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Ion Channels Involvement in Neurodevelopmental Disorders

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Abstract—Inherited and sporadic mutations in genes encoding for brain ion channels, affecting membrane expression or biophysical properties, have been associated with neurodevelopmental disorders characterized by epilepsy, cognitive and behavioral deficits with significant phenotypic and genetic heterogeneity. Over the years, the screening of a growing number of patients and the functional characterization of newly identified mutations in ion channels genes allowed to recognize new phenotypes and to widen the clinical spectrum of known diseases. Furthermore, advancements in understanding disease pathogenesis at atomic level or using patient-derived iPSCs and animal models have been pivotal to orient therapeutic intervention and to put the basis for the development of novel pharmacological options for drug-resistant disorders. In this review we will discuss major improvements and critical issues concerning neurodevelopmental disorders caused by dysfunctions in brain sodium, potassium, calcium, chloride and ligand-gated ion channels.

Key words: epileptic encephalopathy, neurodevelopmental disorders, autism, ion channels, antiepileptic drugs, precision medicine.

INTRODUCTION

Ion channels are widely and selectively distributed in the brain, both in neurons and astrocytes, often forming macromolecular complexes within distinct subcellular compartments with associated proteins or auxiliary subunits. The proper activation of cationic and anionic currents regulates the resting membrane potential, generates the action potential, modulates cell excitability and neurotransmitter release, thus providing subsets of neuronal cells in distinct brain areas with specific electrical features (Hibino et al., 2010; Jan and Jan, 2012; Oyrer et al., 2018; Elorza-Vidal et al., 2019; Catterall et al., 2020). The importance of ion channels in the pathogenesis of brain channelopathies came up with the discovery of one mutation in the *CHRNA4* gene, encoding for the alpha-4 subunit of the nicotinic acetylcholine receptor (nAchR), in patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Steinlein et al., 1995). Since then, ion channel genes have been mapped on human chromosomes and several studies in the past decades ascertained that mutations in brain ion channels genes cause numerous but rare neurodevelopmental disorders (NDDs), including severe forms of epileptic encephalopathy of infancy and childhood (*SCN1A*, Mantegazza and Broccoli, 2019; *SCN2A*, Wolff et al., 2019; *SCN8A*, Gardella and Møller, 2019; *KCNQ2/KCNQ3*, Lee et al., 2018; *KCNA1*; Rogers et al., 2018, Verdura et al., 2019; *KCNA2*; Masnada et al., 2017; *KCNT1,* Hasan et al., 2017; *KCNB1*, Bar et al., 2020; *CACNA1A*, Jiang et al., 2019), autism spectrum disorders (ASD) and psico-motor delay (*SCN1A*, Satterstrom et al., 2020; *SCN2A*, Spratt et al., 2019; *KCNJ10*, Sicca et al., 2011; *KCNQ3*, Sands et al., 2019; *CACNA1C,* Han et al., 2019), ataxias with seizures (*KCNA1*; D'Adamo et al., (2020, Karalok et al., 2018; *KCND3*, Allen et al., 2020; *CACNA1A,* Stendel et al., 2020), cerebellar atrophy and seizures (*KCNMA1*; Tabarki et al., 2016). In most cases, electroclinical seizure

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Abbreviations: ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; AED, antiepileptic drugs; ASD, autism spectrum disorder; ASO, Antisense oligonucleotide; BFNIS, Benign Familial Neonatal Seizures; BFNIS, Benign Familial Neonatal-Infantile Seizures; DS, Dravet syndrome; EA, episodic ataxia; EAST/SeSAME, Epilepsy, Ataxia, Sensorineural deafness, Tubulopathy/Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance; EIMFS, epilepsy of infancy with migrating focal seizures; FHM, familial hemiplegic migraine; GABAA receptor, γ-aminobutyric acid receptor type A; GEFS+, genetic epilepsy with febrile seizures plus; GoF, gain-of-function; iPSCs, induced pluripotent stem cells; LoF, loss-of-function; MLC, Megalencephalic leukoencephalopathy with subcortical cysts; nAChR, nicotinic acetylcholine receptor; NDDs, neurodevelopmental disorders; NGS, next generation sequencing; NMDAR, N-methyl-D-aspartate receptor; SCA, spinocerebellar ataxia; SMEI, Severe Myoclonic Epilepsy in Infancy; SQT3S, Short QT syndrome type 3; SUDEP, sudden unexpected death in epilepsy; TS, Timothy syndrome; WES, whole exome sequencing; WGS, whole genome sequencing.

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patterns are part of complex phenotypes with widespread developmental signs and often with a progressive poor course. The presence of various cognitive and behavioral deficits with pure neurological symptoms pose serious challenges in phenotypes identification. Thorough clinical evaluation of patients using new diagnostic criteria and the genetic revolution allowed important steps ahead in offering a timely and correct clinical and genetic diagnosis in at least 35–40% of people affected by ion channels-related NDDs (McTague et al., 2016). The functional characterization of the mutated channels using homologues and heterologous expression systems, gene-targeted animal models and, more recently, induced pluripotent stem cells (iPSCs)-derived neurons allowed to confirm the pathogenic relevance of the genetic defect, providing important insight into the cellular mechanisms by which ion channel mutations impair neuronal networks (Niday and Tzingounis, 2018; Sanders et al., 2018). The improved understanding of the physiological roles of ion channels in the brain have brought to the important conclusion that seizure occurrence should be inquired by studying the specific time- and space-dependent expression of the mutant ion channel in inhibitory or excitatory neurons within neuronal circuits (Du et al., 2020; Smith and Walsh, 2020). The therapeutic management of affected patients remains one major burden of neurodevelopmental channelopathies. Many antiepileptic drugs (AEDs) are available on the market; though, many patients suffering from NDDs are drug-resistant and therapeutic approaches based on precision medicine are highly desirable (Mesraoua et al., 2019; Perucca and Perucca, 2019; Klein et al., 2020).

This review will discuss up to date research progresses in establishing diagnosis, clarifying pathogenesis and orienting treatment by bringing as examples relevant NDDs-linked mutations in genes coding for voltage-gated and inwardly rectifying ion channels. NDDs caused by mutations in ligand-gated ion channels (γ-aminobutyric acid type A, N-methyl-D-aspartate and nAchRs, $GABA_A$, NMDAR and nAChR) are also briefly mentioned (Table 1).

EMERGING CONCEPTS IN DIAGNOSIS, AETIOLOGY AND TREATMENT OF NDDS

Over recent years, the enhanced access of affected families to health care and the extensive use of next generation sequencing (NGS) and *in silico* tools to predict the pathogenicity of a variant contributed to identify new candidate genes and recognize distinct clinical syndromes, thereby increasing the rate of successful and prompt diagnosis. Single gene testing is no more pursued, and seizure multigene panels or comprehensive sequencing (whole exome sequencing and whole genome sequencing, WES and WGS) are now recommended to establish proper differential diagnosis (McTague et al., 2016). Genetic variants in NDDs comprise small deletions, insertions, frame-shifts, stop codons, missense, splice-site mutations, exon deletions or duplications (Oyrer et al., 2018). If a better comprehension of specific brain phenotypes associated with single ion channel genes has been achieved, the large-scale application of NGS revealed that *de novo* mutations are over-represented in individuals affected by NDDs linked to *SCN1A, SCN2A, KCNT1, KCNA2*, *CACNA1A,* among the others. Therefore, genotype-phenotype correlations may be extremely challenging due to the enormous amount of genetic data obtained from patients' screenings and careful analysis of mutations is of utmost importance to envisage a pathogenic significance and to inform treatment choice (Epi25 Collaborative, 2019; McTague et al., 2016).

Genetic heterogeneity is a characteristic of NDDs. For instance, more than 30 genes have been associated with early-infantile epileptic encephalopathy, which comprise a large and heterogeneous group of severe epilepsies leading to progressive impairment of brain function with developmental slowing and regression (Oyrer et al., 2018; Wolff et al., 2019; Allen et al., 2020). Among ion channels genes, *SCN1A, SCN2A, SCN8A, KCNA2, KCNT1, KCNQ2, CACNA1A, GABRG2, GRIN2B* have been all associated with this heterogeneous disorder (Oyrer et al., 2018).

Other critical issues within NDDs, common to channelopathies, are represented by incomplete penetrance and phenotypic variability, with more than 60% of carriers not displaying a complete disease phenotype (Cooper et al., 2013). It is now clear that phenotypic heterogeneity and seizure evolution might occur within many epilepsy syndromes (McTague et al., 2016). Single ion channel genes can be associated to different epilepsy phenotypes ranging from benign seizure disorder to severe epileptic encephalopathy, as emerged from the observations of *SCN1A* and *SCNA2* or *KCNQ2/3* clinical cases (Oyrer et al., 2018; Sanders et al., 2018). The large phenotypic variability in NDDs is best illustrated in monozygotic twin studies in which identical ion channel variation gives rise to very different clinical course and response to treatment (Rogers et al., 2018). Patients with severe epilepsy also show comorbidities such as cognitive impairments, motor delay, depression, anxiety, migraine and heart and sometimes kidney disease, as occur in *SCN1A, CACNA1C, KCNT1, KCN2A*, *KCNQ2* or *KCNJ10*-associated disorders, complicating diagnosis and drug-response prediction (Hermann et al., 2008; Keezer et al., 2016; Zaccara and Lattanzi, 2019; Frasier et al., 2016; Bockenhauer et al., 2009). Genetic, epigenetic and environmental factors have been suggested as critical modulators of clinical symptoms and responsible for incomplete penetrance, and, although difficult to decipher, some modifier genes have been presumed for *SCN1A* disorders (Glasscock et al., 2007; Gaily et al., 2013; Miller et al., 2014; Manuck and McCaffery, 2014; McTague et al., 2016; Smith and Walsh, 2020; Wong et al., 2018; Gross and Tiwari, 2018). Somatic mosaicism is a concept that is becoming increasingly relevant to explain phenotypic variability in many epileptic encephalopathy syndromes, such as Dravet syndrome (DS) caused by *SCNA1* mutations (Oyrer et al., 2018; de Lange et al., 2019).

