <https://www.omim.org>),
121 172 genes have been identified as causative for epileptic encephalopathy and, among them, 90
122 have been recognized to date as a cause of DEEs (**TABLE 2** and **SUPPLEMENTAL TABLE S1**,
123 https://figshare.com/articles/dataset/Guerrini_et_al_Supplemental_Table_S1/19666521).

124 However, this list may not be exhaustive, as the concept of DEEs is wide and encompasses a large
125 number of conditions and, in up to 8% of individuals are caused by *de novo* copy number variants
126 (CNVs) (17, 18).

127 The study of large cohorts of affected individuals with variable but related phenotypes performed
128 using next generation sequencing (NGS) approaches including targeted gene panels, whole exome
129 and whole genome sequencing, has now demonstrated that 30-50% of DEEs can be attributed to
130 *de novo* pathogenic variants in single genes (9, 16, 19). Low-level somatic mosaicism is observed in
131 about 10% of parents of probands with DEEs (20, 21), with important consequences for recurrence
132 risk estimation. In addition to single *de novo* variants, DEEs pathogenesis has also been associated
133 with recessive mutations in 11-38% of patients (22, 23). Additional genetic mechanisms, that are
134 often not detectable by standard NGS methodology, might also contribute to DEE
135 pathophysiology, including non-exonic variants, brain mosaicism in the patient, oligogenic
136 inheritance, and epigenetic changes.

137 The contribution of non-exonic variants is mainly related to ‘poison’ exons that, when spliced into
138 an RNA transcript, trigger nonsense mediated decay (NMD), a surveillance system that detects and
139 degrades RNAs harboring premature termination codons (PTCs) (**FIGURE 1**). An example of how
140 such mechanism is associated with DEEs has been described by Carvill and collaborators who
141 sequenced 11 non-coding candidate regions of the *SCN1A* gene in 640 individuals with unsolved
142 DEE, selected based on their evolutionary conservation and functional features. The authors
143 identified five variants in intron 20 that promoted inclusion of a “poison” exon and caused a
144 reduction of the amount of full-length *SCN1A* protein (24). Since transcriptome studies on purified
145 neural progenitor cells (NPCs) have identified hundreds of differentially-spliced exons (25),
146 reduced gene expression due to inclusion of poison exons may represent an etiological mechanism
147 of a broad range of neurological diseases, including DEEs.

148 Somatic mosaicism is the result of a variant arisen at postzygotic level, which is inherited by
149 daughter cells via mitotic division, and results in genetically distinct subsets of cells in the same
150 individual. Based on the timing when the variant arises, it can affect one or multiple tissues. Deep
151 sequencing studies in dysplastic brain/blood paired samples have correlated brain-confined
152 mutations with focal malformations of cortical development (MCDs). Mutations in the mTOR
153 pathway genes *AKT1*, *AKT3*, *DEPDC5*, *MTOR*, *NPRL2*, *NPRL3*, *PIK3CA*, *PIK3R2*, *TSC1*, and *TSC2* can
154 now be considered as the major known cause of focal cortical dysplasia type II (FCDII) and
155 hemimegalencephaly (HME) (26), while somatic mutations in *SLC35A2*, encoding a UDP-galactose
156 transporter, have been identified in a limited number of patients with FCD type I, mild
157 malformations of cortical development with oligodendroglial hyperplasia in epilepsy (MOGHE),
158 and with non-lesional focal epilepsy (NLFE) (27–31). In addition, somatic variants - either
159 microdeletions in *SCN1A* or point mutations in *CDKL5*, *PCDH19*, *SCN1A*, and *SCN2A* - have been
160 identified in 1-3.5% of patients with DEE who were mutation-negative to single gene, multigene

161 epilepsy panel or whole-exome sequencing (WES) performed in a clinical diagnostic setting (32,
162 33). Despite this evidence, the impact of somatic mutations in DEEs is likely to be underestimated,
163 as the major challenge in detecting causative somatic mutations is to analyze the right target brain
164 tissue which, for many DEE patients, is not easily accessible. For this reason, recent assays
165 developed from noninvasive protocols for tumor diagnosis and progression monitoring have been
166 attempted to identify low level mosaicism for known or recurrent mutations associated with
167 lesional refractory epilepsy in free DNA extracted from cerebrospinal fluid biopsies (34, 35).

168 Oligogenic inheritance refers to the concept that, in some cases, diseases with complex
169 phenotypes are not inherited as simple single-gene Mendelian disorders, nor they are classic
170 complex traits, but rather fit a model in which mutations in a small number of genes may interact
171 genetically to manifest a phenotype (36). In oligogenic conditions, one of the genes is the major
172 disease-causing gene, while the others act as modifiers. Some DEEs can be attributed to this type
173 of inheritance. Indeed, various studies have demonstrated that co-occurrence of two or more
174 mutations in distinct ion-channel genes can contribute to the epileptic phenotype in both patients
175 and animal models. (37–42). Performing targeted and whole exome sequencing in a boy who
176 presented with profound developmental delay, failure to thrive, ataxia, hypotonia, and tonic-
177 clonic seizures that caused his death, Hasan and collaborators identified a pathogenic variant in
178 *KCNJ10*, encoding the inward rectifying K⁺ channel Kir4.1 and a pathogenic variant in *KCNT1*,
179 encoding Na⁺-activated K⁺ channel known as Slo2.2 or SLACK. Functional studies performed in in
180 *Xenopus laevis* oocytes confirmed the functional effects of the two variants. This finding revealed
181 that, when co-occur, pathogenic variants in Kir4.1 and SLACK result in a fatal disease (40). To gain
182 support for the hypothesis that genetic modifiers can influence clinical presentation in patients
183 with *SCN1A*-derived genetic epilepsy with febrile seizures plus (GEFS+), Hawkins and collaborators
184 (38) combined the *Scn1a*-R1648H allele with either *Scn2a*^{Q54}, which causes spontaneous, adult-

185 onset partial motor seizures or *Kcnq2*^{V182M/+}, which causes increased susceptibility to induced
186 seizures. Double heterozygous mice exhibited early-onset, generalized tonic–clonic seizures and
187 juvenile lethality. These results demonstrate that variants in *Scn2a* and *Kcnq2*, can dramatically
188 worsen the phenotype of mice carrying the *Scn1a*-R1648H mutation (38).

189 A digenic inheritance based on somatic mutations in two mTOR pathway genes (*mTOR* and *RPS6*)
190 has also been demonstrated in association with hemimegalencephaly and intractable epilepsy
191 (43).

192 Aberrant chromatin states leading to altered gene expression patterns (epimutations) have been
193 detected in several conditions. Epimutations can occur secondary to a DNA mutation in a cis- or
194 trans-acting factor (secondary epimutations), or as “true” or primary epimutations in the absence
195 of any DNA sequence change (44). In the brain, such alterations can impair the transfer of
196 information that bind short-lived cellular signals to the whole neuronal activity and the global
197 gene expression (45). Emerging findings in animal models and human brain tissue have
198 demonstrated that DEEs could be ascribed to both classes of epimutations. A classic example of
199 DEE associated with secondary epimutations is Rett syndrome, which can be caused by both
200 nucleotide mutations in, and duplications of *MECP2*, encoding a methylated DNA-binding protein
201 (46).

202 Alterations in DNA methylation have not been explored much in epilepsy yet, but they represent a
203 good example of primary epimutations related to DEEs. Indeed, in the brain, this epigenetic
204 modification regulates cell fate determination and maturation, and plays a fundamental role in the
205 induction of activity-dependent synaptic plasticity, memory, and cognition (47–49). Dynamic
206 alterations in DNA methylation can also contribute to epileptogenesis. For example, various
207 studies have shown that the brain-derived neurotrophic factor (BDNF), whose expression is
208 regulated by a variety of cellular processes, including methylation of its promoter (50), results

209 upregulated in areas implicated in epileptogenesis (51). In addition, in hippocampal neurons, the
210 inhibition of DNA methyltransferases, which mediate demethylation of the BDNF promoter,
211 results in the suppression of neuronal excitability and network activity (52).

212 Various nongenetic etiologies have also been associated with certain DEEs, including hypoxic-
213 ischemic encephalopathy (HIE), cerebrovascular, infectious, or autoimmune disorders, tumors,
214 brain trauma, or metabolic disorders (5–7, 13, 53). While the molecular pathogenesis of these
215 etiologies is more complex and multifactorial than genetic etiologies, they offer a setting where
216 questions of “common pathways or mechanisms” in disease pathogenesis, network dysfunction,
217 and drug-refractoriness can be explored to develop therapies with broader applications. For
218 example, interneuronopathies, as well as mTOR dysregulation, have been implicated in both
219 genetic and nongenetic etiology DEEs (54, 55).

220 Many pathophysiological mechanisms underlie epilepsy and cognitive phenotypes in DEEs, leading
221 to either dysfunction of specific cortical networks or to more generalized epileptogenic changes.
222 These multiple and intersecting mechanisms make genotype/functional phenotype correlations
223 difficult. Cortical and subcortical neuronal networks may interact with each other, and in turn, can
224 cause widespread functional changes in otherwise normal cortex (56). The firing of excitatory
225 cortical neurons is finely regulated by the interplay of sodium and potassium channel activity,
226 which is mediated by chemical and ionic gradients across the cell membrane. If the balance
227 between sodium and potassium gradients is perturbed (for example due to mutations in genes
228 encoding Na^+ or K^+ ion channels or for components of the Na^+/K^+ pump) (**TABLE 2**) abnormal
229 depolarization arises, which in turn causes abnormal neuronal activity and cortical excitability (57).
230 Altered interconnections of glutamatergic neurons are another possible cause of abnormal
231 depolarization and mutations in various genes encoding for glutamate receptors or carriers (**TABLE**
232 **2**) have been associated with different DEEs (58–63). In addition to altered firing in excitatory

233 neurons, epileptogenesis in DEEs may be related to dysfunctions in interneuron networks. An
234 archetype of genetic lesions associated with such mechanism is represented by *SCN1A* mutations,
235 which are associated with Dravet syndrome and a broad category of other epilepsy phenotypes. A
236 series of functional studies have highlighted how changes in membrane properties of one specific
237 cellular population result in altered neuronal network dynamics and widespread cortical
238 dysfunction, in turn leading to an epileptic phenotype. *SCN1A* mutations are mainly associated
239 with loss of function in a subtype of voltage-gated sodium channel, the $\text{Na}_v1.1$ channel, mainly
240 expressed in inhibitory neurons (64). This functional alteration would be expected to predispose to
241 decreased neuronal activity. However, *in vivo*, it is actually associated with increased
242 epileptogenicity as it results in severely impaired sodium currents and action potential (AP) firing
243 in gamma-aminobutyric acid (GABA)ergic inhibitory neurons, without detectable effects on
244 excitatory pyramidal neurons (64, 65). Therefore, the epileptogenic effects of *SCN1A* mutations
245 are primarily mediated by an altered activity of inhibitory interneurons in the cortex rather than
246 by abnormal firing of excitatory neurons (66). Recent single cell RNAseq studies performed in
247 postmortem adult human and rodent brain tissues have confirmed that *SCN1A* (*Scn1a*) is
248 predominantly expressed in inhibitory neurons (67). Conversely, *SCN2A/3A/8A* (*Scn2a/3a/8a*),
249 which also encode for voltage-gated sodium channels, are preferentially expressed in excitatory
250 neurons in multiple brain regions, suggesting that epilepsy due to mutations in these genes is
251 mainly associated with direct alterations of the excitatory conductance (67). Expression and *in*
252 *vitro* and *in vivo* electrophysiological studies are therefore crucial to establish the functional
253 effects of channel mutations which may differ to same genes families or, as we shall see, even
254 with same gene.

255 Mutations in genes encoding glutamate receptors or ion channels can also cause epileptogenic
256 structural brain abnormalities. Using the DREADD (designer receptor exclusively activated by a

257 designer drug) approach, Hurni and collaborators showed that transient embryonic activation of
258 migrating projection neurons (PNs) induced transcriptional changes in a variety of activity-
259 dependent receptors including glutamatergic metabotropic, kainate, NMDA, and AMPA receptors,
260 that were accompanied by premature branching and persistent laminar mispositioning of
261 superficial layer PNs into deep cortical layers, without affecting expression of layer-specific
262 molecular identity markers (68). These findings support the hypothesis that increased intrinsic
263 activity during migration, a condition that can be caused also by mutations in DEE-causing genes,
264 acts as a stop signal for migrating cortical PNs (68).

265 Using a similar DREADD-based approach it has been demonstrated that in the developing mouse
266 neocortex ventricular zone progenitors become more hyperpolarized as they generate successive
267 subtypes of neurons (69). Experimental *in vivo* hyperpolarization shifted the transcriptional
268 programs and division modes of these progenitors to a later developmental status, with
269 precocious generation of intermediate progenitors and a forward shift in the laminar, molecular,
270 morphological, and circuit features of their neuronal progeny (69). These findings indicate that,
271 during development, altered bioelectrical processes can also affect non-excitabile cells, including
272 neuronal progenitors.

273 Genetic background may also alter the genotype-phenotype associations. This is well known in
274 humans and has also been demonstrated in mouse models from different labs. A striking example
275 was presented by Glasscock and collaborators whereby combination of a *Kcna1* knockout and a
276 *Cacna1a* missense mutation masked the absence epilepsy associated with *Cacna1a* and
277 attenuated limbic seizures and death expected from the *Kcna1*-null mutation (37). The C57BL/6
278 background also confers a more severe phenotype in $\text{Na}_v1.1^{+/-}$ mice with targeted deletion of the
279 last encoding exon, than the 129/SvJ background (70). In the C57BL/6 background, $\text{Na}_v1.1^{+/-}$ mice
280 manifest hyperthermia-induced and spontaneous seizures, cognitive and behavioral deficits, early

281 mortality. In contrast, in the 129/SvJ background have hyperthermia-induced seizures, less severe
282 spontaneous seizures and no cognitive deficits.

283 **II. GENERAL CONCEPTS OF EPILEPTOGENESIS AND EPILEPSY**

284 Epileptogenesis is the chronic process by which a brain network is functionally altered toward
285 increased seizure susceptibility, thus having an enhanced probability to generate spontaneous and
286 recurrent seizures. For this reason, epileptogenesis has been traditionally considered in the
287 context of a “latent period” between the causative insult and the first clinical seizure (71). This
288 concept, however, although applicable to several acquired conditions, especially post-traumatic,
289 post-stroke or post-infectious epilepsy, appears to be less apt to describe what happens in the
290 context of genetically determined DEEs where the bases for epileptogenesis are most often
291 imbricated with the altered dynamics of brain development and neural networking. Irrespective
292 from its causes, however, epilepsy is defined by recurrent and unprovoked seizures, and can be
293 divided into different categories, which are defined as generalized, focal (formerly called partial),
294 and combined generalized (57, 71), according to how the epileptogenic process is distributed in
295 the brain. These categories are defined based on the predominant types of seizures, including
296 generalized- or focal-onset (1, 2, 72).

297 A seizure can be conceptualized as the result of a distortion of the normal balance between
298 excitation (E) and inhibition (I), resulting from factors that may alter brain function at many levels,
299 from subcellular signaling cascades to widespread neuronal circuits (1). Genetic factors (i.e.,
300 mutations in specific genes) can affect brain function at any level, from ion channels to receptor
301 function and synaptic connectivity. Acquired cerebral insults (e.g., stroke or traumatic brain injury)
302 are mainly associated with alterations in circuit function (1). Excessive neuronal firing alone does
303 not necessarily cause a seizure, which also requires synchronization of a network of neurons.
304 Glutamatergic interconnections can promote such synchronization. Application of a pro-

305 convulsant agent to hippocampal slices in a feline model precipitates interictal discharges whose
306 intracellular correlate is the paroxysmal depolarizing shift (PDS). PDS is a network-driven burst
307 associated with a sudden depolarization of the membrane potential which lasts hundreds of
308 milliseconds and usually triggers a series of APs on its rising phase. Since cortical pyramidal cells
309 are richly interconnected by excitatory glutamatergic synapses, it has been proposed that the
310 mechanism underlying PDS is a “giant” excitatory postsynaptic potential (73).

311 Gap junctions (GJ) are another possible promoter of neuron synchronization. These specialized
312 intercellular connections allow a ‘low-resistance’ pathway of current flow from one cell to another
313 which, in neurons, may determine a rapid and effective synchronization. In addition, GJ located
314 between proximal axons of principal neurons (axon-axon gap junctions) can promote
315 epileptogenesis by providing pathways for direct spread of APs across neurons. A by-product of
316 such spread is the ability of axonally coupled neurons to generate oscillations at very high
317 frequencies (≥ 70 Hz) (74). Seizure activity, both *in vivo* and *in vitro*, begins with very high-
318 frequency oscillations, suggesting that axon-axon gap junctions may play a major role in seizure
319 initiation (74). Besides interneuronal GJ, inter-glial GJ can also be considered an important
320 mechanism for seizure generation (75). Studying mice with coupling-deficient astrocytes, obtained
321 by crossing conditional connexin-43 deficient mice (connexin-43^{Cx43^{fl/fl}:hGFAP-Cre}) with connexin-30^{-/-}
322 mice, Wallraff and collaborators showed how gap junctions in the impaired astrocytes accelerated
323 potassium clearance, limited potassium accumulation during synchronized neuronal firing, and
324 reduced the threshold for the generation of epileptiform events (76). Other studies, conducted in
325 a similar *in vivo* model, demonstrated that impaired glucose metabolism through astroglial
326 networks can contribute to epileptiform activity (77).

327 A third mechanism of enhanced synchronization is based on impaired inhibition. Individual
328 GABAergic interneurons can effectively phase spontaneous firing in hippocampal pyramidal cells

329 due to the interaction between GABA_A receptor-mediated hyperpolarizing synaptic events and
330 intrinsic oscillatory mechanisms in the pyramidal cells. Since interneurons make numerous
331 connections to pyramidal cells in local areas, a single discharging interneuron can synchronously
332 hyperpolarize a large number of pyramidal cells. Once GABAergic inhibition ceases, there is
333 activation of voltage-dependent currents in pyramidal cells, resulting in a synchronous
334 depolarization of a number of cells which might be high enough to trigger seizures (78).

335 Certain signature seizures and EEG patterns in DEEs also highlight the importance of the
336 corticothalamic network (SWD/atypical absences, generalized seizures) or brainstem structures
337 (tonic seizures, spasms) in the generation or development of generalized seizure activity.

338 The thalamus serves as a gate in trafficking sensory information to and from the cerebral cortex,
339 and its activity is controlled by the basal ganglia (79). The sensory relay thalamic neurons (TC) form
340 reciprocal glutamatergic connections with the cortex, but also project to the GABAergic neurons of
341 the nucleus thalami reticularis (nRT). The nRT neurons receive excitatory input from both the
342 cortex and TC neurons and send inhibitory projections to TC neurons (**FIGURE 2A**). Additional
343 inhibitory input to the TC relay neurons is also stemming from the local GABAergic interneurons
344 (**FIGURE 2A**). The SWDs are generated after a burst of excitatory postsynaptic potentials (EPSPs)
345 from the TC neurons excite the nRT interneurons which in turn send bursts of inhibitory input to
346 the TC neurons causing pronounced inhibitory postsynaptic potentials (GABA_AR and GABA_BR IPSPs,
347 **FIGURE 2A**) leading to activation of hyperpolarization-activated cyclic nucleotide-gated cation
348 channels (HCN), and low threshold calcium channels (T) which cause a calcium spike that triggers a
349 burst of APs. The re-excitation of TC neurons gives rise to a new cycle by eventually engaging also
350 cortical neurons. The typical SWD in humans is ~3 Hz, whereas in models of absence and in many
351 control rodents, typical SWD are 7-8 Hz (79).

352 Atypical absence seizures (AAS) are slower (<2.5 Hz in humans, usually 3-6 Hz in rodents), may
353 have atypical morphology and may not necessarily impair awareness. AAS utilize also the
354 corticothalamic network, although there may be more inputs from the limbic structures which
355 result in augmented cortical excitation. Certain forms of atypical SWD may also be generated from
356 isolated cortex or thalamus (80, 81). The utilization of existing circuitry and networks for the
357 generation of these seizures allows for transitional states, during which SWD may arise from
358 physiological rhythms, such as sleep spindles, when cortical neurons become hyperresponsive to
359 the thalamocortical excitatory input (82).

360 While our knowledge about the functional bases of generalized epileptogenesis and seizures are
361 largely based on experimental models, studies on focal epileptogenesis have taken advantage of
362 intracranial recordings in the setting of neurosurgery for epilepsy patients. These studies have
363 contributed to shape the now widely accepted concept that clinical seizures in focal epilepsies
364 originate in the 'seizure-onset zone' (SOZ), while epileptic seizure activity is generated in the
365 epileptogenic zone (EZ), i.e. the cortical area which is indispensable for seizure generation
366 independent of their clinical manifestation (83). Additional specific cortical areas that can be
367 identified in the epileptic brain are the irritative zone (IZ), representing the area of the cortex
368 generating interictal spikes, the epileptogenic lesion (EL), which may correspond to either a
369 macroscopic epileptogenic lesion (e.g., focal cortical dysplasia) or the hyperexcitable adjacent
370 cortex, and the functional deficit zone (FDZ), representing the area of cortex that does not
371 function normally in the interictal period (84) (**FIGURE 2B**). The EZ may be either more or less
372 extensive than the SOZ. If the EZ is smaller than the SOZ, even its partial resection or
373 disconnection may result in seizure disappearance, as the remaining SOZ could not be sufficient to
374 generate further epileptic seizures. Conversely, if the EZ is larger than the SOZ, total removal of
375 the SOZ cannot ensure seizure disappearance because multiple SOZs with different thresholds

376 may coexist in the same EZ. Indeed, in this case, after the resection of the first SOZ, another SOZ
377 with higher threshold may become clinically evident (83). However, this picture is simplistic.
378 Although seizures tend to have preferential spreading patterns, cortical connections spread in all
379 directions, from any given cortical point, through cortico-cortical and subcortical connections
380 (**FIGURE 2A, B**). Consequently, disconnection of specific networks does not guarantee that seizure
381 activity does not progress via alternative pathways, resulting in a modified clinical semiology, but
382 not in their disappearance (84). Only the complete disconnection or removal of all the potential
383 SOZs can guarantee that seizures disappear.

384 Some of the seizure types observed in epileptic encephalopathies are almost exclusively seen
385 within this category of severe epilepsies and do not fall within the categorization of generalized or
386 focal epileptogenesis. Epileptic spasms and tonic seizures, which are some of the signature
387 seizures in ISS and LGS, are considered to be bilateral seizures. The possible involvement of the
388 brainstem in generating these seizures was demonstrated in 1958 by Kreindler and collaborators
389 who reported bilateral tonic convulsions in cats and rats when stimulating the reticular substance
390 and periaqueductal substance (85). These certainly may explain reports of epileptic spasms in
391 infants with hydranencephaly (86), although they do not certainly exclude the contribution of
392 other higher structures which could activate a broader network generating these tonic seizures.
393 Animal models of ISS for example, have provided evidence that cortical or cortico-hippocampal
394 lesions may suffice to trigger spasms (87, 88). Seizures and epileptic activity may also be
395 multifocal, as in Epilepsy of Infancy with Migrating Focal Seizures (EIMFS), as an expression of a
396 widespread, genetically determined epileptogenesis in immature brains with incomplete
397 myelination and poorly functioning connections (89).

398 **III. MAIN (KNOWN) DETERMINANTS OF NORMAL AND ABNORMAL BRAIN DEVELOPMENT THAT**
399 **INFLUENCE EPILEPTOGENESIS**

400 Normal brain development is a dynamic process that proceeds asynchronously and at different
401 tempos and trajectories across brain regions, cell types and sexes, and is further modified by
402 biological or environmental factors (**FIGURE 3**). This asynchronous and timed maturation also
403 extends to key developmental processes that potentially control the susceptibility to seizures and
404 epileptogenesis: migration and differentiation of progenitors of excitatory and inhibitory neurons,
405 neurogenesis and synaptogenesis of excitatory and inhibitory synapses, morphological changes of
406 various brain regions (**FIGURE 3A**), signaling modes of molecular or electrophysiological signaling
407 cascades that control neuronal excitability, differentiation, function or communication, and
408 survival (**FIGURE 3B**) (90).

409 It is generally considered that around postnatal day 10 (PN10), a rodent is equivalent to a full term
410 human neonate, an assumption based on studies of brain growth spurt (91). Brain growth spurt in
411 these studies included gross brain growth, DNA, cholesterol, and water content. Puberty onset
412 occurs around PN32-36 in female rats and PN35-45 in male rats, whereas in humans it starts
413 around 10-11 years in girls and 11-12 years in boys (92). Distinct processes, such as neurogenesis
414 and migration, synaptogenesis and synaptic pruning, myelination follow different time courses
415 (**FIGURE 3B**) (90, 93–100). For studies that specifically target or relate to specific developmental
416 processes it is therefore important to consider the maturational trajectories of the specific
417 developmental processes of interest across species, when these are known.

418 There are extensive and continuous molecular, morphological or functional changes through
419 development that significantly diversify the effects of epileptogenic processes in the brain, thus
420 rendering the immature brain more amenable to developing the intense and sometimes multifocal
421 epileptic activity associated with DEEs (90). Ontogenetic studies with kindling, a method of
422 repetitive electrical stimulations of limbic structures that results in a progressively more severe
423 seizure phenotype, demonstrated that post-ictal refractoriness to manifest another seizure is

424 shorter in developing animals, which is likely a factor that predisposes them to develop clusters of
425 seizures (101). In addition, kindling antagonism, whereby kindling stimulation at one limbic
426 structure inhibits the kindling development in another, is not operative in immature pups (102).
427 Although the immature brain is more prone to seizures and seizure clusters than adults, it is also
428 more resilient, demonstrating no or less severe injury after prolonged seizures (103, 104).

429 The trajectories of maturation of these developmental processes often follow age, sex, and region-
430 specific patterns. These have been extensively studied for GABA_A receptors (GABA_AR) and include
431 changes in receptor composition and kinetics of, depolarizing/hyperpolarizing postsynaptic GABA_A
432 receptor (GABA_AR) responses or network effects (105–111) (**FIGURE 4**). GABA_AR signaling is usually
433 depolarizing in immature neurons with relatively high intracellular Cl⁻ content and elicits
434 hyperpolarizing responses in mature neurons which have low intracellular Cl⁻ (112–117). The
435 polarity of GABA_AR responses depends upon the relative abundance of cation chloride
436 cotransporters and channels that import [e.g., NKCC1, abundant early on, decreases later in life
437 (118–120)] or export [e.g. KCC2, expression increases postnatally (121, 122)] Cl⁻, in a process that
438 requires energy generated by Na⁺/K⁺ ATPase (**FIGURE 4**). Depolarizing GABA can have
439 neurotrophic effects in immature neurons and is a normal physiological phenomenon needed for
440 normal development (123–125). The developmental shift of GABA_AR signaling from depolarizing in
441 immature neurons to hyperpolarizing in mature neurons has been proposed to follow a rostro-
442 caudal gradient, with earlier maturation in the most caudal regions. In reality, there are significant
443 cell type-, region-, and sex-specific factors that create a more complex temporo-spatial pattern of
444 maturation not only of GABA_ARs, but also of other neurotransmitter and signaling pathways. For
445 example, hyperpolarizing GABA_AR responses may occur earlier in rat CA1 pyramidal neurons than
446 in substantia nigra pars reticulata GABAergic neurons (111, 126); GABA_AR mediated
447 hyperpolarizing responses are already seen in neonatal thalamic neurons, in contrast to cortical

448 neurons which mature later on (127). The maturation to hyperpolarizing GABA_AR signaling may
449 occur earlier in females than in males in certain brain regions (hippocampus, substantia nigra)
450 (108, 111, 125, 126, 128), whereas it may emerge later in females in others (cerebellar Purkinje
451 cells) (129). Premature cessation of depolarizing GABA_AR signaling may disrupt the excitatory
452 synapse formation and dendritic arborization of cortical neurons, leading to neurodevelopmental
453 deficits (130–132). Disruptions of the GABA_AR-sensitive patterns of communication across cells or
454 brain regions may also occur with seizures or epileptogenesis, stressors, genetic variants, drugs or
455 metabolic disorders, altering threshold for ictogenesis or epileptogenesis and predisposing to
456 behavioral or cognitive impairments (109, 125, 126, 133). Depolarizing GABA can also be seen
457 under pathological conditions, e.g. after axonal injury, hypoxia/hypoglycemia, during prolonged
458 seizures, or in epileptic tissue and its significance there can be dual: partly protective to promote
459 neuronal healing, but also potentially epileptogenic as it may promote neuronal excitability and
460 epileptogenesis (133). Further, KCC2 effects are not strictly upon Cl⁻ regulation; the C-terminus of
461 KCC2 may promote synapse formation (134) and the N-terminus can affect neuroprotection (135,
462 136). Loss of function mutations in *KCC2* have been found in epilepsy syndromes, including
463 Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) (137–139).

464 GABA signaling is also an important regulator of cortical-subcortical networks that control
465 fundamental physiological functions, such as learning and memory, but also seizures (110, 140–
466 142). The *modus operandi* of these networks undergoes significant age- and sex-dependent
467 changes through development. The substantia nigra pars reticulata (SNR), largely composed of
468 GABAergic interneurons, acts as a gate through which the cortico-striatal input may activate or
469 inhibit the activity of thalamo-cortical neurons. In adults, activation of GABA_ARs in the SNR exerts
470 anticonvulsant effects in a variety of seizure models (143–145). However, in developing rodents,
471 the outcome of GABA_AR-sensitive SNR-mediated seizure control (anticonvulsant vs proconvulsant

472 vs no effect) varies by region, age, and sex (110, 141, 142). All this knowledge remains too
473 fragmentary, but it represents a promising field to fully understand the human pathology.

474 Excitatory neurotransmitters also undergo significant developmental changes in expression and
475 subunit composition (**FIGURE 3**), which modify their biological effects in regard to both seizure
476 susceptibility and control (110, 146) and neuronal survival (147).

477 An important concept in developmental epileptogenesis is the existence of critical or sensitive
478 periods for the development of specific traits. The effects of biological factors or exogenous insults
479 can be time-locked to specific developmental periods that render the brain sensitive, as shown for
480 the hormonal regulation of the differentiation of seizure-controlling subcortical networks (148).

481 The biological roles of channels may also be age-dependent, as demonstrated for example for M
482 channels (149). M channel activity is essential for the normal morphological development of
483 hippocampus but only during the first postnatal weeks of murine development. Loss of M channel
484 activity at later periods does not have overt morphological sequelae in the hippocampus, although
485 cognitive deficits as well as increased neuronal excitability can still be observed. The therapeutic
486 effects of a treatment can be specific for certain developmental periods when they can modify
487 their desired targets, as shown for neonatal estradiol given to prevent interneuronopathy in an
488 *Arx* knock-in mouse model of epileptic spasms (150). The effects of genetic mutations may differ
489 depending upon the developmental period when these are expressed, as shown for the
490 GABA_AR γ 2(R43Q) epilepsy mutation (151).

491 Beyond gene effects, critical developmental periods are also important in determining the severity
492 of dysfunction conferred by the abnormal excitability. Using bicuculline and penicillin application
493 in the visual cortex of rabbits to induce sporadic epileptiform discharges, Chow and colleagues had
494 shown that the ensuing epileptic activity disrupted the appearance of complex and oriented-
495 directional type cells at the lateral geniculate nucleus in developing, but not in adult, rabbits (152).

496 More recent studies also demonstrated that the induction of epileptiform discharges by electrical
497 stimulation of the hippocampus in developing animals disrupts place cell formation (153, 154).

498 **IV. NON-GENETIC DETERMINANTS OF THE EPILEPTOGENIC RISK**

499 Inflammation and cytotoxic injury may trigger a chronic and evolving ISS phenotype in the multiple
500 hit model, suggesting that the cortical-subcortical network disruption due to the structural lesion,
501 in tandem with neuroinflammation, may trigger spasms and epileptogenesis with
502 neurodevelopmental deficits (88, 155–161). In the same model, dysregulation of the mTOR
503 (mechanistic target of rapamycin) pathway was a critical pathogenic feature and restoration of its
504 activity with rapamycin resulted in partial improvement of the cognitive deficits and reduced
505 epilepsy development, lending further support for the central role of mTOR in epileptogenesis
506 (155, 161). This model also provides evidence for sleep-epilepsy interplay with most adult motor
507 seizures emerging from sleep, as well as evolution to a slow spike-wave EEG, reminiscent of
508 Lennox-Gastaut syndrome (LGS) in adulthood (155).

509 The deficit in parvalbumin (PV) positive interneurons from the contralateral cortex (159) is
510 reminiscent of the interneuronopathy seen in *ARX*-related ISS (150, 162, 163), although the quality
511 of the interneuronopathy under these conditions is different. Mechanistically, these also seem
512 different, since the neonatal estradiol treatment that corrected the *ARX*-related
513 interneuronopathy and epilepsy (150, 163) did not improve the phenotype of the multiple-hit rats
514 (157).

515 Stress has been long advocated as a key pathogenic mechanism of ISS (164, 165). Administration
516 of corticotrophin releasing hormone (CRH) in the cortex or hippocampus of pups induced seizures,
517 but not spasms (166). Exposure to conditions that mimic aspects of early life stressors or stress
518 response, such as prenatal betamethasone or stress (167–169), or that disrupt adrenal function
519 aggravated NMDA induced spasms (170), although some of these conditions (such as prenatal

520 betamethasone) enhanced responsiveness of NMDA-induced spasms to ACTH (168). A more
521 recent model of chronic early life stress due to fragmented nurturing behaviors was reported to
522 manifest a mild spasms phenotype (171).

