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Gene therapy for Dravet syndrome: promises and impact on disease trigger and secondary modifications

Claudia Di Berardino[1], Luca Massimino[2], Federica Ungaro[2], Gaia Colasante[1]

[1]Stem Cell and Neurogenesis Unit, Division of Neuroscience, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy.
[2]Gastroenterology and Digestive Endoscopy Department, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy.

Correspondence to: Dr. Gaia Colasante, Stem Cells and Neurogenesis Unit, Division of Neuroscience, IRCCS San Raffaele Scientific Institute, Via Olgettina 58, Milan 20132, Italy. E-mail: colasante.gaia@hsr.it

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Abstract

Dravet syndrome is a severe epileptic syndrome that begins during the first year of life of otherwise healthy babies. Over the years, the seizure burden changes, and pathology evolves in strong association with behavioral alterations, including cognitive delay and autistic traits. Initially, this aspect was considered a direct consequence of epilepsy severity, and DS was defined as an epileptic encephalopathy. Increasing evidence suggests that these two aspects of the disease, epilepsy and behavioral impairment, might not be so strictly connected. DS is mostly caused by heterozygous loss-of-function mutations in the *SCN1A* gene, which encodes for the alpha-subunit of the voltage-gated sodium channel $Na_v1.1$, responsible for GABAergic interneuron excitability. Interneuron dysfunction is evident at symptom onset in Dravet murine models, but their activity appears to recover in the chronic phase of the disease, when a series of secondary modifications arise and likely drive the phenotype. Given that the genetic basis of the disease is clear, innovative therapies based on the restoration of sufficient expression levels of $Na_v1.1$ to re-establish functional neuronal activity are being developed. In this work, we review such therapeutic approaches, with a specific focus on the existing evidence of their ability to address not only epilepsy but also behavioral alterations, and to recover secondary modifications.

Keywords: Gene therapy, secondary modifications, behavioral alterations

INTRODUCTION

Dravet syndrome (DS, previously known as Severe Myoclonic Epilepsy of Infancy) presents in the first year of life in otherwise healthy children with prolonged, febrile and afebrile, focal clonic (generally hemiclonic), focal to bilateral tonic-clonic, or generalized clonic seizures[1]. Over time, between the ages of 1 and 4 years, other seizure types appear, including myoclonic and atypical absence seizures[1]. Patients are subjected to a high risk of sudden unexpected death in epilepsy (SUDEP), which represents the first cause of death[2,3]. Epilepsy is accompanied by a progressive developmental delay and several comorbidities including cognitive and behavioral deficits, as well as movement and sleep disorders. While with age, the severity and frequency of seizures tend to decrease, cognitive and behavioral alterations worsen and become predominant in adolescent and adult patients, dramatically impacting their quality of life and that of caregivers[4-10]. Over 80% of DS patients carry pathogenic variants in the *SCN1A* gene[1,11]. Primarily, these are loss-of-function (LoF) mutations that affect one copy of the gene, which encodes for the alpha subunit of the voltage-gated sodium channel, $Na_v1.1$[12-16]. $Na_v1.1$ has a pivotal role in the initiation of action potential (AP) firing in neurons and is expressed mainly in inhibitory interneurons (IN)[17,18], leading to IN hypoexcitability[17-19]. This "interneuron hypothesis" was also supported by studies showing that $Na_v1.1$ haploinsufficiency induction only in GABAergic neurons recapitulates most DS phenotypes in mice[20-23] and by the observation that DS patients have impaired intracortical inhibition *in vivo*[24]. Therefore, for a long time, disinhibition has been considered the main mechanism underlying the excitation/inhibition (E/I) imbalance that makes circuits hyperexcitable and thus prone to seizures. More recent findings are contributing to overcoming this simplistic view. First, single-cell RNA sequencing transcriptomic data from humans and mice highlighted that *SCN1A* is not only expressed in inhibitory neurons, but also in pyramidal cells although at lower levels[25]. Second, some classes of pyramidal neurons appear to contribute to the phenotype or circuit dysfunction[26-28], with a defect evident even before IN activity alteration[27,29]. Moreover, vasointestinal peptide (VIP)-expressing disinhibitory INs are also dysfunctional in a DS model[30,31]. Finally, the characteristic firing defect of fast-spiking (FS) parvalbumin (PV) INs is only transient in the cerebral cortex, showing normalized firing already at P35/in the second month of age[32,33]. Nevertheless, DS mice continue to have seizures and behavioral alterations. In view of these findings, the IN defect might be just the substrate for the establishment of other neuronal dysfunctions that sustain DS phenotype in the late phase of the pathology. Hyperexcitability of excitatory neurons in cortico-hippocampal circuits has been proposed as one of those drivers[34]. Thus, the mechanism underlying DS is more complicated than simple circuit disinhibition and a potential role for pyramidal neurons in disease pathogenesis has been proposed.

In this scenario, where new insights into the pathological mechanisms driving DS are continuously emerging, there has been a significant effort toward developing gene therapy strategies aimed at restoring the physiological expression of the *SCN1A* gene. This heightened research activity has been fueled by mounting evidence that DS should not be viewed solely as an epileptic encephalopathy (EE), but also as a condition where *SCN1A* gene haploinsufficiency directly underlies behavioral alterations in patients. Consequently, addressing the root cause of the disease is essential for resolving both epilepsy and behavioral alterations.

In this work, we will first review the main evidence from both human and mouse model studies supporting the notion that behavioral alterations might be independent of the epileptic phenotype. Then, we will analyze the main secondary modifications characterizing the chronic phase of the disease. Finally, we will discuss the possibility that gene therapy might overcome these secondary modifications by examining preclinical data from the most relevant gene therapy studies.