In the last two decades, significant steps ahead have been made also in deciphering the molecular, cellular and

Table 1. Most common ion channels genetic defects causing neurodevelopmental disorders (NDDs)

Gene(Channel)	NDDs (channel defects)	Animal models	Therapy	References
SCN1A (Nav1.1)	GEFS + (LoF); EIMFS (LoF); Dravet syndrome (LoF) Additional: autistic traits, heating sensitivity, ataxia	*Scn1a*$^{+/-}$ mice show epilepsy and autistic traits	Sodium channel blockers avoided; combination of valproate, clobazam stiripentol, topiramate; add-on cannabidiol or fenfluramine; ketogenic diet	Mantegazza and Broccoli (2019), Cross et al. (2019)
SCN2A (Nav1.2)	BFNIS and other early onset epileptic encephalopathy (< 3 months; GoF); infancy-childhood epileptic encephalopathy (> 3 months; LoF); Autism and intellectual disability (LoF) Additional: dyskinesia, ataxia	*Scn2a* GoF mice show ataxia and seizures; *Scn2a*$^{+/-}$ are viable and seizure free	Sodium channel blockers in GoF but avoided in LoF; benzodiazepines, valproate, levetiracetam in LoF; ketogenic diet	Sanders et al. (2018)
SCN8A (Nav1.6)	BFNIS (GoF); epileptic encephalopathy (GoF); Autism (LoF) Additional: dyskinesia, ataxia, sleep disorders and risk of cardiac arrhythmias	*Scn8a*$^{N1768D/+}$ GoF mice show seizure, ataxia and SUDEP	Sodium channel blockers at high doses in GoF; cannabidiol, riluzole, ketogenic diet; GS967	Gardella and Møller (2019)
KCNQ2/3 (Kv7.2/3)	BFNS (LoF); Epileptic encephalopathy (LoF and GoF); autism (GoF)	*Kcnq2* LoF knock-in mice show seizures	Kv7 openers in LoF, retigabine and derivatives; combinations of conventional AEDs and corticosteroids	Lee et al. (2018), Sands et al. (2019)
KCNA1 (Kv1.1)	EA1 (LoF); epileptic encephalopathy (LoF)	*Kcna1*$^{V408A/+}$ LoF mice show ataxia; *Kcna1*$^{-/-}$ mice show epilepsy and SUDEP	Acetazolamide and combinations of conventional AEDs	D'Adamo et al. (2020), Smart et al. (1998)
KCNA2 (Kv1.2)	Epileptic encephalopathy and ataxia (GoF and LoF)	*Kcna2*$^{-/-}$ mice show epilepsy and SUDEP	Acetazolamide and combinations of conventional AEDs	Masnada et al. (2017), Brew et al. (2007)
KCNT1 (K_{Na}1.1)	EIMFS; ADNFLE; epileptic encephalopathy (GoF) Additional: microcephaly, cardiac arrhythmias, behavioral disturbances	*Kcnt1*$^{R455H/R455H}$ mice are embryonic lethal; *Kcnt1*^{R455H} mice show spontaneous seizures, increased seizure susceptibility and mortality	Combinations of conventional AEDs; quinidine in some patients	Gertler et al. (2018)
KCNJ10 (Kir4.1)	Autism and epilepsy (GoF); EAST/SeSAME (LoF)	*Kcnj10*$^{-/-}$ mice show stress-induced seizures, hearing loss, retinal dysfunction, motor impairment	Combinations of conventional AEDs	Guglielmi et al. (2015), Sala-Rabanal et al. (2010)
CACNA1A (Cav2.1)	Absence epilepsy; epileptic encephalopathy (GoF) Additional: cerebellar atrophy and ataxia; autistic traits and intellectual disability	*Cacna1a* LoF mice show absence seizures, ataxia and cerebellar atrophy; *Cacna1a*$^{-/-}$ mice display ataxia but no seizures	Combinations of conventional AEDs	Jiang et al. (2019)

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Table 1 (*continued*)

Gene(Channel)	NDDs (channel defects)	Animal models	Therapy	References
CACNA1C (Cav1.2)	Timothy syndrome (GoF); epileptic encephalopathy Additional: photosensitive seizures; intellectual disability	*Cacna1c*$^{G406R/+}$-neomicine (TS-neo) mice show ASD traits but no cardiac phenotype	Beta-blockers or other antiarrhythmic drugs to treat LQTS; combinations of AEDs to treat epilepsy	Han et al. (2019), Pò et al. (2019)
CLCN2 (ClC-2)	*CLCN2*-related leukoencephalopathy	*Clcn2*$^{-/-}$mice show leukoencephalopath, male infertility and visual impairment	Only supportive therapy	Gaitán-Peñas et al. (2017), Elorza-Vidal et al. (2019)
HCN1 (Hyperpolarization-activated cyclic nucleotide-gated channel 1)	Mild generalized epilepsy; GEFS +; epileptic encephalopathy (GoF and LoF) Additional: heat sensitivity and intellectual disability	*HCN1*$^{-/-}$ mice show hyperexcitability and predisposition to seizures	Combinations of conventional AEDs	Marini et al. (2018), DiFrancesco et al. (2019)
CHRNA2, CHRNA4, CHRNB2 (α2, α4, β2 subunits of nAchR)	ADNFLE (GoF)	GoF knock-in mice show seizures	Carbamazepine at low doses	Steinlein et al. (1995), Becchetti et al. (2015)
GABRA1, GABRB3, GABRG2 (α1, β3, γ2 subunits of GABAA receptor)	Generalized epilepsy; epileptic encephalopathy (LoF) Additional: photosensitive seizures and intellectual disability	LoF knock-in and knock-out mice show generalised epilepsy	Anti-absence AEDs valproic acid and ethosuximide; vigabatrin; corticosteroids; ketogenic diet	Gataullina et al. (2019), Oyrer et al. (2018)
GRIN1, GRIN2A, GRIN2B, GRIN2D (GluN1 and GluN2A-D subunits of NMDAR)	Idiopathic focal epilepsy; epileptic encephalopathy; isolated intellectual disability (LoF and GoF) Additional: language disorders, hyperkinetic movements, cortical defects	Animal models do not show seizures	Memantine useful in GoF; vigabatrin; benzodiazepins; corticosteroids	Gataullina et al. (2019), Oyrer et al. (2018)

ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; AED, antiepileptic drugs; BFNS, benign familial neonatal seizure; BFNIS, benign familial neonatal infantile seizure; EA1, episodic ataxia type 1; EAST/SeSAME, Epilepsy, Ataxia, Sensorineural deafness, Tubulopathy/Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance; GEFS +, genetic epilepsy with febrile seizure plus; LQTS, long QT syndrome; EIMFS, epilepsy of infancy with migrating focal seizures; nAchR, acetylcholine receptor.

circuit mechanisms through which mutations in different ion channels can affect channel activity and contribute to the complex neurodevelopmental changes occurring in rare NDDs. Studies from cellular models of diseases clarified that most mutations alter ion channel biophysics, such as voltage-dependent gating, kinetics, single channel conductance, ion selectivity, affect synthesis and trafficking to the membrane or impact the modulation by signaling pathways, inducing loss-of-function (LoF) or gain-of-function (GoF) of the relevant channel (Imbrici et al., 2016). These studies laid the foundation for precision medicine (Mesraoua et al., 2019; Perucca and Perucca, 2019). Knowledge of ion channels physiology within neuronal circuits and neurobiology studies from animal models and neurons derived from patients' IPSCs greatly helped to assess that disruption of common pathways in cortex and hippocampus might converge to produce ion channels-related epilepsy (McTague et al., 2016). The improved genetics and functional studies overturned the general concept that seizures arises from either GoF mutations in sodium/calcium channels or LoF mutations in potassium channels, instead highlighting that up- or down-regulation of any channel type can underpin epileptic phenotypes (Allen et al., 2020). In addition to the degree and nature of ion channel defect, significant other variables likely contribute to the seizure initiation and heterogeneity, including temporal (prenatal to adulthood) and spatial (brain area and cell-specificity) ion channels expression, cell-type susceptibility (progenitor, migrating, or mature neurons), environment and compensatory mechanisms (Calhoun et al., 2017; Niday and Tzingounis, 2018; Smith and Walsh, 2020; Du et al., 2020). Altered electrical activity of both inhibitory and excitatory neurons within hippocampus and thalamocortical loop can lead to epileptic encephalopathies. Typically, symptoms present at around the age at which ion channels start to be expressed to warrant normal neuronal activity. In this regard, findings from animal models carrying total or partial deletion of *SCN1A, KCNA1* and *KCNA2* genes correlate with patients' disease course (Smart et al., 1998; Brew et al., 2007; Han et al., 2012). A specific develop-

mental pattern and distinct excitatory/inhibitory neuronal expression ratio has been detailed for *SCNA* genes and appears to correlate with the age of seizure onset in carriers of *SCNA* genes mutations (Smith and Walsh, 2020; Du et al., 2020). The almost exclusive expression of *SCN1A* in inhibitory interneurons easily explains that LoF mutation of this channel isoform renders the network hyperexcitable. Conversely, GoF mutations of *SCN2A* and *SCN8A* are expected to have major impact in excitatory neurons where these channels are expressed (Du et al., 2020). The apparent paradox of GoF mutations in potassium channels could be solved by hypothesizing that network synchronization and hyperexcitability may come either from circuit disinhibition, as for reduction of inhibitory interneuron activity, or from elevated firing properties of excitatory cells due to reduced sodium channel inactivation or increased repolarization, or even from enhanced homeostatic plasticity of excitatory pyramidal neurons (Niday and Tzingounis, 2018). Unfortunately, we have little information about GoF mutations in native neuronal systems to draw conclusions. The possibility of potassium channels to heteropolymerize with members of the same family, the interaction of all channels types with other cellular proteins and accessory subunits, as well as posttranslational modifications should all be taken into account when interpreting the impact of mutations in ion channels genes in neuronal excitability and phenotypic pleiotropy (DiFrancesco et al., 2019; D'Adamo et al., 2020). Regarding motor dysfunctions with seizures as a comorbidity, such as ataxia, altered neurotransmission in the cerebellum is more likely to occur, as envisaged from *KCNA1*, *KCNA2* or *CACNA1A* animal models (Hoxha et al., 2018; D'Adamo et al., 2020).

More difficult is to ascertain the molecular and cellular mechanisms underlying ASD with developmental cognitive and motor impairments. Altered expression of ion channels in cortical interneurons may affect excitability and play a role. However, aberrant neuronal proliferation, differentiation and migration, formation of abnormal networks and altered synaptic plasticity during early cortical development likely contribute to disease phenotype, as suggested by studies on *SCN1A, SCN3A, KCNT1, CACNA1C*, *GRIN1* and *GRIN2B* deficits (Mantegazza and Broccoli, 2019; Quraishi et al., 2020). Elucidating the role played by ion channels in prenatal brain is therefore pivotal to understand how early events critically impact disease course and phenotype (Smith and Walsh, 2020).

Dysfunction in astrocytic ion channels, such as *KCNJ10* and *CLCN2*, also suggest that disruption of astrocyte-dependent water and ionic homeostasis appears critical for cognitive dysfunctions, brain malformations and seizure susceptibility (Elorza-Vidal et al., 2019; Guglielmi et al., 2015).

One major burden of NDDs is the therapeutic management of affected patients. A wealth of AEDs are available to treat epilepsy in children affected by developmental epilepsy and encephalopathy (Perucca and Perucca, 2019). These drugs mainly function as ion channels modulators, enhance GABAergic or inhibit glutamatergic transmission. However, drugs targeting specific ion channels defects are lacking and patients are often treated with symptomatic therapies with suboptimal response and side effects (Imbrici et al., 2016). More than 30% of patients suffer from intractable epilepsy (Mesraoua et al., 2019). Responsiveness may be delayed or even changed due to altered expression patterns of ion channels during development (Brunklaus and Lal, 2020). In parallel, pharmacokinetics and pharmacogenomics issues may hamper sustained beneficial effects (Gambardella et al., 2017). In addition, developmental comorbidities are unlikely to be rescued with conventional AEDs, although a quick access to the cure is recommendable (Mesraoua et al., 2019). Therefore, studies of novel genetic variants coming from WES in model systems are fundamental not only for functional validation of mutations pathogenicity, but also to develop drugs able to counteract the specific ion channel dysfunction and to predict drug response. Novel therapeutic approaches are therefore underway to improve precision medicine and the outcome of devastating NDDs in affected individuals including, ion channel-directed gene therapy (Snowball et al., 2019), drug repurposing (such as memantine, quinidine, cannabidiol; Pierson et al., 2014; Gertler et al., 2018; Cross et al., 2019) and antisense oligonucleotides (Mesraoua et al., 2019; Perucca and Perucca, 2019; Lenk et al., 2020). Some of these pharmacological approaches are still at a preclinical stage of development and, considering the rarity and heterogeneity of NDDs, careful studies are required to assess the translational potential of new drugs in the clinical setting. Certainly, unravelling the contribution of distinct gene defects to specific components of developmental delay within NDDs and the identification of the most effective therapy remains tough at present. These emerging concepts are discussed in the next paragraphs.