523 **V. GENETIC DETERMINANTS OF DEE: EXPERIMENTAL MODELS**

524 DEEs causing genes can be grouped into broad functional categories involved in different cellular
525 processes including ions/transmitters/small molecules transport, regulation of synaptic function,
526 cell growth, division and proliferation, cell metabolism, intracellular trafficking and signaling, gene
527 transcription, and protein biosynthesis/degradation. (**TABLE 2** and **SUPPLEMENTAL TABLE S1**,
528 https://figshare.com/articles/dataset/Guerrini_et_al_Supplemental_Table_S1/19666521).

529 We will focus upon gene products which have been extensively studied for their effects on
530 neuronal excitability and epileptogenesis in *in vitro* and *in vivo* studies, using relevant natural or
531 genetically engineered models. They offer clearer targets to design rational therapies to restore
532 normal function in dysfunctional networks which can be easily assessed using physiological tests.
533 We will also discuss more succinctly other genes that emerged as genes of interest for certain
534 DEEs, particularly those associated with migration disorders; however, their mechanisms of action
535 are more complex, involving multiple cellular processes that need to be disentangled before
536 designing safe and effective therapies.

537 Neuronal circuits are formed by principal glutamatergic excitatory neurons and inhibitory
538 GABAergic neurons (**FIGURE 5**). It is thought that in cortical circuits glutamatergic neurons perform
539 computational tasks, whereas GABAergic neurons control and organize the activity of the network
540 and are important for the generation of rhythms of activities, which are the substrate of brain
541 rhythms. Glial cells are important not only for neuron homeostasis and protection but are
542 implicated in synaptic functions. In neurons, the somato-dendritic compartment receives most of
543 the synaptic inputs that are computed in the dendritic tree and integrated in the axon initial

544 segment, where APs are generated. Forward-propagating APs reach presynaptic terminals, where
545 they evoke neurotransmitter release. Back-propagating APs in the somato-dendritic compartment
546 are implicated in dendritic computation and modulation of synaptic inputs. Ion channels are
547 essential in all neuronal sub-compartments and for neuronal signaling. Thus, it is not surprising
548 that channelopathies are implicated in numerous DEEs (172) (**FIGURE 5**).

549 Functional analysis of mutations using a variety of experimental systems is essential for shedding
550 light on the detailed pathomechanisms and clarifying genotype-phenotype correlations, which can
551 in turn facilitate diagnosis, genetic counselling, management, and development of therapeutic
552 approaches. Functional analysis using electrophysiological techniques identifies even subtle
553 modifications in the properties of ion channels. Experimental methods include both *in vitro* and *in*
554 *vivo* systems (173, 174), (**FIGURE 6**).

555 *In vitro* experimental systems often use cells that do not endogenously express the protein of
556 interest, thus simplifying the functional analysis of its properties. They are in general human cell
557 lines (e.g., transfected human embryonic kidney, HEK, cells) or, less frequently, oocytes of the
558 clawed frog *Xenopus laevis* injected with the cRNA of interest that allow massive expression,
559 although a human cellular background is generally preferable. Transfected/transduced neurons in
560 primary cultures are a further *in vitro* system that provides a true neuronal cellular background to
561 evaluate effects on neuronal and network properties. *In vivo/ex vivo* systems are organisms or
562 preparations obtained from them (e.g., brain slices), which should better model the complexity of
563 brain circuits and the actual pathophysiological conditions, as well as provide information of *in*
564 *vivo* phenotypes. The animals more frequently used for generating *in vivo* models of genetic
565 variants are mice and rats (**TABLE 3**), because early site directed mutagenesis techniques
566 exploiting homologous recombination in embryonic stem cells allows easy genetic manipulation of
567 these mammals. The mouse is still the organism of choice for generating animal models of genetic

568 variants, although more recent methods of genome editing (e.g., CRISPR-Cas9) can be used to
569 generate mutant models with other mammals. It is not possible to perform high throughput
570 studies with mammalian models, neither for studying functional effects of variants nor for drug
571 screens. Simpler animal models make it possible to perform relatively large screens, in particular
572 the zebrafish, which has vertebrate features (175). However, findings obtained with these simple
573 systems need to be validated in mammalian models. Neurons differentiated from induced
574 pluripotent stem cells (iPSCs) generated from patients' biopsies are increasingly used to study
575 mutations in human neurons as they bear the patient's genetic background. They can be used for
576 investigations of neuronal properties at the single cell level or can be induced to generate *in vitro*
577 miniature organs resembling the brain (brain organoids) that represent an excellent integrated
578 experimental system to study brain development (176). However, large variability in the
579 properties of these neurons makes studies difficult and reproducibility is still an issue, as observed
580 in studies of *SCN1A* mutations (177–184).

581 **VI. DYSFUNCTIONS IN NEURAL CELL MIGRATION, PROLIFERATION, AND SYNAPTOGENESIS**

582 Mutations in distinct DEE genes can trigger different molecular and biochemical alterations which,
583 depending on the developmental stage involved and the type of alteration, may result in a brain
584 with a grossly abnormal morphology or structurally normal, but functionally abnormal (**FIGURE**
585 **7A-BB**). Disruption of any of the overlapping steps that contribute to the development of the
586 human cerebral cortex that can be recognized as malformations in studies of brain imaging is
587 designated as “malformation of cortical development” (MCD). MCDs can be broadly classified into
588 three major groups that recapitulate the main developmental steps, i.e., malformations of cell
589 proliferation, neuronal migration, or post-migrational cortical organization (185).

590 Among brain morphological abnormalities, MCDs are those most frequently associated with
591 recurrent seizures. In MCDs, altered processes of development involve cells that, under normal

592 circumstances, would participate to the formation of the normal cerebral cortex, and seizures can
593 arise because of neuronal malpositioning, abnormal proliferation or differentiation, or abnormal
594 cortical organization (186).

595 Neuronal malpositioning is the result of altered neuronal migration during brain development. A
596 classic example of DEE associated with neuronal migration deficit is caused by mutations in the
597 *aristaless*-related homeobox (*ARX*) gene, located on chromosome Xp21, which can result in a
598 phenotypic spectrum comprising a nearly continuous series of neurodevelopmental disorders
599 including lissencephaly with ambiguous genitalia (XLAG) (**FIGURE 7A**), Proud syndrome, Partington
600 syndrome, infantile spasms without brain malformations, and syndromic and non-syndromic
601 intellectual disability with epilepsy.

602 The *ARX* protein belongs to the *Aristaless*-related subset of the paired (*Prd*) class of homeodomain
603 proteins. Homeodomain transcription factors play crucial roles in cerebral development and
604 patterning (187). In particular, *ARX* is involved in the normal tangential migration of GABAergic
605 neurons and the occurrence of seizures in most patients carrying mutations in this gene can be
606 ascribed to mislocalization or malfunction of this class of neurons and loss of inhibitory
607 neurotransmission (188). For this reason, *ARX*-related DEEs is considered a “developmental
608 interneuronopathy”, a term coined to differentiate developmental brain disorders caused by
609 impaired development, migration, or function of interneurons from functional deficits or
610 secondary loss of interneurons and from the more common channelopathies (189).

611 Developmental interneuronopathies may also include Dravet syndrome, as discussed above, and
612 classical lissencephaly due to mutations in *PAFAH1B1*, also known as *LIS1* (**FIGURE 7B, C**), or *DCX*
613 (**FIGURE 7D**). The *PAFAH1B1* gene encodes for the regulatory beta-subunit of the cytosolic type I
614 platelet-activating factor (PAF) acetylhydrolase, which is involved in interneuronal migration and
615 survival, whilst *DCX* encodes for a microtubule-associated protein essential for both radial and

616 non-radial interneuronal migration into the cerebral cortex (55). Interneuronopathies have also
617 been described in models of acquired DEEs, such as the multiple hit model of ISS (159), although
618 the underlying interneuronal deficits and mechanisms are likely distinct, requiring different
619 targeted therapeutic approaches (157).

620 The phenotypic continuum of MCDs, which includes focal cortical dysplasia type II (FCDII),
621 hemimegalencephaly (HME), megalencephaly (MEG), and dysplastic megalencephaly (DMEG), is
622 mainly caused by constitutional and somatic mutations in mTOR pathway genes, i.e., *AKT1*, *AKT3*
623 (**FIGURE 7E**), *DEPDC5*, *MTOR* (**FIGURE 7F, G**), *NPRL2* (**FIGURE 7H**), *NPRL3*, *PIK3CA*, *PIK3R2* (**FIGURE**
624 **7I, J**), *PTEN* (**FIGURE 7K, L**), *TSC1*, and *TSC2* (**FIGURE 7M, N**), and represents the paradigm of DEEs
625 caused by abnormal neuronal proliferation or differentiation, in addition to abnormal neuronal
626 migration. FCD is the cause of seizures in almost half of children referred for surgical treatment of
627 medically refractory epilepsy, roughly estimated to near 400,000 people in the United States
628 (Wolters Kluwer UpToDate website - [https://www.uptodate.com/contents/evaluation-and-](https://www.uptodate.com/contents/evaluation-and-management-of-drug-resistant-epilepsy)
629 [management-of-drug-resistant-epilepsy](https://www.uptodate.com/contents/evaluation-and-management-of-drug-resistant-epilepsy)). In addition to abnormal cortical lamination, FCDII also
630 features large dysmorphic neurons without (type IIa) or with (type IIb) balloon cells (190). HME, a
631 condition in which one hemisphere is abnormally larger than the contralateral, and DMEG, a
632 condition in which cortical dysplasia is associated with segmental brain overgrowth, exhibit
633 histopathological features similar to FCDII (185). Tuberous sclerosis complex (TSC), a congenital
634 syndrome characterized by the development of benign tumors (hamartomas) in multiple organs,
635 including the brain, and a neurological phenotype consisting mainly of early-onset seizures,
636 intellectual disability, and at times, autism, is also caused by mTOR pathway dysregulation due to
637 mutations in the tumor suppressor genes *TSC1* or *TSC2* (191).

638 Balloon cells in FCDIIb and TSC exhibit features of both neurons and glia. On histopathological
639 analysis, dysmorphic or cytomegalic neurons are often associated with increased proliferation of

640 normal-appearing glia or abnormal reactive astrocytes, which overexpress specific intermediate
641 filaments and other immature molecular markers. Taken together, these findings strongly
642 implicate primary defects in cellular proliferation and differentiation in the pathogenesis of
643 mTORopathies (192).

644 Electrophysiological recordings in neocortical samples surgically removed from patients with FCD
645 have demonstrated that, under reduced K^+ conductance, dysmorphic cytomegalic neurons, but
646 not balloon cells, generate large Ca^{2+} currents when stimulated, indicating that this aberrant cell
647 type plays an important role in epileptogenesis (193). Both the morphology and size of dysmorphic
648 cytomegalic neurons have been implicated with the origin of aberrant electrical discharges. By
649 using electrophysiology, calcium imaging, morphological analyses, and modeling studies, Williams
650 and collaborators demonstrated that *Pten* (an mTOR pathway inhibitor) knock-out neurons exhibit
651 rapid-onset hypertrophy and higher density of synapses proximal to the soma and that these
652 phenotypic abnormalities promote firing at more hyperpolarized membrane potentials, with
653 greater peak spike rates, and higher sensitivity to depolarizing synaptic input (194).

654 Using mouse models with cell-type-specific mTOR pathway activation obtained by electroporating
655 the *Pik3ca* p.H1047R mutation, which is associated with both FCD and HME, D’Gama and
656 collaborators demonstrated that mTOR pathway activation in excitatory neurons and glia, but not
657 in interneurons, is sufficient to cause abnormal cortical lamination and overgrowth (195). In FCD,
658 epileptic seizures appear to be triggered by a peculiar mechanism which we detail below. By
659 treating *in vitro*-maintained organotypic slice cultures of resected FCD lesions with 4-
660 aminopyridine, a potent convulsant agent acting as potassium channel blocker, Avoli and
661 collaborators demonstrated intrinsically generated ictal-like epileptiform events (196). These
662 epileptiform events were triggered by an amino acid receptor-mediated mechanism which was
663 glutamatergic-independent and mainly attributable to a $GABA_A$ receptor-mediated conductance

664 (196). These findings are in line with the observation that, in some cases, dysplastic tissue of
665 FCDIIb and cortical tubers retain features of immature tissue (197, 198).

666 VII. DYSFUNCTIONS IN INTRINSIC EXCITABILITY

667 1. VOLTAGE-GATED Na⁺ CHANNELS

668 Na⁺ channels are clustered at high density at the axonal initial segment (AIS), which is, for this
669 reason, the primary site for generation of APs in neurons (**FIGURE 5**). In myelinated axons, Na⁺
670 channels are clustered at high density also at the nodes of Ranvier to allow saltatory axonal
671 conduction. Voltage-gated Na⁺ channels are essential for the generation of neuronal excitability
672 because they generate the depolarizing Na⁺ current that initiate and propagate APs. These Na⁺
673 currents have fast activation and inactivate within few milliseconds after opening, although a small
674 fraction of slowly inactivating (persistent) current remains for longer periods during
675 depolarizations (199, 200). Na_v are composed by a principal pore-forming α subunit (nine
676 isoforms: Na_v1.1-Na_v1.9 for the proteins, *SCN1A-SCN11A* for the genes) and auxiliary β subunits
677 (four isoforms: β1-β4 for the proteins, *SCN1B-SCN4B* for the genes) (199, 201). The primary
678 sequence of the α subunits contains four homologous domains (DI-DIV), each comprising six
679 predicted transmembrane segments (S1-S6) that form voltage-sensing modules (S1-S4; S4 is the
680 voltage sensor) and pore modules (S5-S6 and their connecting extracellular loop) in each domain.
681 The β subunits contain a single transmembrane segment. *SCNs/Navs* are targets of clinically used
682 sodium channel blockers, among them several anti-seizure medications (ASMs) (202). Mutations in
683 *SCN1A/Nav1.1*, *SCN2A/Nav1.1*, *SCN3A/Nav1.1* and *SCN8A/Nav1.6* as well as of *SCN1B/β1*, which
684 are expressed in the central nervous system, are important causes of DEEs (199, 203–205).
685 Patients carrying variants in voltage gated Na⁺ channels also exhibit an increased risk of sudden
686 unexpected death in epilepsy (SUDEP) (206). Mutations implicated in DEEs have been found also in
687 genes encoding proteins that can modulate Nav properties, like *FGF12/FHF1* (207, 208).

688 **1.1. Na_v1.1 channels (*SCN1A*)**

689 *SCN1A*, encoding the Na_v1.1 α subunit, is one of the most clinically relevant epilepsy genes, with
690 hundreds of mutations reported thus far, whose associated phenotypes range from Dravet
691 syndrome, a severe form of DEE, to milder GEFS+ and other diseases, such as hemiplegic migraine
692 (4, 209, 210).

693 Dravet syndrome is caused by often *de novo* heterozygous *SCN1A* mutations (211, 212), of which
694 approximately half are missense, and half are predicted to give rise to a truncated non-functional
695 protein. The clinical spectrum of Dravet syndrome does not have firmly established boundaries,
696 but the core phenotype is characterized by intractable seizures precipitated by increased body
697 temperature with onset between six months and one year of age, and subsequent appearance of
698 multiple hyperthermia-induced and hyperthermia-independent seizures. Development is normal
699 in the first year of life but plateaus rapidly, with most patients showing cognitive impairment. In
700 patients with Dravet syndrome, *SCN1A* mutations can also be inherited from a parent with less
701 severe clinical manifestations, at times carrying somatic mosaicism (213). It has been proposed
702 that mutations in other genes can cause Dravet syndrome-like phenotypes (including *SCN1B*,
703 *HCN1*, *KCN2A*, *GABRA1*, *GABRG2*, and *STXBP1*), but with a specific clinical pictures (see below).
704 Thus, the association between *SCN1A* mutations and Dravet syndrome is highly specific. The
705 familial epilepsy GEFS+ syndrome can be also caused by heterozygous missense *SCN1A* mutations
706 and is characterized by febrile seizures plus (FS+: febrile/hyperthermic seizures that extend
707 beyond six years of age) and afebrile generalized tonic-clonic seizures (GTCS), at times including
708 absence, myoclonic, atonic, or focal seizures, and Dravet syndrome. Missense *SCN1A* mutations
709 can also cause sporadic/familial hemiplegic migraine type3 (S/FHM3), a rare form of migraine with
710 aura and onset in adolescence, characterized by hemiparesis as part of the aura phase. Moreover,
711 two missense mutations with gain of function have been associated with an extremely severe

712 early infantile DEE with earlier seizure onset, profound cognitive and motor impairments, and a
713 hyperkinetic movement disorder (214–216).

714 The functional effect of truncating mutations causing Dravet syndrome has since their
715 identification thought to be haploinsufficiency: 50% reduction of functional Na_v1.1 protein in
716 heterozygotes, with complete loss-of-function (LoF) (211), which has been verified in functional
717 studies (217). The effect of missense mutations studied in transfected cell lines has been more
718 controversial, but most of the results point to a LoF, whose severity tends to correlate with that of
719 the phenotype (199, 218). Thus, the severity spectrum of *SCN1A*-related epilepsies could be a
720 continuum and depend on the amount of LoF of the mutant. Some *SCN1A* missense mutations
721 cause LoF because of folding/trafficking defects that lead to channel degradation (219) that can at
722 least partially be rescued by interacting proteins stabilizing the correct folding conformation (220–
723 223).

724 Na_v1.1 is the predominant Na⁺ channel of GABAergic interneurons whose decreased excitability
725 induced by *SCN1A* epileptogenic mutations reduces GABAergic inhibition and causes network
726 hyperexcitability (64). A study with a knock-in model expressing a truncating nonsense DS
727 mutation reported a similar phenotype, showing that Na_v1.1 localizes to the axon initial segment
728 of GABAergic interneurons, in particular fast-spiking parvalbumin (PV)-positive ones (65).
729 Subsequent studies have shown that these mice display also co-morbidities including cognitive
730 and behavioral deficits, ataxia, SUDEP, dysregulated circadian rhythms and sleep dysfunctions
731 (199, 218, 224, 225). Several other studies, including those performed with conditional mouse
732 models that expresses the mutations in specific neuronal subtypes (199, 218) and with a knock-in
733 model of a GEFS+ mutation (*Scn1a*^{R1648H/+}) (226, 227) have confirmed that hypoexcitability of
734 GABAergic neurons is the initial pathological mechanism in Dravet syndrome models and that the
735 amount of LoF can determine the severity of the phenotype. Notably, *Scn1a* mouse models have

736 also allowed to disclose genetic modifiers and cellular remodeling induced by the initial effect of
737 the *Scn1a* mutation, which can modulate the phenotype implementing homeostatic or pro-
738 pathologic modifications, including homeostatic upregulation of Na⁺ channels in GABAergic
739 neurons and seizure-induced hyperexcitability of glutamatergic neurons (199, 218). For instance,
740 the interaction between seizures and the genetic mutation can induce remodeling in the
741 *Scn1a*^{R1648H/+} mouse model transforming a basically asymptomatic phenotype into a Dravet
742 syndrome-like one, because of increased excitability of excitatory glutamatergic neurons (228).
743 Overall, these results show that the initial pathological mechanism in mouse models of human
744 epileptogenic *SCN1A* mutations is LoF and hypoexcitability of at least some subtypes of GABAergic
745 neurons. However, this initial dysfunction triggers both homeostatic and pathologic remodeling,
746 depending on the neuronal types, age, genetic background, and interactions between *SCN1A*
747 mutations and experienced seizures.

748 Although some drugs like stiripentol (229), fenfluramine (230) or cannabidiol (231) have been
749 shown to be partially effective in some patients, Dravet syndrome still remains drug-resistant for
750 most of the patients. *Scn1a* gene targeted mice have been used for testing therapeutic
751 approaches, both classical pharmacological treatments and novel methods, including gene
752 therapy, in some cases obtaining significant amelioration of the phenotype (199, 232). Some novel
753 approaches have shown particularly effective results and have led to clinical trials. For example, an
754 antisense oligonucleotide-based targeted augmentation of nuclear gene output (TANGO)
755 approach has been used to increase the expression of functional Na_v1.1 channels in Dravet
756 syndrome mice, observing a substantial decrease in spontaneous seizures and SUDEP when
757 specific antisense oligonucleotides were injected in newborn mice (233).

758 Mutations causing sporadic/familial hemiplegic migraine (S/FHM) cause Na_v1.1 gain-of-function
759 (GoF) by inducing gating modifications and increasing the slowly inactivating “persistent” Na⁺

760 current (199, 218). Gene targeted mouse models of S/FHM mutations have been generated and
761 they show facilitated/spontaneous generation of cortical spreading depression (CSD), a proposed
762 pathological mechanism of migraine with aura, but no seizures (234, 235). Genetic and acute
763 models have shown that these mutations induce hyperexcitability of GABAergic neurons leading to
764 increase of extracellular K^+ and clamped depolarizing block of neuronal activity (236). A very mild
765 GoF has also been observed for the p.T226M mutation causing an extremely severe early infantile
766 *SCN1A* DEE (216), but it is not clear yet how such a mild change could induce the severe
767 phenotype.

768 **1.2. $Na_v1.2$ channels (*SCN2A*)**

769 *SCN2A* encodes the $Na_v1.2$ sodium channel, which is widely expressed in the central nervous
770 system, particularly in cortical and hippocampal glutamatergic neurons (199). These are the main
771 Na^+ channels of the axonal initial segment (AIS) and the nodes of Ranvier in the first 10 days of
772 postnatal life in rodents, then they are partially replaced by *SCN8A*/ $Na_v1.6$. It is thought that
773 $Na_v1.2$ channels in the proximal AIS promote backpropagation to the soma. In the adult brain,
774 $Na_v1.2$ is also present in thin processes, presumably distal unmyelinated portions of preterminal
775 axons.

776 The first epilepsy syndrome clearly associated with *SCN2A* mutations was benign familial
777 neonatal/infantile seizures (BFNIS) (237), characterized by a mild phenotype. Subsequently, it has
778 been shown that mutations in *SCN2A* cause a wide range of neurodevelopmental disorders,
779 including DEE of varying severity. DEE mutations generally arise *de novo*, and about 80% are
780 missense. About 60% of *SCN2A* DEE have onset in the first three months of life (238, 239). All
781 neonatal-early infantile DEE patients display intellectual disability. About 50% of them have
782 variable seizure types, whereas the others have Ohtahara syndrome (neonatal-onset spasms or
783 tonic seizures and EEG with burst suppression pattern), sometimes evolving into West syndrome,

784 Lennox–Gastaut phenotypes or epilepsy with early infantile migrating focal seizures (EIMFS). DEE
785 with infantile and childhood onset are about 40% of the total (238, 239). The phenotype can be
786 correlated to the age of onset. Patients with onset between three months and one year of age in
787 general have West syndrome, with possible evolution into a Lennox–Gastaut phenotype. Patients
788 with onset after one year of age often show variable seizure phenotypes with
789 developmental/cognitive delay and autistic traits that can appear before seizure onset.

790 Functional analysis in transfected cell lines has shown some genotype-phenotype correlations
791 (199, 240). GoF mutations are related to mild benign neonatal/infantile epilepsy and to
792 neonatal/early infantile DEE, which can be responsive to treatment with Na⁺ channel blockers
793 (239), whereas LoF mutations are linked to infantile/childhood epileptic encephalopathy or
794 neurodevelopmental disorders (autism and intellectual disability) without seizures. However,
795 identifying genotype-phenotype correlations within these GoF and LoF mutants is difficult due to
796 partially overlapping phenotypes.

797 Recordings in brain slices of heterozygous knock-out *Scn2a* (*Scn2a*^{+/-}) mice have shown that
798 Na_v1.2, besides its established axonal role, has important dendritic functions in pyramidal neurons
799 of the prefrontal cortex, where its haploinsufficiency impairs synaptic plasticity and synaptic
800 strength, even when *Scn2a* expression is reduced in single neurons late in postnatal development
801 (241). However, these studies have shown that *Scn2a*^{+/-} mice, differently than *Scn1a* models, have
802 a relatively mild phenotype, including short absence-like seizures, spatial memory deficits but
803 enhanced fear memory, plus autistic traits (199, 242). A *Scn2a* mouse model carrying the severe
804 hypomorphic mutation Δ1898 shows a more severe phenotype, with robust autistic-like features
805 (243). Two additional studies have shown that either complete deletion (244) or reduced
806 expression of *Scn2a* (244, 245) can paradoxically induce hyperexcitability of glutamatergic
807 neurons. Overall, these findings suggest that reduction of *SCN2A* to more than haploinsufficiency

808 could be needed to induce severe phenotypes. A knock-in mouse model of the recurrent GOF
809 mutation R1882Q has been generated (246) showing that heterozygous mice display
810 hyperexcitability of cortical pyramidal neurons, and develop spontaneous seizures at P1 and
811 premature death between P13 and P30. Interestingly, reduction of $\text{Na}_v1.2$ expression by specific
812 antisense oligonucleotides reduced seizures and extended lifespan.

813 **1.3. $\text{Na}_v1.3$ channels (*SCN3A*)**

814 *SCN3A* encodes the $\text{Na}_v1.3$ Na^+ channel, whose functions have not been completely determined
815 yet. *SCN3A* is widely expressed in the brain at high levels during embryonic development and is
816 downregulated afterwards (199).

817 *SCN3A*-related clinical phenotypes comprise a wide spectrum, including mild epilepsy with
818 intellectual dysfunction, early infantile DEE often associated with polymicrogyria, as well as speech
819 and oral motor dysfunctions associated with polymicrogyria without epilepsy. Relatively mild
820 neonatal-childhood onset focal epilepsy (247), would be the mildest phenotype in the spectrum
821 (248, 249). Disrupted cerebral cortical folding and neuronal migration were recapitulated in ferrets
822 expressing the mutant *SCN3A* (249). Functional studies in transfected human cell lines have shown
823 that most of the *SCN3A* mutations associated with severe human phenotypes exhibit prominent
824 GoF, inducing, in particular, large increases of persistent Na^+ current (248–250). LoF caused by
825 reduced current density has been reported for some *SCN3A* variants. Heterozygous adult *Scn3a*^{+/-}
826 knock out mice were investigated as a model of *SCN3A* LoF mutations, but they do not show
827 features of DEE (251).

828 **1.4. $\text{Na}_v1.6$ channels (*SCN8A*)**

829 *SCN8A* encodes $\text{Na}_v1.6$, the main Na^+ channel in the axon initial segment and nodes of Ranvier of
830 myelinated axons in mature excitatory neurons (199).

831 *De novo* heterozygous mutations in *SCN8A* have been identified in a range of phenotypes. Most
832 patients show a DEE with multiple seizure types and early onset, severe intellectual disability and
833 movement disorders and have an increased risk of SUDEP (252). Other patients exhibit milder
834 phenotypes, including benign familial infantile seizures (BFIS) and epilepsies with intermediate
835 phenotypes, or generalized epilepsy with absence seizures (253, 254). Some patients show
836 intellectual disability, autism, or movement disorders without epilepsy (255, 256). About 20% of
837 patients have recurrent mutations.

838 Mutations that cause DEE or milder epilepsy induce GoF with negative shifts of voltage-
839 dependence of activation, positive shifts of voltage-dependence of inactivation, slowed channel
840 inactivation, or increased persistent or resurgent current. These functional changes are all
841 consistent with neuronal hyperexcitability, which has been confirmed in transfected cultured
842 neurons (255, 257). Other mutations causing intellectual disability, autism, or movement disorders
843 without epilepsy induce LoF, which can be complete (255, 256). Massive GoF can induce reduced
844 generation of APs, mimicking LoF mutations (255). LoF mutations have been identified in patients
845 with absence seizures (254), a phenotype reproduced by heterozygous loss of function mouse
846 models.

847 A standard global knock-in (258) and a conditional floxed knock-in (259) mouse models of *SCN8A*
848 DEE mutations are available. Overall, knock-in mice indicate that *SCN8A* GoF mutations are
849 sufficient to induce hyperexcitability of some subtypes of excitatory neurons, generating severe
850 seizures and a lethal phenotype. In contrast, spontaneous mouse models carrying LoF *SCN8A*
851 mutations (260) show a phenotype similar to that of patients with intellectual disability and/or
852 movement disorders without epilepsy.

853 Some patients with *SCN8A* GoF mutations respond to high doses of sodium channel blockers (254,
854 261), but available blockers are not isoform-specific and chronic therapy at high doses can induce

855 adverse effects. A recent study has used antisense oligonucleotides that reduce *Scn8a* expression
856 by 25–50%, showing a delay of seizure onset and lethality in a knock-in *Scn8a* mouse model (262).
857 These genetic approaches are highly specific for *SCN8A*, but problems of oligonucleotide delivery
858 and half-life need to be solved before a translational use.

859 **1.5 Auxiliary subunits of Na⁺ channels**

860 *SCN1B* encodes the “auxiliary” $\beta 1$ Na⁺ channel subunit that is widely expressed in different organs,
861 including the central nervous system. β -subunits were originally named auxiliary because they do
862 not directly form the channel but modulate the properties and the membrane delivery of α -
863 subunits. We now know that they are multifunctional molecules implicated, besides direct
864 modulation of α subunits, in diverse and essential roles in multiple tissues, including cell adhesion
865 and migration, neuronal pathfinding, fasciculation and neurite outgrowth (204). Mutations in β -
866 subunits can alter numerous functions, including modulations of all the α -subunits, and thereby
867 they are involved in epilepsy, neurodegenerative disorders, neuropathic pain, cardiac arrhythmias
868 and cancer (204). Mutations in *SCN1B* have been identified in a range of epilepsy phenotypes.
869 Homozygous *SCN1B* mutations have been identified in DEE patients that were initially included in
870 the Dravet syndrome spectrum (263). However, it is now clear that their clinical features are
871 distinct from Dravet syndrome, showing earlier onset seizures and more severe
872 neurodevelopmental phenotype, including psychomotor, stagnation or regression, and
873 microcephaly (264, 265).

874 Functional analysis of *SCN1B* DEE mutations in transfected cell lines have identified either LoF
875 modulation of co-expressed α subunits or induction of complex gating modifications in different
876 co-expressed α subunits (263–265). Homozygous knock-out null *Scn1b*^{-/-} mice show spontaneous
877 seizures and a high mortality rate (266). Cortical neurons in brain slices obtained from these mice
878 have shown dysfunctions in the excitability of both pyramidal excitatory neurons and GABAergic

879 fast-spiking interneurons. GABAergic interneurons were hypoexcitable, whereas dysfunctions of
880 pyramidal neurons were more complex, with subsets of them exhibiting hyperexcitability at low
881 current injections, as well as hypoexcitability at high stimulation intensities.

882 **2. VOLTAGE-GATED K⁺ CHANNELS**

883 K⁺ channels are the most diverse group of ion channels and can be classified into different families
884 depending on the number of transmembrane domains in each subunit and the gating
885 mechanisms. Voltage-gated K⁺ channels (K_v) are composed by four subunits, each with six
886 transmembrane segments, a voltage sensor module formed by segments S1 to S4, and the pore
887 region formed by S5, S6 and their connecting loop, as in Na_v channels. They generate repolarizing
888 currents that oppose the action of depolarizing currents and are involved in different epileptic
889 encephalopathies.

890 **2.1. M-current K⁺ channels (K_v7, KCNQ)**

891 Five *KCNQ* genes have been identified that encode K_v7 K⁺ channels, which can form both homo-
892 and hetero-tetramers (267, 268). Of the four channels expressed in the nervous system (*KCNQ2*-
893 5), three (particularly *KCNQ2*, but also *KCNQ3* and *KCNQ5*) host DEE mutations. *KCNQ2* and *KCNQ3*
894 are expressed in many brain areas, whereas expression of *KCNQ5* is more limited (269). They
895 generate the M (muscarinic receptor inhibited)-current, a slow non-inactivating K⁺ current that
896 activates at subthreshold membrane potentials (270). *KCNQ2*-linked DEE has its onset in early
897 infancy and features motor impairment and variable intellectual disability (271). Often, the EEG
898 initially shows a burst suppression pattern that may later evolve to multifocal epileptiform activity.
899 *KCNQ3* and *KCNQ5* DEEs display a variable degree of severity and some patients show intellectual
900 disability apparently without epilepsy (9, 267). Mutations in the three *KCNQ* genes can also cause
901 “benign familial neonatal seizures” (BFNS), which is mild and self-limited (268).

902 According to their subcellular localization, K_v channels can regulate specific features of neuronal
903 excitability. Somato-dendritic channels are strongly activated by back-propagating APs, attenuate
904 repetitive firing and contribute to the medium and slow components of the afterhyperpolarization
905 (AHP), determining the refractory period and regulating spiking frequency. In the perisomatic
906 region, slow activation of the M current decreases neuronal firing in response to sustained stimuli,
907 inducing spike-frequency adaptation (269, 272, 273). Axonal K_v channels mostly function by
908 stabilizing the resting membrane potential, leading to increased activation of axonal Na_v channels
909 (274–276) and of presynaptic Ca^{2+} channels, modulating synaptic release (277, 278). Both *KCNQ2*
910 and *KCNQ3* are also expressed in GABAergic interneurons (279).

911 Numerous *in vitro* studies have investigated functional effects of *KCNQ* DEE mutations in cell lines
912 or *Xenopus* oocytes (267). Most studies agree that LoF, often leading to haploinsufficiency, is the
913 pathophysiologic mechanism of mild and self-limited BFNS (267, 268). DEE mutations can induce
914 more severe LoF consistent with a dominant negative effect. A single report showed that the
915 recurrent p.A294V $K_v7.2$ pore mutation does not modify the properties of the current, but induces
916 LoF altering the subcellular localization of K_v channels (280). DEE can also be caused by GoF, in
917 which case, disease severity correlates with that of functional alteration (267). The first transgenic
918 mouse model expressing a *KCNQ2* dominant-negative mutant (149), showed spontaneous focal
919 and generalized tonic-clonic seizures, impaired hippocampus-related memory and pronounced
920 hyperactivity, but also cell loss in the hippocampus, which is not observed in patients. The first
921 knock-in model of *KCNQ2*-DEE (281) carrying the p.T274M mutation displayed an overall 70–80%
922 reduction of the M-current (282). *KCNQ2*^{T274M/+} mice show spontaneous generalized seizures and
923 impairment of spatial learning and memory, and do not show major structural brain defects or
924 neuronal mortality, which is consistent with the clinical features observed in patients. The
925 selective deletion of *KCNQ2* and *KCNQ3* in parvalbumin-positive interneurons increased their

926 firing, leading to homeostatic potentiation of excitatory synaptic activity. Consistently this model
927 shows reduced latency for picrotoxin-induced seizures (283).