EPILEPTIC ENCEPHALOPATHY OR CHANNELOPATHY?

DS was originally considered a mere epileptic encephalopathy, in which cognitive and behavioral symptoms were thought to be the direct consequence of epilepsy severity[35]. This is supported by the observation that developmental regression is more frequent in patients who have suffered status epilepticus with long and persistent crises[5,36,37] and that the severity of epilepsy in the early phase of the pathology correlates with a more severe behavioral impairment in the long-term[38]. In line with this hypothesis, better seizure control was associated with better cognitive outcomes in some patients[39]. In this study, three adult DS patients showed improved seizure control once they had been switched to the most appropriate medication for DS. This lasting reduction in seizures led to a significant additional improvement in cognitive function and quality of life in two patients. Considering this observation, the authors argued that DS is at least in part an epileptic encephalopathy[39].

In parallel, other evidence supports that $Na_v1.1$ haploinsufficiency might be directly responsible for circuit dysfunctions underlying cognitive, social and motor deficits, sustaining the channelopathy versus the EE model[5,6,40,41]. A retrospective study of 34 adolescents and 50 adults over time found that seizures persisted in 73.6% of adolescents and 80% of adults, although epilepsy severity decreased with age. At the last evaluation, 70.5% of adolescents and 80% of adults had moderate or severe intellectual disability. The most severe cognitive and motor impairments were seen in patients with ongoing seizures. The study indicates that while epilepsy severity decreases in adulthood, overall outcomes for DS remain poor, with no corresponding improvement in intellectual and motor abilities, suggesting these unfavorable outcomes are not simply due to epilepsy[7]. Accordingly, several studies that focused mainly on the evaluation of cognitive and behavioral symptoms in DS patients reported that they worsen during the natural course of the disease, despite the attenuation of epileptic phenotype, suggesting that their progression might be independent[9]. In particular, longitudinal studies evaluating cognitive function showed that the intelligence/developmental quotient (IQ/DQ) score decreased with time in DS patients[5,42], although raw scores increased indicating developmental delay but not regression. In line with these results, analysis of association studies revealed that the factor most consistently associated with cognitive outcome was age, with older children presenting lower cognitive scores[9].

In addition to the evidence that cognition worsens with age, cognitive decline has also been reported in patients experiencing an important reduction in seizure burden thanks to the usage of new treatments[5,43,44]. Results from the ENVISION prospective study showed that despite antiseizure medication, children (age < 5 years) develop language/communication delay and stagnation in a follow-up period of 17.5 months, independent of seizure burden. Indeed, monthly countable seizure frequency, number of antiepileptic drugs used, age at first seizure, and convulsive status epilepticus were not predictors of communication raw scores[44].

This is in contrast with previously mentioned studies showing that status epilepticus[37] and age at seizure onset[8] correlate with a worsened cognitive outcome. Although the relationship between epilepsy and comorbidities in DS remains to be better clarified, nowadays, the most widely shared view is that, at least in part, epilepsy can impact neurological functions, and this is the reason why DS is classified as a Developmental and Epileptic Encephalopathy (DEE)[1].

Definitions

In patients, the complete dissection of epilepsy contribution to comorbidities is challenging due to inadequate seizure control, especially in the initial phases of the disease, before diagnosis. Certainly, in the coming years, more information will be available to clarify this point, considering that an increasing

number of patients are achieving good seizure control thanks to new medications[43,45], such as stiripentol[46-48], cannabidiol[49,50], and fenfluramine[51-53].

The generation and characterization of animal models with *Scn1a* haploinsufficiency are shedding light on the relation between the epileptic and non-epileptic phenotypes characteristic of DS.

One of the first evidence in this context suggested that the autism-like symptoms observed in DS mice stem directly from reduced inhibitory neurotransmission due to *Scn1a* haploinsufficiency[54]. A single low dose of benzodiazepine (clonazepam) administered only 30 min before performing the behavioral tests, completely reversed deficits in social interactions and fear-associated contextual memory in DS mice without inducing sedation or anxiety relief[54]. While high doses of benzodiazepines are commonly used to alleviate epileptic seizures and anxiety-related behaviors, they are not typically employed to address core autism-related symptoms due to their sedative effects. Interestingly, in this case, the reversal of cognitive deficits and autism-like behaviors by clonazepam suggests that these conditions in DS mice are not primarily caused by recurrent seizure-induced brain damage but directly by *Scn1a* haploinsufficiency and the consequent decrease in GABAergic transmission.

This theory has been further reinforced by following studies demonstrating that spatially controlled induction of *Scn1a* gene haploinsufficiency in certain neuronal populations or in specific brain regions resulted in behavioral changes that appeared unrelated to epilepsy severity.

Selective deletion of $Na_v1.1$ in PV- or somatostatin (SST)-expressing INs leads to comparable levels of susceptibility to thermally induced seizures, but different impairments in behavior in mice[23]. In fact, while mice with global $Na_v1.1$ haploinsufficiency exhibit autistic-like behaviors, hyperactivity, and cognitive impairment, the restriction of $Na_v1.1$ haploinsufficiency just to PV INs induces autistic-like symptoms but not hyperactivity. Conversely, haploinsufficiency in SST INs leads to hyperactivity without social impairment. Susceptibility to thermally induced seizures is enhanced when $Na_v1.1$ is deleted in both IN types, and this is accompanied by a reduction in long-term spatial memory and context-dependent fear conditioning, with no impact on short-term spatial learning or memory[23].