SODIUM CHANNELS IN NDDS

SCN1A (Nav1.1)

Several sodium channels genes (*SCN1A*, *SCN2A, SCN3A* and *SCN8A*) have been widely implicated in the pathophysiology of epilepsy associated with neurobehavioral comorbidities, such as cognitive impairment, psychiatric disorders and compromised social interactions (Brunklaus and Lal, 2020). *SCN1A*, which encodes for the Nav1.1 neuronal sodium channel, is the most recurrently mutated gene in epileptic encephalopathies. DS, also termed Severe Myoclonic Epilepsy in Infancy (SMEI), is the most frequent (1:15,000) drug-resistant epileptic encephalopathy of infancy, together with West syndrome with disease onset at 6 months. DS is associated with dramatic effects on psychomotor (ataxia and dysarthria), visuospatial, language and social development (autistic traits) initiating after 1–2 years of age (Brunklaus and Lal, 2020). Intellectual disability is reported in the majority of cases as well as sleep disturbances and sudden unexpected death in epilepsy (SUDEP; Mantegazza and Broccoli, 2019). Approximately 80% of DS patients carry LoF missense or truncations/deletions mutations in *SCN1A* (Escayg and Goldin, 2010). Besides DS, *SCN1A* mutations underlie also

milder epileptic phenotypes, including simple febrile episodes and self-remitting genetic epilepsy with febrile seizures (GEFS +), as well as severe epilepsy of infancy with migrating focal seizures (EIMFS) (Catterall, 2014; Mantegazza and Broccoli, 2019). To further complicate the matter, a large-scale WES study of autistic individuals also identified *SCN1A* as a risk-gene for ASD (Satterstrom et al., 2020). DS provides a clear example of genetic heterogeneity in epilepsy. Indeed, mutations in other ion channels genes can recapitulate DS-like phenotypes, including *GABRA1, GABRG2, HCN1, KCNA2* and *SCN1B* ($Na_V1.1$ β subunits); therefore, genetic screening is essential to frame the therapeutic approach and suggest lifestyle modification. Some patients are supposed to have DS with a polygenic basis, as envisaged by the observation that some probands reported severe symptoms, whereas, other family members only have mild-to moderate disease manifestations. Furthermore, genetic modifiers have been suggested by animal models studies (McTague et al., 2016). Mosaicism has also been proposed as an important modifier of *SCN1A*-related phenotypes, the pathogenic outcome depending on the proportion and cell types affected by the mutation (Depienne et al., 2010). In a study of 128 carriers of *de novo SCN1A* variants, quantitative allele specific assays demonstrated that mosaicism occurs in 7% of DS patients and that mosaic patients have on average milder phenotypes (de Lange et al., 2019).

The results obtained from *in vitro* and *in vivo* studies confirmed that the initial pathophysiologic mechanism underlying the *SCN1A* mutation-induced epileptogenesis is LoF (Han et al., 2012; Ito et al., 2013; Sun et al., 2016; Kim et al., 2018). Truncating mutations were associated with slightly earlier age of myoclonic and absence seizure onset compared with missense mutations (Wolff et al., 2019). Heterozygous and knock-in animals suggested that Nav1.1 LoF leads to hypo-excitability of some types of cortical and hippocampal GABAergic interneurons (including parvalbumin-positive and somatostatin-positive ones) leaving almost unaffected hippocampal excitatory pyramidal neurons (Mantegazza and Broccoli, 2019). *SCN1A* is almost exclusively expressed at the axon initial segment of inhibitory GABAergic fast-spiking cells in the neocortex and hippocampus and its expression is lowest prenatally and rises constantly towards adult age. Consistently with age- and cell-dependent Nav1.1 expression, *SCN1A* LoF mutations reduce GABA release thereby leading to disinhibition and hyperexcitability of the relevant neuronal networks, triggering seizures (Bender et al., 2012; Catterall, 2014). The inhibitory neurons deficit and mutation-specific disease severity have been recently confirmed using iPSCs technology from DS patients (Liu et al., 2013; Sun et al., 2016; Kim et al., 2018). Animal models also helped to elucidate the severe cognitive and behavioral features of DS. Actually, GABAergic interneurons are critical for the spatiotemporal synchronization of neural activity which is fundamental for strengthening cognitive processes during the first critical weeks of postnatal life (Murray et al., 2011). Conditional heterozygous knock-out *Scn1a* mice (an animal model of DS), in which the number of $Na_V1.1$ channels are specifically reduced in subpopulations of cortical and hippocampus GABAergic inhibitory interneurons), develop seizure and ASD behavioral phenotype such as hyperactivity, stereotyped behaviors, social interaction deficits and weak context-dependent spatial memory, suggesting that impairment in these fast-spiking cells also recapitulates the developmental comorbidities typical of the DS phenotype (Hermann et al., 2008; Bender et al., 2012; Han et al., 2012; Satterstrom et al., 2020). Emerging evidences suggest that additional factors – besides the electrical deficit in interneurons – are involved in the occurrence of comorbidities and in the remarkable phenotypic heterogeneity reported in DS patients. Phenotype severity in *Scn1a*$^{+/-}$ mice is strongly dependent on strain background (Miller et al., 2014). Moreover, homeostatic and pathological circuits remodelling may occur during development. Accordingly, cognitive symptoms may result from an additional sodium-mediated neurodevelopment process, regardless of seizures (Hermann et al., 2008; Nabbout et al., 2013; Mantegazza and Broccoli, 2019). Studies from digenic mice and patients, aimed at identifying genetic modifiers in DS, also suggest that concurrent *SCN9A* or *SCN8A* mutations can lower or raise seizure threshold, respectively, compared with *SCN1A*-only mutants. These findings highlight the co-existence of variants that may modulate the clinical phenotypes through compensatory or worsening mechanisms (Martin et al., 2007; Depienne et al., 2010; Meisler et al., 2010). Similarly, *Scn1a*$^{+/-}$ mice with decreased *Cacna1g* (Cav3.1) expression show partial amelioration of disease phenotypes with improved survival and reduced spontaneous seizure frequency, suggesting *Cacna1g* as a possible genetic modifier of DS and Cav3.1 as a potential drug target for DS patients (Calhoun et al., 2017). Finally, subjects harbouring both *SCN1A* and *CACNA1A* variants develop absence seizures more frequently and earlier than patients with only *SCN1A* mutations (Ohmori et al., 2013).

Even though a selective channel opener would be an obvious strategy to treat Nav1-associated syndromes due to LoF, no such drug has been developed to date (de Lera Ruiz and Kraus, 2015). In children with *SCN1A*-related epilepsies, seizure control is particularly critical as they are at high risk for SUDEP (Cross et al., 2019). In addition, clinical evidences indicate that having seizures controlled by medication reduces the subsequent brain damage and limit cognitive impairment (Mesraoua et al., 2019). Genetic testing in DS, as well as in other *SCN1A*-related seizure disorders, is therefore of paramount importance to address therapy and life style modifications. Sodium channel blockers, such as carbamazepine, lamotrigine and phenytoin, are contraindicated as they may exacerbate seizures frequency and severity also in animal models (Catterall, 2014). Increased body temperature should be avoided as it is the most important seizure trigger (Cross et al., 2019). In agreement with reduced GABAergic tone in *SCN1A*-related seizures, the potentiation of GABAergic transmission in inhibitory interneurons represents the most beneficial therapeutic intervention. Indeed, treatment of conditional heterozygous knock-out *Scn1a* mouse with

low-dose of clonazepam protected against myoclonic and generalized tonic-clonic seizures and completely rescued the abnormal social behaviors and deficits in fear memory (Han et al., 2012). At present, the standard treatment of children with DS in Europe and USA is based on a combination of valproate, clobazam, stiripentol or topiramate (Cross et al., 2019). A ketogenic diet has been proposed, albeit with caution, as well as potassium bromides, as possible therapeutic alternatives for some resistant patients (Oyrer et al., 2018; Perucca and Perucca, 2019). Newer agents including cannabidiol and the amphetamine analogue, fenfluramine, proved beneficial as add-on in clinical trials (Cross et al., 2019; Perucca and Perucca, 2019; Lagae et al., 2020). However, the mechanistic basis of efficacy of these drugs remains unclear. Cannabidiol has been shown to be a nonselective inhibitor of recombinant voltage-gated sodium channels at concentrations that could be relevant, therapeutically (Ghovanloo et al., 2018). At higher doses, cannabidiol protects against thermally-induced seizures in the *Scn1a*$^{+/-}$ mouse model of DS (Kaplan et al., 2017). Furthermore, an antagonist of the atypical cannabinoid receptor GPR55 blocked the action of cannabidiol, raising the possibility that cannabidiol may exert an anti-seizure action in DS through GPR55 receptors (Kaplan et al., 2017). Recently, by using inhibitory and excitatory neurons derived from DS patient's stem cells, it was shown that therapeutic concentrations (50 nM) of cannabidiol reduce the excitability of excitatory neuron and increase the firing rate of inhibitory cells, suggesting a cell type-dependent mechanism for the therapeutic action of cannabidiol, independent from sodium channel activity (Sun and Dolmetsch, 2018). An exploratory approach to treat *SCNA1*-related disorders, selectively, is based on Nav1.1 expression enhancement. Antisense oligonucleotide (ASO) designed to block a RNA-based regulatory mechanism rescued at least in part the seizure phenotype in a DS mouse model (Hsiao et al., 2016). Evidences from an animal model with haploinsufficiency for *Scn8a* (Nav1.6) suggest that reduction in the activity of Nav1.6 channels might increase survival and seizure resistance in *Scn1a* mutant mice, and be an alternative useful drug target for Nav1.1-associated seizures (Martin et al., 2007; Wong et al., 2018; de Lera Ruiz and Kraus, 2015). A single treatment with an antisense oligonucleotide (ASO) against *Scn8a* extended survival of heterozygous *Scn1a* knock-out mice from 3 weeks to >5 months (Lenk et al., 2020). In addition, CRISPRa-mediated correction of *Scn1a* defect restores inhibitory interneuron excitability and ameliorates seizures in DS mice (Colasante et al., 2020a). Rescuing approaches aimed at promoting the surface expression of sodium channel with folding-defective mutations associated with GEFS+ and DS hold promise for future treatment (Terragni et al., 2018).

SCN2A (Nav1.2)

Another overrepresented gene in the genetics of NDDs is *SCN2A*. Dominant mutations in *SCN2A*, encoding for the Nav1.2 sodium channel, were initially associated to Benign Familial Neonatal-Infantile Seizures (BFNIS), a mild seizure phenotype that responds positively to treatment with typical AEDs and generally remits by the age of 1–2 years (Berkovic et al., 2004). *De novo* mutations are now known to cause a wide spectrum of severe epileptic neurodevelopmental phenotypes, including Ohtahara syndrome, epilepsy of infancy with migrating focal seizures (EIMFS), infantile spasms (West syndrome), Lennox-Gastaut syndrome, Dravet-like syndrome, or autism, intellectual disability, ataxia, with or without epilepsy (Sanders et al., 2018; Wolff et al., 2019). In patients with epilepsy and *SCN2A* mutations, seizures may initiate from the first week of life, possibly due to the timing of Nav1.2 channel expression, which peaks during early development and is then gradually replaced by Nav1.6 (*SCN8A*), during the first months of life (Smith and Walsh, 2020; Du et al., 2020).