928 **2.2. Delayed rectifier K⁺ channels (K_v1.1/KCNA1; K_v1.2/KCNA2; K_v2.1/KCNB1; K_v8.2/KCNAV2)**

929 K_v1.1 (KCNA1) and K_v1.2 (KCNA2). KCNA2 encodes the K_v1.2 shaker-type voltage-gated K⁺ channel
930 subunit, which is highly expressed in both excitatory and inhibitory neurons, particularly in the
931 axon (284–286). The K_v1.2 channel generates the delayed rectifier K⁺ current, an important
932 component of the repolarizing currents during APs. K_v1.2 channels have high homology to K_v1.1
933 channels but require stronger depolarization to activate. K_v1.1 and K_v1.2 subunits can produce
934 heteromeric potassium channels with intermediate properties between the respective homomers
935 (287). K_v1.1 and K_v1.2 subunits are associated with cell adhesion molecules (CAMs), including
936 LGI1, which contributes to their subcellular localization (288). KCNA2-DEE clinical features have
937 been correlated with effects of mutations studied *in vitro* and can result in strong GoF, mixed GoF
938 and LoF, and LoF with negative dominance (289, 290). Patients with strong GoF variants have
939 onset between five and 15 months of age with intellectual disability, ataxia, and cerebellar
940 atrophy, often with generalized seizures. Patients with variants showing mixed GoF and LoF effects
941 have onset from the neonatal period to six months of age with refractory generalized and focal
942 seizures and developmental delay. Patients with dominant negative LoF variants can have less
943 severe features. The LoF mechanism is consistent with the classical role of K⁺ channels and the
944 phenotype of K_v1.2 mutant mice. Homozygous knock-out mice show severe seizures and early
945 mortality, and heterozygous knock-out mice are more sensitive to convulsants, although they do
946 not show spontaneous seizures (291). Both heterozygous and homozygous “Pingu” mice, which
947 carry a chemically induced LoF missense mutation, show motor abnormalities (292). Since K_v1.2
948 channels are expressed in both excitatory and inhibitory neurons, the cellular mechanism of
949 KCNA2 mutations is not completely clear. Their repolarizing role can help both sustaining high

950 firing rates and setting the resting membrane potential. Recordings from LoF mouse models have
951 shown contrasting modifications of neuronal firing, with hypoexcitability of glycinergic neurons in
952 *Kcna2* knock-out (291) and hyperexcitability of cerebellar basket cells in *Pingu* mice (292).
953 Although the detailed pathological mechanism is still elusive, the identification of GoF or LoF
954 effects can provide important information for orienting the therapy in a precision medicine
955 approach. In fact, it has been recently shown that the K⁺ channel blocker 4-aminopyridine
956 antagonized GoF defects caused by variants in *KCNA2* *in vitro* and was effective in reducing
957 symptoms in patients carrying GoF *KCNA2* mutations (293). *KCNA1* LoF mutations have also been
958 identified in some DEE patients (294, 295) extending the pathological spectrum of this gene to
959 severe epilepsy.

960 K_v2.1 (*KCNB1*) and K_v8.2 (*KCNV2*). *KCNB1* encodes the K_v2.1 pore-forming and voltage-sensing α-
961 subunit, which contributes to generate the delayed-rectifier K⁺ current (268). It is expressed in
962 both excitatory and inhibitory neurons of the mammalian brain, and is localized to the soma,
963 proximal dendrites, and axon initial segments (296). K_v2.1 is important for sensing and
964 homeostatically regulating excitability, because its activity can be inhibited by phosphorylation;
965 increased neuronal activity induces dephosphorylation, which results in increased delayed rectifier
966 current leading to reduced neuronal excitability (297, 298). Mutations in *KCNB1* have been
967 recently reported in patients with early-onset DEE (299–301). *KCNB1*-related DEEs encompass a
968 wide spectrum of neurodevelopmental disorders with different types of epileptic seizures,
969 predominant language difficulties and behavioral impairment. Most variants occur *de novo* and
970 mainly consist of missense variants located on the voltage sensor and the pore domain. Truncating
971 variants in the C-terminal domain are associated with a less-severe epileptic phenotype, although
972 cognitive/behavioral impairment is still severe. Functional studies in transfected cells have shown
973 a variety of defects, including loss of ion selectivity, reduced conductance, and dominant negative

974 effects, as well as milder effects on gating properties (299, 302, 303), which induce reduced
975 function and increased neuronal excitability. *Kcnc1*^{-/-} mice show hyperexcitable hippocampal
976 circuits but do not show spontaneous seizures, although they exhibit increased seizure
977 susceptibility (298). The voltage-gated K⁺ channel subunit K_v8.2, encoded by the *KCNV2* gene, is a
978 silent subunit when expressed as a homotetramer, but it increases the K_v2 current when co-
979 assembled as a hetero-tetramer with other K_v2 channels, for example in hippocampal pyramidal
980 neurons, in which it co-localize with K_v2.1 and contributes to the generation of the delayed-
981 rectifier K⁺ current (304, 305). *De novo* LoF variants in the *KCNV2* gene, encoding the voltage-
982 gated K⁺ channel subunit K_v8.2, have been identified in few DEE patients and contribute to
983 epilepsy susceptibility in mice (305).

984 **2.3. Fast K⁺ channels**

985 K_v3.1 (*KCNC1*) and K_v4.1-4.3 (*KCND*). *KCNC1* encodes the K_v3.1 channel, which is preferentially
986 expressed in neurons that generate high frequency firing, including parvalbumin-containing
987 GABAergic interneurons in the cerebral cortex, hippocampus and amygdala, auditory brainstem
988 neurons, cerebellar granule cells, and neurons of the reticular nucleus of the thalamus (306). In
989 fast spiking GABAergic neurons, K_v3.1 channels are localized to the proximal dendrites, somata,
990 axon hillock, and synaptic terminals, but are not found in distal dendrites (306, 307). Specific
991 functional properties that distinguish K_v3.1 channels from other K_v channels are the very fast
992 kinetics of activation and deactivation, and the voltage dependence of activation shifted towards
993 depolarized potentials (306). These properties are optimized for promoting the generation of high
994 firing rates, up to hundreds of Hz (308). Mutations in *KCNC1* have been identified in early onset
995 DEE and in patients with intellectual disability without seizures (309, 310). Mutants have been
996 functionally characterized in the *Xenopus laevis* oocytes. All characterized mutations resulted in
997 partial or complete LoF, with some of them inducing negative dominance leading to >50%

998 reduction of current in heterozygosis (309–311). These results are consistent with hypo-
999 excitability of fast spiking neurons as the main pathogenic mechanism of *KCNC1* DEE. Knock-out
1000 *Kcnc1*^{-/-} mice have a subtle phenotype (312) and there are no mouse models engineered to carry
1001 human *KCNC1* mutations.

1002 *KCND1-3* encode the K_v4.1-3 alpha subunits of the Shal family of the A-type voltage-gated K⁺
1003 channels, which generate a rapidly inactivating outward K⁺ current and are important in
1004 membrane repolarization in excitable cells (313). The *de novo* heterozygous missense variant
1005 p.V404M of *KCND2* has been associated with DEE with onset at 2 months (314). Functional
1006 analysis in *Xenopus laevis* oocytes showed modified current kinetics and reduced inactivation,
1007 consistent with GoF, but effects on neuronal excitability were not investigated (314).

1008 **2.4. Non-inactivating K⁺ channels**

1009 The ether-a-go-go (EAG) K⁺ channel family is formed by *KCNH1* and *KCNH5*, which encode the
1010 K_v10.1 (EAG1) and K_v10.2 (EAG2) channels, respectively. They generate non-inactivating voltage-
1011 dependent K⁺ currents and in expression systems can form heteromeric channel complexes, in
1012 which the slow activation of K_v10.1 is dominant (315). *KCNH1* heterozygous missense mutations
1013 have been identified in patients with Zimmermann-Laband and Temple-Baraitser syndromes, as
1014 well as in patients with unclassified syndromes and a broad phenotypic spectrum with intellectual
1015 disability and epilepsy (316–320). Epilepsy is a key phenotypic feature in most patients with
1016 *KCNH1*-related syndromes, who show both generalized and focal tonic-clonic seizures (319, 320).
1017 Functional studies of *KCHN1* mutants in cell lines have shown left-shifted voltage-dependence of
1018 activation and slower deactivation kinetics, consistent with GoF (316, 317), although it is not clear
1019 how this effect could cause the observed phenotypes. *De novo* heterozygous missense variants of
1020 *KCNH5* have been identified in few DEE patients (321, 322). Functional analysis in transfected cell

1021 lines has shown a strong hyperpolarizing shift of voltage-dependence of activation and an
1022 acceleration of activation, consistent with GoF.

1023 **2.5. Ca²⁺ activated K⁺ channels**

1024 *KCNMA1* encodes the pore-forming α -subunit of the large-conductance $K_{Ca1.1}$ Ca²⁺ activated K⁺
1025 channel (“Big K⁺”, BK), which is activated by depolarizations and intracellular Ca²⁺ (323). $K_{Ca1.1}$ is
1026 widely distributed in excitable and non-excitable cells. Expression levels are highest in brain and
1027 muscle, where BK channels are critical regulators of neuronal excitability and muscle contractility.
1028 In both excitatory and inhibitory neurons, BK channels are implicated in AP repolarization and
1029 after-hyperpolarization, influencing the shape, frequency, and propagation of APs (323).
1030 Heterozygous *de novo* missense mutations in *KCNMA1* are associated with a wide phenotypic
1031 spectrum primarily defined by brain and muscle dysfunction (324–326). *KCNMA1*-linked DEE is
1032 characterized by a heterogeneous combination of epilepsy, dyskinesia, and intellectual disability.
1033 Functional analysis in cell lines transfected with *KCNMA1* mutations have shown both LoF and GoF
1034 effects that correlate with phenotypic features (325). While seizures do not show differential
1035 distribution between patients carrying GoF and LoF variants, neurodevelopmental and structural
1036 brain abnormalities are prevalent in patients with LoF mutations. There are no animal models
1037 carrying mutations identified in patients. *Kcnma1*^{-/-} mice show ataxia, tremor, impaired
1038 coordination and spatial learning (327, 328). Pharmacological inhibition of $K_{Ca1.1}$ channels induce
1039 tremor and ataxia in animals (329), but they might be used for treatment of GoF mutations.

1040 **2.6. Na⁺-activated K⁺ channels**

1041 *KCNT1* encodes the $K_{Na1.1}$ subunit, which has a classical six transmembrane segments structure
1042 and forms a tetrameric Na⁺-activated K⁺ channel (also called Slack, $KCa4.1$ or Slo2.2). $K_{Na1.1}$ is
1043 activated by both intracellular Na⁺ and voltage and is expressed in different organs, including the
1044 nervous system, heart, and kidney. In the central nervous system, it has a distinct expression

1045 pattern with respect to $K_{Na}1.2$ (encoded by the gene *KCNT2*), but the two subunits can co-localize
1046 and form heteromeric channels (330, 331). K_{Na} channels can modulate intrinsic excitability and
1047 firing properties of different types of neurons. In particular, they contribute to generate the slow
1048 after-hyperpolarization that follows AP discharges, which induce Na^+ influx through Na_v channels
1049 (332).

1050 Mutations in *KCNT1* cause different phenotypes, including DEEs. *De novo* heterozygous mutations
1051 were first identified in epilepsy of infancy with migrating focal seizures (EIMFS) also called
1052 malignant migrating partial seizures of infancy (MMPSI) (333). EIMFS is characterized by refractory
1053 focal seizures arising and status epilepticus with onset in the first six months of life. The
1054 neurological status progressively deteriorates, with progressive hypotonia, and severe
1055 development arrest. *De novo* heterozygous mutations have been also identified in severe
1056 autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), sleep-related hypermotor epilepsy
1057 SHE (334) and other less common DEE phenotypes (335).

1058 The identified mutations induce large GoF, increasing the $K_{Na}1.1$ current amplitude (333, 336),
1059 while one heterozygous LoF variant (p.F932I) from a patient with severe generalized seizures and
1060 delayed myelination causes impaired $K_{Na}1.1$ trafficking (337). Knock-in mice of the p.Y796H GoF
1061 *KCNT1* mutation show early-onset seizures (338). Although $K_{Na}1.1$ increases K_{Na} currents in both
1062 excitatory and inhibitory cortical neurons in primary cultures, an increase of the current in the
1063 subthreshold voltages is only found in inhibitory neurons, resulting in inhibitory neuron-specific
1064 impairments in excitability and AP generation. GoF *KCNT2* mutations also cause DEE (339).
1065 Blocking *KCNT* GoF mutants with quinidine, a class I antiarrhythmic drug, has shown variable
1066 success in patients because of dose-limiting off-target effects, poor blood-brain barrier (BBB)
1067 penetration, and low potency (335).

1068 **3. Ca^{2+} CHANNELS**

1069 Voltage-gated Ca^{2+} channels are important for numerous physiological functions; in neurons, they
1070 contribute to neuronal excitability and, at the synaptic level, mediate synchronous transmitter
1071 release. The voltage-dependent Ca^{2+} currents are generated by monomeric $\alpha 1$ subunits that have
1072 the canonical four domains of six transmembrane segments, (340, 341). The 10 cloned $\alpha 1$ -subunits
1073 can be grouped into three families according to the membrane potential range at which the
1074 channel is activated and the sensitivity to drugs: the high-voltage activated dihydropyridine-
1075 sensitive channels (L-type; *CACNA1S*/ $\text{Ca}_v1.1$, *CACNA1C*/ $\text{Ca}_v1.2$, *CACNA1*/ $\text{Ca}_v1.3$, and
1076 *CACNA1F*/ $\text{Ca}_v1.4$), the high/moderate-voltage activated dihydropyridine-insensitive channels
1077 (P/Q-, N- and R- type; *CACNA1A*/ $\text{Ca}_v2.1$, *CACNA1B*/ $\text{Ca}_v2.2$ and *CACNA1E*/ $\text{Ca}_v2.3$), and the low-
1078 voltage-activated (T-type; *CACNA1G*/ $\text{Ca}_v3.1$, *CACNA1H*/ $\text{Ca}_v3.2$ and *CACNA1I*/ $\text{Ca}_v3.3$) channels. It
1079 has been proposed that L-type channels may be implicated in the generation of epileptiform
1080 paroxysmal depolarization shifts (342) and that they may be therapeutic targets for epilepsy (343).
1081 However, besides Timothy syndrome, a severe multiorgan disorder caused by *CACNA1A*/ $\text{Ca}_v2.1$
1082 GoF mutations (in which surviving patients can develop epilepsy and autism) (344), there are no
1083 reports of their direct involvement in DEE. At least for high-voltage activated channels, functional
1084 properties, and intracellular trafficking of $\alpha 1$ subunits can be modulated by accessory subunits,
1085 including the β -, $\alpha 2\delta$ -, and γ -subunits (345).

1086 **3.1 T-type channels**

1087 T-type channels (*CACNA1G/H/I*, $\text{Ca}_v3.1-3$) are widely expressed throughout the nervous system
1088 (346). Voltage-dependent opening of T-type channels occurs at comparatively negative membrane
1089 potentials and, depolarizing the cell membrane, facilitates the generation of APs. This function is
1090 especially relevant in several central neurons in which T-type channels are particularly abundant in
1091 dendrites, where they enhance subthreshold postsynaptic potentials and facilitate the
1092 propagation of the electrotonic signal to the cell body (347). They are also involved in the firing of

1093 rebound burst of APs that support various forms of neuronal pacemaking, particularly in the
1094 thalamocortical network, whose dysfunction is implicated in absence seizures (348).
1095 Polymorphisms in *CACNA1G*/ $\text{Ca}_v3.1$ and *CACNA1H*/ $\text{Ca}_v3.2$ causing in general mild GoF have been
1096 identified as risk factors for idiopathic generalized epilepsy (349). *De novo* heterozygous missense
1097 variants in *CACNA1G*/ $\text{Ca}_v3.1$ and *CACNA1I*// $\text{Ca}_v3.1$ have been recently identified in patients with
1098 infantile-onset DEE (350, 351) and variable neurodevelopmental phenotypes including DEE with
1099 cognitive impairment, hypotonia, and epilepsy (352). Functional effects are consistent with GoF
1100 (including persistent Ca^{2+} current at resting membrane potential for *CACNA1I*// $\text{Ca}_v3.1$ variants), or
1101 mixed GoF and LoF. Evidence for the involvement of *CACNA1H*/ $\text{Ca}_v3.2$ in DEE is more limited.
1102 Gene targeted animal models of T-type channels DEE mutations are not available yet.

1103 **3.2. P/Q-type Ca^{2+} channels**

1104 *CACNA1A* encodes the $\text{Ca}_v2.1$ Ca^{2+} channel, which generates the high voltage-activated P/Q-type
1105 Ca^{2+} current with moderate voltage-dependent inactivation. *CACNA1A* is widely expressed in the
1106 central nervous system and is essential for fast and synchronous neurotransmitter release in
1107 numerous neuronal subtypes (353). *CACNA1A* mutations have been associated with episodic
1108 ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6), familial hemiplegic migraine type 1
1109 (FHM1) (354–356) and, more recently, with severe intellectual disability, motor impairment, and
1110 episodic ataxia (61, 357). Functional characterization of *CACNA1A* DEE mutations performed in
1111 transfected cell lines has generated controversial results because both LoF (decreased channel
1112 targeting at the cell membrane) and GoF (hyperpolarized shift of the activation curve) effects were
1113 reported (358). A mouse model of *CACNA1A* DEE has not been generated yet.

1114 **3.3. N-type Ca^{2+} channels**

1115 *CACNA1B* encodes the $\text{Ca}_v2.2$ N-type Ca^{2+} channel, which generates the high voltage-activated Ca^{2+}
1116 current with moderate voltage-dependent inactivation. *CACNA1B* is expressed throughout the

1117 central nervous system and acts synergistically or complementarily with *CACNA1A* for generating
1118 presynaptic Ca^{2+} fluxes that mediate neurotransmitter release (353). Expression of *CACNA1B* is
1119 thought to be crucial for neurotransmission in the early postnatal period as $\text{Ca}_v2.2$ channels are
1120 replaced by $\text{Ca}_v2.1$ channels in most mature synapses (353). In particular, $\text{Ca}_v2.2$ is implicated in
1121 asynchronous synaptic release, which occurs up to tens of seconds after the AP (353). $\text{Ca}_v2.2$ may
1122 also have a role in synaptic plasticity, synaptogenesis, gene transcription, neuronal survival, and
1123 migration of immature neurons (340). *CACNA1B* variants have been identified in a severe form of
1124 autosomal recessive DEE featuring developmental delay, microcephaly, inability to walk or speak,
1125 early onset refractory seizures, myoclonus/dyskinesia, frequent feeding difficulties and high risk of
1126 premature demise (359). The identified variants are predicted to truncate the $\text{Ca}_v2.2$ protein,
1127 leading to LoF and haploinsufficiency, although the genotype/phenotype relationships are not
1128 clear. There are no gene targeted mouse models of the identified human variants, but *Cacna1b*^{-/-}
1129 mouse models show overt neurodevelopmental abnormalities, including abnormal locomotor
1130 activity and memory impairment (360).

1131 **3.4. R-type Ca^{2+} channels**

1132 *CACNA1E* encodes the moderate voltage-activated $\text{Ca}_v2.3$ Ca^{2+} channel, which generates the R-
1133 type Ca^{2+} current with fast voltage-dependent inactivation. It is highly expressed in the central
1134 nervous system and involved in generating the Ca^{2+} influx in synaptic terminals that initiates rapid
1135 release of neurotransmitters. Mutations in *CACNA1E* cause severe autosomal dominant DEE with
1136 macrocephaly, hypotonia, early-onset refractory seizures, profoundly impaired neurodevelopment
1137 and hyperkinesia (361). Functional analysis of *CACNA1E* variants in transfected cell lines mostly
1138 showed GoF effects with facilitated activation and slowed inactivation (361), consistent with
1139 increased neurotransmitter release and network hyperexcitability. Other variants were predicted

1140 to generate truncated non-functional proteins and haploinsufficiency. *Cacna1e*^{-/-} null knock-out
1141 mice do not show overt neurological phenotypes (362).

1142 **4. OTHER CATION CHANNELS**

1143 **4.1 Voltage-independent Na⁺ channels**

1144 The sodium leak NALCN channel (Na_V2.1) is predominantly expressed in neurons where it is
1145 important for setting resting potential and controlling neuronal excitability. *NALCN* encodes for a
1146 voltage-independent, non-inactivating cation channel permeable to Na⁺, K⁺, and Ca²⁺ that
1147 generates the background TTX resistant Na⁺ leak current (363, 364). Autosomal-recessive missense
1148 and nonsense *NALCN* mutations have been identified in a DEE with onset at birth or in early
1149 infancy, characterized by variable degrees of hypotonia, speech impairment and intellectual
1150 disability (infantile hypotonia with psychomotor retardation and characteristic facies, IHPRF) (365).
1151 A subsequent study found heterozygous *de novo* *NALCN* missense variants to cause congenital
1152 contractures of the limbs and face, hypotonia, and developmental delay (CLIFAHDD) (366, 367).
1153 Functional studies in transfected cell lines have generated controversial results, showing GoF, LoF
1154 or dominant-negative effects for both syndromes (367, 368). *Nalcn*^{-/-} knock-out mice show
1155 severely disrupted respiratory rhythm and die within 24 hours of birth.

1156 **4.2 Hyperpolarization-activated cyclic nucleotide-gated channels (HCN)**

1157 HCN are Na⁺/K⁺ permeable channels that are activated by hyperpolarization at voltages more
1158 negative than -50 mV (369). cAMP and cGMP directly bind to the intracellular cyclic nucleotide-
1159 binding domain and shift the activation curve of HCN channels to more positive voltages,
1160 increasing channel activity. The four known HCN isoforms (HCN1-4) have secondary structure that
1161 resembles that of K⁺ channels. HCN1 and HCN2 are the main isoforms expressed in the brain with
1162 a predominant somato-dendritic expression. The hyperpolarization-activated currents generated
1163 by HCN channels in neurons, named *I_h* or *I_q*, are depolarizing and contribute to set the resting

1164 membrane potential, shape synaptic inputs and generate rhythmic and synchronized neuronal
1165 activity implicated in pacemaking and somato-dendritic oscillations (370).

1166 *De novo* dominant missense *HCN1* variants were initially identified in DEE patients with a Dravet-
1167 like phenotype (371), but have subsequently been related to a wider phenotypic spectrum ranging
1168 from mild generalized epilepsy or GEFS+ to severe early onset DEE (372). Functional studies in cell
1169 lines identified GoF as the major pathophysiological mechanism and a LoF with dominant negative
1170 effect for a limited number of variants (371). Knock-out *HCN^{-/-}* mice show deficits in motor
1171 learning and modifications of spatial memory, but not a DEE phenotype (373) while the knock-in
1172 mouse for the p.M305L mutation shows DEE (374). Functional analysis in CHO cells and pyramidal
1173 neurons demonstrated that the p.M305L mutation causes a constitutive activation of the channel
1174 (372).

1175 **5. The Na⁺/K⁺-ATPase pump**

1176 The Na⁺/K⁺-ATPase (NKA) ion pump is a ubiquitous transmembrane enzyme responsible for active
1177 exchange of Na⁺ and K⁺ ions across the plasma membranes in higher eukaryotic cells and is
1178 composed of a large catalytic α -subunit and smaller β - and γ -subunits that modulate the
1179 membrane exposure and activity of α -subunits (375, 376). In neural tissue, NKA generates an
1180 outward current that contributes to the resting membrane potential, and powers secondary active
1181 transports, including Na⁺/H⁺ and Na⁺/Ca²⁺ exchanges, K⁺/Cl⁻ cotransport, and Na⁺-dependent
1182 neurotransmitter uptake (375).

1183 The four known human isoforms of the α -subunit (α 1-4), encoded by four paralogous genes
1184 (*ATP1A1-4*) have developmental and tissue expression specificity. The α 2- and α 3-isoforms,
1185 encoded by *ATP1A2* and *ATP1A3*, are predominantly expressed in the central nervous system.
1186 Constitutional heterozygous mutations in *ATP1A2/A3* have been associated with several
1187 autosomal dominant neurological disorders including familial hemiplegic migraine (FHM), rapid-

1188 onset dystonia-parkinsonism (RDP), alternating hemiplegia of childhood (AHC), cerebellar ataxia-
1189 areflexia-progressive optic atrophy (CAPOS), and relapsing encephalopathy with cerebellar ataxia
1190 (RECA) (377–379). Epilepsy and intellectual disability may co-occur with FHM and AHC, and severe
1191 epilepsies have been described in rare patients with *ATP1A2* or *ATP1A3* mutations (380, 381).
1192 Early lethal hydrops fetalis, intrauterine growth restriction, arthrogryposis, microcephaly,
1193 polymicrogyria, and lack of respiratory drive have been associated with homozygous truncating
1194 mutations in *ATP1A2* (382, 383). Investigating the genetic causes of DEEs variably associated with
1195 malformations of cortical development in a large cohort of patients, Vetro and collaborators
1196 identified 22 patients carrying *de novo* or inherited heterozygous *ATP1A2/A3* mutations. Most
1197 patients manifested early, often neonatal, onset seizures with a multifocal or migrating pattern. A
1198 distinctive, ‘profound’ phenotype, featuring polymicrogyria or progressive brain atrophy and
1199 epilepsy, resulted in early lethality. Neuropathological analysis of the whole brain in two
1200 individuals with polymicrogyria revealed a mainly neural pathogenesis, compounded by vascular
1201 and leptomenigeal abnormalities (384). Functional studies performed in COS-1 cells transfected
1202 with different mutations demonstrated a LoF mechanism, with a wide *continuum* of severity
1203 distributed across mutations that variably impaired NKA pump activity. Mutations associated with
1204 most severe phenotypes cause lack of COS-1 cell-survival (384). Interestingly, *PRRT2*, a gene
1205 whose deficiency is associated with paroxysmal disorders including epilepsy (see next section) is
1206 co-expressed with and is a physiological activator of $\alpha 3$ -NKA in several brain areas (385).

1207 **VIII. DYSFUNCTIONS IN SYNAPTIC TRANSMISSION AND PLASTICITY**

1208 Although the most straightforward causes of hyperexcitability have been historically and widely
1209 associated with mutations in ion channel genes, dysfunctions of synaptic transmission are
1210 emerging as primary causes of epilepsy and DEE. That is why the term “*synaptopathies*” was
1211 coined, inherently meaning that anomalies of information transfer at the synapse can profoundly

1212 affect network excitability, excitatory/inhibitory (E/I) balance and nervous system development,
1213 triggering epileptogenesis.

1214 In the last 30 years, the machinery of synaptic transmission has been largely clarified in the most
1215 subtle molecular details. Several physiologically important molecular actors orchestrate
1216 neurotransmitter (NT) release at the presynaptic side and decode its message into a biological
1217 response at the postsynaptic side. Biology teaches that the more important a process is, the higher
1218 the complexity is involved in its control. Thus, there is a relatively large probability that gene
1219 mutations hits some of these numerous actors.

1220 Neurotransmitter release evoked by APs reaching nerve terminals is based on the exocytotic
1221 fusion of small organelles, synaptic vesicles (SVs), real nanomachines that sense Ca^{2+} entry through
1222 presynaptic voltage-dependent channels and trigger exocytosis by activating a SNARE-mediated
1223 fusion machine that incorporates the SV in the presynaptic membrane and releases its content
1224 into the synaptic cleft. The SV-competent membrane patch is eventually retrieved by distinct
1225 endocytotic mechanisms that take place at distinct activity patterns. Endocytosis regenerates new
1226 SVs that are loaded by NT by an active proton gradient generated by the SV-associated vacuolar
1227 H^+ -adenosine triphosphatase (vATPase) (386, 387). Synaptic vesicles are organized into distinct
1228 functional pools, including (i) the Readily Releasable Pool (RRP), comprising SVs immediately
1229 recruited for exocytosis, (ii) a Recycling Pool (RP) made by SVs that can be rapidly recruited to
1230 replenish the RRP depleted upon activity, and (iii) a Resting Pool (ResP) representing a reserve of
1231 SVs that are not immediately releasable upon activity (388, 389).

1232 When referring to alterations in synaptic transmission and plasticity, some issues should be
1233 considered. Except for postsynaptic receptors and scaffolding proteins, the presynaptic
1234 components of excitatory and inhibitory synapses share most of the actuators of NT release and,
1235 in principle, should be equally affected by mutation-induced protein dysfunction, without net

1236 changes in the excitatory/inhibitory balance. However, differentially expressed proteins or the
1237 different impact of protein dysfunction in excitatory and inhibitory neurons very often imply an
1238 overt phenotype affecting network excitability and/or the excitatory/inhibitory balance. For
1239 example, a protein involved in SV recycling and refilling of RRP will be more essential for the
1240 proper functionality of high-frequency firing neurons (e.g., the PV-positive interneurons) than for
1241 excitatory neurons.

1242 Another important issue is the respective weight of basal synaptic strength and short-term
1243 plasticity mechanisms. Short-term plasticity is a fundamental determinant of network activity and
1244 excitability and thereby plays a central role in epileptogenesis. As neurons do not fire single action
1245 potentials (APs), but rather trains of APs, short-term plasticity phenomena, such as depression or
1246 facilitation, have a strong impact on the network computational activities, including frequency
1247 band filtering of synaptic inputs or pattern detection activities (390, 391).

1248 Synaptic transmission is a major determinant of excitability at the neuronal network level. Intrinsic
1249 excitability at single neuron level is mostly affected by the size of the neuron and its input
1250 resistance, and by the level of expression and functionality of voltage-dependent ion channels,
1251 particularly at the axon initial segment, the AP initiation spot. However, at the level of a
1252 population of synaptically connected neurons, intrinsic excitability is not the only factor defining
1253 the firing and bursting behavior of the network, as well as its synchrony. While inhibitory synapses
1254 act to confine the excitation waves temporally and spatially, excitatory synaptic connections play a
1255 prominent role in establishing the activity of the network, owing to their short-term plasticity
1256 properties (392).

1257 Mutations in numerous genes affect synaptic functions, and the number of so-called
1258 “synaptopathies” is continuously raising. Strong evidence, accumulated in the last two decades,
1259 has shown that mutations in fundamental actors of the complex process of NT release can result in

1260 neurodevelopmental disorders with epilepsy (**FIGURE 8**). In this section, we will only consider
1261 mutations that affect purely synaptic genes, leaving mutations in the genes of presynaptic and
1262 postsynaptic ion channels to the dedicated chapter. Among genes encoding for *bona fide* synaptic
1263 proteins, a distinction should be made between expression/function alterations affecting
1264 fundamental proteins for the synaptic transmission machinery, and genes that play a modulatory
1265 action on the process by impacting more on synaptic plasticity than on the essential synaptic
1266 machinery. In addition, the targets of mutations can participate in distinct processes contributing
1267 to synaptic transmission. According to the view of the tetrapartite synapse, four distinct entities
1268 are involved in the physiological regulation of synaptic transmission: the presynaptic neuron, the
1269 target postsynaptic neuron, the extracellular matrix and the perisynaptic astrocyte. While the
1270 impact of mutations in astrocyte-specific genes is still poorly understood, we can consider three
1271 main classes of synaptopathies, namely: (i) *presynaptic synaptopathies*, including defects of the
1272 post-docking SV priming/fusion processes, as well as defects in the processes regulating SV
1273 trafficking or NT synthesis and loading into SVs; (ii) *postsynaptic synaptopathies*, including defects
1274 in postsynaptic receptors and their scaffold/transduction systems; and finally (iii) *extracellular*
1275 *synaptopathies*, including defects in trans-synaptic and extracellular matrix (ECM) proteins at the
1276 synaptic cleft and secreted synaptic proteins (**FIGURE 8**). For all these classes of synaptic
1277 pathologies, the identification of gene mutations in patients has catalyzed an extensive research
1278 activity that often contributed to clarifying the pathomechanism of the disease and elucidating the
1279 physiological role of the synapse-specific gene products. A substantial contribution to
1280 physiopathology of synapse-bases DEEs came from the exploitation of murine knockout or knock-
1281 in experimental models, recapitulating at least some traits of the clinical phenotypes, as well as
1282 from mutation studies in transfected cell lines and primary neurons, up to the studies performed
1283 in iPSC-derived neurons either containing the patient's mutation in the patient's own genetic

1284 background or in which the mutation under study has been reproduced by CRISPR-Cas9 gene
1285 editing in WT cells. There is a wide spectrum of phenotypic DEE features stemming from
1286 alterations of synaptic function, although genotype-phenotype correlations are not always
1287 straightforward (393). We will follow the aforementioned classification to list the main DEE-
1288 related genes.

1289 **1. Presynaptic synaptopathies**

1290 Mutations in many genes encoding proteins involved in the multi-step process of NT release cause
1291 epilepsy. Starting from the machinery essential for regulated exocytosis, mutations in genes
1292 encoding t-SNARE proteins Syntaxin 1B, SNAP25b and VAMP/synaptobrevin, the SNARE-associated
1293 proteins STXBP1 (Munc18-1) and PRRT2, the α -subunit of the P/Q type voltage-dependent Ca^{2+}
1294 channel, and the critical SV Ca^{2+} sensor synaptotagmin have been identified and associated with
1295 epilepsy and DEEs (9, 358, 359, 394–400). In mice, constitutive knockout of these genes greatly
1296 hinders AP-driven NT release, with a compensatory increase of spontaneous release and synaptic
1297 facilitation (401–407). However, it remains unclear how an impairment of ubiquitous players of
1298 evoked NT release can trigger epileptogenesis, presumably by their different impact on distinct
1299 neuronal populations, resulting in E/I imbalance and circuit instability. Similar mechanisms are
1300 likely to take place in case of mutations in genes encoding proteins not directly actuating NT
1301 release, but supporting trafficking, maintenance, and integrity of the SV pools. Loss of function in
1302 these proteins, which include the SV proteins synapsins and SV2 and the proteins actuating the
1303 endocytotic retrieval of SVs after each round of exocytosis, such as dynamin or amphiphysin, may
1304 induce network hyperexcitability by affecting, to a larger extent, neurons undergoing high
1305 frequency activity, such as parvalbumin (PV)-positive inhibitory neurons.