$Na_v1.1$ is also detected in disinhibitory INs expressing VIP, which exhibit reduced excitability in DS mice[30]. Selective deletion of *Scn1a* in VIP-INs replicates core autistic-like behaviors along with deficits in VIP-IN function at cellular and circuit levels, albeit without inducing epilepsy, SUDEP, or avoidance behaviors observed in the global model[31].

Local reduction in $Na_v1.1$ levels in the hippocampus of adult rats was sufficient to induce a dysregulation of hippocampal oscillations and impairment in spatial recognition, although in the absence of any epileptic phenotype[55]. Similarly, *Scn1a* silencing in the medial septum induced a specific working memory deficit without affecting reference memory[56], again in the absence of seizures. This set of experiments strongly supported that loss of $Na_v1.1$ function in specific brain networks might be associated with specific cognitive and/or behavioral impairments that arise independently of any epileptic manifestation.

Conversely, when similar experiments were performed in mice by genetic deletion of the *Scn1a* gene in the hippocampus, both epileptic seizures[57-59] and cognitive impairment appeared[57], making it again difficult to discriminate the impact of epilepsy on cognitive dysfunction.

Result interpretation was not straightforward when temporally controlled induction of *Scn1a* gene haploinsufficiency was achieved, by inducing the expression of a specific LoF mutation in the *Scn1a* gene (A1783V) at various postnatal developmental stages (postnatal day 30 (P30) and P60)[60]. This approach provided valuable insights into the necessity of maintaining physiological levels of $Na_v1.1$ expression also beyond the critical period of neuronal circuit development. In fact, as soon as *Scn1a* gene haploinsufficiency was induced, seizures appeared at all analyzed time points[60]. A different output was obtained when spatial learning was tested, with P30-induced mice showing a significant amelioration and with P60-displaying a complete rescue in comparison to DS mice. However, at the time of cognitive testing (3-3.5 months), P30- and P60-induced mice had experienced less time in seizures compared to P2-induced mice, and thus, the better cognitive function could also be interpreted as a direct effect of that. Finally, dysfunction of $Na_v1.1$ activity in the medial prefrontal cortex (mPFC) of adolescent mice increased the local E/I ratio, leading to epileptic activity, cognitive deficits, and depressive-like behavior in adulthood. Again, these effects did not correlate with the severity of epilepsy[61].

In support of the EE model, there is evidence that the mild phenotype induced by a specific *Scn1a* gene mutation can be transformed by the induction of recurrent seizures early in life into a severe DS-like phenotype with frequent spontaneous seizures and cognitive and behavioral deficits[62]. In these mice, exacerbation of the phenotype was not related to gross morphological alterations in the brain, but possibly to pathological remodeling of circuits, as increased excitability of hippocampal granule cells was observed.

Altogether, several studies in mouse models of DS sustain the channelopathy theory, pointing out a direct role for $Na_v1.1$ haploinsufficiency and IN dysfunction in the pathogenicity of non-epileptic symptoms. This evidence underlies the necessity to adopt efficient *SCN1A*-directed therapies to improve both the epileptic phenotype and other DS symptoms. However, some studies still underline the deleterious impact of epilepsy severity on the manifestation of behavioral alterations and on cognitive functions. For this reason, DS is mainly considered a DEE, in which the combination of *SCN1A* gene haploinsufficiency itself and epilepsy severity contributes to comorbidities. Both factors lead to secondary modifications in disease progression. In the next section, we will discuss the main secondary modifications described in DS models.

SECONDARY MODIFICATIONS IN DS PATHOGENESIS

A growing body of literature indicates that mechanisms other than disinhibition take place as DS progresses. They might influence the pathological development during the chronic phase, which is characterized by a less severe epileptic phenotype and a prevalence of comorbidities.

With secondary modifications, we refer to all those remodeling effects that arise as adaptive, maladaptive, or compensatory mechanisms in response to the original genetic defect. In DS models, the synergistic effort of different research groups helped to dissect the changes in brain physiology at different stages of disease progression, and at different levels, including intrinsic neuronal properties and brain circuit remodeling, gene expression, proteomic and metabolomic alterations, and even structural and functional rearrangements [Table 1].

At the functional level, besides the primary deficit in IN excitability that directly derives from cell-autonomous *Scn1a* gene haploinsufficiency[17-19,63], alterations in the intrinsic excitability of other neuronal types have been described[27,29,64], together with changes in synaptic transmission[23,33,34,54,65,66], and rhythmic activity in different brain areas[59]. This remodeling can be dynamic and evolve with the course of the pathology, with transient alterations that can be found just in a precise phase of the disease and long-term modifications that persist.

Table 1. Secondary modifications assessed in different DS mouse models at functional and gene expression levels