Among ion channel-associated NDDs, a genotype-phenotype correlation has been outlined for *SCN2A* syndromes. Out of 140 *SCN2A* identified variants, about 40 have been functionally characterized, classified according to their functional GoF or LoF defects and related to three main underlying diseases and drug response. GoF defects mainly recapitulate neonatal-infantile seizure phenotypes (<3 months; BFNIS or EIMFS or Ohtahara) and better responsiveness to sodium channel blockers. LoF truncating or missense mutations have been associated to infantile-childhood severe seizures (>3 months) or ASD and intellectual disability, with poor response to sodium channel block (Ben-Shalom et al., 2017; Wolff et al., 2019; Sanders et al., 2018; Brunklaus and Lal, 2020). Seizure severity is consistent with the degree of GoF and nature of the genetic variant, with *de novo* high-impact mutations causing infantile epileptic encephalopathy, and inherited less defective variants leading to remitting BFNIS without apparent neurological perturbation (Sanders et al., 2018; Hedrich et al., 2019). *SCN2A, SCN3A* and *SCN8A* are all expressed predominantly, but not exclusively, in excitatory neurons. Diverse animal models recapitulating Nav1.2 GoF diseases are available and have shown increased excitability in hippocampal excitatory pyramidal neurons, neonatal seizures and ataxia, in agreement with this prominent expression of *SCN2A* (Kearney et al., 2001; Schattling et al., 2016).

As said before, *SCN2A* is highly expressed in fetal brain and is in part replaced by *SCN8A* during development at axon initial segment in hippocampus and cortex and nodes of Ranvier. The co-existence of *SCN2A* and *SCN8A* genes in excitatory neurons claims for some degree of compensation of neuronal excitability, which, albeit not bringing to complete remission of phenotypes, might set the threshold that distinguishes severe epilepsy from milder forms (Van Wart and Matthews, 2006; Sanders et al., 2018). Another mechanistic hypothesis has been proposed to explain the impact of LoF *SCN2A* variants in NDDS. Namely, a decreased excitability of cortical and hippocampal excitatory neurons during development would be the early event that triggers a persistent later alteration in neuronal activity, leading to ASD and severe epilepsy phenotype (Ben-Shalom et al., 2017; Ogiwara et al., 2018). *SCN2A* is also

present at unmyelinated axons which may innervate inhibitory interneurons in the adult brain. Reduced functioning of GABAergic interneurons due to LoF Nav1.2 dysfunction, later in development, cannot be ruled out as well (Sanders et al., 2018; Brunklaus and Lal, 2020). Very recently, Nav1.2 was shown to be important for regulating excitability and synaptic strength not only in the developing axons but also in dendrites of mature cortical pyramidal cells. In fact, heterozigous *Scna2* knock-out mice show, in addition to impaired action potential initiation, reduced back-propagation of action potentials into distal dendrites of neocortical pyramidal cells. This event would likely hamper synaptic strength and plasticity even later in development, and might reinforce the cortical dysfunctions and behavioral abnormalities predicted to occur in ASD (Spratt et al., 2019). While homozygous *Scn2a* knock-out mice die within 1–2 days of birth, heterozygous animals are seizure-free, suggesting the need to develop reliable knock-in mice carrying human mutations to model properly LoF diseases associated with *SCN2A* mutations (Planells-Cases et al., 2000). Interestingly, *SCN2A* can exist in a neonatal and an adult form with distinct biophysical properties, but whether these two isoforms have a pathophysiological significance is unknown (Hedrich et al., 2019).

Drug response in the presence of a *SCN2A* mutation is highly dependent on the clinical phenotype and molecular defects of probands. This reinforces the need and utility of the biophysical characterization of novel genetic variants prior to therapy initiation. Despite sodium channel blockers are not recommended in carriers of *SCNA1* and *SCNA2* LoF mutations, as they are mostly not effective or aggravate seizures, some of these drugs are reasonably useful in ameliorating disease caused by GoF *SCNA*2 and *SCN8A* mutations (Wolff et al., 2019; Boerma et al., 2016). In neonatal-early infantile epilepsy caused by GoF *SCN2A* mutations, high dose of phenytoin generally ensures seizure freedom. Also, the sodium channel blockers carbamazepine, lacosamide, or zonisamide can be used as first-line medications and are beneficial in a significant number of cases. Of note, phenobarbital and levetiracetam, which are commonly used in neonatal seizure treatment, are largely ineffective in *SCN2A*-related neonatal-early infantile developmental epileptic encephalopathies (Wolff et al., 2019). West syndrome patients carrying LoF *SCN2A* mutations are typically resistant to treatment, including steroids or adrenocorticotropic hormone, suggesting that this *SCN2A*-related disease is particularly difficult to treat. Among the patients with other LoF infantile/childhood onset epilepsy phenotypes, seizure reduction or freedom occurred frequently with levetiracetam, benzodiazepines, and valproic acid (Wolff et al., 2019).

SCN8A (Nav1.6)

De novo mutations in *SCN8A,* encoding for the Nav1.6 isoform, have been identified in children affected by early-onset epileptic encephalopathy characterized by developmental delay, seizure onset within the first 12 months of life and intractable epilepsy, dyskinesia and risk of cardiac arrhythmias (Epi25 Collaborative, 2019; Gardella and Møller, 2019). As for *SCN1A* and *SCN2A*, autosomal dominantly inherited *SCN8A* mutations have been also associated with self-remitting BFNIS without cognitive delay (Brunklaus and Lal, 2020; Gardella and Møller, 2019). SCN8A is expressed throughout the brain in excitatory and, at lower level, in inhibitory neurons and at nodes of Ranvier of myelinated axons. Commonly, SCN8A mutations associated with epileptic encephalopathy produce GoF defects with enhanced persistent sodium current and incomplete channel inactivation (Brunklaus and Lal, 2020). A knock-in mouse model carrying the GoF mutation N1768D recapitulates the seizure phenotype, ataxia and SUDEP seen in the human carrier and, consistently, shows increased excitability in both the cortex and hippocampus, gliosis and abnormal function of the Na^+/Ca^{2+} exchanger (Lopez-Santiago et al., 2017; Meisler, 2019). This animal model also shows myocytes hyperexcitability with lower threshold for action potential generation, increased resting calcium, parasympathetic neuron hyperexcitability and high risk of fatal cardiac arrhythmias (Frasier et al., 2016). The conditional activation of R1872W, specifically in excitatory neurons of the forebrain, is sufficient to initiate seizures and leads to death in both developing and adult mice. These effects are consistent with the principal expression of Nav1.6 channels at the axon initial segment of excitatory neurons (Meisler, 2019). As mentioned before, Nav1.6 replaces Nav1.2 as the dominant sodium channel in excitatory neurons in infancy, which may explain the later age of seizure onset caused by *SCN8A* mutations with respect to *SCN2A* ones (Du et al., 2020). Notably, *SCN8A* LoF mutations result in intellectual disability without epilepsy in both humans and mouse models (Gardella and Møller, 2019; Brunklaus and Lal, 2020). The specific loss of Nav1.6 in pyramidal neurons appears to be protective against convulsive seizures (Makinson et al., 2017). As anticipated above, reduced Nav1.6 function was also able to ameliorate seizure severity in a *Scn1a* mouse model of DS, suggesting *SCN8A* as a modifier of disease (Martin et al., 2007).

All GoF *SCN8A*-related epilepsies exhibit better seizure control with sodium channel blockers, usually at supratherapeutic doses in the severe cases (Meisler, 2019). Extensive work is underway in order to reduce GoF defects by targeting the persistent sodium current with riluzole or cannabidiol in drug-resistant seizures. Reduction of *SCN8A* transcript is another promising approach for the treatment of intractable childhood epilepsies. Interestingly, reducing the abundance of the *Scn8a* transcript with ASO delayed seizure onset and prolonged survival in a mouse model carrying the *SCN8A* GoF mutation R1872W associated with early onset encephalopathy in humans (Lenk et al., 2020). The polyciclic selective Nav1.6 blocker GS967 demonstrated efficacy in reducing spontaneous seizures and prolonging survival time both in heterozygous N1768D *Scn8a* mice and *Scn1a* DS and *Scn2A* mice (Anderson et al., 2017; Meisler, 2019). Phase 1 clinical trials of this compound are in progress. Repurposing of drugs targeting the Na^+/Ca^{2+} exchanger, including amiodarone and bepridil might also be a potential therapeutic opportunity (Oyrer et al., 2018). In severe

SCN8A NDDs, ketogenic diet often has a good effect, whereas levetiracetam has a negative effect, if any (Meisler, 2019).

SCN3A (Nav1.3)

SCN3A and *SCN9A* encoding for the Nav1.3 and Nav1.8 isoforms have been linked to similar severe NDDs (Brunklaus and Lal, 2020). Consistent with the expression of *SCNA3* during embryonic development, GoF Nav1.3 mutations are associated with heterogeneous phenotypes with malformation of cortical development and oral motor deficits but not seizures (Smith and Walsh, 2020; Oyrer et al., 2018). LoF and GoF mutations in the same *SCN3A* gene have also been found in patients with neonatal onset seizures and epileptic encephalopathy without clear sign of cortical malformations (Smith and Walsh, 2020). The expression of *SCN3A* is highest in the cortex at the prenatal stage and decreases with age, suggesting that disruption of this latter gene underpins prenatal disorders and cortical malformations, similarly to some *GRIN* genes (Brunklaus and Lal, 2020). Future work is needed to elucidate the subtle role of sodium flux in the developing cortex and the impact of *SCNA3* modifications in pre- and postnatal development, resulting in malformation in cortical development versus infantile epilepsy (Smith and Walsh, 2020).

POTASSIUM CHANNELS IN NDDS

KCNQ2/3 (Kv7.2/Kv7.3)

Several heterogeneous epilepsy syndromes with developmental delay support the notion that both LoF and GoF defects occur in potassium channel genes. Mutations in the *KCNQ2* genes, coding for Kv7.2 channels, have been associated with a broad spectrum of human epilepsies ranging from benign familial neonatal convulsions (LoF; BFNS1) to more severe neonatal onset epileptic encephalopathy characterized by tonic seizures and profound developmental impairment (Steinlein et al., 2007; Weckhuysen et al., 2012; Lee et al., 2018). More than 80 *KCNQ2* mutations have been identified in BFNS families (database for *KCNQ2* mutations: http://www.hgmd.cf.ac.uk). It has been proposed that the *KCNQ2* mutation is "pan-ethnic", since there are more case reports on Kv7.2-channelopathy in Asia rather than in other regions of the globe. Notably, some patients with *de novo KCNQ2*-associated neonatal epileptic encephalopathy display global neurodevelopmental disability and autistic traits.