1306 **1.1. Perturbation of priming/fusion event and of Ca^{2+} sensitivity**

1307 SNARE PROTEINS. Mutations in the large family of genes encoding membrane proteins mediating
1308 vesicle fusion, the so-called SNARE proteins SNAP-25, Syntaxin-1 and VAMP2, constituting the
1309 fusion machine actuating exocytosis have been reported. Mutations in the two SNARE motifs of
1310 SNAP-25, particularly in its splicing isoform SNAP-25b cause a combination of epilepsy and
1311 cognitive deficits that are reproduced by the selective KO of SNAP-25b in mice (395, 408–410). The
1312 second presynaptic SNARE protein, syntaxin-1, has been less implicated in epilepsy and DEE.
1313 However, some mutations associated with febrile epilepsy have been mapped to the H_{abc} region of
1314 the Syntaxin-1b isoform (STX1B) that controls its open/close conformation permitting the
1315 assembly of the SNARE complex. In mice, the a and b isoforms of Syntaxin-1 are redundant, so that
1316 the single KO mice are viable. However, the genetic “freezing” of the b isoform in the open
1317 conformation in the syntaxin-1a KO generates severe seizure activity and premature death (402,
1318 411). Mutations in the SNARE motif of VAMP2 were also identified in patients with intellectual
1319 disability, epilepsy and hyperkinetic movement disorder (396). While it is conceivable that
1320 inactivation of SNARE proteins, profoundly altering neuronal signaling at the synapse, can cause
1321 neurodevelopmental deficits, the mechanism of the frequently associated hyperexcitability and
1322 epilepsy is more elusive. Notably, the three SNARE proteins are the specific targets of Tetanus and
1323 Botulinum neurotoxins (412) and either toxin-mediated or genetic inactivation of either SNARE
1324 protein irreversibly blocks evoked neurotransmitter release. The different sensitivities of
1325 excitatory and inhibitory neurons to SNARE inactivation is believed to generate an E/I imbalance
1326 resulting in the developmental deficits.

1327 CALCIUM SENSING/TRIGGERING MACHINERY. Synaptotagmin1/2 is the fundamental Ca²⁺-binding
1328 sensor for evoked fast and synchronous NT release. Synaptotagmin binds to SNARE proteins
1329 together with complexins limiting spontaneous release and, bearing two C2 domains, acts as a low
1330 affinity Ca²⁺ sensor that is exposed to high concentrations of Ca²⁺ entering through activated

1331 voltage-gated Ca^{2+} channels and upon Ca^{2+} binding trigger membrane deformation and fusion
1332 (413, 414). Five heterozygous mutations were reported in Synaptotagmin-1 located at the Ca^{2+} and
1333 phospholipid binding motifs of the second C2 domain (C2B) in patients presenting with various
1334 degrees of developmental delay and EEG abnormalities, but no epilepsy (415). Synaptic
1335 transmission was impaired in neurons expressing the synaptotagmin mutants, with graded
1336 dominant-negative effects that could be rescued by K^+ channel antagonists (400). Presynaptic
1337 voltage-gated Ca^{2+} channels (VGCC) Cav2.1 and Cav2.2, corresponding to the P/Q- and N-type
1338 VGCCs are the essential electro-chemical transducers concentrated in the nano- and micro-
1339 domains of the active zones and converting APs into synchronous exocytosis of SVs.

1340 PRRT2 is a neuron-specific, type-2 membrane protein with a C-terminal anchor that concentrates
1341 in synaptic and axonal domains, where it interacts with key components of the fusion/ Ca^{2+} sensing
1342 machinery (synaptotagmin 1/2, SNAP-25 and VAMP2) boosting the Ca^{2+} sensitivity of NT release
1343 and suggesting a function in the Ca^{2+} -dependent transition from SV priming to fusion (398). As a
1344 consequence, the probability of release at excitatory synapses is dramatically decreased, with a
1345 parallel marked increased synaptic facilitation that results in an E/I imbalance and lack of network
1346 stability (406, 416, 417). This role of PRRT2 has recently been supported by the discovery of a
1347 direct interaction of PRRT2 and P/Q VGCCs that contributes to concentrate them at the
1348 nanodomain where the machinery for synchronous release is assembled. In the absence of PRRT2,
1349 membrane targeting and concentration of P/Q channels at active zones is impaired, decreasing
1350 Ca^{2+} influx in response to APs (418). PRRT2 has been identified as the causative gene for several
1351 paroxysmal neurological disorders including epilepsy, paroxysmal kinesigenic dyskinesia, but also
1352 migraine and ataxia. These disturbances have been associated with a severe encephalopathic
1353 phenotype with intellectual disability in the few patients with homozygous or compound
1354 heterozygous mutations (398).

1355 Another SV protein playing an important role in regulating the initial release probability of SVs and
1356 neural network synchronous activity is the Synaptic Vesicle glycoprotein-2 (SV2). SV2 is encoded
1357 by three paralog genes (*SV2A*, *SV2B*, *SV2C*) with distinct patterns of expression in neuronal
1358 populations. SV2 plays multiple roles as a catalyzer of evoked NT release: it accelerates SV priming,
1359 increases the size of the RRP, regulates the stability and trafficking of synaptotagmin, boosting the
1360 Ca^{2+} sensitivity of release. Interestingly, *SV2A* is essentially expressed in inhibitory neurons and its
1361 loss-of-function impairs synaptic inhibition. Accordingly, individuals bearing point mutations in the
1362 *SV2A* gene experience epilepsy and cognitive deficits (419, 420).

1363 SV PRIMING MACHINERY. The preparation of SVs for fusion after docking is an essential
1364 mechanism for allowing fast and synchronous release. Two proteins are essential for this
1365 progression, Munc-18 (*STXBP1*) and Munc-13 (*UNC13A*), while other nerve terminal proteins such
1366 as RIM1, SV2 and synapsin I have a modulatory role. The Munc-18 and Munc-13 tandem is needed
1367 for a correct assembly of the SNARE complex, whereby Munc-18 regulates the participation of
1368 syntaxin-1 to the complex, while Munc-13 activates syntaxin-1, stabilizes the active zone-SNARE-
1369 SV assembly and protects the SNARE complex from disassembly by the ATPase NSF (421, 422).

1370 While a nonsense mutation in *UNC13A* was identified in a single patient with microcephaly and
1371 interictal multifocal epileptiform EEG activity (423), mutations in *STXBP1* were repeatedly
1372 identified in a series of epileptic encephalopathies including Ohtahara, West, and Rett syndromes.
1373 Over 85 distinct *STXBP1* mutations are known and *de novo* *STXBP1* mutations are among the most
1374 frequent causes of DEEs of synaptic origin with severe intellectual disability (424).

1375 ORGANELLE ACIDIFICATION AND NT LOADING. As mentioned above, vATPase plays a fundamental
1376 role in the active loading of SVs with the surprisingly reproducible amount of NT that constitute
1377 the “*quantum*”. Quantal reproducibility relies on the fact that the amount of NT is an important
1378 determinant of the probability of release, so that only fully filled SVs are likely to be released (425,

1379 426). This mechanism is based on the dissociation of the V1 catalytic cytosolic domain from the V0
1380 transmembrane proton transfer domain upon complete build-up of the proton gradient and
1381 corresponding NT loading. The synaptic protein encoded by the *DMXL2* gene, rabconnectin-3a, is
1382 strictly involved in the assembly and incorporation of vATPase into SVs (427, 428). The role of v-
1383 ATPase in pH homeostasis and intracellular signaling pathways is ubiquitous, with a prominent role
1384 in the nervous system. In humans, mutations in 16 of the 22 genes encoding for vATPase subunits
1385 are associated with a variety of congenital disorders with neurological impairment. Recently, four
1386 different *de novo* missense mutations in *ATPV1A* have been associated with a clinical spectrum of
1387 DEE ranging from rapidly progressive early encephalopathies to mild intellectual disability with
1388 epilepsy (429). Unexpectedly, rather than NT loading, the identified pathomechanism involves
1389 dysfunctions of lysosomal homeostasis impacting on neuronal connectivity. Missense pathogenic
1390 variants of *ATPVOA1*, which participates in the transmembrane proton pumping machinery of
1391 vATPase, are also associated with DEE (430). Heterozygous copy number variations and loss-of-
1392 function biallelic mutations in *DMLX2*, regulating the trafficking and activity of v-ATPase, are
1393 associated with a severe DEE (Ohtahara syndrome) with a superimposable pathomechanism
1394 consisting of defective endolysosomal homeostasis and autophagy resulting in synaptic loss (431).

1395 **1.2. Perturbation of synaptic vesicle trafficking within nerve terminals**

1396 Synaptic vesicles undergo a restless cycle within nerve terminals aimed at preserving their
1397 availability in a sufficient amount in the RRP and maintaining synaptic transmission also during
1398 high frequency activity. Thus, loss-of-function of any of the vast cohort of nerve terminal proteins
1399 operating SV endocytosis and maintaining the recycling pool of SVs are predicted to impact on SV
1400 availability and impair NT release in the tonic high frequency discharging neurons (387). Since
1401 neurons with these characteristics are often inhibitory interneurons (such as the PV-positive
1402 neurons) a diffuse impairment in SV endocytosis/recycling often results in an E/I imbalance.

1403 A large array of gene products mediating various steps of the endocytosis process has been
1404 associated with epilepsy and cognitive impairment. These include the membrane adaptor AP-2,
1405 synaptophysin (SYP), dynamin-1 (DNM1), the coat protein clathrin (CLTC), as well as proteins
1406 involved in activity-dependent bulk endocytosis such as Rab11, TBS1D24 and AP-1. In the case of
1407 AP-2, four patients bearing a point mutation in the μ 2 subunit of the protein presented with
1408 epilepsy and developmental delay (432). Synaptophysin is an integral SV protein interacting with
1409 VAMP2 and regulating its availability and retrieval during endocytosis (433). Nonsense and
1410 missense mutations in the X-linked *SYP* gene have been associated with epilepsy and cognitive
1411 defects (434, 435). *De novo* mutations in the *DNM1* gene result in DEEs including West and
1412 Lennox-Gastaut syndromes (436). Dynamin-1 is the essential GTPase that mediates SV fission by
1413 assembling in a collar around the neck of the budding vesicle and inducing constriction thanks to
1414 the mechanical force produced by GTP hydrolysis (437). Most of the *DNM1* mutations identified
1415 thus far cluster within the GTPase domain (436). A series of missense and nonsense mutations in
1416 the *CLTC* gene encoding for clathrin, the building block of the endocytic coat that allows to
1417 increase the curvature of the vesicle bud to form the endocytic vesicle, have been identified in
1418 patients with epilepsy and intellectual disability (438, 439). Impairment in bulk endocytosis also
1419 occurs with mutations in *AP-1*, *Rab11* and *TBC1D24*. This gene, whose mutants are associated with
1420 epilepsy and DOORS (Deafness, Onychodystrophy, Osteodystrophy, mental Retardation and
1421 Seizures) syndrome affect several processes linked to neural development and synaptic
1422 transmission, such as endocytosis, endosome/lysosome flux and neurite growth (440–444).
1423 Synapsins (Syns) constitute a family of three synaptic genes (*SYN1*, *SYN2* and *SYN3*) that encode
1424 for SV-associated proteins regulating SV formation, SV pool maintenance and SV trafficking
1425 between the functional SV pools. While Syn3 is a developmental isoform and is downregulated
1426 after birth, Syn1 and Syn2 represent the adult isoforms (445–448). Knockout mice for *Syn1*

1427 (homozygous females or hemizygous males), *Syn2*, *Syn1/Syn2* and *Syn1/Syn2/Syn3* all exhibit an
1428 epileptic and autistic-like phenotype with impairment of social interactions and cognitive functions
1429 (449, 450). As observed in other mouse models (e.g., *Grin1* and *Grin2b*), heterozygous mice do not
1430 show an overt phenotype. An array of nonsense and missense mutations have been identified in
1431 patients with epilepsy and/or ASD. Most nonsense mutations in the X-linked gene *SYN1* have been
1432 identified in epilepsy patients, whose seizures were typically triggered by contact with water
1433 regardless of the water temperature. Developmental delay of a variable degree was frequently
1434 associated (451–456). Owing to their role in regulating network activity and stability, presynaptic
1435 proteins can also represent a target for antiepileptic drugs, such as levetiracetam that binds to SV2
1436 on SVs (457).

1437 **2. Postsynaptic and extracellular synaptopathies**

1438 Neurotransmitters released by the pre-synaptic terminals diffuse in the extracellular space and
1439 transfer information downstream by interacting with specific post-synaptic receptors. Mutations
1440 have been identified in genes encoding receptors for the main excitatory (glutamate) and
1441 inhibitory (GABA) neurotransmitters, as well as in transporters involved in ionic homeostasis and
1442 neurotransmitter reuptake, receptor-associated scaffolding proteins and extracellular trans-
1443 synaptic proteins.

1444 **2.1 Ionotropic neurotransmitter receptors**

1445 NMDA GLUTAMATE RECEPTORS. NMDA glutamate receptor subunits are encoded by the *GRIN*
1446 gene family, formed by seven genes: *GRIN1* (encoding the GluN1 subunit), *GRIN2A-D* (encoding
1447 the GluN2A to D subunits), and *GRIN3A-B* (encoding the GluN3A and B subunits). Receptor
1448 activation requires binding of both glutamate and glycine, which are often considered co-agonists.
1449 Functional NMDA receptors are heterotetramers composed of two *GRIN1* subunits binding glycine
1450 and two *GRIN2* or *GRIN3* subunits binding glutamate. They are essential for numerous

1451 physiological functions, including neuronal migration, synaptic connectivity, neuronal pruning and
1452 survival, and synaptic plasticity. They are Ca^{2+} -permeable and generate a slow voltage-dependent
1453 synaptic current.

1454 Among NMDA receptor subunits, *GRIN1*, *GRIN2A* and *GRIN2B* are the target of most variants
1455 identified in patients with neurodevelopmental disorders. Epilepsy in patients carrying *GRIN*
1456 mutations has onset from birth to few years of early childhood. Mutations can be either inherited
1457 or *de novo* and generate a spectrum of phenotypes ranging from mild intellectual disability to
1458 severe DEE (458). The *GRIN1* spectrum includes LoF of varying severity with a dominant negative
1459 effect and is characterized by severe intellectual disability with absent speech, seizures (in about
1460 65% of patients), hypotonia, dyskinesia, cortical blindness and generalized cerebral atrophy (63).
1461 *GRIN2A* mutations have either LoF and GoF effects and are associated with epilepsy and
1462 intellectual disability with normal brain imaging (58, 459, 460). Seizures often originate in the
1463 temporo-rolandic regions, and EEGs often show centrotemporal spike-wave discharges, which may
1464 be continuous during slow-wave sleep (CSWS or CSWSS). Affected individuals exhibit a range of
1465 language/speech problems and, at times, complete aphasia (458). The *GRIN2B* spectrum,
1466 characterized by various GoF and LoF mechanisms, is similar to the *GRIN1* spectrum and includes
1467 developmental delay, hypotonia, epilepsy (in about 50% of patients), movement disorders and, at
1468 times, cortical malformations (461). The phenotypic range of *GRIN2D* missense GoF variants
1469 includes developmental delay with failure to thrive, intellectual disability, hypotonia, and
1470 hyperreflexia (59).

1471 Heterozygous and homozygous knock-out (null) mouse models have been generated for each of
1472 the seven *GRIN* genes (458). Mice carrying homozygous null mutations for *Grin1* and *Grin2b* are
1473 postnatal lethal, whereas heterozygous mice survive normally, but they have not been
1474 characterized in detail. Also, knock-in mice carrying mutations identified in patients have been

1475 generated (462–464). *Grin2a*^{+S/644G} and *Grin2a*^{+/N615K} mice show perinatal lethality in homozygosis,
1476 increased seizure propensity, behavioral and cognitive deficits in heterozygosis. *Grin2b*^{+/C456Y} mice
1477 show anxiety-like behavior with strongly reduced Grin2b levels and NMDA currents.

1478 AMPA GLUTAMATE RECEPTORS. AMPA glutamate receptors are composed by four types of
1479 subunits: GluA1–GluA4 subunits (also named GluR1-4) encoded by the *GRIA1–GRIA4* genes. Most
1480 AMPARs are heterotetrameric, consisting of symmetric 'dimer of dimers' of GRIA2 and either
1481 GRIA1, GRIA3 or GRIA4 (465, 466). AMPA receptors interact with multiple accessory proteins (e.g.,
1482 TARP and cornichon). Glutamate released from the presynaptic terminal triggers the rapid opening
1483 of AMPA receptor channels that generate an inward cation current. The *GRIA2* mRNA is often
1484 edited at the p.Q607 residue that confers Ca²⁺ impermeability to mature receptors containing the
1485 edited GluA2 subunit. The AMPA current underlies most of the excitatory synaptic signaling in the
1486 central nervous system. It is typically brief (on the order of a few ms) because glutamate rapidly
1487 unbinds and is removed from the synaptic cleft and leads to a brief depolarization of the
1488 postsynaptic neuron. *De novo* heterozygous *GRIA2* mutations have been found in patients with
1489 different associations of seizures, speech impairment, intellectual disability and autistic features
1490 (467) with both GoF and LoF mechanisms. The most severe phenotypes were associated with the
1491 p.A639S mutation, which caused DEE with death in infancy. There are no mouse models available
1492 for human mutations. *Gria2*^{-/-} mice exhibit increased mortality, impaired motor coordination and
1493 behavioral abnormalities, whereas heterozygous mice do not show an overt phenotype (468). *De*
1494 *novo* heterozygous missense mutations in *GRIA3*, as well as genomic deletions, have been initially
1495 identified in a form of X-linked intellectual disability with dysmorphic features, a relatively mild
1496 phenotype due to a LoF mechanism (469). However, *GRIA3* mutations have been associated with a
1497 larger phenotypic spectrum that also includes severe early onset DEE (470). There are no animal
1498 models that carry human mutations. Finally, *de novo* heterozygous variants in *GRIA4* have been

1499 identified in a range of phenotypes characterized by variable developmental delay, ranging from
1500 mild to severe, with absent speech, epilepsy, gait abnormalities, and behavioral problems (471).

1501 GABA RECEPTORS. Ionotropic GABA (GABA_A) receptors are pentameric assemblies of up to 3 of 19
1502 subunits encoded by distinct *GABR* genes (*GABRA1–6*, *GABRB1–3*, *GABRG1–3*, *GABRR1–3*, *GABRD*,
1503 *GABRE*, *GABRP*, and *GABRQ*). The combination of two α -subunits (*GABRA*), two β -subunits
1504 (*GABRB*) and one γ -subunit (*GABRG*) is the most common functional receptor in the brain. GABA_A
1505 receptors are cys-loop ligand-gated chloride/anion channels that, at low intracellular chloride,
1506 implement an outward inhibitory current generating hyperpolarization and decrease in membrane
1507 impedance by a shunting effect. The GABAergic synaptic action is essential for reducing excitability
1508 in neuronal networks and generating rhythms of activity. The synaptic GABA_A receptors provide
1509 brief but strong phasic inhibition, whereas those extrasynaptic receptors can induce long-lasting
1510 tonic effects in response to ambient GABA. *GABRA1*, *GABRA2*, *GABRA3*, *GABRA5*, *GABRB1*,
1511 *GABRB2*, *GABRB3* and *GABRG2* genes have been identified as targets of *de novo* heterozygous DEE
1512 mutations (472). Mutations in *GABRA2*, *GABRA3*, *GABRA5*, and *GABRB1* have been mostly
1513 associated with severe phenotypes, whereas variants in other GABA_A genes include, in addition to
1514 DEE, phenotypes associating moderate/mild intellectual disability with epilepsy, and familial
1515 epilepsy without intellectual disability (16, 473–475). The severe phenotypes of *GABRA1* DEE
1516 mutations share some of the clinical features of Dravet syndrome (476). Most GABA_A mutations
1517 induce LoF (with dominant negative effects), by decreasing membrane targeting or modifying
1518 gating properties or GABA sensitivity (474, 475, 477–479). Overall, LoF of GABA_A receptors reduce
1519 the inhibitory tone in neuronal networks and thus generate hyperexcitability; the effect has some
1520 similarity to that of *SCN1A* mutations that reduce the intrinsic excitability of GABAergic neurons. A
1521 different functional effect has recently been proposed for mutations in *GABRD*, which encodes the
1522 GABA_A δ -subunit found in extra-synaptic receptors generating tonic GABA_A currents (480). DEE

1523 patients carrying mutations in this gene have generalized epilepsy, intellectual disability, and
1524 behavioral problems.

1525 Functional analysis of the identified mutations showed GoF of GABA_A receptors containing the δ-
1526 subunit with increased GABA_A current. Thus, increased tonic GABA_A-evoked current may be a
1527 novel pathological mechanism in DEE and neurodevelopmental diseases. Electroclinical findings in
1528 these patients resembled those reported in patients carrying LoF mutations in the GABA uptake
1529 transporter *SLC6A1/GAT1* (481), consistent with a similar pathogenic mechanism (see below).
1530 Heterozygous *Gabra1* knockout mice show spontaneous electrographic spike-wave discharges
1531 with behavioral absence-like seizures and develop myoclonic seizures later in life, consistent with
1532 a relatively mild IGE phenotype (482). *Gabrb2* knockout mice do not show spontaneous epilepsy,
1533 but are more susceptible to seizures and exhibit behavioral disturbances (483). Heterozygous
1534 *Gabrb3* knockout mice show epileptic seizures, EEG abnormalities and a range of behavioral
1535 deficits (484, 485). Homozygous *Gabra3* knockout mice do not show seizures (486). The mouse
1536 line carrying the human *GABRG2* p.Q390X DEE mutation, which has a dominant negative effect,
1537 show a more severe phenotype compared to *Gabrg2*^{-/-} mice (487). Mutations in *GABBR2*, that
1538 induce LoF with reduced slow GABAergic inhibition (488), have been identified in DEE (11, 16).

1539 **2.2 Synaptic transporters**

1540 DEE variants have also been identified in genes that although not directly implicated in synaptic
1541 transmission are involved in synaptic functions.

1542 *SLC12A5* AND *CLCN4*. Cl⁻ is essential for numerous physiological functions, including GABAergic
1543 inhibition. It is actively transported, and its concentration tightly regulated in neurons and virtually
1544 all cells types. Cation-chloride co-transporters are postsynaptic plasma membrane proteins that
1545 determine the intracellular Cl⁻ homeostasis and are thereby directly implicated in GABA_A current
1546 generation (489). The gene family includes Na⁺-Cl⁻ cotransporter (NCC), the Na⁺-K⁺-2Cl⁻

1547 cotransporters (NKCC) and the K^+ - Cl^- cotransporters (KCC). The *SLC12A5* gene, exclusively
1548 expressed in the central nervous system, encodes the neuronal KCC2 K^+ - Cl^- cotransporter, which is
1549 the major extruder of intracellular chloride in mature neurons. Low KCC2 activity can lead to
1550 increased intracellular Cl^- and to depolarizing GABAergic transmission (490).

1551 *SLC12A5* recessive *de novo* mutations have been identified in a spectrum of epileptic disorders
1552 (491, 492). The most severe phenotype is a DEE with features of epilepsy of infancy with migrating
1553 focal seizures (EIMFS) caused by LoF mutations that decrease KCC2 surface expression and reduce
1554 protein glycosylation (139).

1555 The *CLCN* gene family contains nine members in mammals, four of which encode plasma
1556 membrane chloride channels (*CLCN1*, *CLCN2*, *CLCNKA*, *CLCNKB*) and five intracellular $2Cl^-/H^+$
1557 exchangers (*CLCN3–7*) (493). Their function is not completely clear, but *CLCN* Cl^- channels are
1558 involved in the regulation of excitability by controlling extra- and intracellular ion homeostasis.
1559 Dysfunction of some *CLCN* genes leads to severe neurological disorders, in particular LoF
1560 mutations in *CLCN4* cause a spectrum of phenotypes including severe DEE with drug-resistant
1561 seizures, cognitive and behavioral disorders (494, 495).

1562 *SLC1A2* AND *SLC6A1*. *SLC1A* are plasma membrane glutamate transporters expressed by glial cells
1563 and/or glutamatergic presynaptic terminals. They are essential for the removal and termination of
1564 action of glutamate released from the synapses. Mutations in *SLC1A2*, encoding the astrocytic
1565 EAAT2 glutamate transporter selectively expressed in astrocytes, have been identified in DEE
1566 patients (496). Functional studies in transfected cell lines showed LoF and negative dominance
1567 (496), consistent with impaired clearance of extracellular glutamate. *Slc1a2* knockout mice show a
1568 severe epilepsy phenotype only in homozygosity (497).

1569 The *SLC6A1* gene, encoding the GAT1 GABA transporter, is responsible for the reuptake of GABA
1570 into presynaptic terminals and astrocytes, are emerging as a common cause of DEE (498, 499).

1571 Functional studies have shown that *SLC6A1* DEE mutations induce LoF of GAT1 possibly associated
1572 with negative dominance and reduced GABA reuptake (481). *Slc6a1*^{-/-} mice recapitulate some
1573 features of the human phenotypes, including motor and cognitive impairment, whereas
1574 heterozygous *Slc6a1*^{+/-} mice do not show an overt phenotype (498). Overall, the mechanism may
1575 be similar to that of *GABRD* mutations, which increase the extrasynaptic GABA_A current (480).

1576 **2.3 Other postsynaptic proteins**

1577 Other mutations in genes encoding for receptor-associated proteins, such as the GTPase SynGAP1
1578 (500) and Stargazin (501) are also associated with dysfunctions in circuit excitability and epileptic
1579 encephalopathies. Mutations in trans-synaptic adhesion protein genes, including neurexin-1,
1580 ILRAPL1, and Caspr2 were also reported to cause epilepsy in a limited number of cases (502–504).

1581 **2.4 Extracellular synaptic proteins**

1582 An interesting group of epilepsy-related genes encodes for proteins that are secreted at the
1583 synaptic cleft and contribute to trans-synaptic communication and synapse maintenance. Loss-of-
1584 function mutations in *LGI1* gene (or LGI1 neutralizing autoantibodies) are associated with epilepsy
1585 with cognitive impairment (505). LGI1 is a protein secreted by both the presynaptic and
1586 postsynaptic neurons that is part of a trans-synaptic structural/functional bridge linking the
1587 integral proteins ADAM22 and ADAM23, exposed on the opposite sides of the synaptic cleft, that
1588 regulates the assembly and organization of AMPA glutamate receptors by the scaffold protein
1589 PSD95 (**FIGURE 8**) (140, 506). Other genes encoding for the synaptically secreted proteins SRPX2
1590 and Reelin can cause epilepsy if mutated. These proteins play a role in synaptogenesis and in the
1591 neuronal development of the cerebral cortex by acting as extracellular synaptic organizers (507–
1592 510). In case of Reelin, the mutation impairs reelin secretion and the reelin variant is retained and
1593 eventually degraded through the autophagic way.

1594 **IX. DYSFUNCTIONS IN NEURONAL HOUSEKEEPING: mTOR AND AUTOPHAGY**

1595 Mature neurons are post-mitotic cells that do not replicate and have a very high workload
1596 throughout life. The housekeeping of their structure/function is therefore of paramount
1597 importance. Autophagy, a highly conserved structural turnover process that directs dysfunctional
1598 macromolecules and organelles to lysosomal degradation is an indispensable mean for neurons to
1599 maintain their integrity and functionality over time (511–513). Besides neuronal survival,
1600 autophagy plays a key role in neural development and in the formation and maintenance of
1601 synaptic connectivity. It is therefore conceivable that dysfunctional autophagy, due to mutations
1602 in genes controlling its multi-step processes, can cause severe neurodevelopmental disorders
1603 including DEEs.

1604 One of the main pathways controlling autophagy and cell homeostasis is the mTOR
1605 (Mammalian/mechanistic target of rapamycin) pathway. mTOR is an atypical serine/threonine
1606 protein kinase that is activated by a very complex pathway integrating intra- and extra-cellular
1607 signals and strictly controlling matter and energy balance within the cell. The most important
1608 mTOR effectors are the p70 ribosomal S6 protein kinase-1 (p70S6K1) and the eukaryotic initiation
1609 factor 4E-binding proteins (4E-BPs) that transduce extracellular signals, such as neurotransmitters,
1610 growth factors, hormones, into an activation of translation. In neurons, these targets implicate
1611 mTOR in cell growth, neurite outgrowth and synaptic formation, all fundamental activities for
1612 neuronal functions and plasticity. The final synergistic effectors mTOR cascade are two mTOR
1613 complexes, mTORC1, mainly implicated in cell growth and proliferation, and mTORC2, mainly
1614 regulating cytoskeleton and dendrite growth (514, 515). Here we will focus on mTORC1, since
1615 virtually all pathogenic mutations identified thus far affect the mTORC1 cascade, and the
1616 implication of mTORC2 in DEEs is still limited. The activation of mTORC1 is tightly controlled by
1617 two inhibitory switches, the Tuberous Sclerosis Complex (TSC) and the GATOR1 complex (**FIGURE**
1618 **9**). Both complexes are under the inhibitory control of two upstream complexes, GATOR2 that

1619 inhibits GATOR1 and the phosphoinositide-3 kinase (PI3K)/Akt pathway that inhibits TSC. The
1620 canonical mTOR pathway starts with the activation of the PI3K/Akt pathway in response to
1621 extracellular signals (that is subjected to a further inhibitory control by PTEN) that relieves the
1622 mTOR complex from the tonic TSC inhibition. To be active, mTOR needs to attach to intracellular
1623 organelles, which requires the G-proteins Rheb and Rag (part of the Ragulator complex) to be in an
1624 active, GTP-bound state. The Ragulator complex, present on the membrane through an interaction
1625 with the proton pump vATPase, is a guanine nucleotide exchange factor (GEF) that activates Rag
1626 and allows mTOR membrane binding and activation. On the other hand, TSC and GATOR1
1627 inhibitory complexes act as GTPase activating proteins (GAP) to deactivate Rheb and Rag,
1628 respectively, and thereby block mTOR activation. In addition to the stimulation of protein
1629 synthesis, activated mTOR inhibits autophagy by inhibiting the ULK1 complex that, through the
1630 sequential activation of the Beclin complex and the WD-repeat phosphoinositide-interacting
1631 protein 4 (WIPI4), activate the maturation and fusion of autophagosomes with acidic lysosomes to
1632 form autolysosomes (512). A central role in the regulation of mTOR activation, as well as in the
1633 autophagy progression, is played by the proton pump vATPase and its ancillary proteins that
1634 allows docking of Ragulator to the organelle membrane which in turn recruits and activates the
1635 mTORC1 complex (**FIGURE 9**).