Secondary modification		Brain area/cell type	Disease stage	DS model	Reversible?	Reference
Neural function	Normalization of IN excitability	Somatosensory cortex/ PV+ INs	Early chronic (P35-56)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Favero *et al.*, 2018[32]
		Hippocampus (CA1)/ Horizontal stratum oriens INs (partial)	Early chronic (P33-35)	*Scn1a*$^{+/A1783V}$ mouse	Not assessed	Almog *et al.*, 2021[27]
		Hippocampus (CA1)/ FS (partial) and non-FS INs	Early chronic (P45-55)	Reversible *Scn1a*$^{+/stopflox}$ mouse	No; Increased excitability upon $Na_v1.1$ levels restoration at P30	Valassina *et al.*, 2022[66]
		Hippocampus (DG)/ PV+ INs (partial)	Chronic (P48-91)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Mattis *et al.*, 2022[34]
	Altered (distal) location of AP initiation site	Somatosensory cortex/ PV+ INs	Early chronic (P35-56)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Favero *et al.*, 2018[32]
	Hyperexcitability of excitatory neurons	Acutely dissociated hippocampus/ Pyramidal neurons	Onset (P21-24)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Mistry *et al.*, 2014[64]
		Hippocampus (DG)/ Granule cells	Chronic (P60)	*Scn1a*$^{+/R1648H}$ mouse + 10-days seizure induction protocol since P21	Not assessed	Salgueiro-Pereira *et al.*, 2019[62]
	Increased E/I ratio	Cortico-hippocampal circuit (EC-DG synapses) Granule cells	Onset (PW4)	*Scn1a*$^{+/A1099X}$ mouse	Not assessed	Tsai *et al.*, 2015[65]
			Chronic (P54-75)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Mattis *et al.*, 2018[34]
		Hippocampus (CA3-CA1 synapses) Pyramidal cells	Onset (P18-20)	*Scn1a*$^{+/-}$	Not assessed	Jones *et al.*, 2022[29]
			Onset (P20-25)	*Scn1a*$^{+/A1783V}$ mouse	Restored in P21 DS mice upon CAV2-*Scn1a* gene therapy in the hippocampus (Fadila *et al.*, 2023[97])	Almog *et al.*, 2022[69]
		Hippocampus (CA1)/ Spontaneous transmission onto pyramidal cells	Chronic (10 month old mice)	*Scn1a*$^{+/-}$ (targeted deletion of the last exon)	Not assessed	Han *et al.*, 2012[54]
	Reduced synaptic conductance	Hippocampus (CA1)/ Excitatory and inhibitory synapses onto pyramidal cells	Onset (P20-25)	*Scn1a*$^{+/A1783V}$ mouse	Not assessed	Almog *et al.*, 2022[69]
	Increased release probability at excitatory synapses	Hippocampus (DG)/ Excitatory synapses onto granule cells	Onset (PW4)	*Scn1a*$^{+/A1099X}$ mouse	Not assessed	Tsai *et al.*, 2015[65]
		Hippocampus (DG)/ EC synapses onto granule cells	Chronic (P54-75)	*Scn1a*$^{+/-}$ (targeted deletion of the first exon)	Not assessed	Mattis *et al.*, 2022[34]

		Hippocampus (CA1)/ Excitatory synapses onto SO cells	Onset (P20-25)	*Scn1a*$^{+/A1783V}$ mouse	Not assessed	Almog *et al.*, 2022[69]
	Reduced theta-gamma coupling	Primary visual cortex Hippocampus	Onset (P23)	*Scn1a*$^{+/-}$ (targeted deletion of the exon 8)	Not assessed	Jansen *et al.*, 2021[59]
Cell and synaptic morphology	Reduced dendritic arborization and increased spine density	DG granule cells	Onset (PW4)	*Scn1a*$^{+/A1099X}$ mouse	Not assessed	Tsai *et al.*, 2015[65]
Gene expression	Altered transcriptomic profile	Hippocampus	Onset (P24)	*Scn1a*$^{+/-}$	Not assessed	Hawkins *et al.*, 2019[70]
		Cortex and hippocampus	Chronic (4 months)	*Scn1a*$^{+/STOP}$ mouse	Yes (cortex); Partially (hippocampus)	Valassina *et al.*, 2022[66]
	Altered proteomic profile	Hippocampus	Presymptomatic (PW2) onset (PW4)	*Scn1a*$^{+/A1783V}$ mouse	Not assessed	Miljanovic *et al.*, 2021[73]

Although during the initial characterization of the disease, no change in the intrinsic excitability of excitatory neurons was detected[17,67,68], in the following years, they were described to develop hyperexcitability, at least when recorded in acutely-dissociated hippocampal neurons from DS mice at P21-24[64].

A seminal discovery in the field was the finding that IN dysfunction is transient in mouse models of DS[32]. Indeed, INs of the PV subclass in layer 2/3 of the somatosensory cortex completely recover from the firing defect characteristic of disease onset during the chronic phase (P35-55). This recovery might be explained by compensatory recruitment of other Nav subunits in the axonal initial segment, enabling the normalization of firing properties, as proposed by Favero *et al.*[32]. However, in these later stages, DS mice still experience seizures and display behavioral abnormalities. This could be explained by persistent firing defects in other interneuron subclasses (SST, CCK, and VIP) and/or in PV INs located in brain areas different from the somatosensory cortex. In the hippocampus, FS-PV INs show a marked tendency toward a recovery of their firing ability in the chronic phase of disease[34,66]. Although they still present subtle deficits in spike generation and impairment in repetitive firing, they retain inhibitory reserve that is sufficient to correctly inhibit the circuit[34]. Similarly, the excitability of non-FS INs in this brain area appears ameliorated[27] or restored[66]. The dissolution of the first two possibilities suggests that IN dysfunction does not underlie the phenotypic deficits characteristic of the chronic phase of the disease.