In general, haploinsufficiency caused by the LoF of a single *KCNQ2* allele is the most common cause of *KCNQ2*-related BFNS. LoF via the dominant-negative effect of the mutated *KCNQ2* gene or GoF are presumed to be the major mechanisms underlying *KCNQ2* encephalopathy and comorbidities (Lee et al., 2018). Heteropolymerization of the potassium channel subunit Kv7.2 (*KCNQ2*) with Kv7.3 (*KCNQ3)* generates "M" channels which are the principal mediator of muscarine-induced depolarization in neurons (Wang et al., 1998). M channels generate a typical sub-threshold potassium conductance that contributes to setting the resting membrane potential of neurons, thus controlling cell excitability and preventing repetitive firing. The expression of this channel type is finely tuned during development, as the relevant current density undergoes enhancement during postnatal development along with the occurrence of more organized forms of electrical activity, such as theta and gamma rhythms (Buzsáki and Draguhn, 2004). This property could explain some developmental-related clinical features of the disease associated with *KCNQ2* mutations, such as the onset of the epilepsy during second and eighth day of life and the spontaneous disappearance later in life.

Rare mutations in the *KCNQ3* gene have been detected and associated with benign familial neonatal seizures-2 (BFNS2) (Neubauer et al., 2008). Usually, brain imaging and psychomotor development are normal in the reported cases. However, *de novo* interstitial deletion or reciprocal translocation in the *KCNQ3* have been identified in children with a broad spectrum of congenital abnormalities, psychomotor delay, convulsions and autistic traits (Verheij et al., 2009; Gilling et al., 2013). Recently, several clinical cases have been reported that are characterized by seizures-freedom during the neonatal period, although, within the first 2 years of life demonstrated global neurodevelopment disability, ASD, autistic traits and abundant sleep-activated discharges recorded with EEG. These patients carried *KCNQ3* mutations resulting in GoF of Kv7.3 channels (Sands et al., 2019). Given the importance of M current in the development of neuronal identity and regulation of excitability, its impairment in immature neurons could delay the definition of complex neuronal rhythms that possibly results in NDDs.

Most cases of BFNS can be controlled with phenobarbital, oxcarbazepine, vigabatrin, topiramate and valproate (Neubauer et al., 2008; Weckhuysen et al., 2012). Clobazam, ethosuximide, lacosamide, levetiracetam, amotrigine, oxcarbazepine, rufinamide and ketogenic diet have been prescribed to treat children carrying GoF mutations in *KCNQ3*. Moreover, high dose of diazepam (1 mg/kg) and corticosteroids have been administered to control their sleep-activated discharges with inconsistent effect on behavior (Sands et al., 2019). Retigabine (Ezogabine) and flupirtine, are both able to activate Kv7 channel and ameliorate the functional defects caused by the mutations, *in vitro* and *in vivo*, although their toxicity prevents clinical exploitation (Raol et al., 2009; Porter et al., 2012). Recently, it has been shown that gabapentin activates the heteromeric *KCNQ2/3* potassium channel (Manville and Abbott, 2018). These findings could pave the way for the synthesis of a new class of *KCNQ* channel activators with higher specificity and potency for therapeutic use. Importantly, the preclinical antiepileptogenesis evaluation of potentially repurposable molecules or their combinations should be promoted (Klein et al., 2020).

KCNA1 and *KCNA2* (Kv1.1 and Kv1.2)

Belonging to the same *Shaker* subfamily of voltage-gated potassium channels, *KCNA1* and *KCNA2* genes,

encoding for Kv1.1 and Kv1.2 channels, are involved in ataxia and epileptic phenotypes. *KCNA1*-associated diseases provides another clear example of phenotypic variability as the clinical characteristics of mutations in this gene can span from simple myokymia to epileptic encephalopathy. *KCNA1* is the only known gene causatively associated with episodic ataxia type 1 (EA1), a potassium channelopathy characterized by constant myokymia and episodic contractions of the skeletal muscles with loss of motor coordination. Despite initially considered an almost exclusive motor disease of cerebellar origin, the screening of more affected families has made now clear that EA1 individuals are more prone to develop epilepsy and may also present cognitive dysfunction (D'Adamo et al., 2020). The definite association of *KCNA1* with human epilepsy and developmental deficits came up with the identification of one heterozygous recessive and three *de novo* mutations within the H loop and PVP motif (V368L and P405S/L and P403S) of the Kv1.1 channel in children with drug resistant seizures, motor and cognitive impairment (Rogers et al., 2018; Verdura et al., 2019). In particular, twins carrying the same mutation displayed very different phenotypes and response to AEDs (Rogers et al., 2018). Recently, LoF and GoF heterozygous missense mutations in *KCNA2* gene have been also identified and characterized, in patients presenting with early infantile epileptic encephalopathy, intellectual disability, delayed speech development and sometimes ataxia (Masnada et al., 2017).

In vitro and *in vivo* functional studies provided valuable information linking Kv1.1 and Kv1.2 to the occurrence of ataxia and epilepsy in humans. Several mutations in the *KCNA1* gene have been well characterized at molecular level and all induce LoF defects in Kv1.1 channels biophysical properties, membrane expression and modulation, regardless of the clinical phenotype (D'Adamo et al., 2015, Imbrici et al., 2007, 2011, 2017). Therefore, a genotype-phenotype correlation is far from being drawn. Kv1.1 channels are located at the cerebellar basket cells terminals where they promote GABA release onto Purkinje cells, in the hippocampus where their expression confers seizure protection and at the nodes of Ranvier of myelinated nerves where they ensure safe neuromuscular transmission, and can heteropolimerize with other Kv1 subunits (D'Adamo et al., 1998, 2020). A rodent knock-in mouse, carrying the episodic ataxia mutation V408A, allowed to clarify that ataxic gaits result from altered GABAergic neurotransmission in the cerebellum, whereas myokymia occurs as a consequence of increased excitability of myelinated axons during high electrical activity or ischemic insult (Herson et al., 2003; Brunetti et al., 2012; Begum et al., 2016). The homozygous *Kcna1* knock-out mouse, instead, clarified the origin of seizures in humans as it displays spontaneous early onset seizures and premature death, likely due to impaired network excitability in the hippocampus (Smart et al., 1998; Simeone et al., 2013). Seizures onset parallel the developmental expression of *KCNA1* in the brain as they manifest at about day 15 after birth. This mouse is considered a model to study SUDEP, and *KCNA1* is therefore a candidate gene to screen when SUDEP occurs (Klassen et al., 2014). Protective or deleterious interactions between co-existing pathogenic ion channel variants may markedly alter the clinical expression of epilepsy in affected individuals. Interestingly, impairin *Cacna1a* function in *Kcna1*-null mice attenuated limbic seizures and reduced risk of sudden death, by decreasing network excitability. Similarly, removing *Kcna1* channels masked the absence epilepsy in a mouse model carrying a missense mutation in the *Cacna1a* gene (tottering mice), by increasing axon terminal excitability and improving synaptic transmission (Glasscock et al., 2007). The same *Kcna1* mice with partial genetic ablation of *Scn2a* exhibited reduced duration of spontaneous seizures and significant improvement of survival rates, confirming that sodium channel blockers may have some treatment value (Mishra et al., 2017).

KCNA2 mutations can bring to both GoF, LoF and even mixed functional defects (Masnada et al., 2017). A genotype-phenotype correlation has been tempted suggesting that different pathophysiological mechanisms correspond to distinct clinical presentations. Indeed, less severe phenotypes, occurring in the infantile and childhood age, are associated with LoF mutations (such as I263T and P405L), whereas *de novo* GoF mutations (such as R297Q, L298F, E157K) are linked to more severe epileptic phenotypes beginning at neonatal age and followed by cognitive delay and cerebellar atrophy (Syrbe et al., 2015; Masnada et al., 2017; Niday and Tzingounis, 2018). The cellular basis of epilepsy due to either LoF or GoF *KCNA2* mutations is not clear, as Kv1.2 is expressed in both excitatory and inhibitory neurons. The association between Kv1.2 LoF mutation and neuronal hyperexcitability is more obvious and would be supported by the neuronal phenotype of *Kcna2* knock-out mice, which exhibit spontaneous severe seizures by postnatal day 15 and even shorter lifespan than *kcna1* knock-out mice, with premature death occurring approximately at 19 days of age (Brew et al., 2007). Mice carrying a point mutations in *Kcna2* (Pingu mice) show altered cerebellar Purkinje cells output that could explain the observed ataxic phenotype (Xie et al., 2010). In excitatory glutamatergic pyramidal neurons, Kv1.2 LoF mutations would likely impair neuronal repolarization and activate repetitive firing. GoF mutations of Nav1.2 channels, expressed in inhibitory interneurons, could as well drive epilepsy by favoring membrane hyperpolarization and silencing neuronal firing, finally leading to reduced GABA release and network disinhibition, a mechanism similar to that underlying DS (Masnada et al., 2017; Niday and Tzingounis, 2018). As for Kv1.1, mutations in Kv1.2 channels could also impact the activity of heteromeric Kv1.2-containing channels, alter the excitability of specific cell types and thus cause variable phenotypes (D'Adamo et al., 1999, 2020; Masnada et al., 2017). Future studies using knock-in mice and patients' iPSCs-derived neurons are needed to determine the exact mechanisms by which Kv1.1 and Kv1.2 mutations lead to complex epileptic encephalopathy (Schwarz et al., 2019). How Kv1.1 and Kv1.2 channel defects result in cognitive dysfunction

and developmental disabilities is as well difficult to postulate to date.

Among patients presenting Kv1.1 and Kv1.2 mutations linked to NDDs, most are often unresponsive to current antiepileptic therapies. Indeed, in some patients epilepsies originating from Kv1.1 and Kv1.2 mutations only improve with a combination of different AEDs such as lamotrigine and valproic acid, oxcarbazepine, clobazam, whereas in others convulsive episodes are severe and drug-resistant. Acetazolamide also remit successfully ataxia and myoclonic epilepsy caused by *KCNA2* mutations in some patients (Pena and Coimbra, 2015; Masnada et al., 2017). The development of Kv1.2 blockers would be greatly appreciated for the treatment of epilepsy caused by GoF defects and actually the approved potassium blocker 4-aminopyridine is being trialed in patients carrying GoF mutations (Allen et al., 2020; Oyrer et al., 2018). Animal models, again, provided compelling evidence to help addressing the best treatment opportunity for a given patient. Homozygous Kv1.1 knockout mice treated with a ketogenic diet showed reduced seizure frequency and extended the life span (Ren et al., 2019), suggesting this as a therapeutic options for some probands. The well-known ability of Kv1.1 in dampening neuronal excitability has been recently exploited by preclinical gene-therapy approaches to reduce seizure frequency in refractory epilepsy (Snowball et al., 2019; Colasante et al., 2020b). Using a non-integrating lentiviral vector and an excitatory neurons-specific promoter, Kv1.1 overexpression was shown to reduce seizures in a rat model of focal epilepsy (Snowball et al., 2019). In addition, CRISPRa-mediated overexpression of Kv1.1 channels in mouse hippocampal excitatory neurons successfully decreased spontaneous generalized tonic-clonic seizures and recovered cognitive deficits in a mouse model of intractable chronic temporal lobe epilepsy (Colasante et al., 2020b). Of course, further preclinical studies are essential to validate long term efficacy and safety of gene-based therapy before translation in humans. Interestingly, everolimus, an mTOR inhibitor recently approved for the treatment of focal seizures associated with tuberous sclerosis complex, has also been shown to normalize Kv1.1 expression in a mouse model of cortical dysplasia with epilepsy, indicating the possibility to further explore this drug in Kv1.1-related disorders (Nguyen and Anderson, 2018).