1636 Not surprisingly, dysregulation, particularly hyperactivation, of the mTOR pathway caused by
1637 mutations in the various components of the PI3K-mTOR pathway and regulatory cascades, result
1638 in DEEs, the so-called mTORopathies, that are often associated with malformations of cortical
1639 development. mTORopathies include a spectrum of drug-resistant epilepsy syndromes ranging
1640 from apparently non-lesional focal epilepsy to Tuberous Sclerosis Complex (TSC), FCDII, HME that
1641 are associated with brain malformations (515, 516). The complexity of the architecture of the
1642 mTOR pathway, while it guarantees a full control over key processes for cell survival and

1643 adaptation, also offers several genes whose mutations impact on critical neuronal processes and
1644 are causative of DEEs (517). The first identified, prototypical mTORopathy is the TSC linked to
1645 germline/somatic mutations in *TSC1* or *TSC2* genes that remove the inhibitory brake of TSC on the
1646 downstream mTOR complex 1 (mTORC1) and determine mTOR hyperactivity in several tissues
1647 including the brain. Subsequently, high throughput sequencing has discovered an array of
1648 germline and *de novo* somatic mutations causing mTOR hyperactivity that involved either loss-of-
1649 function of inhibitory complexes (*PI3K*, *PTEN*, *AKT3*, *TSC1*, *TSC2*, *RHEB*, *DEPDC5*, *NPRL2*, and
1650 *NPRL3*) or gain-of-function hyperactive variants of *MTOR*. Animal models of mTORopathies are
1651 constantly characterized by an epileptic phenotype. *Pten*, *Tsc1*, *Tsc2* and *Depdc5* knockout mice,
1652 as well as *Mtor* knock-in mice recapitulate the human phenotype of mTORopathies by displaying
1653 dysplastic cortical areas with enlarged cortex, ectopic and hypertrophic neurons, and epilepsy.
1654 Constitutive and generalized mTOR hyperactivity is embryonic lethal, so that conditional models
1655 have been used to effectively reproduce human pathology. In addition to the dysplastic
1656 phenotype, mTOR hyperactivity causes hyperexcitability (515, 518, 519). This is not only
1657 attributable to the intrinsic instability of dysplastic circuits with dendritic and somatic hypertrophy
1658 and formation of reverberant connections, but also depends on an augmented intrinsic excitability
1659 of principal cortical neurons and potentiation of excitatory synaptic transmission at both pre- and
1660 post-synaptic levels promoting E/I imbalance. Another interesting feature of mTORopathies
1661 regards the relative frequency of somatic mutations that are likely to occur predominantly during
1662 brain development and that establish a condition of mosaicism in the neuronal populations of the
1663 cerebral cortex. The intersection between inherited germline mutations and acquired somatic
1664 ones (“second hit”) causes loss of heterozygosity or mosaic compound heterozygosity that can
1665 convert a latent deficit into an overt phenotype in restricted brain areas. This phenomenon was
1666 clearly demonstrated for *DEPDC5* mutations both in humans and in experimental models in which

1667 the “somatic” knockdown of *Depdc5* was reproduced by *in utero* electroporation and/or RNA
1668 interference, generating focal cortical dysplasia (29, 520, 521). One of the mechanisms by which
1669 mTOR hyperactivity causes epileptic encephalopathies is through a dysregulation of autophagy, a
1670 process strongly implicated in neuronal survival and plasticity (511, 512, 520). As mentioned
1671 above, neuronal autophagy is a highly regulated process in which mTOR inhibition releases the
1672 ULK1 activator which, together with AMPK, activates the autophagy initiator Beclin complex
1673 (composed of VPS34, VPS15, Ambra1 and Beclin1), that is recruited to the phagophore, stimulates
1674 the production of PI3P and the binding of WIPI proteins. This allows the conversion of LC3-I into
1675 phosphatidylethanolamine-bound LC3-II that is the fundamental signal activating autophagy
1676 (**FIGURE 9**). mTOR and AMPK represent a yin-yang mechanism controlling autophagy: not only the
1677 two pathways exert an opposite control onto the underlying autophagic process, but AMPK also
1678 stimulates the TSC complex, keeping mTOR inactive during autophagy activation (522). The final
1679 common pathway of autophagy is the fusion of mature autophagosomes with lysosomes to form
1680 the autolysosomes, where the vesicular content is finally degraded and released into the
1681 cytoplasm. This event is regulated by the autophagic proteins EPG5 and SNX14 and requires
1682 internal acidification of the organelle to allow for proteolysis. Biallelic mutations of *EPG5* and
1683 *SNX14* cause respectively the Vici syndrome, a rare and severe congenital multisystem disorder
1684 characterized by failure of the corpus callosum, cataracts, oculocutaneous hypopigmentation,
1685 cardiomyopathy and combined immunodeficiency (523) and a form of cerebellar ataxia and
1686 intellectual disability (524). Acidification is provided by vATPase, a multifunctional proton pump
1687 that regulates multiple cellular processes including membrane trafficking, receptor-mediated
1688 endocytosis, SV cycling and NT loading. Acidification is necessary for autophagy progression and
1689 drugs or vATPase variants impairing the build-up of the proton gradient block autophagy (525).
1690 vATPase is composed of a V1 cytosolic domain that hydrolyses ATP and a V0 transmembrane

1691 domain that transfers protons to the organelle's interior (526). While the proton pumping activity
1692 of vATPase occurs in all tissues, the brain is particularly vulnerable to vATPase defects for its
1693 multiple functions in exocytosis (426, 527) and autophagy. In man, as many as 22 genes encode for
1694 the multiple and redundant subunits of the V1 and V2 complexes, allowing the composition of
1695 different v-ATPase complexes with specific properties and tissue expression.

1696 Several, heterozygous or biallelic mutations in *ATP6V1A* coding for the V1 subunit A have been
1697 recently described in patients with DEEs of variable severity, ranging from moderate intellectual
1698 disability with seizures to an early-onset DEE with premature lethality (429, 528). *ATP6V1A*
1699 variants affect lysosomal homeostasis and autophagy and, when expressed in postsynaptic
1700 neurons, impair neurite development and the formation/maintenance of excitatory synaptic
1701 connections (429). *ATP6V1A* deficits have also been recently associated with neuronal impairment
1702 and neurodegeneration. *ATP6V1A* silenced neurons show reduced network activity and alteration
1703 of synaptic proteins consistent with a key role of *ATP6V1A* in neuronal maturation and activity
1704 (529).

1705 In addition, mutations in ancillary proteins of the v-ATPase complex, known to regulate its
1706 function and trafficking to the organelles, also cause severe neurodevelopmental disorders with
1707 epilepsy and impair brain development when modelled in mice (430, 530). A *de novo* variant of
1708 *ATP6AP2*, a vATPase accessory protein, was identified in a patient with neurodevelopmental
1709 disorder and fulminant degeneration. In murine models and patient neurons, loss-of-function of
1710 *ATP6AP2* results in lysosomal and autophagic defects with impaired neuronal survival, revealing a
1711 key role of this V-ATPase modulator in brain function (530). Homozygous recessive and compound
1712 heterozygous mutations in the *DMXL2* gene were recently found to cause a severe and rapidly
1713 progressing DEE associated with Ohtahara syndrome and premature death (431). The gene
1714 encodes for the vesicular protein DMXL2 (also known as rabconnectin-3a), a member of the WD40

1715 repeat (WDR) protein family that is highly expressed in brain tissue, regulates the trafficking and
1716 activity of V-ATPase and interacts with the SV-associated G-protein Rab3A (531, 532). Altered
1717 lysosomal homeostasis and defective autophagy were recapitulated in *Dmnl2*-silenced mouse
1718 hippocampal neurons that exhibit impaired neurite elongation and synaptic loss (431). *Dmnl2*^{-/-}
1719 mice are embryonic lethal (533) while heterozygous *Dmnl2* mice show brain malformations,
1720 uncovering a penetrant role of *Dmnl2* in brain development (534).

1721 These data confirm a primary role of autophagy dysregulation in alterations of cortical
1722 development and epileptogenesis and suggest that the severity of the clinical phenotypes and the
1723 extent of neurodegeneration depend on the stage of neuronal development and on the specific
1724 consequences of the impairment of synaptic transmission and neuronal survival due to the
1725 stressful condition of impaired autophagy (535).

1726 **X. THE SEARCH FOR PERSONALISED TREATMENT APPROACHES**

1727 Currently, most therapies for DEEs target individual symptoms such as seizures, and not the
1728 underlying disease mechanisms. For many individuals with DEEs, seizure control is not achieved,
1729 even when antiseizure medications (ASMs) are optimized for the underlying etiology, while in
1730 patients in whom seizure control is achieved, developmental impairments and other comorbidities
1731 often remain severe (4, 397). In addition, since patients with the same type of clinical seizures may
1732 differently respond to ASMs, the pathophysiological events that underlie epileptic seizures
1733 apparently not only differ between unique seizure syndromes and specific etiologies, but may also
1734 be multifactorial for the same types (536). The remarkable growth of animal models of DEEs has
1735 enabled preclinical studies that tested several experimental drugs have been tested in different
1736 animal models of DEEs (**TABLE 4**). Both acute and chronic models of epileptic spasms have been
1737 used to study effects on spasms prevention using protocols that administer drugs prior to the
1738 induction (acute models and certain chronic models) (537–544), spasms control / cessation

1739 (chronic models: multiple-hit, TTX) (150, 155–158, 160, 161, 545), hypsarrhythmia (TTX model)
1740 (546), disease modification or antiepileptogenesis (chronic models: multiple-hit, ARX KI) (150,
1741 155). Among the existing models, resistance to existing therapies for ISS (ACTH, vigabatrin) is
1742 shown at the multiple-hit rat model of ISS due to structural lesions (88). The only model that has
1743 been used to study effects on hypsarrhythmia is the TTX model which allows testing of effects on
1744 the EEG using multiple electrodes in post-pubertal animals. This is technically challenging in rodent
1745 models that manifest spasms only during restricted early developmental periods, when the skull is
1746 small and fragile. The prenatal betamethasone/postnatal NMDA model suggested that prenatal
1747 betamethasone increases the sensitivity to ACTH, even though it increases the severity of NMDA-
1748 induced spasms and has been used to explore pharmacologically therapeutic effects of pathways
1749 thought to mediate the ACTH effects, i.e., mineralocorticoid receptor signaling (538). Among the
1750 treatments tested in these preclinical trials, two drugs eventually acquired orphan status for ISS
1751 (carisbamate, CPP-115). CPP-115 has also been tested in a case report on an infant with drug-
1752 resistant ISS with significant improvement in spasms and seizure control compared to vigabatrin
1753 (547). In support of the promising effects of the mTOR inhibitor rapamycin on spasms in the
1754 multiple-hit model, recent clinical case reports suggested a possible benefit in infants with
1755 tuberous sclerosis related ISS treated with the mTOR inhibitor everolimus (548).

1756 There has also been a remarkable progress in screening for new treatments for Dravet syndrome,
1757 using both rodents (233, 384, 549–558) as well as high throughput studies in zebrafish (391, 559–
1758 564). A noteworthy development has been the recent approval by the Food and Drug
1759 Administration of fenfluramine, a reuptake inhibitor of 5-OH-tryptamine, for the treatment of
1760 Dravet syndrome, based on evidence provided by both preclinical (560, 561) and clinical trials
1761 (230, 565–569).

1762 A challenge in these preclinical drug trials has been the comparison of findings across models,
1763 given the different species (rats vs other animal models), different induction protocols,
1764 developmental periods of exposure to the drugs, and treatment protocols (pre- vs post-
1765 treatments) (150, 155, 157, 161, 537, 542, 545). Hopefully identification of biomarkers guiding
1766 treatment implementation would help de-risk the process of selecting promising candidates for
1767 transitioning in clinical trials, as well as optimizing treatment protocols and designs.

1768 The choice of ASM for DEE in clinical practice is mainly influenced by cumulative experience in
1769 open label studies and only a limited number of drug trials in etiologically homogeneous
1770 conditions (570). For most DEE, treatment choices rely on the hypothesized drug action on clinical
1771 and EEG phenomenology and remain confined to symptom relief and general principles of epilepsy
1772 management. Commercially available ASMs are typically grouped by their principal mode of action
1773 (drugs that affect voltage-dependent sodium channels, calcium currents, GABA activity, the
1774 glutamate receptors, and drugs with other mechanisms of action - **TABLE 5**), although for many
1775 drugs the precise mechanism of action remains unknown, or multiple actions are hypothesized. As
1776 for other conditions, treatments for DEEs may include conventional drugs or repurposed therapies
1777 (i.e., with specific actions that may have been used in entirely unrelated conditions).

1778 Only recently, the enormous amount of knowledge generated by molecular genetic findings in the
1779 DEE and increased knowledge of the underlying disease mechanisms has allowed designing
1780 etiology-specific trials for genetic DEEs (<https://clinicaltrials.gov/ct2/home>), (see **TABLE 6** and
1781 **SUPPLEMENTAL TABLE S2**,
1782 https://figshare.com/articles/dataset/Guerrini_et_al_Supplemental_Table_S2_xlsx/17694728, for
1783 examples).

1784 When clinical, EEG, and imaging findings suggest focal localization of the epileptogenic zone, and if
1785 any resulting neurologic deficit is not more severe than epilepsy itself, surgical treatment of

1786 epilepsy remains one of the best options. For example, for patients with TSC, epilepsy surgery is
1787 considered after failure of two ASMs even when multiple cortical lesions are present and TSC
1788 operated patients, including those with infantile spasm, have a 50%–60% chance of long-term
1789 seizure freedom after surgery for epilepsy (570).

1790 A promising alternative approach to treat DEEs is represented by gene therapies. The growth in
1791 gene therapies in medicine has partly been realized through the development of safe and effective
1792 means of gene delivery using viral vectors. These vectors are engineered to avoid their replication
1793 in human cells and to deliver the wild type form of the gene mutated in a given patient into the
1794 right cell target. The expression of the gene product delivered by viral vectors in the cells where
1795 the genetic defect needs to be corrected is ensured using a specific promoter. For DEEs, this
1796 treatment approach has some drawbacks, as most viral vectors can only carry a limited amount of
1797 DNA (and thus cannot be used to replace big genes such as *SCN1A*). In addition, to bypass the
1798 blood brain barrier (BBB), they need to be injected in the brain through intraventricular injections.
1799 Gene therapy based on vectors derived from adeno-associated virus (AAV) has the potential to
1800 overcome the latter limitation since it has been demonstrated that different AAV serotypes are
1801 able to cross the BBB and thus can be used to implement gene delivery strategies based on them
1802 to treat CNS diseases (571). Gene therapy, including AAV for DEE carries several major technical
1803 limitations (572). Delivery would need to target the whole brain as most DEE are related to
1804 widespread brain dysfunction. Diffusely and irreversibly altering the genetic make-up of neurons
1805 may be a worrisome perspective since some of the genetic defects causing DEE result from both
1806 loss and gain of function effects an overdosing can cause its own pathology. Dosing and
1807 distribution are further complicated by the X-linked or somatic mosaic genetic alteration that
1808 underlies the disorder, with only half or small percentages of cells needing the supplemental

1809 transgene delivery. Additional limitations are related to the fact that delivery should be realized in
1810 a critical period, before mature networks are established.

1811 Another possible way to correct the effects of a mutation at intracellular level using genetic
1812 material is the use of antisense oligonucleotides (ASOs), single stranded deoxyribonucleotides
1813 which are complementary to a specific target mRNA and that, upon binding it alters its splicing,
1814 impede its translation, or promote its degradation. ASOs are usually administered intrathecally.

1815 Studies on a conditional mouse model with Cre-dependent expression of the pathogenic patient
1816 *SCN8A* mutation p.Arg1872Trp (R1872W) have recently demonstrated that administration of an
1817 ASO directed against the *Scn8a* mutant transcript delayed seizure onset and increased survival,
1818 suggesting that reduction of *SCN8A* transcript is a promising approach to treatment of intractable
1819 childhood epilepsies (262). The effectiveness of ASOs has been confirmed in an additional study
1820 carried out by Li and collaborators (246). These authors demonstrated that targeted reduction of
1821 *Scn2a* mRNA expression by central administration of a gapmer ASOs in *Scn2a* Q/+ mice reduced
1822 spontaneous seizures and significantly extended life span in treated animals. These results suggest
1823 that human *SCN2A* gapmer ASOs could likewise impact the lives of patients with *SCN2A* gain-of-
1824 function DEE (246). Because ASOs can regulate splicing, it is possible to use them to increase the
1825 production of translated mRNA. TANGO (targeted augmentation of nuclear gene output)
1826 approach, which modulates naturally occurring, nonproductive splicing events to increase target
1827 gene and protein expression, has recently been used in animal models of DS to increase *Scn1a*
1828 transcript and Na_v1.1 protein expression. In these models, a single intracerebroventricular dose of
1829 a lead ASO at postnatal day 2 or 14 reduced the incidence of electrographic seizures and sudden
1830 unexpected death in epilepsy (SUDEP), suggesting that TANGO may provide a unique, gene-
1831 specific approach for the treatment of DS (233).

1832 ASOs can also be used to regulate the activity of miRNAs, which are ‘multi-pathway’ regulatory
1833 molecules. Mature miRNAs are generated via a multistep process. They are initially transcribed as
1834 relatively large (even more than 1 kb) hairpin structures known as pri-miRNA. This undergoes
1835 cleavage in the nucleus by the enzyme Drosha to produce a 60–70-nucleotide (nt) stem loop pre-
1836 miRNA, which is subsequently transported from the nucleus to the cytoplasm. The enzyme Dicer
1837 recognizes pre-miRNA and cleaves the stem loop, leaving an imperfect ~21–23-nt miRNA duplex
1838 with a ~2-nt 3’ overhang at each end. The less thermodynamically stable end of the pre-miRNA
1839 duplex is then uploaded to a binding pocket within an Argonaute (Ago) protein to form the RNA-
1840 induced silencing complex (RISC). The miRNA-loaded RISC then traffics along mRNAs searching for
1841 complementary binding sites and, upon finding mRNA targets containing a ~7–8-nt seed match
1842 [typically within the 3’ untranslated region (UTR)], triggers either target degradation or
1843 translational repression (573). Since there have been more than 300 studies on miRNA and
1844 epilepsy, and over 100 different miRNAs have found to be altered in experimental models and
1845 human samples [EpimiRBase (574)], miRNA could represent a novel class of molecules to be
1846 targeted using ASOs to treat epilepsy in DEEs.

1847 Gene therapy can be also pursued correcting a specific genetic defect in patients’ cells through
1848 endogenous gene editing. This approach is not based on expressing the wild type form of a given
1849 gene in cells that do not express it properly, but on the substitution of a specific portion of the
1850 endogenous mutated gene (e.g. that flanking a mutation) with the wild type sequence. Clustered
1851 regularly interspaced palindromic repeats (CRISPR)/Cas9 is a gene-editing technology that makes it
1852 possible to correct errors in the genome and turn on or off genes in cells and organisms quickly,
1853 cheaply and with relative ease. This gene-editing technology has a number of laboratory
1854 applications including rapid generation of cellular and animal models, functional genomic screens,
1855 live imaging of the cellular genome, and gene therapy (575, 576). CRISPR/Cas9 involves two

1856 essential components: a guide RNA to match a desired target gene, and Cas9 (CRISPR-associated
1857 protein 9) - an endonuclease which causes a double-stranded DNA break, allowing modifications
1858 of the genome. One of the most exciting applications of CRISPR/Cas9 is its potential use to treat
1859 genetic disorders caused by single gene mutations. Examples of such diseases include cystic
1860 fibrosis (CF), Duchenne's muscular dystrophy (DMD) and haemoglobinopathies (576). A modified
1861 version of the CRISPR-Cas9 system, called dead Cas9 (dCas9), can be tailored to obtain a robust
1862 and highly specific activation of the *Scn1a* gene both in cultured neurons and in the brain tissue of
1863 a DS mouse model, suggesting that dCas9 may be an effective and targeted approach to DS and
1864 other disorders resulting from altered gene dosage (232). A number of challenges remain before
1865 the potential of CRISPR/Cas9 can be translated to effective treatments at the bedside. Indeed, its
1866 clinical translation has been hampered by varying efficiency, off-target effects, and, on occasion,
1867 insufficient vector size for the necessary genetic material (570). In addition, a suitable vector is
1868 needed to safely deliver Cas9-nuclease encoding genes and guide RNAs *in vivo* without any
1869 associated toxicity (576). To overcome this problem, the use of AAV vectors has been proposed.
1870 However, this delivery system may be too small to allow efficient transduction of the Cas9 gene. A
1871 smaller Cas9 gene could be used, but this has additional implications on efficacy (575).

1872 **XI. CONCLUSIONS**

1873 Multiple genetically determined or, at times, acquired etiologies may severely alter the balance
1874 between excitatory and inhibitory neuronal activity and result in widespread epileptogenesis in
1875 the developing brain. If the causative defect imposes serious consequences on physiological brain
1876 function, the superimposed epilepsy will add a clinical burden to the already compromised
1877 neurodevelopmental processes. If the causative defect only mildly impacts physiological brain
1878 development and function, any superimposed severe epileptogenic process will cause
1879 considerable neurodevelopmental deterioration (577). Development of higher cortical functions is

1880 the most complex and vulnerable process and will be the most severely affected. Impairment may
1881 be relatively selective, in the context of DEE in which for example, language, memory, attention or
1882 executive functions are impaired to a different extent. Generalized severe cognitive impairment or
1883 autistic features may be the result of a widespread epileptogenic process. Although DEE have a
1884 multitude of causes and variable clinical patterns, with many genes involved whose altered
1885 expression may affect different aspects of neural cell functioning, mechanisms whereby epileptic
1886 activity may interfere with brain function and produce such patterns, tend to be relatively limited.
1887 Moderate levels of hyperexcitability can disrupt cortical processing, with temporal and anatomic
1888 specificity (578). Disruption is time-locked with the EEG event accompanying hyperexcitability and
1889 specific to the modality represented in the anatomic area involved (579–581). Redundant and
1890 frequent EEG abnormalities as observed in DEE may cause widespread cortical dysfunction that
1891 can be manifested with signs of early cognitive regression even when seizure activity is not yet
1892 overly manifested (582, 583). The timing of onset, network distribution and duration of the
1893 epileptogenic process influence how the DEE will be manifested, at times in close relationship with
1894 its specific etiology, at times irrespective from it similar anatomoclinical backgrounds may be
1895 accompanied by very different forms of DEE in different individuals. In this perspective, while
1896 efforts towards development of precision treatment approaches in DEE shall ideally target the
1897 causative mechanisms, traditional approaches with antiseizure medications addressed at
1898 mitigating the consequences of redundant epileptic activity on physiological brain function still
1899 have a relevant role.

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3770 Tables

3771 Table 1. Developmental and epileptic encephalopathies (DEEs) by age

	Age of onset	Gender	Etiology	Electroclinical characteristics
<u>Neonatal – Infantile onset</u>				
Early infantile DEEs (previously Ohtahara syndrome or early myoclonic epilepsy)	≤3mo of life	M=F	Genetic or structural/metabolic	Clinical: Abnormal neurological findings, neurodevelopmental deficits. Seizures: tonic (independent of sleep), myoclonic, epileptic spasms, sequential. EEG: Burst-suppression pattern; multifocal epileptiform discharges; hypsarrhythmia may appear. Seizure patterns are bilateral or focal onset, depending on seizure types.
Epilepsy of Infancy with Migrating Focal Seizures (EIMFS)	First year of life	M=F	Mainly genetic	Clinical: Neurodevelopmental delay, focal motor tonic or clonic seizures. EEG: Migrating EEG patterns during ictal events, multifocal discharges
Infantile spasms syndrome (ISS)	3-12mo (1- 24mo)	M>F	Structural/metabolic, genetic, unknown	Clinical: Epileptic spasms, other seizures may occur; neurodevelopmental disorders, intellectual disabilities. EEG: Hypsarrhythmia, electrodecremental responses (ictal or interictal), multifocal epileptiform discharges.
Dravet syndrome	3-9mo (1-20mo)	M=F	Genetic	Clinical: Neurodevelopmental deficits. Prolonged hemiclonic seizure with fever in the absence of infectious / structural brain lesion; myoclonic, focal impaired awareness, atypical absences, atonic seizures, non-convulsive status epilepticus, tonic and tonic-clonic seizures in sleep.

	Age of onset	Gender	Etiology	Electroclinical characteristics
				<i>EEG</i> : Focal or multifocal epileptiform abnormalities and seizures, photoparoxysmal responses.
Etiology-specific syndromes: - <i>KCNQ2, CDKL5,</i> <i>PCDH19, SCL2A1,</i> Pyridoxine and Pyridox(am)ine 5'- Phosphate Dependent Epilepsy, Glucose Transporter 1 Deficiency syndrome (Glu1DS), Sturge- Weber syndrome	First year of life	M=F, M<F (<i>PCDH1</i> 9, <i>CDKL5</i>)	Genetic	<p><i>KCNQ2-DEE</i>: Sequential or focal tonic seizures, burst suppression; autonomic symptoms, epileptic spasms. Burst suppression or multifocal EEG.</p> <p><i>CDKL5-DEE</i>: Tonic seizures, epileptic spasms. hypermotor-tonic-spasms; movement disorders</p> <p><i>PDE, PSPDE</i>: Intrauterine or early life seizures; focal seizures, spasms or generalized tonic-clonic; response to pyridoxine or P5P. EEG: burst suppression or multifocal discharges.</p> <p><i>Glut1DS</i>: Intellectual disability, low CSF/plasma glucose ratio, generalized seizures (myoclonic, myoclonic-atonic, generalized tonic-clonic, absences). EEG: 2.5-5Hz spike-wave.</p> <p><i>PCDH19-DEE</i>: Intellectual disability, autism spectrum disorder; focal impaired aware to tonic or atypical absence seizures. EEG: focal onset seizures.</p> <p><i>Sturge-Weber</i>: Facial port-wine stain, progressive neurological course, epilepsy, hemiparesis, psychomotor delay, stroke-like events, psychiatric disorders, glaucoma. Focal motor seizures, febrile seizures, infantile spasms, myoclonic-atonic, gelastic seizures. EEG: Asymmetric, focal epileptiform activities.</p>

	Age of onset	Gender	Etiology	Electroclinical characteristics
- Gelastic Seizures with Hypothalamic Hamartoma	First year of life	M=F	Structural, Genetic-structural	Clinical: Normal neurological exam initially, deficits appear later; precocious puberty; gelastic seizures with mirthless laughter (mandatory), gelastic and dacrystic seizures, focal impaired awareness or generalized seizures, other types of seizures may occur. EEG: Focal or generalized
<u>Childhood onset</u>				
Myoclonic-atonic epilepsy	2-6y	M>F	Genetic	Clinical: Seizures: myoclonic-atonic (mandatory), atonic, myoclonic, absence, generalized tonic-clonic. EEG: 3-6Hz (poly)spike-slow wave discharges, generalized, activated in sleep; generalized paroxysmal fast
Lennox-Gastaut syndrome	18mo – 8y	(M>F)	Structural/metabolic, genetic	Clinical: Tonic seizures in sleep (mandatory), atypical absence, atonic, myoclonic, focal impaired awareness, generalized tonic-clonic, epileptic spasms EEG: Slow spike-wave (≤ 2.5 Hz), generalized; generalized paroxysmal fast; focal or multifocal slow spike-wave may be seen.
DEE with SW activation in sleep (D/EE-SWAS)	2-12y	M=F	Structural, genetic	Clinical: Neurocognitive / behavioral deficits which ameliorate / resolve with resolution of SWAS; seizure types depend on etiology: focal or focal to bilateral, typical, or atypical absences, atonic, negative myoclonus. EEG: Slow background; focal or multifocal discharges; marked activation of diffuse epileptiform discharges in sleep (>50% of sleep, 1.5-2Hz spike-wave runs).

	Age of onset	Gender	Etiology	Electroclinical characteristics
Febrile Infection Related Epilepsy Syndrome (FIRES)	2-17	(M>F)	Infectious/post-infectious	<p>Clinical: Developmental regression, intellectual disabilities, attention or behavioral problems, motor dysfunction. Focal or multifocal seizures, super-refractory status epilepticus.</p> <p>EEG: Slow background, multifocal epileptiform discharges; extreme delta brushes. Increasing frequency of focal onset seizures (focal >10Hz evolving to rhythmic spike-waves).</p>
Hemiconvulsion-Hemiplegia-Epilepsy syndrome (HHE)	<4y	M=F	Unknown, structural/metabolic, genetic	<p>Clinical: Focal (clonic) febrile status epilepticus and persistent hemiparesis, aphasia when dominant hemisphere involved; focal or focal to bilateral motor seizures.</p> <p>EEG: Focal or lateralized rhythmic ictal delta, focal recruiting (10Hz) rhythms.</p>
<u>Variable age onset</u>				
Progressive myoclonus epilepsy (PME)				
- Unverricht-Lundborg (EPM1)	7-13y	M=F	Genetic	<p>Clinical: progressive course; myoclonus induced by touch / photic stimulation, more pronounced upon awakening; other generalized seizures may occur.</p> <p>EEG: Photosensitivity; generalized polyspike and wave (ictal).</p>
- Lafora disease (EPM2)	6-19y	M=F	Genetic	<p>Clinical: Vision loss, cognitive decline, cerebellar symptoms. Myoclonic and generalized tonic-clonic seizures.</p> <p>EEG: Photosensitivity; spike wave and polyspikes, no activation in sleep.</p>

	Age of onset	Gender	Etiology	Electroclinical characteristics
- Neuronal Ceroid Lipofuscinosis (NCL)				
- CLN2 (late infantile)	2-4yo	M=F	Genetic	Clinical: Language delay, progressive course, multiple seizures febrile and afebrile, including myoclonic. EEG: Photoparoxysmal response at low frequencies.
- Juvenile CLN3	4-10y	M=F	Genetic	Clinical: Visual loss progressive, macular degeneration, optic atrophy, retinitis pigmentosa. EEG:
- Adult NCL (Kufs)	11-50y	M=F	Genetic	Type A: PME with dementia and ataxia. Type B: Dementia with cerebellar or extrapyramidal motor symptoms but not PME.

- 3772 The list of DEEs follows the 2021 proposal of the International League Against Epilepsy (ILAE) Task Force on Nosology and Definitions for epilepsy syndromes (5–7). DEE:
- 3773 Developmental and epileptic encephalopathies; D/EE-SWAS: DEE with SW activation in sleep; EIMFS: Epilepsy of Infancy with Migrating Focal Seizures; EPM: epilepsy with
- 3774 progressive myoclonus; F: female; Glut1DS: Glucose Transporter 1 (*SLC2A1*) Deficiency syndrome; HHE: Hemiconvulsion-Hemiplegia-Epilepsy syndrome; M: male; mo: months;
- 3775 NCL or CLN: neuronal ceroid lipofuscinosis; PME: progressive myoclonic epilepsy; PDE: Pyridoxine dependent epilepsy; P5PDE: Pyridox(am)ine 5'-Phosphate (P5PD) Dependent
- 3776 Epilepsy; SW: spike-and-slow-wave; y: years.

3777 **TABLE 2. Genes involved in epileptic encephalopathy and DEE pathogenesis**

	Approved Symbol	Gene/Locus name
Ion channels		
	<i>KCNA2</i>	Potassium channel, voltage-gated, Shaker-related subfamily, member 2
	<i>CACNA1E</i>	Calcium channel, voltage-dependent, alpha 1E subunit
	<i>KCNT2</i>	Potassium channel, subfamily T, member 2
	<i>SCN3A</i>	Sodium channel, voltage-gated, type III, alpha polypeptide
	<i>SCN2A</i>	Sodium channel, voltage-gated, type II, alpha subunit
	<i>SCN1A</i>	Sodium channel, voltage-gated, type I, alpha polypeptide
	<i>SCN9A</i>	Sodium channel, voltage-gated, type IX, alpha subunit
	<i>CACNA2D2</i>	Calcium channel, voltage-dependent, alpha-2/delta subunit 2
	<i>HCN1</i>	Hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
	<i>KCNQ5</i>	Potassium channel, voltage-gated, KQT-like subfamily, member 5
	<i>KCNT1</i>	Potassium channel, subfamily T, member 1
	<i>CACNA1B</i>	Calcium channel, voltage-dependent, L type, alpha 1B subunit
	<i>SCN8A</i>	Sodium channel, voltage gated, type VIII, alpha polypeptide
	<i>CACNA1G</i>	Calcium channel, voltage-dependent, T type, alpha-1G subunit
	<i>CACNA1A</i>	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit
	<i>SCN1B</i>	Sodium channel, voltage-gated, type I, beta polypeptide
	<i>KCNB1</i>	Potassium voltage-gated channel, Shab-related subfamily, member 1
	<i>KCNQ2</i>	Potassium voltage-gated channel, KQT-like subfamily, member 2
	<i>CLCN4</i>	Chloride channel-4

	Approved Symbol	Gene/Locus name
Receptors		
	<i>GABRA2</i>	Gamma-aminobutyric acid (GABA) A receptor, alpha-2
	<i>GABRB1</i>	Gamma-aminobutyric acid (GABA) A receptor, beta-1
	<i>GABRB2</i>	Gamma-aminobutyric acid (GABA) A receptor, beta-2
	<i>GABRA1</i>	Gamma-aminobutyric acid (GABA) A receptor, alpha-1
	<i>GABRG2</i>	Gamma-aminobutyric acid (GABA) A receptor, gamma-2
	<i>NTRK2</i>	Neurotrophic tyrosine kinase, receptor, type 2
	<i>GABBR2</i>	Gamma-aminobutyric acid B receptor 2
	<i>GRIN1</i>	Glutamate receptor, ionotropic, N-methyl D-aspartate 1
	<i>GABRB3</i>	Gamma-aminobutyric acid (GABA) A receptor, beta-3
	<i>GABRA5</i>	Gamma-aminobutyric acid (GABA) A receptor, alpha-5
	<i>GRIN2A</i>	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A
	<i>GRIN2D</i>	Glutamate receptor, ionotropic, N-methyl-D-aspartate 2D
Transporters		
	<i>ATP1A2</i>	ATPase, Na ⁺ /K ⁺ transporting, alpha-2 polypeptide
	<i>SLC2A1</i>	Solute carrier family 2 (facilitated glucose transporter), member 1
	<i>ARV1</i>	ARV1 homolog, fatty acid homeostasis modulator
	<i>SLC1A4</i>	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
	<i>SLC25A12</i>	Solute carrier family 25 (mitochondrial carrier, Aralar), member 12
	<i>SLC6A1</i>	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1
	<i>SLC25A22</i>	Solute carrier family 25 (mitochondrial carrier, glutamate), member 22
	<i>SLC1A2</i>	Solute carrier family 1 (glial high affinity glutamate transporter),

	Approved Symbol	Gene/Locus name
		member 2
	<i>SLC13A5</i>	Solute carrier family 13 (sodium-dependent citrate transporter), member 5
	<i>SLC25A10</i>	Solute carrier family 25 (mitochondrial carrier), member 10 (dicarboxylate ion carrier)
	<i>SLC25A42</i>	Solute carrier family 25, member 42
	<i>ATP1A3</i>	ATPase, Na ⁺ /K ⁺ transporting, alpha-3 polypeptide
	<i>SLC12A5</i>	Solute carrier family 12, (potassium-chloride transporter) member 5
	<i>SLC35A2</i>	Solute carrier family 35 (UDP-galactose transporter), member 2
	<i>SLC9A6</i>	Solute carrier family 9 (sodium/hydrogen exchanger), member 6
Synapse related		
	<i>CPLX1</i>	Complexin 1
	<i>PPP3CA</i>	Protein phosphatase 3, catalytic subunit, alpha isoform (calcineurin A alpha)
	<i>SYNGAP1</i>	Synaptic Ras GTPase activating protein 1
	<i>ADAM22</i>	ADAM metallopeptidase domain 22
	<i>STXBP1</i>	Syntaxin-binding protein 1
	<i>DNM1</i>	Dynamin-1
	<i>NECAP1</i>	NECAP endocytosis-associated protein 1
	<i>DMXL2</i>	DMX-like 2
	<i>AP3B2</i>	Adaptor-related protein complex 3, beta 2 subunit
	<i>STX1B</i>	Syntaxin 1B
	<i>SYNJ1</i>	Synaptojanin 1
	<i>NRXN1</i>	Neurexin 1
Cell growth, division, and proliferation related		