A third possibility is that IN hypofunctionality during a specific critical period may lead to alterations in neuronal circuits, which sustain DS phenotype in the chronic phase. In parallel to the changes in the intrinsic excitability of different neuronal populations, altered synaptic transmission has also been described in several circuits in both the acute and chronic phases. At symptom onset, reduced GABAergic neurotransmission, associated with an increase in the E/I balance in DS circuits, has been reported[23,29,65]. While in most of these studies, the spontaneous excitatory inputs were shown to be increased as a consequence of disinhibition[23,29], at some excitatory synapses, an increase in neurotransmitter release probability was observed[65,69]. In the dentate gyrus (DG) of the hippocampus, this functional alteration was accompanied by changes in dendritic arborization and spines maturation already detectable at P28, indicating that

circuit remodeling starts early after IN dysfunction[65]. Interestingly, CA1 pyramidal neurons receive excitatory inputs from the CA3 with reduced synaptic strength, which could represent an attempt to compensate for the decreased inhibitory tone[69]. However, the E/I ratio was still imbalanced toward excitation in DS mice compared to control, suggesting that this acute secondary event is not sufficient to restore physiological activity in the CA1 circuit.

Disruption of E/I balance persists in adult DS circuits despite the IN firing normalization observed in different brain areas. In fact, inhibitory synaptic transmission is not recovered in young adult DS brain[33] and the reduced inhibitory function is accompanied by an increased excitation, resulting in a hyperexcitable and hyperactive network, in both cortex and hippocampus[23,33,54,66]. However, in adult DS cortico-hippocampal circuit, it has been proposed that an increased excitatory input rather than a dysfunctional inhibition might be the prevailing mechanism of E/I imbalance[34]. In fact, increased synaptic strength at excitatory synapses from the entorhinal cortex (EC) onto DG granule cells (GCs) was detected, which was attributable to a presynaptic change in excitatory transmission, while as aforementioned, IN ability to provide feedforward disynaptic inhibition was preserved[34].

This study was the first to clearly suggest that secondary mechanisms might not only ameliorate or exacerbate the phenotype caused by IN dysfunction, but could also become the primary drivers of DS phenotype in the chronic phase. It emphasizes the importance of the crosstalk between brain networks and reminds us that while focusing on individual cell types or circuits can help us dissect the contribution of specific mechanisms to altered circuit functionality, understanding the disease pathogenesis requires caution. We cannot simply extrapolate findings from a specific circuit to the entire brain or assume that observations in one circuit reflect the coordinated activity of different brain areas *in vivo*.

At the gene expression level, multiple studies reported an altered transcriptional profile in the brain of DS models after symptom onset[66,70,71]. Of note, at P24, very few genes were differentially regulated between *Scn1a* haploinsufficient mice and control littermates, suggesting that at this stage, secondary modifications are just starting to arise and evolve in parallel with symptom appearance. However, by separating DS mice into two groups based on whether they had experienced a spontaneous seizure 24 h before RNA collection, researchers were able to identify gene deregulation specifically induced by epileptic activity. While some of these genes are shared with other epileptic models, such as those associated with astrogliosis, DS-specific enriched genes are linked to processes like calcium signaling, ligand-receptor interaction, and synaptic plasticity. This suggests that mechanisms of homeostatic plasticity are activated in the hippocampus as an early response to seizures. This finding supports the hypothesis that homeostatic plasticity occurs in response to epileptic activity, and that its failure to adequately rebalance excitability contributes to the establishment of an epileptic circuit[72].

The proteomic profile of DS mice is also altered[73]. An analysis of protein expression in the hippocampus of DS mice was conducted in both presymptomatic and symptomatic mice at 4 weeks. Consistent with observations from transcriptomics studies, proteomic analysis revealed more pronounced alterations after the onset of epilepsy[73]. Proteins involved in neurotransmitter dynamics, ion channel and receptor function, synaptic plasticity, and astrogliosis were particularly enriched in DS mice. Following the manifestation of epilepsy, a significant downregulation of pathways linked to synaptic transmission and both GABAergic and glutamatergic signaling was found[73]. Not only were structural synaptic proteins downregulated, but functional proteins, including GABA, glutamate and dopamine receptors and transporters, were also affected. The expression of potassium and calcium channels was reduced, indicating that both synaptic transmission and neuronal excitability undergo remodeling in the hippocampal circuit. Depending on the

cell type in which these changes occur, the net result could differ. Therefore, functional studies are needed to better understand whether these alterations in protein expression correspond to maladaptive mechanisms that contribute to circuit hyperexcitability or compensatory mechanisms that try to counterbalance it.

THE PROMISES OF GENE THERAPY FOR DS

As previously discussed, it is difficult to understand to what extent comorbidities and the epileptic phenotype in DS are interdependent. While several studies have indicated that inducing $Na_v1.1$ haploinsufficiency in specific brain circuits can cause specific neurological alterations, supporting the channelopathy model, others have reported that early exposure to epilepsy correlates with a worse phenotype, both epileptic and behavioral, in the chronic phase of the disease. Additionally, as reviewed in the previous section, many adaptive changes arise as the disease advances, secondary to the original defect in IN excitability. Both of these factors could impact the actual efficacy of novel precision medicine approaches being developed for DS treatment. By targeting the underlying *SCN1A* haploinsufficiency, these strategies hold the potential to alter disease progression[74]. However, if the pathogenetic mechanism evolves with the disease, targeting the original defect might not be as effective in later phases of the pathology.

During the last decade, several strategies have been developed to restore functional levels of $Na_v1.1$ in brain circuits. These strategies exploit different technologies but essentially rely on one of two approaches: *SCN1A* gene supplementation, in which a functional copy of the gene is delivered by viral vectors, or upregulation of the endogenous healthy allele. Each of these strategies comes with specific challenges and limitations, mainly related to their specific efficacy, delivery methods, off-target effects, and long-term sustainability of treatment effects. While most of those aspects have been previously reviewed[74-79], we will focus on the evidence attesting their ability to impact both epilepsy and behavioral alterations and rescue secondary modifications characteristic of DS [Table 2].