KCNT1 (Slack or $K_{Na}1.1$)

Over the past few years, a wide spectrum of epileptic phenotypes has been associated with inherited and *de novo* mutations in the *KCNT1* gene, encoding for a sodium-activated potassium channel subfamily T member 1 (also termed Slo2.2 or Slack or $K_{Na}1.1$), involved in the slow hyperpolarization that follows repetitive neuronal firing (Hasan et al., 2017; Allen et al., 2020). Commonly, affected individuals develop intractable seizures at a younger age, or epilepsy of infancy with migrating focal seizures (EIMFS; 40% of these patients carry a mutation in *KCNT1*) or ADNFLE, have cognitive and motor comorbidities and display psychiatric and behavioral problems. Occasionally, cardiac arrhythmia has been reported (Gertler et al., 2018; Borlot et al., 2020).

KCNT1-related epilepsy is reported to occur through diverse GoF molecular mechanisms including enhanced sodium sensitivity, channels cooperativity or open probability (Niday and Tzingounis, 2018). Of note, the magnitude of GoF has been proposed to positively correlate with disease severity (Ohtahara syndrome 13-fold, EIMFS fivefold, severe ADNFLE threefold compared to wild type) (Gertler et al., 2018). At present, the cellular mechanisms underlying phenotypes caused by *KCNT1* mutation are still unclear, as the physiological role of this channels type is not fully understood. $K_{Na}1.1$ channels are expressed at higher levels in glutamatergic neurons of the mouse and rat cerebral cortex, whereas little or no expression has been detected in inhibitory interneurons (Quraishi et al., 2020). Faster activation carried by GoF mutants following sodium influx during an action potential may therefore sustain rapid repolarization and enhance action potential firing rate in excitatory neurons (Bhattacharjee and Kaczmarek, 2005; Niday and Tzingounis, 2018). This hypothesis has been supported by functional studies using iPSC-derived neurons that carry an EIMFS mutation. Indeed, these cells exhibit shorter action potentials and larger afterhyperpolarizations allowing more rapid firing (Quraishi et al., 2020). Further mechanistic clues may arise from the recent generation of *KCNT1* animal models. Interestingly, *Kcnt1* knock-out mice show deficits in open field behavior and motor skill learning but have no spontaneous seizure and show strong seizure protection. Conversely, embryonic lethality has been observed in homozygous *Kcnt1* mice carrying the GoF EIMFS mutation R455H. Instead, heterozygous $Kcnt1^{+/R455H}$ animals exhibit spontaneous seizures, increased sensitivity to pentylenetetrazole-induced seizures and increased mortality but normal functioning in behavioral and motor learning tasks (Quraishi et al., 2020). These findings are consistent with the hypothesis that larger $K_{Na}1.1$ currents correlates with epilepsy phenotype in patients, and provide important knowledge to the understand SUDEP susceptibility and protection. More importantly, the striking discrepancy between the abnormal behavior and learning abilities displayed by homozygous *Kcnt1* knock-out mice and the epileptic phenotype with normal behavior observed in $Kcnt1^{+/R455H}$ mice suggests that two different pathways account for such different phenotypes. The behavioral dysfunction reported in some patients could be due to frequent seizures, aberrant development from abnormal firing patterns, altered synaptic connectivity, or impaired interaction of $K_{Na}1.1$ channels with cellular proteins essential for proper synaptic plasticity and development (Quraishi et al., 2020). Further studies using patients' cell lines and younger animal models are however required to better clarify these points.

KCNT1-related epilepsy is often refractory to conventional anticonvulsants. Stiripentol, benzodiazepines, levetiracetam, although well tolerated, had limited success. Quinidine has been used as an off-label anticonvulsant with success in some, but not all, individuals with GoF defect in *KCNT1* and *KCNT2*

(Gertler et al., 2018). A recent randomized trial in individuals with ADNFLE failed to show efficacy and safety, suggesting that caution must be taken when interpreting results of single-case reports and strengthening the need for careful preclinical evaluations of efficacy before translating results of novel therapies into human settings (Mesraoua et al., 2019). Despite contrasting results and side effect, this drug could be, however, a good lead compound for the development of more specific and better tolerated medications targeting $K_{Na}1.1$. Cannabidiol and ketogenic diet also seem to reduce seizures, although, evidence-based practice is still unavailable (Borlot et al., 2020).

KCNJ10 (Kir4.1)

Neurodevelopmental symptoms have been shown as a consequence of mutations in the inwardly-rectifying potassium channels Kir4.1 (*KCNJ10*) (Guglielmi et al., 2015). Notably, digenic mutations causing LoF in Kir4.1 (*KCNJ10*) and GoF in $K_{Na}1.1$ (*KCNT1*) have been associated with severe-disabling seizures, NDD and lethality (Hasan et al., 2017). LoF mutations in Kir4.1 have also been identified in patients with brain and renal complications named EAST (Epilepsy, Ataxia, Sensorineural deafness, Tubulopathy) or SeSAME (Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance) syndrome (Bockenhauer et al., 2009; Sala-Rabanal et al., 2010; Williams et al., 2010). Kir4.1 mutations identified in patients with EAST/SeSAME syndrome reduce the activity of both homomeric Kir4.1 and heteromeric Kir4.1/Kir5.1 channels through distinct mechanisms that can account for the kidney and neuronal clinical phenotype (Sala-Rabanal et al., 2010). Indeed, Kir4.1 channels are also expressed in the kidney, where they contribute to maintain a negative membrane potential that drives ion pumps and exchangers, and in the inner hear, where they play a role in potassium regulation and generation of the endocochlear potential, essential for normal development of audition (Hibino et al., 2010).

Interestingly, three heterozygous *KCNJ10* mutations (R18Q, V84M and R348H) have been found in children with a characteristic autism-epilepsy phenotype (AEP) and NDD (Sicca et al., 2011, 2016). Kir4.1 R18Q, V84M and R348H mutant channels associated with autism, epilepsy and NDD features, expressed in Xenopus oocytes and in astrocyte-like cells, exert a GoF effect by enhancing Kir4.1 membrane expression, single channel conductance and decreasing pH sensitivity, respectively. These evidence could be considered as putative molecular mechanisms underlying the disorder (Sicca et al., 2011, 2016).

However, the pathophysiologic mechanisms responsible for the increased susceptibility to ASD and seizure underlined by Kir4.1 channel dysfunction are not fully understood, and only preliminary hypothesis can be formulated. In the brain, Kir4.1 channels are expressed in oligodendrocytes and in astrocytes surrounding synapses and blood vessels where they contribute to the clearing and spatial buffering of K^+ released by neurons during action potential propagation, an essential mechanism ensuing proper synaptic activity (Chever et al., 2010; Hibino et al., 2010). The causative link between Kir4.1 alteration and both seizure susceptibility and cognitive dysfunctions, as discussed before for other channel types, might be at least in part attributed to timely channel expression and function in neurons and astrocytes during development. Dysfunction in the astrocytic-dependent K^+ buffering during postnatal development, resulting in abnormal synaptic transmission and electrical discharge, might thus contribute both to epilepsy and ASD traits in affected children, and may represent a new target for therapeutic approaches (Sicca et al., 2011; Guglielmi et al., 2015; Sibille et al., 2015). Kir4.1 GoF may allow neurons to fire for longer periods of time or at higher frequencies by hyperpolarizing membrane potential and preventing sodium channel inactivation. Several evidences from animal models of ASD come in support of this hypothesis. Indeed, an up-regulation of Kir4.1 has been found in *locus coeruleus* neurons of the *MECP2*-null mice, an animal model of Rett syndrome (Zhang et al., 2010). Here, Kir4.1 over-expression might impair noradrenergic modulation, leading to the autistic behaviors (Zhang et al., 2010). Relevant clues can be drawn from Kir4.1 knock-out mice, which display seizure, hearing loss and retinal dysfunction, altered K^+ transport in hippocampus, impaired glutamate uptake by astrocytes and motor impairment due to hypomyelination (Kofuji et al., 2000; Neusch et al., 2001). Furthermore, conditional astrocytic ablation of Kir4.1 channels in mice result to: (*i*) small brain, (*ii*) lethargy, ataxia, and stress-induced seizures, (*iii*) severe depolarization of astrocyte resting potential, (*iv*) reduced astrocytic uptake of both K^+ and glutamate, (*v*) reduced spontaneous excitatory post-synaptic currents in hippocampal CA1 pyramidal cells, and (*vi*) elevated hippocampal post-tetanic and short-term potentiation, supporting the role of Kir4.1 in astrocyte development, astrocyte-neuronal cross-talk, and synaptic strength (Djukic et al., 2007).

It has been also reported on identical tweens displaying ASD and Short QT3 syndrome (SQT3S) who carried GoF mutations in both Kir4.1 (R18Q) and Kir2.1 (K346T) channels (Ambrosini et al., 2014). Although it is formally possible that the *KCNJ2* mutation *in cis* with *KCNJ10* contributes separately to SQT3S or ASD, each playing a clear distinctive role, this conclusion could underscore the potential contribution of GoF mutation in Kir2.1 in NDD. Indeed, Kir2.1 channels are highly expressed in the brain, particularly in hippocampus, caudate, putamen, nucleus accumbens, habenula and amygdala, all areas implicated in cognition, mood disorders and ASD.

OTHER POTASSIUM CHANNELS GENES

Mutations in other potassium channels have been involved in NDDs including GoF mutations in *KCNT2* (Ambrosino et al., 2018), LoF in *KCNB1* (Kv2.1) (de Kovel et al., 2017; Niday and Tzingounis, 2018; Bar et al., 2020), mutations in *KCNC1* (Kv3.1), in *KCND2* (Kv4.2) (Lin et al., 2018) and LoF mutations in *KCND3* (Kv4.3) (Allen et al., 2020).

GoF *KCNMA1* (K_{Ca}1.1 or BK) mutations cause early onset epilepsy and paroxysmal dyskinesia (Tabarki et al., 2016; Allen et al., 2020), whereas, LoF have been associated with ataxia (Bailey et al., 2019). The consequences of these mutations on neuronal activity are still difficult to predict. Of relevance to this matter is the notion that calcium influx, occurring during the action potential of pyramidal neurons, activates BK channels which promote faster afterhyperpolarizations, faster recovery from inactivation of sodium channels and shortened action potential width (Niday and Tzingounis, 2018). Notewhorty, some GoF mutations in BK channels increase the coupling between calcium binding and channel opening that could lead to increased repolarization of the soma and axon initial segment, resulting in higher firing frequency in excitatory neurons (Niday and Tzingounis, 2018).