	Approved Symbol	Gene/Locus name
	<i>MTOR</i>	Mechanistic target of rapamycin
	<i>AKT3</i>	AKT serine/threonine kinase 3
	<i>NPRL2</i>	NPR2-like protein, GATOR1 complex subunit
	<i>STAG1</i>	Stromal antigen 1
	<i>RNF13</i>	RING finger protein 13
	<i>PIK3CA</i>	Phosphatidylinositol 3-kinase, catalytic, alpha
	<i>PPP2CA</i>	Protein phosphatase-2 (formerly 2A), catalytic subunit, alpha isoform
	<i>ACTL6B</i>	Actin-like 6B
	<i>RHOBTB2</i>	Rho-related BTB domain-containing protein 2
	<i>TSC1</i>	Hamartin
	<i>AKT1</i>	AKT serine/threonine kinase 1
	<i>NPRL3</i>	Nitrogen permease regulator-like 3
	<i>TSC2</i>	Tuberin
	<i>PIK3R2</i>	Phosphatidylinositol 3-kinase, regulatory subunit 2
	<i>DEPDC5</i>	DEP domain-containing protein 5
Cell metabolism related		
	<i>MTHFR</i>	Methylenetetrahydrofolate reductase
	<i>ST3GAL3</i>	ST3 beta-galactoside alpha-2,3-sialyltransferase 3
	<i>PARS2</i>	Prolyl-tRNA synthetase 2
	<i>HNRNPU</i>	Heterogeneous nuclear ribonucleoprotein U
	<i>CAD</i>	CAD trifunctional protein of pyrimidine biosynthesis
	<i>MDH1</i>	Malate dehydrogenase, soluble
	<i>UGP2</i>	Uridyl diphosphate glucose pyrophosphorylase-2
	<i>BOLA3</i>	bolA family member 3
	<i>ST3GAL5</i>	Sialyltransferase 9

	Approved Symbol	Gene/Locus name
	<i>GAD1</i>	Glutamate decarboxylase-1, brain, 67kD
	<i>GLS</i>	Glutaminase
	<i>D2HGDH</i>	D-2-hydroxyglutarate dehydrogenase
	<i>UGDH</i>	UDP-glucose dehydrogenase
	<i>MANBA</i>	Mannosidase, beta A, lysosomal
	<i>NUS1</i>	NUS1 dehydrodolichyl diphosphate synthase subunit
	<i>MDH2</i>	Malate dehydrogenase, mitochondrial
	<i>DENND5A</i>	DENN domain-containing protein 5A
	<i>NARS2</i>	Asparaginyl-tRNA synthetase 2
	<i>ALG9</i>	ALG9 alpha-1,2-mannosyltransferase
	<i>FCSK</i>	Fucose kinase
	<i>PNPO</i>	Pyridoxamine 5'-phosphate oxidase
	<i>ITPA</i>	Inosine triphosphatase-A
	<i>ALG13</i>	ALG13 UDP-N-acetylglucosaminyltransferase subunit
Intracellular trafficking related		
	<i>TRAK1</i>	Trafficking protein, kinesin-binding 1
	<i>AP2M1</i>	Adaptor-related protein complex 2, mu 1 subunit
	<i>CAMK2G</i>	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
	<i>TBC1D24</i>	TBC1 domain family, member 24
	<i>NSF</i>	N-ethylmaleimide-sensitive factor
	<i>CLTC</i>	Clathrin, heavy polypeptide (Hc)
	<i>ARX</i>	Aristaless-related homeobox, X-linked
Intracellular signaling related		
	<i>SZT2</i>	SZT2 subunit of KICSTOR complex
	<i>DOCK7</i>	Dedicator of cytokinesis 7
	<i>YWHAG</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase

	Approved Symbol	Gene/Locus name
		activation protein, gamma isoform
	<i>GNAO1</i>	Guanine nucleotide-binding protein (G protein), alpha-activating activity
	<i>PLCB1</i>	Phospholipase C, beta-1
	<i>SIK1</i>	Salt-inducible kinase 1
Transcription and gene expression related		
	<i>PUM1</i>	Pumilio RNA binding family member 1
	<i>TSEN2</i>	tRNA splicing endonuclease, subunit 2
	<i>PURA</i>	Purine-rich element binding protein A
	<i>MEF2C</i>	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
	<i>KMT2E</i>	Lysine (K)-specific methyltransferase 2E
	<i>CELF2</i>	CUGbp- and ELAV-like family, member 2
	<i>CUX2</i>	Cut-like homeobox 2
	<i>FOXG1</i>	Forkhead box G1B
	<i>IRF2BPL</i>	Interferon regulatory factor 2-binding protein like
	<i>CHD2</i>	Chromodomain helicase DNA binding protein-2
	<i>NEUROD2</i>	Neurogenic differentiation 2
	<i>MECP2</i>	Methyl-CpG-binding protein-2
Protein biosynthesis/degradation related		
	<i>DHDDS</i>	Dehydrolipoyl diphosphate synthase
	<i>ATP6V1A</i>	ATPase, H ⁺ transporting, V1 subunit A
	<i>UBA5</i>	Ubiquitin-like modifier activating enzyme 5
	<i>GUF1</i>	GUF1 homolog, GTPase
	<i>VAR51</i>	Valyl-tRNA synthetase 1
	<i>PLAA</i>	Phospholipase A2-activating protein
	<i>CARS2</i>	Cysteinyl-tRNA synthetase 2

	Approved Symbol	Gene/Locus name
	<i>AARS1</i>	Alanyl-tRNA synthetase 1
	<i>EEF1A2</i>	Eukaryotic translation elongation factor-1, alpha-2
	<i>PIGP</i>	Phosphatidylinositol glycan, class P
	<i>PIGA</i>	Phosphatidylinositol glycan, class A
Cytoskeletal proteins		
	<i>CYFIP2</i>	Cytoplasmic FMRP interacting protein 2
	<i>PHACTR1</i>	Phosphatase and actin regulator 1
	<i>SPTAN1</i>	Spectrin, alpha, nonerythrocytic-1 (alpha-fodrin)
Mitochondria proteins		
	<i>MFF</i>	Mitochondrial fission factor
	<i>FARS2</i>	Phenylalanyl-tRNA synthetase 2, mitochondrial
	<i>RMND1</i>	Required for meiotic nuclear division 1 homolog
	<i>BRAT1</i>	BRCA1-associated ATM activator 1
	<i>PMPCB</i>	Peptidase, mitochondrial processing, beta
	<i>TWINK</i>	Twinkle mtDNA helicase
	<i>DNM1L</i>	Dynamin 1-like
	<i>POLG</i>	Polymerase (DNA directed), gamma
	<i>GOT2</i>	Glutamic-oxaloacetic transaminase 2, mitochondrial
	<i>TIMM50</i>	Translocase of inner mitochondrial membrane 50
Other/multiple functions proteins		
	<i>FBXO11</i>	F-box only protein 11
	<i>MBD5</i>	Methyl-CpG-binding domain protein 5
	<i>PTPN23</i>	Protein-tyrosine phosphatase, nonreceptor-type, 23
	<i>DALRD3</i>	DALR anticodon-binding domain-containing protein 3
	<i>SERPINI1</i>	Protease inhibitor 12
	<i>FGF12</i>	Fibroblast growth factor-12
	<i>CSNK2B</i>	Casein kinase-2, beta polypeptide

	Approved Symbol	Gene/Locus name
	<i>CNPY3</i>	Canopy 3, zebrafish, homolog of
	<i>CDK19</i>	Cyclin-dependent kinase 19
	<i>TRRAP</i>	Transformation/transcription domain-associated protein
	<i>FRRS1L</i>	Ferric-chelate reductase 1-like
	<i>COQ4</i>	Coenzyme Q4, <i>S. cerevisiae</i> , homolog of
	<i>ATN1</i>	Atrophin 1
	<i>PACS2</i>	Phosphofurin acidic cluster sorting protein 2
	<i>PIGB</i>	Phosphatidylinositol glycan, class B
	<i>PIGQ</i>	Phosphatidylinositol glycan anchor biosynthesis class Q protein
	<i>ROGDI</i>	Rogdi atypical leucine zipper
	<i>WWOX</i>	WW domain-containing oxidoreductase
	<i>PIGS</i>	Phosphatidylinositol glycan, class S
	<i>TCF4</i>	Transcription factor-4 (immunoglobulin transcription factor-2)
	<i>PNKP</i>	Polynucleotide kinase 3' phosphatase
	<i>CDKL5</i>	Cyclin-dependent kinase-like 5 (serine/threonine protein kinase 9)
	<i>CASK</i>	Calcium/calmodulin-dependent serine protein kinase
	<i>SMC1A</i>	Segregation of mitotic chromosomes 1 (SMC1, yeast, homolog of; DXS423E; SB1.8)
	<i>FGF13</i>	Fibroblast growth factor-13
	<i>ARHGEF9</i>	Rho guanine nucleotide exchange factor 9
	<i>PCDH19</i>	Protocadherin 19

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3780 Genes gathered from OMIM (<https://www.omim.org/>) interrogated on October 27, 2021, using the keywords

3781 'epileptic encephalopathy' and 'developmental and epileptic encephalopathy'. See Supplemental Table S1

3782 (https://figshare.com/articles/dataset/Guerrini_et_al_Supplemental_Table_S1_xlsx/17694713) for more details.

3783 TABLE 3. Selected animal models of DEEs

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
A. Models of epileptic spasms (ES)								
A1. Acute								
NMDA	Rat, mouse C57	NMDA i.p., PN7-18	PN7-18	NR	<i>Rat:</i> Learning and coordination deficits; <i>3days – 7days post NMDA (mouse):</i> Increased anxiety, impaired motor coordination and poor memory retention	High dose ACTH ₁₋₃₉ ; Vigabatrin	Spasms	(167, 170, 543, 584–587)
NMDA variants								
- Prenatal betamethaso	Rat	Betamethasone i.p. G15;	PN12-15	NR	NR after spasms induction	Low dose ACTH ₁₋₃₉ ;	Spasms, ACTH	(168, 537, 538, 542)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
ne / postnatal NMDA		NMDA i.p., PN12-15				Vigabatrin	sensitivity	
- Prenatal stress / postnatal NMDA	Rat	Forced restraint (FR) (G15 or Forced swim test (FST) (G1-parturition); NMDA i.p., PN15	PN15	NR	NR after spasms induction	<u>FST/NMDA</u> : Responds to ACTH ₁₋₃₉ ; <u>FR/NMDA</u> : Responds to repeated low dose ACTH ₁₋₃₉	Spasms, stress	(167, 169)
- Prenatal MAM / postnatal NMDA	Rat	MAM (2 doses, G15); NMDA i.p., PN12-15 (1 or 3 doses)	PN12-15	NR	NR after spasms induction	No effect of low dose ACTH; Responds to vigabatrin	Spasms, dysplasias	(588)
- Adrenalecto	Rat	Adrenalectomy (PN10); NMDA i.p.	PN11	NR	NR after spasms induction	High dose ACTH ₁₋₃₉	Spasms	(170)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
my / postnatal NMDA		(PN11)						
- <i>Tsc1</i> ^{gfap-/+} mouse, postnatal NMDA,	Mouse, <i>Tsc1</i> ^{flox/flox} -GFAP-Cre knockout C57Bl/6 and SV129			As in <i>Tsc1</i> ^{gfap-/+}	NR after spasms induction	NR	Spasms, induced on a genetic background	(589)
Down syndrome / GBL	Mouse (Ts65Dn), C57Bl/6JEiXC3H/HesnJ	γ-butyrolactone (GBL) i.p.	1wk – 2mo	NR	NR after spasms induction	Responds to ACTH ₁₋₂₄ but not to ACTH ₁₋₃₉ ; Responds to vigabatrin	Spasms, induced on a genetic background	(539, 590, 591)
A2. Chronic								
Tetrodotoxin (TTX)	Rat	Intrahippocampal or intracortical TTX, unilateral	≥PN21	Yes	NR	Sensitive to ACTH, vigabatrin	ISS structural, hypsarrhyth	(87, 546, 592–594)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
		(PN10-38)					mia; drug-sensitive	
Multiple-hit	Rat	PN3: Right intracortical LPS, right intraventricular doxorubicin PN5: PCPA i.p.	PN4-13	Yes	Sociability deficits, learning/memory deficits, stereotypies	Resistant to ACTH; partial/transient response to vigabatrin	ISS structural, drug-resistant	(88, 155–161)
Arx cKO	Mouse, CD1 and C57Bl/6	Arx deletion from ganglionic eminence neuronal progenitors	Adulthood	Yes	NR	NR	ISS, genetic	(162)
Arx KI [Arx^{(GCG)10+7}]	Mouse, 75% C57BL/6; 25%	Expansion of 1 st polyalalanine	PN7-11	Yes	Low anxiety, impaired learning	NR	ISS, genetic	(150, 163, 595)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
	129S5/SvEvBrd	tract repeat (PA1) of <i>Arx</i>			and sociability			
<i>Arx</i> with PA1 or PA2 expansion	Mouse, C57BL/6N-Hsd	PA1 or PA2 expansion of <i>Arx</i>	≥PN10 to adulthood (myoclonic seizures)	NR	1-2mo: Sociability, neuromuscular strength deficits, anxiety and fear	NR	ISS, genetic	(545)
Adenomatous polyposis (<i>Apc</i>)	Mouse	<i>Apc</i> gene knockout from excitatory CamKII neurons	Peak at PN9	Yes	Reduced social interest, increased repetitive behaviors	NR	ISS, genetic	(596)
<i>Tsc1</i>^{+/-}	Mouse, C57BL/6	Heterozygous <i>Tsc1</i> ^{+/-}	PN12-16; Observed for 1 day/pup	NR	NR	NR	ISS, genetic	(597)
Aged <i>CDKL5</i>, heterozygous females	Mouse, C57BL/6J	<i>Cdkl5</i> ^{R59X/+} or <i>Cdkl5</i> ^{KO/+}	>PN300 female	No (only spasms seen)	NR in females; Sociability deficits in males	NR	ISS, genetic	(598)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
Chronic early life stress	Rat	Unpredictable and fragmented nurturing behaviors in dams (PN2-9 period)	PN17-35; Last for 1 or several days	NR	NR	NR	ISS, unknown	(171)
B. Models of Lennox-Gastaut syndrome, atypical absence seizures								
AY9944	Rat	AY9944 7.5mg/kg SC (PN2, 8, 14, PN20)	NR	Slow SWD	Cognitive deficits, hyperactivity, anxiety, spatial learning, olfactory recognition deficits	Responsive to DZP, ETH, CGP35348; Worse with CZP, baclofen, γ -OH-butyrate	Chronic atypical absence seizures	(599–605)
MAM - AY9944	Rat	Prenatal MAM / postnatal AY9944	NR	Slow SWD	NR	Refractory to ETH, VPA, CGP35348, CBZ	Refractory atypical absence seizures	(606)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
PValb-Dnm1^{Ftfl/flox}	Mouse, C57BL/6J	<i>Dnm1</i> ^{Ftfl/flox} in PV cells	NR	PN19-50: SWD, lethal seizures	Tremor	NR	LGS	(607)
GABABR1a	Mouse, C57BL/6	<i>GABABR1a</i> overexpression in forebrain	NR	Slow SWD	Impairment in learning, spatial memory	Responsive to ETH, CGP35348	Chronic atypical absence seizures	(608)
GABABR1b	Mouse	<i>GABABR1b</i> overexpression in forebrain	NR	Slow SWD	Mild impairment in learning, spatial memory	Responsive to ETH, CGP35348	Chronic atypical absence seizures	(609)
NHE1	Mouse, SJL/J and C57BL/6J	Na ⁺ /H ⁺ exchanger null	NR	Slow SWD (3Hz) (at 4-5wk); Lethal tonic or tonic-clonic	Ataxia; Early mortality	NR	Chronic atypical absence seizures	(610)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
				seizures				
Multiple-hit	Rat	PN3: Right intracortical LPS, right intraventricular doxorubicin PN5: PCPA i.p.	PN4-13	Adulthood: slow SWD (5-6Hz), motor seizures in sleep	Sociability deficits, learning/memory deficits, stereotypies	NR	ISS with LGS features	(155)
C. Models of Dravet syndrome								
<i>Scn1a</i> KO	Mouse	Exon 26 deletion, global constitutive		Convulsive seizures, Hyperthermia seizures, mortality	Hyperactivity, stereotypies, sociability, and spatial memory deficits	Tested	Dravet	(64)
<i>Scn1a</i> CKO	Mouse, C57BL/6 and	Exon 25 deletion,		Motor	NR		Dravet	(611)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
	129Sv	forebrain GABAergic interneurons		seizures, Hyperthermia seizures				
Scn1a CKO	Mouse, B6.SJL-Tg(ACTFLPe)9205Dy m/J and C57BL/6	Conditional deletion of exon 7	Inhibitory neurons	≥PN16 seizures, occasional death	Hypoactive, jerks, death		Dravet	(612)
			Forebrain excitatory neurons	No	NR			(612)
			Forebrain excitatory neurons and haploinsufficiency in inhibitory	Improved lethality from seizures	NR		Dravet	(612)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
			neurons					
			PV interneurons	≥PN14 seizures, death	Ataxia (PN10)		Dravet	(612)
Scn1a KI, R1407X	Mouse, 129/SvJ and C57BL/6J	Human R1407X nonsense mutation		≥1mo: Seizures	Hyperactivity, stereotypies, sociability and spatial memory deficits		Dravet	(65, 224, 225)
Scn1a KI, S1231R	Drosophila	S1231R mutation, loss of function		Seizures	NR		Dravet	(613)
Scn1Lab (<i>didy</i> ^{s552})	Zebrafish	Scn1Lab mutation (low expression)		Increased locomotor activity, epileptiform activity,	Impaired exploration, decreased mobility	Tested	Dravet	(614)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
specific models of DEE								
<i>Kcnq2</i> <i>KI</i>	Mouse, C57BL/6J	<i>Kcnq2</i> -Y284C/+, <i>Kcnq2</i> -A306T/+		NR	NR	Retigabine reduces KA-seizures	KCNQ2 DEE	(616)
<i>Kcnq2</i> - <i>Thr274Met</i> /+	Mouse, 129Sv, C57BL/6N	<i>Kcnq2</i> - <i>Thr274Met</i> /+		Yes (>PN20)	Spatial learning and memory deficits. Death by 3 rd mo (25%)	NR	KCNQ2 DEE	(281)
<i>Kcna1</i> -/-	Mouse	<i>Kcna1</i> -/-		Yes	NR	Retigabine reduces	KCNA1 epilepsy	(617)
						spontaneous seizures		
<i>Kcnq1</i> -	Mouse	<i>Kcnq1</i> -		Rare	NR	Retigabine:	KCNQ1	(617)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
<i>A340E/A340E</i>		<i>A340E/A340E</i>		spontaneous seizures		adverse cardiac effects	epilepsy	
<i>Pcdh19 KO and heterozygous females</i>	Mouse, 129S5.C57BL/6	<i>Pcdh19 KO and heterozygous females</i>		NR; Increased susceptibility to 6Hz and flurothyl seizures		NR	<i>PCDH19</i> <i>DEE</i>	(618)
<i>Pcdh19-HET</i>	Mouse, C57BL/6N	<i>Pcdh19-HET</i>	Mossy fiber deficits	NR	Pattern completion and separation deficits	NR	<i>PCDH19</i> <i>DEE</i>	(619)
<i>Pcdh19 KO</i>	Mouse, C57BL/6N	<i>Pcdh19-KO</i>		NR	Increased exploratory behavior, reduced anxiety	NR	<i>PCDH19</i> <i>DEE</i>	(618)

Models	Species	Induction method	Spasms, age of onset	Subsequent epilepsy	Behavioral / neurodevelopmental deficits	Response to ACTH/vigabatrin	Model of:	References
<i>Cdkl5 CKO</i>	Mouse, CD1	<i>Cdkl5 CKO in glutamatergic or GABAergic neurons</i>	Defective dendritic arborisation and spine maturation	Yes when deleted in glutamatergic neurons.	Autistic symptomatology, motor coordination, memory and breathing abnormalities	Epigallatocatech in-3-gallate (EGCG) corrects synaptic deficits	<i>CDKL5 DEE</i>	(620, 621)

3784 ACTH: adrenocorticotropic hormone; Apc: adenomatous polyposis colon; Arx : aristaless X-linked homeobox protein; AY9944; trans-1,4-bis-

3785 cyclohexane dihydrochloride, cholesterol biosynthesis inhibitor; CDKL5: cyclin-dependent kinase-like 5; CGP35348: GABAB receptor inhibitor; DEE:

3786 DEE: Developmental and epileptic encephalopathy; ES: epileptic spasms; CKO: conditional knockout; CZP: clonazepam; Dnm1: dynamin 1; DZP:

3787 diazepam; EGCG: Epigallatocatechin-3-gallate; ETH: ethosuximide; GBL: gamma butyrolactone; FR: forced restraint; FST: Forced swim test; G:

3788 gestational day; GABABR: GABAB receptor; Gfap: glial acidic fibrillary protein; HET: heterozygous; ISS: infantile spasms syndrome; Kcna1:

3789 Potassium Voltage-Gated Channel Subfamily A Member 1); KCNQ: Potassium Voltage-Gated Channel Subfamily Q; KI: knockin; KO: knockout; LGS:

3790 Lennox-Gastaut syndrome; LPS: lipopolysaccharide; MAM: methyl-azoxy-methanol acetate; NHE1: Na⁺/H⁺ exchanger; NMDA: N-methyl-D-

- 3791 aspartate; NR: not reported; PA: polyalanine; PCPA: p-chlorophenylalanine (inhibits serotonin synthesis); PN: postnatal; PV or PValb: parvalbumin;
- 3792 Scn1a: sodium channel 1 alpha; SWD: spike and slow wave discharge; Tsc: tuberous sclerosis complex; TTX: tetrodotoxin; VPA: valproic acid.

3793 **TABLE 4. Experimental drugs tested in models of DEE**

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
A. ES models					
CGP35348	GABA _B R antagonism	Mouse, Down/GBL	Pre-treatment	Shortens EDRs	(538)
Baclofen	GABA _B R agonist	Mouse, Down/GBL	Pre-treatment	Prolongs EDRs	(538)
5-OH-tryptophan	Serotonin increase	Mouse, Down/GBL	Pre-treatment	Prolongs EDRs	(538)
GIRK2 knockout	Deletion of GABA _B R associated inward rectifying potassium channel	GIRK2 KO Mouse, Down/GBL	GIRK2 Knockout mice	GIRK2 knockout confers resistance to GBL induced spasms	(539)
Rapamycin	mTOR inhibitor	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment	No effect, no evidence of target relevance	(537)
Ganaxolone	Allosteric	Rat, Prenatal	Pre-treatment	Reduces number,	(540)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
	activator of GABA _A R, synaptic and extrasynaptic	betamethasone / postnatal NMDA		delays onset of NMDA spasms	
ACTON PROLONGATUM ®	Synthetic ACTH based on porcine ACTH	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment	Reduces number, delays onset of NMDA spasms after 2doses but not after single dose	(541)
AQB-565	ACTH1-24 linked to melanocyte stimulating hormone, acts on MC3, MC4 melanocortin receptors	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment	Reduces number of NMDA spasms after 8 doses	(538)
Estradiol, diethylstilbestrol	Gonadal hormone,	Rat, Prenatal betamethasone /	Pre-treatment (PN3-10)	No effect on spasms; increased	(542)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
	estrogen analogue	postnatal NMDA		GAD67 cells in neocortex	
β-OH-butyrate	Ketoacid	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment	Reduces spasms and delays latency to NMDA spasms after repeat but not single dose administration	(543)
β-OH-butyrate	Ketoacid	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment (200mg/kg ip)	No effect	(544)
2-deoxyglucose	Metabolic inhibition of glycolysis	Rat, Prenatal betamethasone / postnatal NMDA	Pre-treatment	No effect	(544)
B. ISS models					
Rapamycin, pulse	mTOR inhibitor	Rat, multiple hit	Treatment after spasms onset, 3days	Decreases spasms within 2h; stops	(155, 161)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
				spasms with repeat dosing; improves spatial learning; prevents adult epilepsy; reverses mTOR dysregulation	
CPP-115	High affinity vigabatrin analog	Rat, multiple hit	Treatment after spasms onset, repeated (PN4-12)	Reduces spasms from the first hour and for up to 3 days; better efficacy and tolerability than vigabatrin	(156)
Carisbamate	Unknown; effect on spasms not due to sodium channel blockade	Rat, multiple hit	Treatment after spasms onset, single dose (PN4 or PN6-7)	Reduces spasms within the first hour	(160)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
NAX-5055	Galanin receptor 1 (GalR1) agonist	Rat, multiple hit	Treatment after spasms onset, single dose (PN4 or PN6-7)	No effect; low expression of GalR1 in pups	(158)
VX-765	Caspase 1 inhibitor	Rat, multiple hit	Treatment after spasms onset, single dose (PN4)	No effect	(157)
CGP 35348	GABA _B R antagonism	Rat, multiple hit	Treatment after spasms onset, single dose (PN4)	No effect	(157)
17β-estradiol	Gonadal hormone	Rat, multiple hit	Treatment started after induction (PN3- 10)	No effect	(157)
17β-estradiol	Gonadal hormone	Mouse, <i>Arx</i> KI [<i>Arx</i> (GCG ¹⁰⁺⁷)	Pre-treatment (PN3- 10)	Prevents spasms and other seizures, restores interneuronopathy	(150)
17β-estradiol	Gonadal hormone	Mouse, <i>Arx</i> with PA1 or PA2 expansion	Pre-treatment (PN3- 10)	Reduces seizures but not the	(545)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
				abnormal behavior	
C. Dravet syndrome models					
Soticlestat	Cholesterol 24-hydroxylase inhibitor	Mouse, <i>Scn1a</i> ^{tm1Kea} with exon 1 deletion	Treatment after hyperthermia priming	Reduced seizures, protected against hyperthermia seizures, prevented SUDEP	(549)
Medium chain triglyceride diet (decanoic C10, octanoic acid C8 mix)	Ketogenic diet metabolite	Mouse, <i>Scn1a</i> KI, R1407X	4wk treatment prior to hyperthermia	C10/C8 (80:20) reduce both seizures and mortality; C10 reduces mortality	(551)
<i>Gabra2</i> repair	<i>GABRA2</i> expression restoration (increase)	Mouse, <i>Scn1a</i> ^{+/-}	Genetic repair of <i>Gabra2</i>	Rescues epilepsy phenotype	(550)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
Trpv1 receptor deletion	<i>Trpv1 receptor deletion</i>	<i>Mouse, Scn1a^{+/-}</i>	<i>Trpv1 receptor deletion</i>	No effects on hyperthermia seizures, frequency of spontaneous seizures or survival	(552)
SB-705498	<i>Trpv1 selective antagonist</i>	<i>Mouse, Scn1a^{+/-}</i>		No effect on seizures or survival	(552)
Cannabigerolic acid	<i>Phytocannabinoid</i>	<i>Mouse, Scn1a^{+/-}</i>	Pre-treatment	Potentiated clobazam effects on hyperthermia induced and spontaneous seizures,	(554)
				anticonvulsant in MES, proconvulsant in 6Hz test.	

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
Cannabichromene, 5-Fluoro-Cannabichromene	<i>Phytocannabinoid</i>	<i>Mouse, Scn1a^{+/-}</i>	Pre-treatment	Anticonvulsant	(553)
Ketogenic diet	Ketogenic diet	<i>Mouse, Scn1a</i> KI, R1407X	Ketogenic diet, 14 days	Decreases SUDEP, protects against seizure induced respiratory arrest	(555)
SCN1A transfer in the brain (adenoviral)	SCN1A expression in the brain	Mouse, SCN1A-A1783V KI	Adenovirus expressing SCN1A, intracerebral injection	Protected from death, attenuation of epilepsy, hyperactivity persisted, cognitive effects variable	(556)
Naltrexone	Opioid antagonist	Zebrafish, <i>scn1Lab</i>	Pretreatment, 30min prior to PTZ	Anticonvulsant effects	(559)
Fenfluramine, Norfenfluramine	Reuptake inhibitor of 5-OH-	Zebrafish, <i>scn1Lab</i> -/-	Treatment exposure	Anticonvulsant effects	(560)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
enantiomers	tryptamine				
Fenfluramine	Reuptake inhibitor of 5-OH-tryptamine	Zebrafish, <i>scn1Lab</i>	Treatment exposure	Anticonvulsant effects	(561)
Dimethadione	Calcium channel blocker	Zebrafish, <i>scn1Lab</i>	Treatment exposure	Anticonvulsant effects	(561)
Clemizole	Serotonergic	Zebrafish, <i>scn1Lab</i>	Treatment exposure	Anticonvulsant effects	(562)
PK11195	Pck1 activator and translocator protein ligand	Zebrafish, <i>scn1Lab</i>	Treatment exposure	Anticonvulsant effects	(563)
GR-46611	5HT1D receptor agonist	Mouse, 129S-Scn1Atm1Kea/Mmjax	Pretreatment	Protects from hyperthermia seizures, decreases seizure severity, improves survival	(557)
ASO, increases	ASO, increases	Mouse, F1:129S-	PN2 or 14,	Reduces seizures	(233)

Drug	Mechanism	Model / species	Treatment Protocol	Effect	Reference
Scn1a	<i>Scn1a</i> (TANGO method)	Scn1a+/- \hat{A} [~] C57BL/6J	intracerebroventricular injection	and SUDEP	
CRISPR/dCas9-based Scn1a gene activation in inhibitory neurons	CRISPR/dCas9-based Scn1a gene activation in inhibitory neurons	Mouse, <i>Scn1a</i> ^{Rx/+} , floxed dCas9-VPR ^{VPR/+} , Vgat-Cre ^{Cre/+} ,	Intravenous AAV injection, 4wk	Improved behavioral deficits, ameliorated febrile seizures	(558)
MV1369, MV1312	Nav1.6 inhibitor	Zebrafish, <i>Scn1Lab</i> KO	Treatment	Reduced spontaneous burst movements and seizures	(564)
AA43279	Nav1.1 activator	Zebrafish, <i>Scn1Lab</i> KO	Treatment	Reduced spontaneous burst movements and seizures	(564)

3794 AQB-565: ACTH1-24 linked to melanocyte stimulating hormone; ASO: antisense oligonucleotides; CPP-115: high affinity GABA aminotransferase
3795 inhibitor; CRISPR: clustered regularly interspaced short palindromic repeats; EDRs: ectrodecremental responses; GABRA2: GABA_AR A2 subunit;
3796 CGP35348: GABA_B receptor inhibitor; DEE: Developmental and epileptic encephalopathy; ES: epileptic spasms; GABA_BR: GABA_B receptor; GalR:

3797 galanin receptor; GBL: gamma butyrolactone; GIRK: inward rectifying potassium channel; 5-HT: serotonin; KI: knockin; mTOR: mechanistic target
3798 of rapamycin; MC: melanocortin receptor; Nav: Sodium channel; NMDA: N-methyl-D-aspartate; PCK1: Phosphoenolpyruvate Carboxykinase 1; PN:
3799 postnatal; *Scn1a* sodium channel 1; SUDEP: sudden death in epilepsy; *Trpv1*: transient receptor potential cation channel subfamily V member 1;
3800 Vgat: vesicular GABA transporter.
3801

	Excitatory transmission	Na ⁺ channels	Ca ⁺⁺ channels	K ⁺ channels	Glutamate receptors	5-HT receptors	Sigma-1 receptors	Inhibitory transmission	GABA-A receptors	GABA turnover	GABA-B receptors	SV2A binding	Carbonic anhydrase	Clinical efficacy
														ABS
Levetiracetam	YES		YES	YES				YES	YES			YES		BS
Oxcarbazepine	YES	YES	YES	YES										FS, GTCS
Perampanel	YES				YES									FS, GTCS
Phenobarbital	YES		YES		YES			YES	YES					FS
Phenytoin	YES	YES	YES											FS, GTCS
Pregabalin	YES		YES					YES						FS
Primidone	YES		YES					YES	YES					FS, GTCS
Retigabine	YES			YES										FS
Rufinamide	YES	YES												LGS
Stiripentol								YES	YES					DS
Tiagabine								YES		YES				FS
Topiramate	YES	YES	YES	YES	YES			YES	YES				YES	BS
Valproate	YES	YES	YES					YES	YES	YES				BS
Vigabatrin								YES		YES				FS, ES

	Excitatory transmission	Na ⁺ channels	Ca ⁺⁺ channels	K ⁺ channels	Glutamate receptors	5-HT receptors	Sigma-1 receptors	Inhibitory transmission	GABA-A receptors	GABA turnover	GABA-B receptors	SV2A binding	Carbonic anhydrase	Clinical efficacy
Zonisamide	YES	YES	YES										YES	FS, GTCs, MYO

3803 BS: broad spectrum; GABA:Gamma-aminobutyric acid; 5-HT: 5-hydroxytryptamine; SV2A: synaptic vesicle protein 2^o; FS: focal seizures; LGS: Lennox Gastaut syndrome; DS:

3804 Dravet syndrome; GTCs: generalized tonic-clonic seizures; ABS: absence; SGS: secondary generalized seizures; ES: epileptic spasms; MYO: myoclonic.

3805 Adapted from <https://www.uptodate.com/contents/antiseizure-drugs-mechanism-of-action-pharmacology-and-adverse-effects>.