The first gene therapy set for DS was based on an activatory CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeats/CRISPR-associated protein, CRISPRa) system aimed at boosting endogenous *Scn1a* gene transcription specifically in GABAergic INs[80]. This strategy successfully rescued IN firing ability in the hippocampus of DS mice treated at birth, and improved the epileptic phenotype, attenuating thermally induced seizures. However, seizures were not completely suppressed, likely due to the low efficiency of IN co-infection (~20%) with the two viral vectors used. Although this work did not evaluate the potential for also rescuing comorbidities, it provided the first proof of concept that increasing $Na_v1.1$ expression levels in GABAergic INs could be a therapeutic approach for DS.

A similar strategy was pursued by crossing DS mice with a mouse line expressing an activatory Cas9 specifically in GABAergic interneurons[81]. In addition to reduced susceptibility to thermally induced seizures, treated mice exhibited mild improvements in some behavioral symptoms, achieving intermediate hyperactive and anxious-like phenotypes compared to control and DS mice. Moreover, treatment was delivered at four weeks of age, indicating that the *Scn1a* upregulation strategy might hold therapeutic potential even after symptom onset.

Another transcriptional activator system, ETX101, used zinc finger binding proteins to enhance *Scn1a* expression in GABAergic INs. This system targeted a sequence in the upstream regulatory region of *SCN1A* that is highly conserved in mammals, facilitating translation to clinical settings after validation in mice and non-human primates (NHP)[82,83]. Tanenhaus *et al.* reported a significant decrease in the number of spontaneous and hyperthermia-induced seizures in DS mice treated perinatally[83], along with prolonged survival for over one year. Although the potential of ETX101 in improving comorbidities has not been

Table 2. Ability of $Na_v1.1$ restoration approaches to rescue different DS symptoms as well as primary and secondary alterations

$Na_v1.1$ restoration approach	Study phase	Time of delivery	Rescue of epilepsy & mortality	Rescue of comorbidities	Rescue of primary deficit	Rescue of secondary modifications	Reference
Gene therapies based on *Scn1a* upregulation							
CRISPRa-based *Scn1a* upregulation in GABAergic INs	Preclinical	P1 DS mice, prior to onset	Reduced susceptibility to thermally induced seizures and mortality	Not assessed	Increased *Scn1a* mRNA and protein levels Rescue of IN firing ability in hippocampal slices	Not applicable	Colasante *et al.*, 2020[80]
CRISPRa-based *Scn1a* upregulation in GABAergic INs	Proof of principle	4-week-old triple transgenic DS mice, at disease onset	Reduced susceptibility to thermally induced seizures	Mild amelioration of hyperactive and anxious-like behavior	Increased *Scn1a* mRNA and protein levels IN function not assessed	Not assessed	Yamagata *et al.*, 2020[81]
Zinc finger-based *Scn1a* upregulation in GABAergic INs	Preclinical	P1 DS mice, prior to onset	Reduced spontaneous and hyperthermia-induced seizures Prolonged survival	Not assessed	Increased *Scn1a* mRNA and protein levels IN excitability not assessed	Not applicable	Tanenhaus *et al.*, 2022[83]
ASO-mediated *Scn1a* upregulation in cells naturally expressing *Scn1a*	Preclinical	Single dose in P2 or P14 DS mice, prior to symptom onset	Reduced spontaneous seizures number and increased latency Prolonged survival	Not assessed	Increased *Scn1a* mRNA and protein levels Rescue of sodium current density and firing properties in cortical INs Rescue of spontaneous GABAergic transmission in somatosensory cortex	Not applicable	Han *et al.*, 2020[84] Yuan *et al.*, 2023[111]
	Phase I/II Clinical trials (ADMIRAL, UK and MONARCH, US)	Multiple doses in 2-18-year-old DS patients	Reduced convulsive seizure frequency	Data on clinical and caregivers' global impression of change and quality of life assessment not available yet	Not assessed	Not assessed	Stoke Therapeutics 2024[91,94]
	Open-label extension study (LONGWING, UK and SWALLOWTAIL, US)	Continued treatment with multiple doses in patients who completed phase I/II clinical trial (preliminary data from first 12-month period of SWALLAWTAIL)	Durable reduction in convulsive seizure frequency	Improvement in expressive and receptive communication, motor skills, and executive function	Not assessed	Not assessed	Cross *et al.*, 2023[92] Desurkar *et al.*, 2023[93] Stoke Therapeutics, 2024[91,94]