CALCIUM CHANNELS IN NDDS

CACNA1C (Cav1.2)

Mutations in the *CACNA1C* gene, encoding for the Cav1.2 voltage-gated L-type calcium channel, have been identified in children affected by Timothy syndrome (TS) (Liao and Soong, 2010; Napolitano et al., 2016). Despite TS is considered a very rare form of long QT syndrome, ventricular tachyarrhythmia and the principal cause of death, affected patients also display complex multi-organ dysfunctions including autism and developmental delay, syndactyly, dysmorphism, intermittent hypoglycemia, immunodeficiency (Han et al., 2019). The most severe form (TS2) results from two *de novo* mutations, G406S and G402R, occurring in the alternatively spliced exon 8 of Cav1.2 isoform, expressed by 80% in brain and heart (Splawski et al., 2004; Napolitano et al., 2016). Several cardiac-only mutations in *CACNA1C* have been reported, as well as mutations in other exons of the same gene associated with severe central phenotypes (Han et al., 2019). The A1473G in exon 36 was linked to early onset intractable epilepsy, developmental delay and stroke (Gillis et al., 2012). Very recently a *de novo* missense mutation in exon 33 (V1363M) has been identified in a child with neonatal onset intractable epilepsy with severe cognitive and psychomotor delays (Bozarth et al., 2018). In another family, a splice site variant in one member caused late onset epilepsy with learning difficulties (Bozarth et al., 2018). Recently, it has been described a children with TS and photosensitive epilepsy due to the *de novo* mutation E407G in exon 9 of the same gene (Pò et al., 2019). These new case reports greatly expand the spectrum of *CACNA1C*-related phenotypes and suggest that children with ASD, epilepsy with photoparoxysmal response and LQTS should undergo gene sequencing of the entire coding region of *CACNA1C* gene.

Cav1.2 channels is predominantly expressed in the hippocampus, thalamus, cerebral cortex, and cerebellum and play a key role in long-term synaptic plasticity, gene expression, muscle contraction and hormone release (Simms and Zamponi, 2014; Catterall et al., 2020). The variable clinical spectrum reported in TS patients may be accounted for by the numerous Cav2.1 isoforms generated by alternatively spliced exons, variable expression of different transcripts in tissues, and the position of the mutation in the structural domain of the Ca_v1.2 channel. The functional characterization of Cav1.2 TS mutations in heterologous expression systems revealed a GoF of the channel due to loss of voltage-dependent inactivation (Splawski et al., 2004). This functional defect easily explain the prolonged cardiac action potential and abnormally long QT interval typically observed in patients, but may also contribute to the observed widespread dysfunction of some probands (Liao and Soong, 2010; Bidaud and Lory, 2011). Important insights into the complex pathogenesis of TS come from recent studies in animal models and iPSCs-derived neurons or cardiomyocytes of affected patients. iPSCs-derived cardiac cells from TS patients revealed calcium overload, prolonged action potential duration and irregular contraction in ventricular-like cells and demonstrated the ability of calcium, sodium and beta-blockers to inhibit abnormal calcium influx and restore Cav1.2 channel functions (Yazawa and Dolmetsch, 2013; Napolitano et al., 2016). The generation of a TS mouse model carrying the G406R mutation, engineered to survive to adulthood (TS2-neo mice), confirmed the involvement of Cav1.2 in psychiatric disorders. Indeed, TS2-neo mice exhibited typical ASD traits, namely impaired social interaction and communication, restricted and repetitive behavior, and enhanced fear memory (Bader et al., 2011). Recently, studies from cortical neurons derived from iPSCs of TS patients showed defects in calcium signaling and altered activity-dependent gene expression counteracted by the cyclin-dependent kinase (CDK) inhibitor, roscovitine. These and other findings provide evidence that GoF Cav1.2 mutations likely alter the differentiation, migration and development of cortical neurons (corticogenesis) and affect neurotransmitter availability, thus contributing to the presentation of the autistic traits (Paşca et al., 2011; Panagiotakos et al., 2019; Kamijo et al., 2018). Further studies are however required to assess whether altered activity of Cav1.2 in the thalamus or hippocampus could mimic epilepsy as recently observed in some probands.

Owing to cardiac arrhythmia being the major life threatening risk for TS children, TS treatment includes the use of beta-blockers and/or other antiarrhythmic drugs to maintain QT interval stability and avoid ventricular tachyarrhythmia. Pacemakers are frequently used to prevent AV block and an implantable cardioverter defibrillator to elude sudden cardiac death is considered in all affected patients (Napolitano et al., 2016). Sodium channel blockers such as mexiletine and ranolazine have been reported to be effective in some TS patients, and reduce their risk of ventricular arrhythmia (Han et al., 2019). Combination of AEDs have been employed to remit seizures in single cases including valproic acid, clonazepam, clobazam, topiramate and ethosuximide (Pò et al., 2019; Bozarth et al., 2018).

CACNA1A (Cav2.1)

Among calcium channels, much attention has been given to high-threshold Cav2.1 P/Q-type voltage-gated calcium

channel encoded by the *CACNA1A* gene (Catterall et al., 2020). This gene has been conventionally associated with three neurologic conditions, Episodic Ataxia type 2 (EA2; LoF defects), Familial Hemiplegic Migraine 1 (FHM; GoF defects) and spinocerebellar ataxia type 6 (LoF defects) (Catterall et al., 2020). Early reports pointed out that EA2 patients also display epilepsy (Imbrici et al., 2004). Very recently, inherited deletion, truncation or de novo mutations in CACNA1A have been identified in children affected by severe early-infantile epileptic encephalopathy, severe intellectual disability, motor impairment and episodic ataxia (Damaj et al., 2015; Epi4K Consortium, 2016; Jiang et al., 2019; Oyrer et al., 2018). Thus, *CACNA1A* variants may be considered significant and recurring cause of severe NDDs.

Among the number of *CACNA1A* mutations associated with developmental epileptic encephalopathies and signs of cerebellar dysfunction (ataxia, nystagmus) reported to date, only few ones have been functionally characterized. One biophysical study of four *de novo CACNA1A* mutations has shown both GoF (A713T and V1396M) and LoF (G230 and I1357S) defects (Jiang et al., 2019). In the case of LoF mutations, a strong cytoplasmic inclusion has been reported, claiming that a trafficking defect and a dominant-negative effect on wild-type subunit could provoke the more severe phenotype of the *de novo* LoF mutations carriers, with respect to that caused by inherited variants (Jiang et al., 2019). As seen for mutations in other ion channels, the mechanisms by which both LoF and GoF mutations in *CACNA1A* trigger network hyperexcitability and cause the same epileptic phenotype is unknown and might depend on the impact of specific mutations in different neuronal cell types and neuronal circuits, as assessed through *in vitro* studies and animal models. Cav2.1 channels have been shown to mediate synaptic release from a variety of neuronal cell types, both excitatory and inhibitory, in the cortex, hippocampus, thalamus and cerebellum (Hoxha et al., 2018; Catterall et al., 2020). In agreement with the functional role of calcium channel and patients' phenotype, spontaneous mouse models carrying LoF defect in Cav2.1 channels (*e.g.* leaner, tottering, rolling, rocker), all of them show dystonia, ataxia, premature death and epilepsy (Hoxha et al., 2018). Functional studies suggest that the selective loss of Cav2.1 channels at parvalbunine-positive GABAergic interneurons in cortex and hippocampus appears sufficient to drive seizures as a result of impaired GABA release (Rossignol et al., 2013). Animal models also highlighted that different calcium currents are critical for maintaining normal thalamo-cortical oscillations and motor control and that loss of Cav2.1 triggers thalamic excitability during development and adulthood (Bomben et al., 2016; Miao et al., 2020). *CACNA1A*-dependent Purkinje cells loss is more likely expected to recapitulate the cerebellar features seen in patients (Rossignol et al., 2013; Damaj et al., 2015). Less obvious is to decipher the mechanisms underlying epilepsy resulting from GoF mutations. Clues can be drawn from the observation that epilepsy frequently occurs in families carrying GoF FHM mutations (Prontera et al., 2018). In addition, while heterozygous knock-in mice carrying the S218L FHM variant do not develop seizures, homozygous mice may develop fatal seizures caused by spreading depolarization in the brainstem and respiratory and cardiac arrest, likely reflecting gene dosage effects (van den Maagdenberg et al., 2010; Loonen et al., 2019). The magnitude of GoF effect exerted by Cav2.1 genetic variants inducing epileptic encephalopathy may correlate with seizure susceptibility. Furthermore, calcium excitotoxicity could be hypothesized as one of the causative mechanisms (Jiang et al., 2019).

Progressive cognitive deficits have been reported both in patients and in the heterozygous leaner and Nagoya mutant mice (Alonso et al., 2008; Takahashi and Niimi, 2009). The mechanisms underlying cognitive and behavioral dysfunction due to *CACNA1A* mutations are uncertain, but altered transmission in GABAergic interneurons and cerebellum could be postulated (Rossignol, 2011). Indeed, disruption within the cerebellum have been involved in learning and cognition deficits (Galliano et al., 2013). In addition, as discussed before, cortical and limbic GABAergic interneurons regulate the synchronous neuronal firing in populations of neurons and participate in the generation of high frequency gamma oscillations involved in cognitive processes (Murray et al., 2011).

The rarity and clinical heterogeneity of epilepsy due to variants in *CACNA1A* pose challenges to clinical diagnosis and treatment. Thus, the functional characterization of genetic variants is essential to address proper therapeutic intervention. It could be predicted that patients with GoF mutations are responsive to Cav2.1 antagonists, whereas, patients carrying LoF mutations may benefit from pharmacochaperones designed to improve trafficking of the channels to the plasma membrane (Terragni et al., 2018). In a child with epileptic encephalopathy, ataxia, cognitive impairment, and significant social-behavioral abnormalities, due to the *de novo* pathogenic variant S1373L in the *CACNA1A* gene, lamotrigine improved seizure frequency and severity, while zonisamide and sodium valproate were ineffective. This suggests that lamotrigine also modulates the activity of the P/Q-type calcium channel, making it a candidate for precision therapy for patients with epileptic encephalopathy due to *CACNA1A* pathogenic variants (Byers et al., 2016). Children affected by developmental epileptic encephalopathy carrying *CACNA1A de novo* variants are generally treated with a combination of conventional AEDs to attain seizure freedom such as felbamate, phenytoin, lorazepam, nitrazepam, phenobarbital, phenytoin, rufinamide, vigabatrin or topiramate, levetiracetam and, valproic acid (Jiang et al., 2019).

Other calcium channels genes

GoF effects with facilitated voltage-dependent activation and slowed inactivation, have been revealed for several partially recurring *de novo* variants identified in $Ca_V2.3$ channel (*CACNA1E)* associated with human epilepsy and developmental disorders (Helbig et al., 2019). Five individuals carrying *CACNA1E* mutations achieved seizure freedom upon administration of the anti-epileptic drug

topiramate, which blocks R-type calcium channels (Helbig et al., 2019). Over recent years, it has been highlighted that a number of chronic neurological disorders could arise from defects in T-type channel function (Weiss and Zamponi, 2020). Indeed, mutations in *CACNA1H*, encoding for the Cav3.2 T-type calcium channel, could increase the susceptibility to develop generalized or focal epilepsy of varying severity despite the fact that a clear causative association is still lacking. Phenotypic features involving other organ systems (immune, gastrointestinal) and developmental delay and autism can occur in addition to epilepsy (Weiss and Zamponi, 2020). The *CACNA2D2* gene, encoding for the alpha-2/delta subunit of the voltage-dependent calcium channel complex, has been associated with early-onset epileptic encephalopathy and with cerebellar atrophy with seizures and variable developmental delay. Notably, this accessory subunit is a receptor for the AED gabapentin (Butler et al., 2018).