3806

3807 TABLE 6. Clinical trials on DEEs.

1	<p>Study Title: Study to Evaluate NBI-921352 as Adjunctive Therapy in Subjects With SCN8A Developmental and Epileptic Encephalopathy Syndrome (SCN8A-DEE)</p> <p>Recruiting: Not yet</p> <p>Has results: No</p> <p>Conditions: SCN8A DEE Syndrome</p> <p>Interventions: Drug: NBI-921352, Placebo</p> <p>Locations: Neurocrine Clinical Site, Washington, District of Columbia, United States</p>
2	<p>Study Title: XEN496 (Ezogabine) in Children With KCNQ2 Developmental and Epileptic Encephalopathy</p> <p>Recruiting: Yes</p> <p>Has results: No</p> <p>Conditions: Epilepsy, Epilepsy in Children, Epilepsy; Seizure Disease, Brain Diseases, Central Nervous System Diseases, Nervous System Diseases. Epileptic Syndromes</p> <p>Interventions: Drug: XEN496, Placebo</p> <p>Locations: Children's Hospital of Colorado, Aurora, Colorado, United States. Northwest Florida Clinical Research Group, Gulf Breeze, Florida, United States. Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, Illinois, United States. Columbia University Irving Medical Center, New York, New York, United States. The Cleveland Clinic Foundation, Cleveland, Ohio, United States. Oregon Health and Science University, Portland, Oregon, United States. MultiCare Health System - Mary Bridge Pediatrics - Tacoma, Tacoma, Washington, United States. Sydney Children's Hospital, Sydney, New South Wales, Australia. Children's Health Queensland Hospital and Health Service, South Brisbane, Queensland, Australia. Austin Health, Heidelberg, Victoria, Australia.</p>

3	<p>Study Title: An Open-Label Extension of the Study XEN496 (Ezogabine) in Children With KCNQ2-DEE</p> <p>Recruiting: Yes</p> <p>Has results: No</p> <p>Conditions: Epilepsy, Epilepsy in Children, Seizure Disease, Brain Diseases, Central Nervous System Diseases, Nervous System Diseases, Epileptic Syndromes</p> <p>Interventions: Drug: XEN496, Placebo</p> <p>Locations: MultiCare Health System - Mary Bridge Pediatrics - Tacoma, Tacoma, Washington, United States.</p>
4	<p>Study Title: Study of TAK-935 as an Adjunctive Therapy in Participants With Developmental and/or Epileptic Encephalopathies</p> <p>Recruiting: No</p> <p>Has results: Yes</p> <p>Conditions: Developmental and/or Epileptic Encephalopathies</p> <p>Interventions: Drug: TAK-935, Placebo</p> <p>Locations: Xenoscience, Phoenix, Arizona, United States Medsol Clinical Research Center, Port Charlotte, Florida, United States. University of South Florida, Tampa, Florida, United States. Center for Integrative Rare Disease Research, Atlanta, Georgia, United States. Bluegrass Epilepsy Research, Lexington, Kentucky, United States. Mid-Atlantic Epilepsy and Sleep Center, Bethesda, Maryland, United States. The Comprehensive Epilepsy Care Center for Children and Adults, Saint Louis, Missouri, United States. Northeast Regional Epilepsy Group, Hackensack, New Jersey, United States. Thomas Jefferson University, Philadelphia, Pennsylvania, United States. Medical University of South Carolina, Charleston, South Carolina, United States. University of Virginia Health Sciences Center, Charlottesville, Virginia, United States.</p>
5	<p>Study Title: A Phase 2, Multicenter, Randomized, Double-blind, Placebo-controlled Study to Evaluate the Efficacy, Safety, and Tolerability of TAK-935 (OV935) as an Adjunctive Therapy In Pediatric Participants With Developmental and/or Epileptic Encephalopathies</p> <p>Recruiting: No</p>

Has results: Yes

Conditions: Epilepsy, Dravet Syndrome, Lennox-Gastaut Syndrome

Interventions: Drug: TAK-935, Placebo

Locations: Phoenix Children's Hospital, Phoenix, Arizona, United States. Children's Hospital Los Angeles, Los Angeles, California, United States. Colorado Children's Hospital, Aurora, Colorado, United States. Nicklaus Children's Hospital, Miami, Florida, United States. Pediatric Neurology PA, Orlando, Florida, United States. Rare Disease Research, LLC, Atlanta, Georgia, United States. Center for Rare Neurological Diseases, Norcross, Georgia, United States. Ann and Robert H Lurie Children's Hospital of Chicago, Chicago, Illinois, United States. Mayo Clinic - PPDS, Rochester, Minnesota, United States. Northeast Regional Epilepsy Group, Hackensack, New Jersey, United States. Children's Hospital at Saint Peter's University Hospital, New Brunswick, New Jersey, United States. Columbia University Medical Center, New York, New York, United States. Wake Forest Baptist Medical Center, Winston-Salem, North Carolina, United States. Medical University of South Carolina, Charleston, South Carolina, United States. Cook Children's Medical Center, Fort Worth, Texas, United States. Monash Children's Hospital, Clayton, Victoria, Australia. Austin Hospital, Heidelberg West, Victoria, Australia. Hospital For Sick Children, Toronto, Ontario, Canada. Peking University First Hospital, Beijing, China. Capital Medical University (CMU) - Beijing Children's Hospital, Beijing, China. Beijing Children's Hospital, Capital Medical University, Beijing, China. Xiangya Hospital Central South University, Changsha, China. Children's Hospital of Fudan University, Shanghai, China. Shenzhen Children's Hospital, Shenzhen, China. Sheba Medical Center-PPDS, Tel Hashomer,, Ramat Gan, Israel. Soroka University Medical Centre, Beer Sheva, Israel. Bnai Zion Medical Center, Haifa, Israel. Edith Wolfson Medical Center, Holon, Israel. Hadassah Medical Center, Jerusalem, Israel. Schneider Children's Medical Center of Israel, Petach Tikva, Israel. Tel Aviv Sourasky Medical Center, Tel Aviv, Israel. Uniwersyteckie Centrum Kliniczne - PPDS, Gdansk, Pomorskie, Poland. NZOZ Centrum Neurologii Dziecięcej i Leczenia Padaczki, Kielce, Świętokrzyskie, Poland. Szpital Kliniczny im. H. Świecickiego Uniwersytetu Medycznego im. Karola Marcinkowskiego w Poznaniu, Poznan, Wielkopolskie, Poland. Centrum Medyczne Plejady, Krakow, Poland. Samodzielny Publiczny Dziecięcy Szpital Kliniczny w Warszawie, Warsaw, Poland. Instytut Pomnik Centrum Zdrowia Dziecka, Warsaw, Poland. Centro Hospitalar Lisboa Central- Hospital Dona Estefania, Lisboa, Portugal. Centro Hospitalar Lisboa Norte, E.P.E. Hospital de Santa Maria, Lisboa, Portugal. Largo da

Maternidade de Julio DinisCentro Materno Infantil do Norte, Porto, Portugal. Clinica Universidad Navarra, Pamplona, Navarra, Spain. Hospital Vithas La Salud, Granada, Spain. Hospital Ruber Internacional, Madrid, Spain. Hospital Universitari i Politecnic La Fe de Valencia, Valencia, Spain.

3808 Clinical Trials gathered from ClinicalTrials.gov (<https://clinicaltrials.gov/ct2/home>) interrogated on October 27, 2021 using the keywords

3809 'developmental and epileptic encephalopathy'.

3810 **Figures Legends**

3811 **FIGURE 1. Schematic representation of an example of 'poison exon'-mediated protein**
3812 **degradation.** Panel A shows a hypothetical gene encoding a transmembrane protein with four
3813 transmembrane segments. The gene comprises nine coding exons (1-9) and a potential poison
3814 exon (P). In the canonical splicing, the poison exon is not included in the mRNA, which is translated
3815 into the wild type protein. After translation, the protein is correctly integrated into the plasma
3816 membrane, where it exerts its normal function. In panel B, the presence of an intronic mutation,
3817 which can introduce a novel splicing acceptor site, activate an exonic splicing enhancer (ESE, i.e. a
3818 sequence that promotes the inclusion of an exon in an mRNA) or disrupt an exonic splicing silencer
3819 (ESS, i.e. a sequence that inhibits the inclusion of an exon in an mRNA), promoting the inclusion of
3820 the poison exon in the mRNA. The poison exon alters protein amino acid sequence and introduces
3821 a premature stop codon (PTC). The PTC is recognized by cellular surveillance systems and the
3822 mutant protein is degraded.

3823

3824 **FIGURE 2. Schematic representation of the cortical and subcortical zones and mechanisms**
3825 **involved in epileptic seizures generation and spreading. A.** Schematic model of networks involved
3826 in spike-and-wave generation in generalized epilepsies. Thalamic relay neurons in the
3827 thalamocortical circuit can activate cortical pyramidal neurons and *vice versa*. Thalamus-mediated
3828 cortical activation is largely controlled by thalamic reticular neurons. They hyperpolarize thalamic
3829 relay neurons through gamma-aminobutyric acid type B (GABA_B)-mediated signals and are
3830 themselves inhibited by neighboring reticular neurons through GABA type A (GABA_A)-mediated
3831 signals. Cortical pyramidal neurons can, in turn, activate thalamic reticular neurons in a
3832 glutamate-mediated feed-forward loop. The neuronal basis of the EEG spike-and-wave in this
3833 reverberating loop derive from an alternance of the summated outside-negative excitatory

3834 membrane events (each spike) and the summated outside-positive inhibitory membrane events
3835 (each slow wave). Spike and waves appear as negative (upward going) events due to a dipole
3836 effect as the soma and apical dendrites maintain opposite polarity. **B.** In the epileptic brain, focal
3837 epileptic seizures are generated in the epileptogenic zone (EZ), while clinical seizures are
3838 generated in the seizure onset zone (SOZ). If the EZ is larger than the SOZ, as in the case depicted
3839 in the figure, its complete removal is required to guarantee seizures disappearing as multiple SOZs
3840 with different thresholds may coexist in the same EZ. The complete EZ disconnection or removal is
3841 also required to ensure that seizures do not spread to other areas connected to it via cortico-
3842 cortical and subcortical (i.e. thalamocortical) connections (purple arrows), which can cause
3843 secondary generalization. Additional specific cortical areas that can be identified in the epileptic
3844 brain are the epileptogenic lesion (EL), which may correspond to either a macroscopic
3845 epileptogenic lesion (e.g. focal cortical dysplasia, as shown in the figure) or hyperexcitable
3846 adjacent cortex, the irritative zone (IZ), representing the area of the normal cortex generating
3847 interictal spikes, and the functional deficit zone (FDZ), representing the area of the cortex that
3848 does not function normally in the interictal period.

3849

3850 **FIGURE 3. Trajectories of developmental processes in normal brain development. A.** The
3851 temporal trajectories of selected developmental processes that are important for normal brain
3852 development in rodents (upper panel) and humans (lower panel) are shown. Birth (B), weaning
3853 (W), puberty (P) and adulthood are indicated separately in each panel for the rodent or human
3854 development. The different timescales used across species (23 days in rodents, 9 months in
3855 human) highlight the significant differences in the speed of maturation across species. The time of
3856 brain growth spurt in humans (full term birth) and rodents [around postnatal day (PN) 10] has
3857 been used to indicate the ages across species that correspond to a full term newborn human baby

3858 (91). Brain growth spurt in these studies included gross brain growth, DNA, cholesterol, and water
3859 content. Puberty onset occurs around PN32-36 in female rats and PN35-45 in male rats, whereas
3860 in humans it starts around 10-11 years in girls and 11-12 years in boys (92). Distinct processes,
3861 such as neurogenesis and migration, synaptogenesis and synaptic pruning, myelination follow
3862 different time courses (90, 93–100). However, the staging of the equivalence of developmental
3863 stages across species is only approximate and each developmental process needs to be considered
3864 individually. **B.** Significant changes occur during development in the expression or function or
3865 various signaling processes. A schematic depiction of the age-related changes in GABA_AR and
3866 glutamatergic signaling in rats is presented here, however cell type, regional and sex-specific
3867 differences also exist (90, 105, 622). Early in development, there is less effective GABA_AR-mediated
3868 inhibition, due to the presence of depolarizing GABA_AR signaling (see also **FIGURE 4**), more tonic
3869 and less phasic GABA_AR inhibition. In contrast, glutamatergic receptors, such as NMDAR or kainate
3870 receptors also show age-related expression patterns.

3871

3872 **FIGURE 4. Depolarizing and hyperpolarizing GABA_AR signaling in normal development and**
3873 **disease. A.** GABA_AR signaling is depolarizing early in life due to the higher intracellular Cl⁻
3874 concentrations ([Cl⁻]_i) that force Cl⁻ efflux upon GABA_AR activation. Although the GABA_AR
3875 depolarizations render GABA inhibition less efficient, as it relies upon shunt inhibition, they are
3876 critical for normal brain development. GABA_AR depolarizations may activate L-type voltage
3877 sensitive calcium channels and may release the Mg⁺⁺ block of NMDARs, triggering intracellular Ca⁺⁺
3878 rises that are important for neuronal survival, migration, differentiation and integration (123, 130,
3879 132, 623). The [Cl⁻]_i in immature neurons is a result of increased expression and/or activity of Cl⁻
3880 importers, like NKCC1 (a Na⁺/K⁺/Cl⁻ cotransporter) over Cl⁻ exporters, like KCC2 (a K⁺/Cl⁻
3881 cotransporter). The Na⁺/K⁺ ATPase provides the energy to maintain the cation chloride

3882 cotransporter function. During development, there is a gradual switch in the relative dominance of
3883 these cation chloride cotransporters at specific timepoints that follows cell type, region, and sex
3884 specific patterns (108, 122, 125, 126). As a result, mature neurons demonstrate hyperpolarizing
3885 GABA_AR responses that allow for effective inhibition to occur. **B.** Normal brain development
3886 depends upon the age, cell type, region, and sex appropriate presence of depolarizing and
3887 hyperpolarizing GABA_AR signaling. Genetic variants, drugs, perinatal or postnatal insults that
3888 trigger precocious presence of hyperpolarizing GABA_AR signaling may result in
3889 neurodevelopmental deficits or abnormalities that could increase the risk for epilepsy (130, 132,
3890 623). Conversely, pathological persistence or re-appearance of depolarizing GABA has been
3891 described in epileptogenic pathologies and may predispose to increase excitability (133).

3892

3893 **FIGURE 5. Simplified diagram of a cortical microcircuit with interconnected Glutamatergic and**
3894 **GABAergic neurons, and an astrocyte, and cellular/subcellular distribution of ion channels and**
3895 **transporters.** A cortical neuronal microcircuit is illustrated as a pre-synaptic GABAergic neuron
3896 (green) and a presynaptic myelinated glutamatergic neuron (ocher) that form synaptic
3897 connections on the dendrites of a myelinated glutamatergic neuron (ocher). Glial cells are
3898 displayed as an astrocyte (light blue) in proximity of the glutamatergic synapses, and as the myelin
3899 sheets around the axons of the glutamatergic neurons formed by oligodendrocytes (violet; the
3900 soma is not displayed), allowing saltatory conduction at the nodes of Ranvier. The upper insets
3901 show in more detail a GABAergic (left) and a glutamatergic (right) synapse. The ion channels and
3902 transporters targeted by DEE mutations are indicated with their protein name (see text for details)
3903 and their known cellular/subcellular distribution, according to the neuronal sub-compartments
3904 (dendrites, soma, axon initial segment, nodes of Ranvier of the myelinated axon, pre-synaptic
3905 terminal, and post-synaptic membrane).

3906 **FIGURE 6. Schematic representation of the different *in vitro* and *in vivo* models that can be used**
 3907 **to study functional effects of mutations affecting DEEs causative genes.** Regardless of the
 3908 starting point, researchers can move from a model to another based on the type of functional
 3909 assay they want to apply and the physiological process they want to study. EPS:
 3910 electrophysiological studies; MRI: magnetic resonance imaging; BEH: behavioral studies; ICC:
 3911 immunocytochemistry; LI: live imaging; TR: transcriptomics; PR: proteomics; IHC:
 3912 immunohistochemistry; ISH: *in situ* hybridization.

3913

3914 **FIGURE 7. Brain MRI of patients with different malformations of cortical development. A.** T1-
 3915 weighted (T1W) coronal section. Lissencephaly in a boy with *ARX* mutation. The ventricles are
 3916 severely dilated, the corpus callosum is absent, the basal ganglia are severely hypoplastic. **B, C.**
 3917 Coronal T1W and Axial T2W sections of a brain with posterior > anterior pachygyria and increased
 3918 cortical thickness. Boy with *LIS1* mutation. The white asterisk in B indicates the point of more
 3919 severe cortical thickening. White arrows in C point to areas of more severely smooth and thick
 3920 cortex. **D.** T1W coronal section. Diffuse subcortical band heterotopia in a girl with *DCX* mutation.
 3921 The white circle surrounds the subcortical laminar heterotopia which forms an almost continuous
 3922 band beneath the cortex, separated from it by white matter. **E.** Axial T2W section. Right occipital
 3923 cortical dysplasia (surrounded by a white circle) in a girl with a very low-level mosaic mutation in
 3924 *AKT3* (0.67% in brain, not detectable in blood). **F, G.** Axial FLAIR and coronal T1W sections in two
 3925 patients carrying mosaic mutations in the *MTOR* gene with different percentages of mosaicism [**F:**
 3926 p.Thr1977Ile, 20% of mosaicism in blood, **G:** p.Ser2215Phe, 5.5% of mosaicism in the surgically
 3927 removed dysplastic brain tissue]. In **F**, the patient has megalencephaly with large ventricles and
 3928 multiple areas of abnormal cortex alternating infoldings with smooth surface. This pattern is
 3929 suggestive of polymicrogyria (white arrows). In **G**, the white circle highlights an area of cortical

3930 dysplasia with increased volume of the brain parenchyma, blurring of the gray-white matter
3931 junction and irregular cortical folding. **H.** T2W coronal section. Left parieto-temporal focal cortical
3932 dysplasia in a girl with *NPRL2* mutation. The circle surrounds the parietal portion of the cortical
3933 abnormality. **I, J.** T1W axial sections in two patients carrying the p.Gly373Arg *PIK3R2* gene
3934 mutation with different percentages of mosaicism (**I:** 13% of mosaicism in blood, 43% in saliva, **J:**
3935 10% of mosaicism in blood, 29% in saliva). Both patients have bilateral perisylvian polymicrogyria
3936 (white circles). **K, L.** Axial FLAIR and coronal T1W sections showing right posterior quadrantic
3937 dysplasia (white circle) in a boy with a constitutional *PTEN* mutation. **M, N.** T2W coronal and
3938 sagittal sections in two patients with constitutional *TSC2* mutations (**M:** p.Thr1623Ile, **N:**
3939 p.Pro1202His) showing right posterior quadrantic dysplasia caused by a large cortical tuber (**M,**
3940 white circle) and an extensive dysplastic area involving most of the right frontal lobe (**N,** white
3941 arrowheads). **O, P.** T2W axial and T1W sagittal sections. Lissencephaly with normally thick cortex
3942 and cerebellar hypoplasia (**P,** asterisk) in a girl with *RELN* mutation. The white circle surrounds a
3943 hypoplastic brainstem. **Q, R.** Axial and sagittal T1W sections. Thickened cortex with simplified gyral
3944 pattern and cerebellar hypoplasia in a boy with *TUBA1A* mutation. The circles surround the
3945 hypoplastic cerebellum and brainstem. The asterisk indicates the area below a hypoplastic
3946 cerebellar vermis and the black arrow points to a hypoplastic corpus callosum lacking its most
3947 posterior part. **S.** T1W axial section. Diffusely simplified gyral pattern with prominent thickening
3948 and infolding of the sylvian fissures in a boy with *TUBB2B* mutation. The arrows point to an area of
3949 smooth cortex. **T.** T2W axial section. Severe dysgyria with simplified gyral pattern in a girl with
3950 *SCN3A* mutation. **U.** T1W axial section. Classical bilateral periventricular nodular heterotopia in a
3951 girl with *FLNA* mutation. Bilateral nodules of subependymal heterotopia (white arrowheads) are
3952 contiguous, extensively lining the ventricular walls. **V.** T1W axial section. Diffuse polymicrogyria,
3953 more prominent posteriorly (white arrows) in a boy with *ATP1A2* mutation. **W.** T2W coronal

3954 section. Polymicrogyria with abnormal cortical infoldings and packed microgyria (black arrows),
 3955 combined with abnormal sulcation in a boy with *ATP1A3* mutation. **X**. T2W axial section. Bilateral
 3956 frontoparietal cortical thickening and diffusely abnormal cortical pattern in a boy with biallelic
 3957 *GPR56* mutations. **Y, Z**. T1W axial and sagittal sections. Pachygyria and perisylvian polymicrogyria
 3958 in a girl with *DYNC1H1* mutation. Asterisks in **Y** are located where there is maximum cortical
 3959 thickening, in the posterior cortex. The asterisk in **Z** is located beneath a hypoplastic cerebellar
 3960 vermis. **AA**. T2W axial section. Diffuse polymicrogyria in a boy with a *GRIN2B* mutation. **BB**. T1W
 3961 axial section. Diffuse abnormality of the cortical pattern with smooth cortex and areas of
 3962 abnormal infolding, suggestive of polymicrogyria in a boy with biallelic *WDR62* mutations.

3963

3964 **FIGURE 8. Main actors of synaptic transmission and mapping of the synaptic gene products**
 3965 **causing synaptic encephalopathies with epilepsy.** Schematic representation of a symbolic synapse
 3966 containing excitatory and inhibitory synaptic components. The main targets of synaptopathies are:
 3967 (i) at the presynaptic level, gene products involved in the post-docking SV priming/fusion
 3968 processes (SNAREs and SNARE-associated proteins: Munc-13, IM1, Munc-18, PRRT2, SNARE
 3969 proteins, synaptotagmin-1/2, voltage-gated Ca^{2+} -channels), SV trafficking (trafficking proteins:
 3970 Synapsins I/II, vATPase, synaptophysin, SV2A, VAMP2, synaptojanin-1, AP-2, dynamin-1, TBC1D24)
 3971 and NT synthesis and loading into SVs (transport proteins: GAD1, vATPase); (ii) at the postsynaptic
 3972 level, postsynaptic receptors and their scaffold/transduction systems (GABA_A and NMDA
 3973 receptors, gephyrin, collybistin, PSD-95, Homer, Shank-3, SynGAP-1, DLG-1); (iii) at the synaptic
 3974 cleft level, trans-synaptic and extracellular matrix proteins and their receptors (neurexin-1,
 3975 neuroligin, IL1RAPL1, ADAM 22/23, LGI1), as well as secreted proteins (SRPX2, reelin). Other
 3976 presynaptic voltage-gated channels that affect the dynamics of nerve terminal activation and NT
 3977 release are also shown. In green, the actin-based cytoskeleton that regulates trafficking and

3978 maintenance of SV pool in the nerve terminal and concentrates postsynaptic receptors on the
3979 postsynaptic side.

3980 **FIGURE 9. Schematic representation of the mTOR and autophagy intracellular cascades and their**

3981 **interrelationships.** The complex regulatory cascade triggering the activation of the mTORC1

3982 complex is initiated by extracellular signals (growth factors, neurotransmitter, hormones). To be

3983 activated, mTORC1 needs to bind to the organelle membrane, a process that depends on the

3984 active form of the small G-protein Rheb and by the presence of a docking complex on the

3985 membrane formed by the GEF Ragulator and an appropriate combination of GTP- and GDP-bound

3986 Rag G-proteins. The membrane location of the latter complex depends on the presence of the

3987 vATPase on the membrane, which is favored by Dm α 12. Since the small G-proteins are the

3988 molecular switches for mTOR activation, they are also the targets of the two main upstream

3989 inhibitory complexes that act as GTPase activating proteins (GAPs), namely the TSC and Gator1

3990 complexes that inactivate Rheb and Rags, respectively. These inhibitory TSC and Gator1 complexes

3991 are in turn subjected to inhibition by the PI3K/AKT pathway, activated by extracellular signals and

3992 Gator 2, respectively, that therefore catalyze release of mTORC1 from inhibition. Activation of

3993 mTORC1 favors anabolism, protein synthesis, cell growth and, in neurons, outgrowth of neuronal

3994 processes and formation of synaptic connections. On the other hand, activation of mTORC1

3995 silences the autophagy chain by inhibiting the ULK1 complex which is required to recruit the Beclin

3996 complex to the phagophore for its activation by AMPK. This results in the following steps of

3997 autophagy flux, including LC3 conversion and binding, formation of the autophagosome and

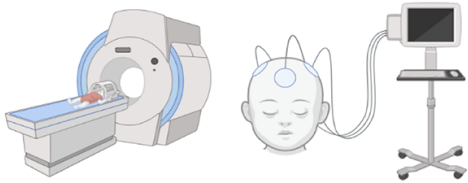
3998 subsequent fusion with lysosomes to form autolysosomes. In these processes, the proton gradient

3999 established by vATPase is essential, as well as the activity of accessory proteins such as DMXL2,

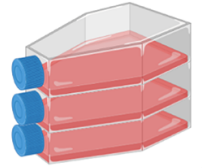
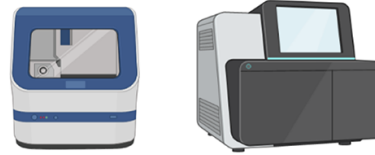
4000 EPG5 and SNX14.

4001

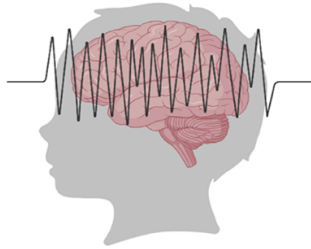
DIAGNOSIS



GENETIC STUDIES



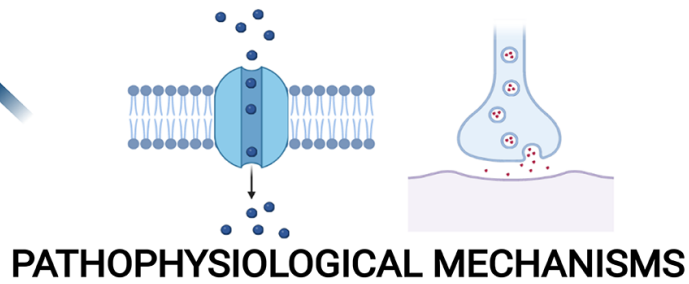
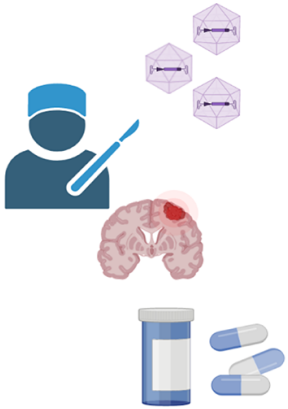
FUNCTIONAL STUDIES



DEE



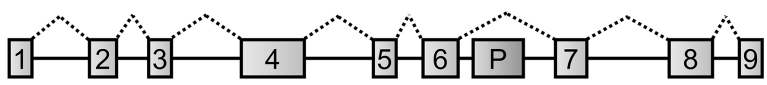
PERSONALIZED TREATMENTS



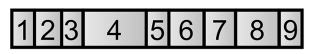
PATHOPHYSIOLOGICAL MECHANISMS



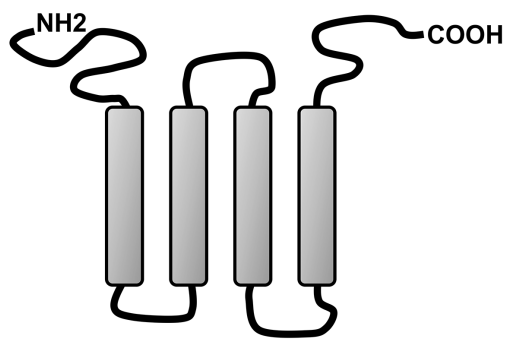
A



↓ **CANONICAL SPLICING**



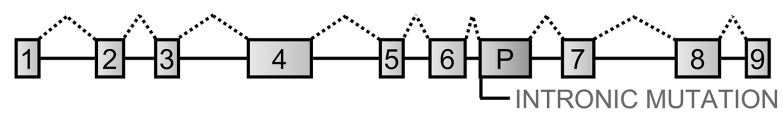
↓ **TRANSLATION INTO THE WILD TYPE PROTEIN**



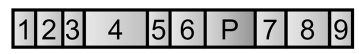
↓

INTEGRATION INTO THE PLASMA MEMBRANE AND NORMAL FUNCTIONING

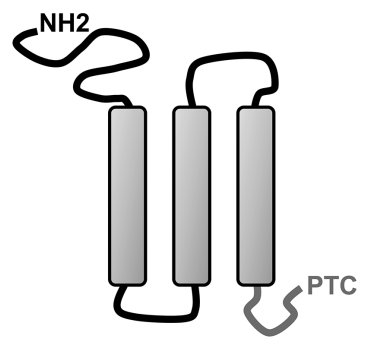
B



↓ **ABERRANT SPLICING**

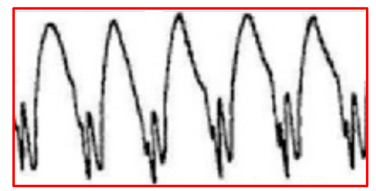
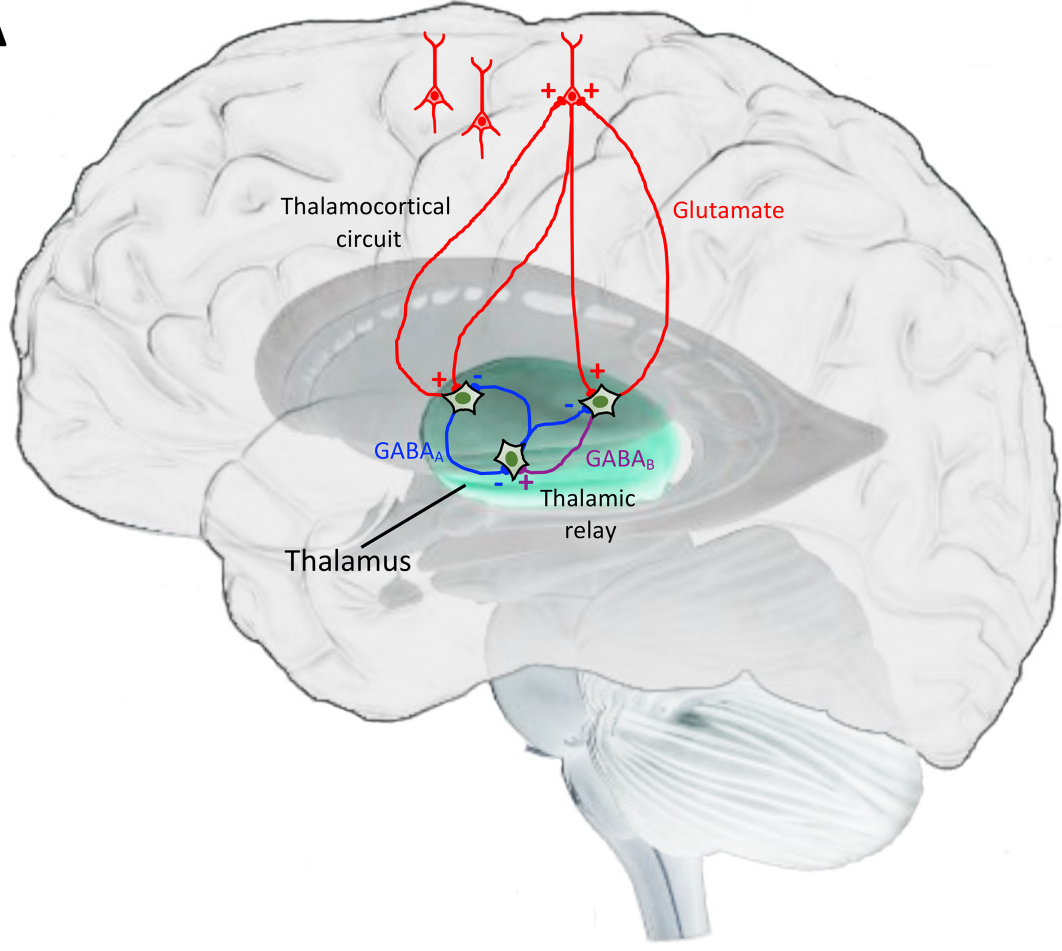
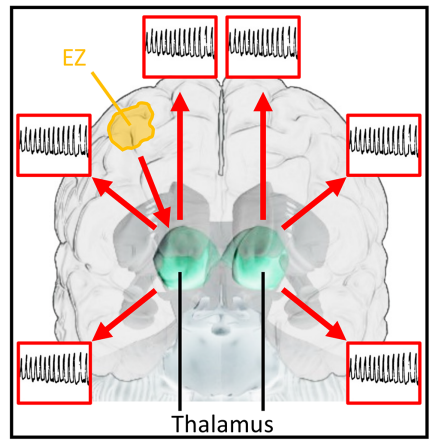
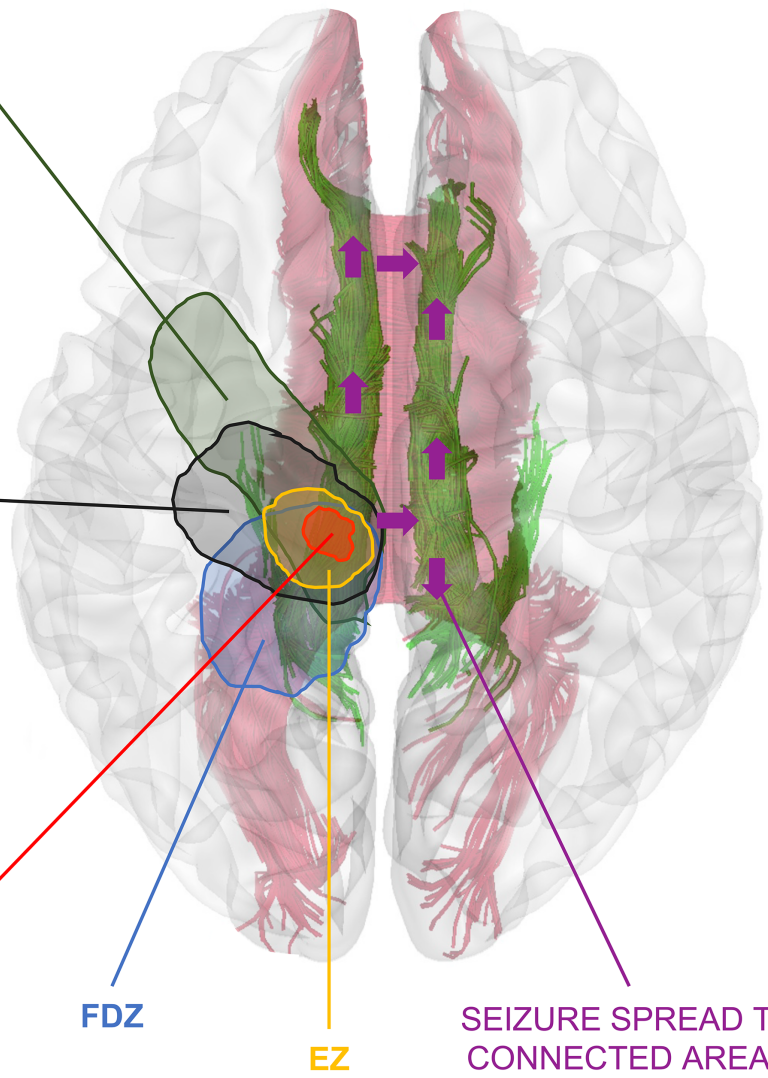
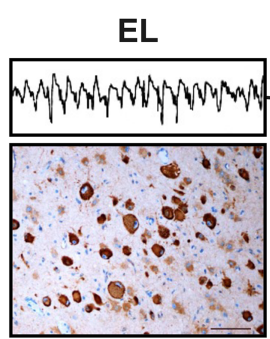
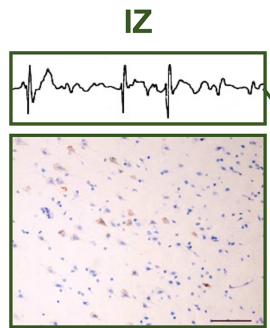