Gene therapies based on *Scn1a* supplementation							
HC-AdV-based *Scn1a* delivery to prefrontal cortex, basal ganglia, and cerebellum for ubiquitous expression	Preclinical	P30 DS mice, after symptom onset	Reduced interictal epileptiform discharges Decreased susceptibility to thermally induced seizures Increased survival	Amelioration of compulsive behavior, cognitive and motor dysfunction No correction of hyperactivity and spatial reference memory	Increased *Scn1a* mRNA and protein levels IN function not assessed	Not assessed	Mora-Jimenez *et al.*, 2021[96]
HC-AdV-based *Scn1a* delivery to hippocampus and thalamus for expression in GABAergic neurons	Preclinical	P21 DS mice, at disease onset	Reduced susceptibility to thermally induced seizures Mild improvement in survival	Not assessed	Not assessed	Not assessed	Ricobaraza *et al.*, 2023[102]
CAV2-based *Scn1a* delivery in the hippocampus and/or thalamus for neuronal expression	Preclinical	P35 DS mice, early chronic stage	Reduced susceptibility to thermally induced seizures Decreased interictal spikes frequency	Mild amelioration in working memory No correction of hyperactivity	Not assessed	Not assessed	Fadila *et al.*, 2023[97]
		P21-24 DS mice, at disease onset	Reduced susceptibility to thermally induced seizures Decreased interictal spikes frequency Increased survival	Mild amelioration in working memory and correction of hyperactivity (targeting hippocampus) Rescue of spatial memory (combined targeting of hippocampus and thalamus)	Rescued spontaneous GABAergic transmission onto CA1 pyramidal neurons (targeting hippocampus)	Restored E/I ratio at CA3-CA1 synapses (targeting hippocampus)	
Split-intein-based *Scn1a* reconstitution in GABAergic neurons	Preclinical	P0-3 DS mice, prior to onset	Reduced susceptibility to spontaneous and thermally induced seizures SUDEP protection	Not assessed	Not assessed	Not applicable	Mich *et al.*, 2023[98]
Genetic model							
Scn1a$^{stopflox/+}$ reversible model, with Cre-dependent restoration of $Na_v1.1$ in cells naturally expressing *Scn1a*	Proof of principle	P1, prior to onset	Prevention of SUDEP and susceptibility to thermally induced seizures	Not assessed	Restored *Scn1a* mRNA and protein levels	Not applicable	Valassina *et al.*, 2022[66]
		P30, after symptom onset	Full protection from SUDEP, spontaneous and hyperthermia-induced seizures	Rescue of hyperactivity and social interaction deficits, as well as of working and spatial reference memory	Increased $Na_v1.1$ protein levels in cortex and hippocampus Rescued spontaneous	Increased excitability of both FS and non-FS INs in the hippocampus (CA1) Full rescue of	

				GABAergic transmission onto CA1 pyramidal neurons	transcriptomic profile in the cortex; almost full in the hippocampus
	P90, chronic stage	Protection from seizures	Not assessed	$Na_v1.1$ protein restoration in cortex and hippocampus	Not assessed

determined, the broad transgene expression and good safety profile achieved in NHPs led to the approval for safety and efficacy assessment in clinical trials (https://clinicaltrials.gov/study/NCT05419492?cond=Dravet%20Syndrome&page=2&rank=12; https://clinicaltrials.gov/study/NCT06112275?cond=Dravet%20Syndrome&rank=9; https://classic.clinicaltrials.gov/ct2/show/NCT06283212).

The first precision medicine-based therapy able to reach clinical trial for DS patients is STK-001[84]. This antisense oligonucleotide (ASO) differs from previously described therapies, by increasing $Na_v1.1$ levels through reduced RNA degradation rather than boosting gene transcription. It utilizes Targeted Augmentation of Nuclear Gene Output (TANGO) technology, which targets a naturally occurring, non-productive alternative splicing event, or "poison exon", in *SCN1A*[85-87]. Preclinical studies demonstrated the efficacy of STK-001 in increasing $Na_v1.1$ mRNA and protein levels, after administering a single dose in DS mice, either at P2 or P14 (close to disease onset)[84]. While seizure frequency was significantly reduced and survival increased to 97% in P2-treated mice, the survival improvement was less robust in P14-treated mice (~85%), suggesting that providing therapy as early as possible might be more effective and protective against SUDEP. ASO-mediated *Scn1a* upregulation in P2-treated mice was associated with a complete recovery of the sodium current density in cortical INs, which rescued their excitability and spontaneous GABAergic transmission, indicating that early treatment with ASO might resolve the primary deficit of DS[88]. The effect on cognitive and behavioral symptoms was not examined; however, after assessing biodistribution, safety and effective *SCN1A* upregulation in NHPs[89], STK-001 was approved for testing in *SCN1A*-related DS patients. Recently completed phase I/II clinical trials, MONARCH (NCT04442295, US) and ADMIRAL (2020-006016-24, UK), evaluated the safety and tolerability of STK-001, with its efficacy as an adjunctive therapy as a secondary objective. While results have not been published yet, a recent disclosure from Stoke Therapeutics reported encouraging preliminary data showing a good safety profile, with 30% of patients experiencing a treatment-emergent adverse event such as CSF protein elevation or procedural vomiting, and just one patient showing an unexpected serious adverse reaction considered related to the study drug[90,91]. At the higher dose tested (70 mg), STK-001 demonstrated substantial and sustained reduction in convulsive seizure frequency on top of the best available antiseizure medication (in most cases a combination of three or four drugs), with a median reduction of 85% at 3 months and 74% at 6 months after the last dose. As a secondary outcome, these studies also evaluated clinical and caregivers' global impression change (CGIC and CaGIC, respectively) as well as patients' quality of life through the EQ-5D-Y test, aiming to provide a first-row estimate of the potential improvement of behavioral and psychological comorbidities in parallel to the amelioration in seizure burden, although results are not yet available (https://clinicaltrials.gov/study/NCT04442295?tab=table#outcome-measures).

More importantly, patients completing the trial were eligible to continue treatment in SWALLOWTAIL (US) and LONGWING (UK) open-label extension (OLE) studies, which evaluate long-term safety and efficacy as well as potential impact on the reduction of comorbidities. Promising results from a subset of patients who participated in SWALLOWTAIL for 12 months indicated a durable reduction in convulsive seizure frequency and a substantial improvement in multiple assessments of cognition and behavior, including expressive and receptive communication, motor skills, and executive function[90,92-94]. Notably, these same parameters were evaluated in the BUTTERFLY observational study in patients subjected to treatment with available antiepileptic drugs, and a recent *ad interim* analysis reported only a slight improvement in the communication score but no changes in executive function, which was instead enhanced by STK-001 treatment, over a 12-months period[95]. This represents the first report in humans of a gene therapy strategy able to improve cognitive symptoms in DS patients. While these results are very encouraging, they further support the need for better evaluation of cognitive and behavioral parameters not only in observational and follow-up studies but also as main trial outcomes, and prior to this in preclinical studies.