CHLORIDE CHANNELS IN NDDS

Defects in ClC-2 chloride channel (*CLCN2*), in the astrocytic protein MLC1 and in the glial cell adhesion molecule (GlialCAM), underlie human leukoencephalopathies, degenerative disorders affecting the white matter of the brain and associated with myelin and astrocyte vacuolization (Depienne et al., 2013; Hoegg-Beiler et al., 2014; Elorza-Vidal et al., 2019). In particular, the molecular mechanisms underlying megalencephalic leukoencephalopathy with subcortical cysts (MLC) supported the involvement of glial cells and chloride homeostasis in the aetiology of NDDs. MLC is a genetic disease of infantile onset characterized by macrocephaly, white matter edema and delayed-onset neurologic deterioration, including cerebellar ataxia, epilepsy and mild cognitive decline, due to mutations in either MLC1 or GlialCAM (López-Hernández et al., 2011). Recessive mutations in *CLCN2* gene cause very rare *CLCN2*-related leukoencephalopathy, also known as leukoencephalopathy with ataxia characterized by intramyelinic edema (Gaitán-Peñas et al., 2017). More variable abnormalities may include visual field defects, headaches and learning disabilities (Depienne et al., 2013). To date, only 14 cases have been reported in details, with the majority of patients showing mild clinical phenotype with prolonged survival (Depienne et al., 2013; Guo et al., 2019).

Despite the direct involvement of ClC-2 chloride channels in epilepsy is still questionable, its contribution to MLC and leukoencephalopathy has been ascertained. In the CNS, chloride homeostasis, ensuring the chloride gradient for proper inhibitory signaling, is crucial for normal development and tightly controlled by various transporters and channels (Auer et al., 2020). ClC-2 is expressed in pyramidal hippocampal neurons and interneurons, but also in astrocytes, in the endfeet surrounding blood vessels as well as in oligodendrocytes (Elorza-Vidal et al., 2019). *In vitro* and *in vivo* studies supported the idea that in astrocytes ClC-2 forms a ternary complex with MLC1 and GlialCAM. MLC1 is involved in astrocytic volume regulation while GlialCAM ensures the correct membrane localization of MLC1. GlialCAM is also known to act as an auxiliary subunit for ClC-2 channels, targeting the channel to cell–cell junctions under depolarizing condition and modifying ClC-2 rectification. In particular, whereas in basal conditions ClC-2 displays outwardly rectifying currents, promoting chloride efflux, upon depolarization, as occurs after a train of action potentials and potassium siphoning, the MLC1/GlialCAM complex binds to ClC-2, allowing GlialCAM to modify ClC-2-mediated currents and abolishing the rectification. Thus, chloride influx into the cells is generated, that may compensate the excess of potassium (Elorza-Vidal et al., 2019). In neurons, ClC-2 chloride channel is thought to control intracellular chloride concentrations in inhibitory GABAergic neurons, thus preventing chloride accumulation and consequent depolarization at GABAergic synapses (Ratté and Prescott, 2011). Alternatively, ClC-2 could contribute to neuronal hyperpolarization mediating chloride influx. Therefore, despite the pathophysiology of MLC remains unclear, all the studies from different genetic animal models suggest that current reduction or change in ClC-2 biophysics may at least in part account for the impaired glial ion homeostasis in both MLC forms (Blanz et al., 2007).

Interestingly, human harbouring LoF *CLCN2* mutations and *Clcn2* knockout mice share overlapping phenotype, mainly including leukoencephalopathy, male infertility and visual impairment. A reduction in the ClC-2-mediated current was observed in oligodendrocytes of *Clcn2* knockout mice suggesting that ClC-2 disruption might result in the disturbance of the compensation of ion shifts induced by action potential, finally leading to osmotic intramyelinic edema. Accordingly with the reported *in vivo* GlialCAM, MLC1 and ClC-2 interaction (Brignone et al., 2015), additional disruption of GlialCAM may aggravate the vacuolization in *Clcn2* knockout mice (Hoegg-Beiler et al., 2014).

No ClC-2 selective modulators exist. In this regard, cation-chloride cotransporters and other key proteins setting chloride gradient have gained interest as promising and alternative novel target for modulating chloride homeostasis (Ben-Ari, 2017). Particularly, the Na-K-2Cl cotransporter isoform 1 (NKCC1), present in neurons, glia and endothelial cells of blood–brain-barrier, shows an expression profile widely depending on the developmental stage, which lead to profound changes in synaptic signaling (Auer et al., 2020). Recently, in consideration of the safety profile bumetamide, an inhibitor of NKCC usually used as diuretic drug, attracted attention as prime drug for the treatment of neurological and psychiatric diseases where dysregulated chloride homeostasis seems to be implicated (Ben-Ari, 2017; Blaesse et al., 2009). Several promising bumetamide derivatives with better pharmacokinetic profiles are under investigation (Auer et al., 2020).

HYPERPOLARIZATION-ACTIVATED, CYCLIC NUCLEOTIDE-GATED CHANNELS IN NDDS

Inherited and *de novo* mutations in the brain *HCN1* gene, encoding for the hyperpolarization-activated, cyclic nucleotide-gated channel 1, have been recently

identified in patients with a spectrum of brain disorders spanning from early-onset epileptic encephalopathy to benign idiopathic generalized epilepsies and GEFS+. Yet, causative epilepsy-linked mutations appear to occur in a small number of epileptic patients (Marini et al., 2018; DiFrancesco et al., 2019). Both GoF and LoF defects have been reported for *HCN1* mutations, but a clear genotype-phenotype correlation between the dysfunction of the HCN1 channels and epilepsy is still challenging (Marini et al., 2018). HCN channels in the brain seem to play a role in setting the resting membrane potential and neuronal firing rate, and functional studies and evidences from animal model could claim for a contribution of HCN dysfunction in epilepsy (Oyrer et al., 2018). HCN1 knock-out mice show a significant increase of excitability, with a predisposition to seizures development. Similarly, spontaneous and genetically-modified LoF HCN2 animal models show spontaneous generalized epilepsy. Dysfunctions of HCN2, of the pacemaker HCN4 channels and accessory HCN subunits appear also to predispose to the development of epilepsy with variable phenotypes (DiFrancesco et al., 2019). So far, the screening of a large population of patients and deeper insight into the role played by HCN channels in neurons are necessary to ascertain the clear role of these channels in the development of epilepsy.

LIGAND-GATED ION CHANNELS IN NDDS

Since the discovery of the first mutation in the *CHRNA4* gene in patients with ADNFLE (Steinlein et al., 1995), other mutations have been discovered in the alpha (*CHRNA2, CHRNA4*) and the beta (*CHRNB2*) subunits of the nAchR with a penetrance of about 70–80% (Oyrer et al., 2018). Differently from ADNFLE resulting from *KCNT1* channels dysfunction, mutation in AchR subunits are not associated with severe psychiatric and cognitive symptoms, suggesting that cellular effects beyond altered excitability can produce variable phenotypes (Quraishi et al., 2020). All the identified mutations increase the sensitivity to the nAchR to acetylcholine and knock-in mouse models bearing human mutations show increased acetylcholine synaptic release at GABAergic interneurons and increased seizure activity (Becchetti et al., 2015). Despite being identified over 25 years ago, no targeted therapies for mutations in nAchR exist. The mainstay of therapy in patients with nAChR mutations is carbamazepine at low doses, with approximately 70% showing remission (Oyrer et al., 2018).

Mutations in the alpha (*GABRA1*), beta (*GABRB3*), or gamma (*GABRG2*) subunits of $GABA_A$ receptor segregate with sporadic and familial idiopathic generalized epilepsies. More recently, LoF mutations in these genes have been identified in patients with early infantile epileptic encephalopathies, including Ohtahara, West syndrome and Dravet-like phenotypes (Gataullina et al., 2019). Both heterozygous *GABRA1* knock-out and knock-in mice show absence-like seizures and are good preclinical models of generalized epilepsy. Consistently, $GABA_A$ receptor agonists, like valproic acid and vigabatrin, successfully control epilepsy in *GABRA1* mutations carriers (McTague et al., 2016; Klein et al., 2020). Knock-in and knock-out *GABRG2* animal models recapitulates the absence and febrile seizures and support the hypothesis that the degree of *GAGRB2* haploinsufficiency seen in mice correlates with seizure severity in patients. The conditional *Gabrg2* knock-in mice, carrying the human R43Q mutation from birth, show increased seizure-sensitivity, highlighting that early mutational events can compromise long term network stability producing neurodevelopmental consequences (Reid et al., 2013). Given *GABR3* expression in embryonic brain and its role in synaptogenesis and neuronal migration, pharmacological intervention during early development would be advisable to blunt seizures and rescue neurodevelopmental regression (Oyrer et al., 2018).

The phenotypical spectrum of *GRIN1* (LoF) and *GRIN2A* (LoF and GoF) mutations, encoding for the GluN1 and GluN2A subunit of the NMDAR, includes isolated intellectual disability, idiopathic focal epilepsy or epileptic encephalopathies, with language disorders and hyperkinetic movements being very common and a genotype-phenotype correlation being unclear (Gataullina et al., 2019). A spectrum of disorders, from mild epilepsy to early onset epileptic encephalopathy and isolated intellectual disability, may occur also as a consequence of GoF and LoF mutations in *GRIN2B* (GluN2B) (Gataullina et al., 2019). A recurrent *de novo* GoF mutation in *GRIN2D* (GluN2D) has been associated with epileptic encephalopathy, intellectual disability and movement disorders (Oyrer et al., 2018). Animal models of NR1 and NR2A-2D subunits mimic only partially patients' phenotypes, as they generally do not display seizure, thus hampering further insight into the pathophysiology of diseases and development of targeted therapies (Oyrer et al., 2018; Smith and Walsh, 2020). Indeed, selective loss of *Grin1* determines impaired synaptic plasticity and memory deficits but do not recapitulate seizures. Similarly, no seizures have been reported for *Grin1* knock-out mice that die soon after birth. Whereas the mechanisms underlying LoF is less obvious, GoF defects of NMDA receptors can increase brain excitability and be amenable to specific channel block (Oyrer et al., 2018). Indeed, in a child with GoF *GRIN2A* encephalopathy, the NMDAR antagonist memantine has successfully reduced seizure frequency, but not cognitive dysfunction (Pierson et al., 2014). Calcium overload inducing cell death has been proposed as the molecular mechanism underlying GoF *GRIN2D* mutation, supporting the beneficial effect of NMDAR blockers, such as memantine and ketamine, in affected carriers. Yet, these observations in single cases should be confirmed in additional probands and a clinical trial with memantine is underway (Mesraoua et al., 2019). Both *GRIN2B* and *GRIN1* are expressed prenatally or during early development, with GRIN2B being replaced by GRIN2A, suggesting a role for GluN subunits in brain development (Smith and Walsh, 2020). Indeed, *GRIN2B* and *GRIN1* GoF mutations have been associated with polymicrogyria, a malformation occurring during fetal development of the cortex that is characterized by an overfolded cerebral cortex likely due to calcium overload and excitotoxicity (Smith and Walsh, 2020).

Over the last two decades, the collaborative efforts of basic scientists, clinical experts, pharmaceutical companies and patients' associations have contributed to face some challenges in the diagnosis and pathophysiology of rare NDDs. It emerges that NDDs and refractory epilepsy have still unmet medical needs. Pointing to new neuroprotective pathways, repurposing commercial drugs or developing new compounds that target ion channels defects as well as specific gene therapy, are all therapeutic options undertaken to offer patients a precision medicine, with the aim to improve not only seizures, but also the developmental outcome and associated comorbidities (Perucca and Perucca, 2019).

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Corrigendum to "Ion Channels Involvement in Neurodevelopmental Disorders" [Neuroscience 440C (2020) 337–359]

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