↓ **TRANSLATION INTO A TRUNCATED PROTEIN**

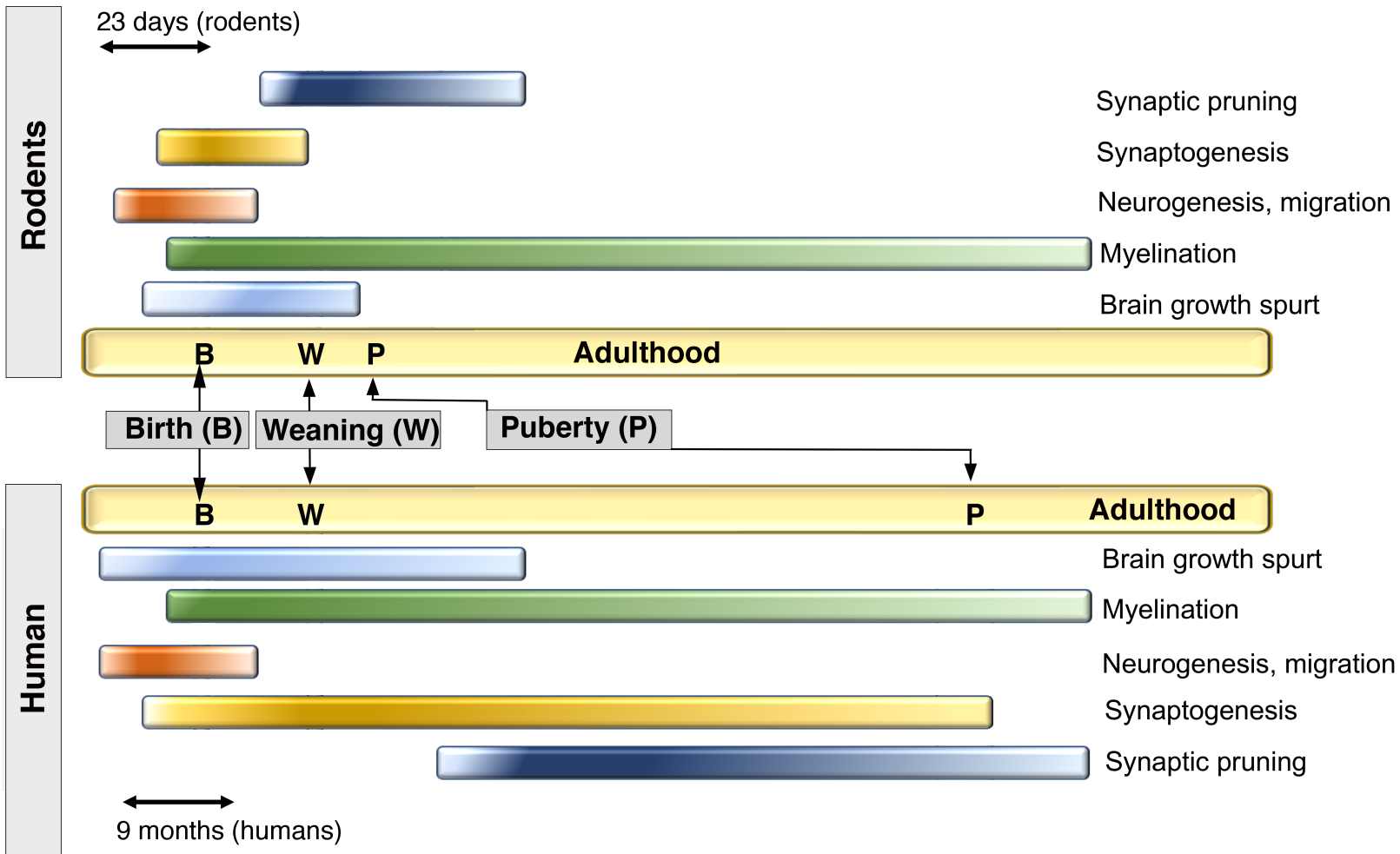


↓

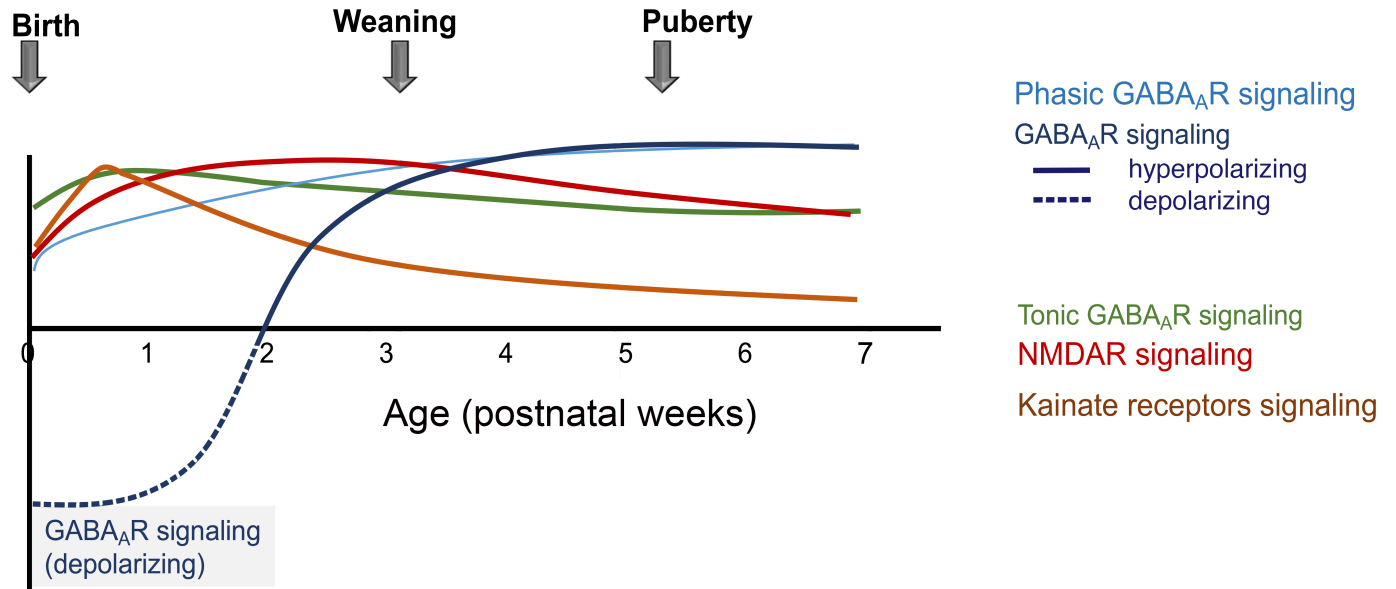
RECOGNITION OF A PTC AND DEGRADATION OF TRUNCATED PROTEIN

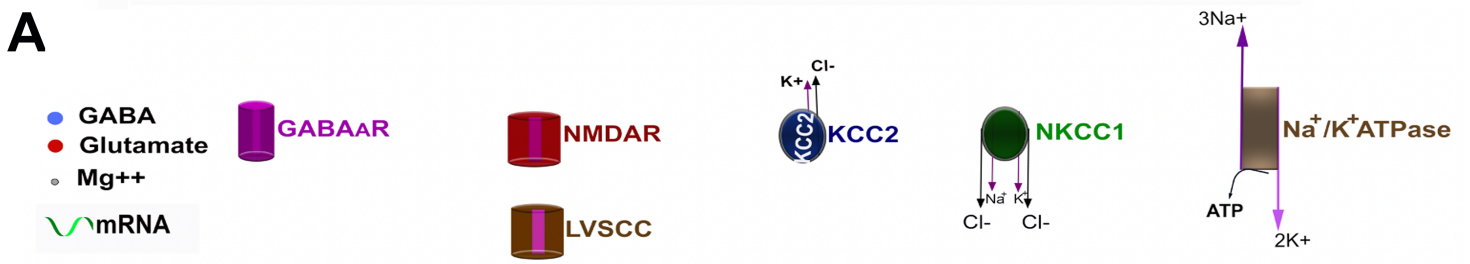
A**Spike and wave EEG****B**

SECONDARY GENERALIZATION

A**B**

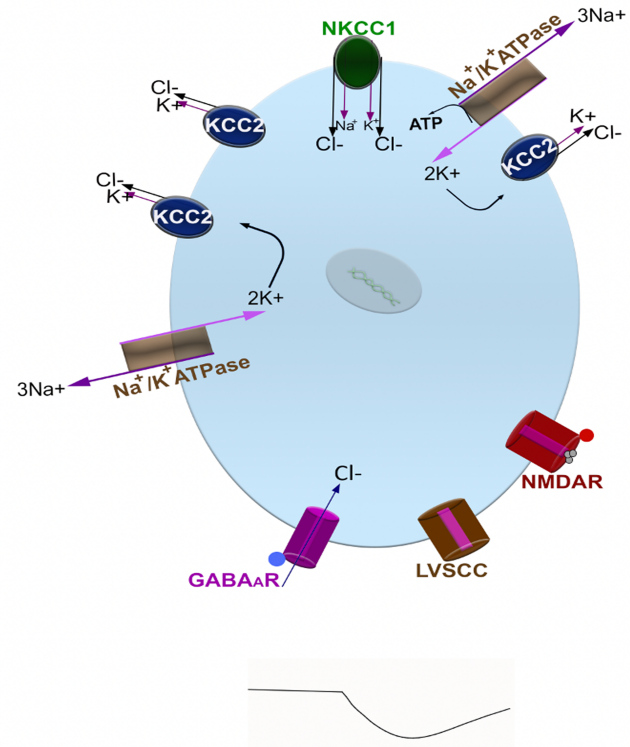
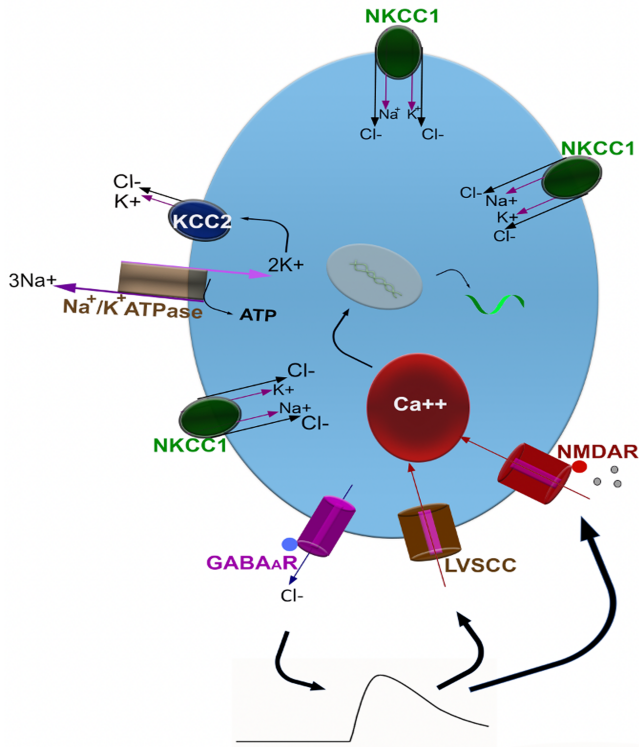
Developmental changes in GABA / glutamate receptor signaling in rodents





**Immature neurons,
High intracellular [Cl⁻]**

**Mature neurons
Low intracellular [Cl⁻]**

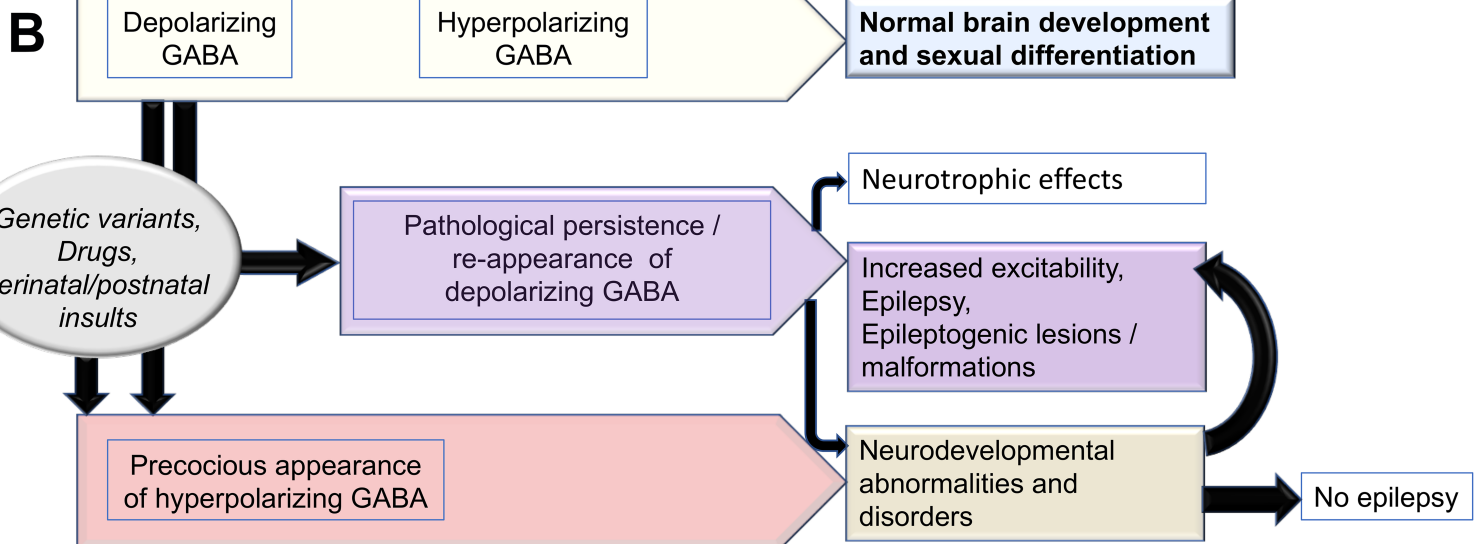


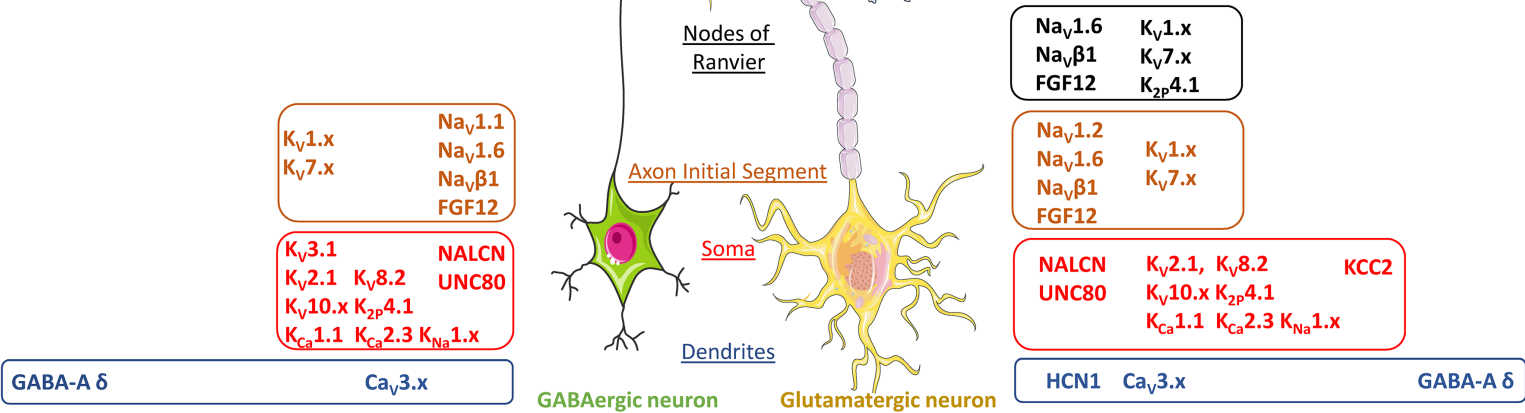
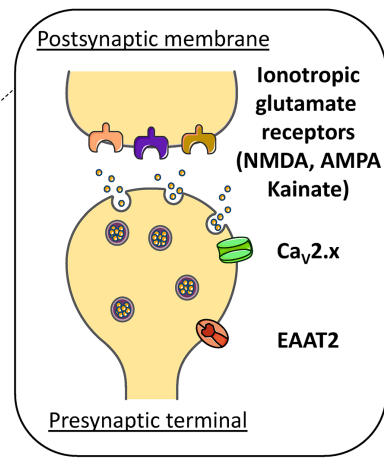
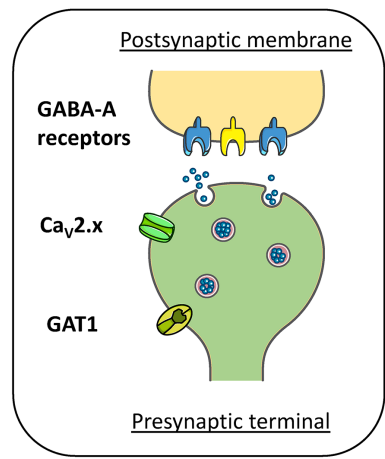
Depolarization

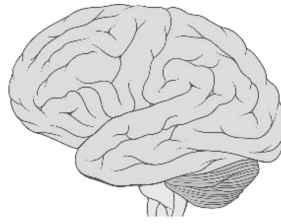
Hyperpolarization

DNA synthesis
 Proliferation
 Migration
 Survival
 Synaptogenesis
 Neuronal differentiation
 Increased excitability (?)
 Shunt inhibition

Effective inhibition







Human brain
EPS, MRI, BEH



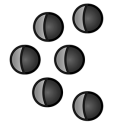
Human brain slices cultures
EPS, LI, IHC, ISH, TR, PR



2D cell cultures
EPS, ICC, LI, TR, PR



Brain organoids
EPS, LI, IHC, ISH, TR, PR, BEH



Frog oocytes
EPS, ICC, LI, TR, PR



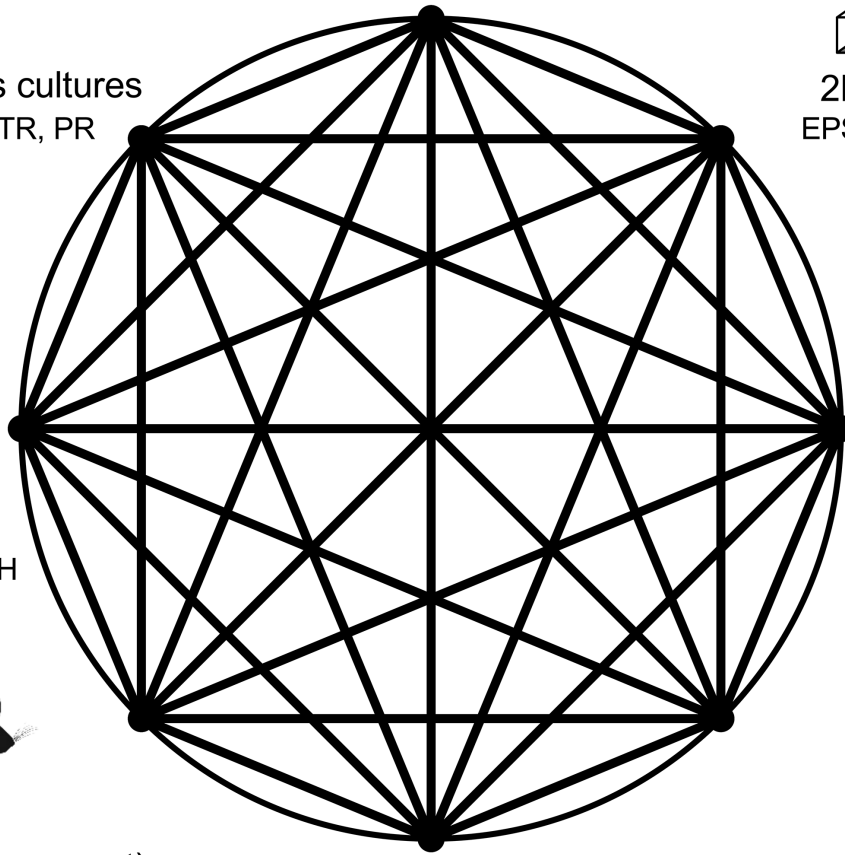
Rodent models (mouse, rat)
EPS, MRI, LI, IHC, ISH, TR, PR, BEH

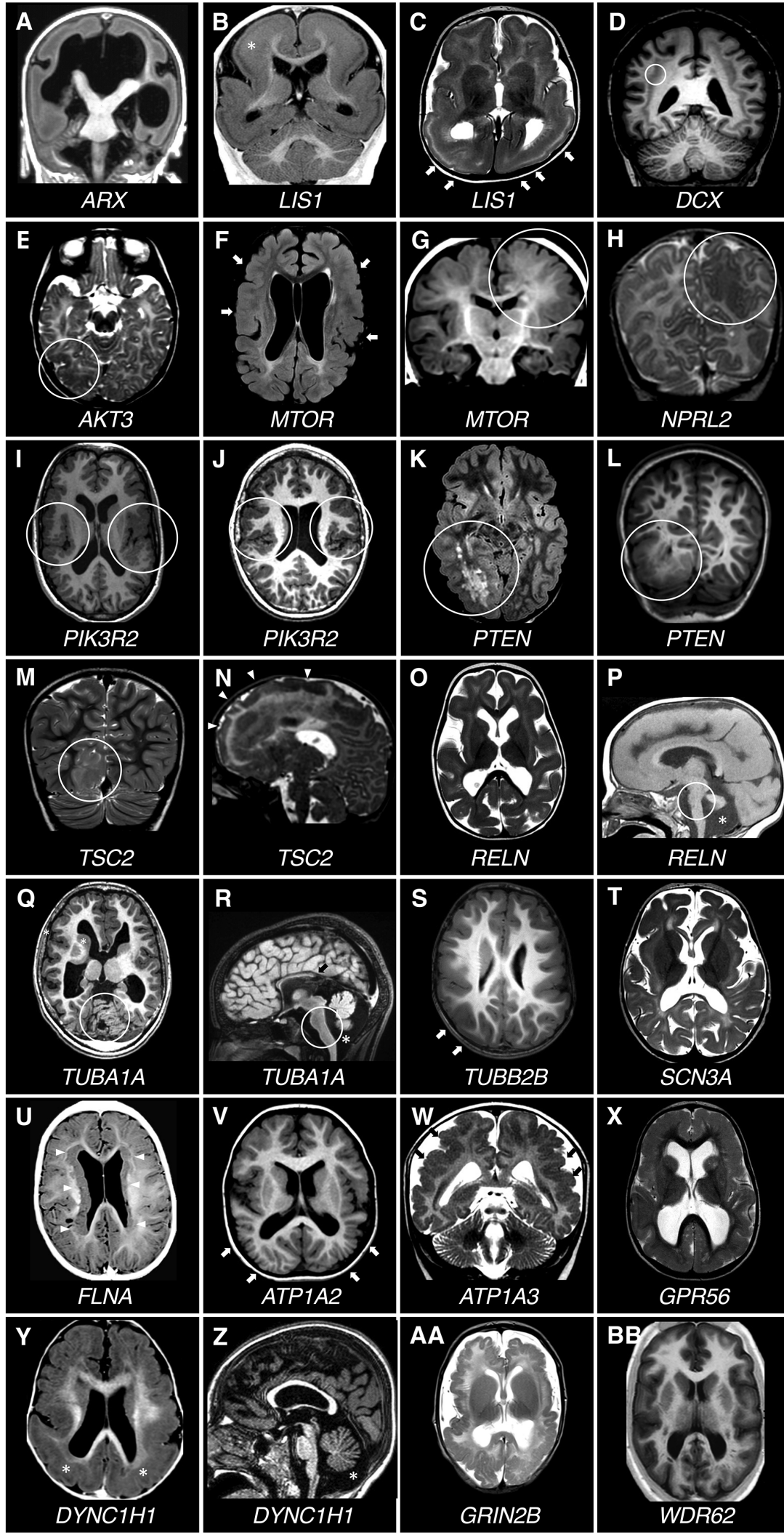


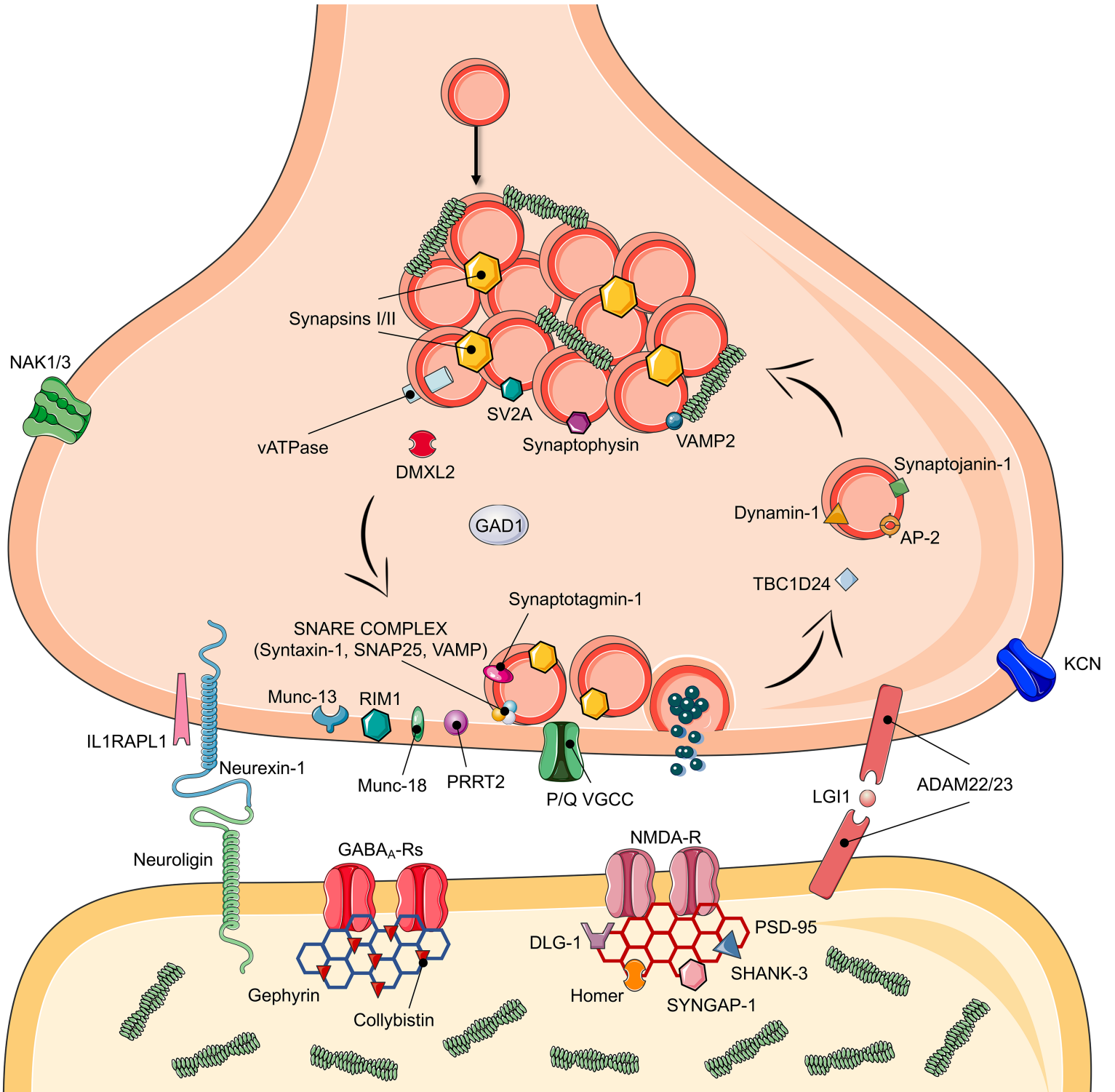
Fruit fly
EPS, MRI, LI, IHC, ISH, TR, PR, BEH

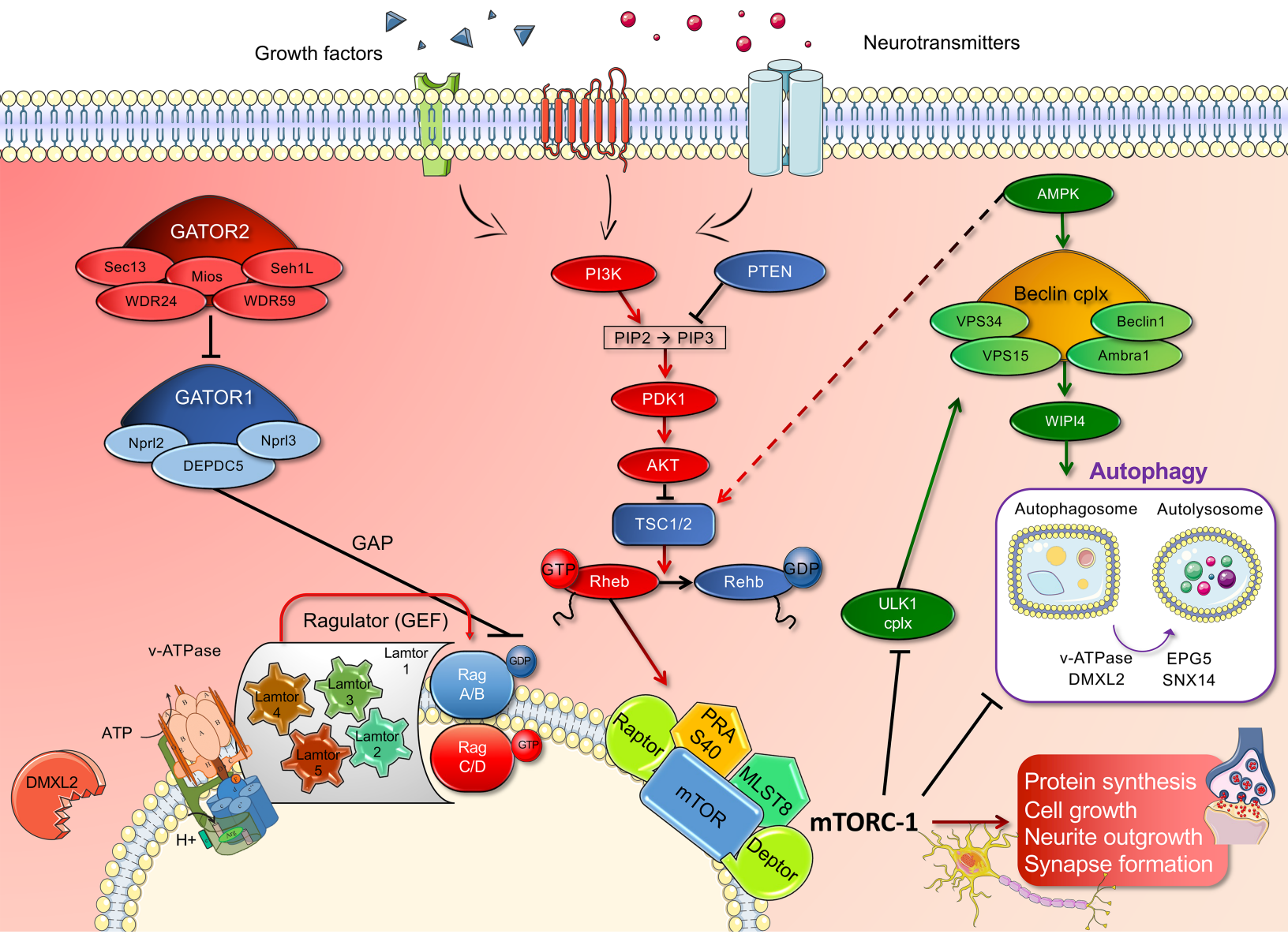


Lower vertebrates (zebrafish, frog)
EPS, MRI, LI, IHC, ISH, TR, PR, BEH









Growth factors

Neurotransmitters

GATOR2

GATOR1

PI3K

PTEN

PIP2 → PIP3

PDK1

AKT

TSC1/2

Rheb (GTP)

Rehb (GDP)

GAP

Ragulator (GEF)

v-ATPase

ATP

H+

Lamtor 1

Lamtor 4

Lamtor 3

Lamtor 5

Lamtor 2

Rag A/B (GDP)

Rag C/D (GTP)

Raptor

PRA S40

MLST8

mTOR

Deptor

mTORC-1

Protein synthesis
Cell growth
Neurite outgrowth
Synapse formation

Autophagy

Autophagosome

Autolysosome

v-ATPase

DMXL2

EPG5

SNX14

ULK1 cplx

Beclin cplx

VPS34

VPS15

Beclin1

Ambra1

WIPI4