As for therapeutic strategies based on gene replacement, they have progressed slowly compared to those based on *SCN1A* upregulation, mainly because of technical issues regarding the limited packaging capacity of adeno-associated viral (AAV) vectors and the nature of *SCN1A* coding sequence (CDS), which is highly prone to rearrangement. However, recent works that exploited a codon-optimized *SCN1A* CDS and different strategies to overcome the AAV packaging size, showed promising results and, in some cases, good translational potential[96-98].

The first alternative was proposed by using high-capacity adenoviral (HC-AdV) vectors, which can harbor up to 37 kb of exogenous sequence, for *SCN1A* gene supplementation[99,100]. By employing a ubiquitous promoter, the authors achieved good transduction of both excitatory and inhibitory neurons, as well as glial cells[96]. However, to achieve an amelioration of DS symptoms, they needed to target multiple brain regions, obtaining better results by concomitant injection in the prefrontal cortex, basal ganglia, and cerebellum. Notably, they obtained complete protection from SUDEP in DS mice treated after symptom onset (P30). Thermally induced seizures were also ameliorated, as well as some behavioral symptoms, including compulsive behavior, cognitive and motor dysfunction. However, these symptoms were not fully recovered, likely due to the poor ability of the HC-AdV to diffuse from the injected area to other brain regions, such as the hippocampus, which has been implicated in both seizure susceptibility and spatial memory disruption[55-58,101]. This study provided the first evidence that *SCN1A* gene supplementation could be an effective strategy to treat DS and that it can ameliorate symptoms, even if administered after their onset, indicating that broad targeting of the brain might be required to achieve correction of both epilepsy and comorbidities.

In a subsequent study, the authors attempted to refine their strategy by including part of the endogenous *SCN1A* regulatory region in their HC-AdV vector to restrict *SCN1A* expression to GABAergic INs, targeting the hippocampus and thalamus of three-week-old DS mice[102]. However, this led to only a partial rescue of survival and reduced seizure susceptibility, further supporting the necessity to target broad brain areas and indicating that $Na_v1.1$ function might be required in neuronal populations different from INs.

As an alternative to HC-AdV vectors of human origin, Fadila *et al.* recently proposed the use of a canine-derived HC-AdV, the canine adenovirus type 2 (CAV-2), which reduces the risk of previous immunization in patients[97,103]. This vector presents the additional advantages of preferentially transducing neurons and spreading through retrograde axonal transport to broad areas, allowing for high diffusion of the transgene

in specific brain networks following local injection into the parenchyma[97,104,105]. After local injection of a CAV-2 carrying *SCN1A* CDS in the hippocampus, thalamus, or both regions, the authors observed the presence of transduced cortical cells projecting to the injected areas. Notably, both survival and seizure susceptibility to hyperthermia were improved in DS mice injected in either the hippocampus or the thalamus, with the protective effect on seizures being more evident when both regions were targeted. One of the most promising results was the amelioration obtained in both the epileptic phenotype and in cognition (spatial memory) by treating mice after symptom onset, highlighting the potential of CAV-2-mediated *SCN1A* gene supplementation both in the severe phase of the disease, in juvenile mice (P21-24), and in the chronic phase, in adolescent mice (P35). Importantly, improvement in cognition in juvenile mice was associated with the restoration of the E/I balance in the hippocampal circuit, providing evidence for the possibility of also recovering a functional secondary modification, albeit in the early phase of the disease.

A novel strategy with therapeutic potential for *SCN1A* gene replacement in DS has recently been developed. To circumvent AAV packaging size constraints, *SCN1A* CDS was split into two halves, which, when injected in combination, could be fused back together by intein-mediated ligation to reconstitute a functional full-length protein[98,106]. Specifically, restoring $Na_v1.1$ expression in approximately 50% of GABAergic INs was effective in protecting DS mice against mortality and significantly reduced both spontaneous and thermally induced seizures. Notably, the study showed that providing $Na_v1.1$ channel also to excitatory neurons (by expressing the transgene under a pan-neuronal promoter) decreased the efficacy of DS treatment and resulted in pre-weaning mortality, further supporting the idea that GABAergic INs are the main population to be targeted in DS. However, in this study, mice were treated perinatally, so the efficacy of such an approach after symptom onset remains to be determined. Moreover, the effect on comorbidities was not assessed; hence, further studies should clarify these two important aspects to define the real potential of this approach.

To summarize, while therapeutic strategies aimed at restoring physiological levels of $Na_v1.1$ appear very promising for DS, many of them have only been tested in proof-of-principle studies validating their ability to rescue IN hypoexcitability and improve the epileptic phenotype and survival, without focusing on their effect on comorbidities. Moreover, many of them are delivered perinatally, testing their efficacy of treatments in the presymptomatic phase, and thus are not suitable for evaluating the modification of secondary events occurring in the chronic phase of DS. This limitation is partly justified by the constraints of currently available technology, particularly regarding the challenge of targeting the entire brain, as well as the complexity of the disease itself. Many aspects of DS remain to be clarified, such as which brain regions and cell types need to be targeted to restore altered brain functions, to what extent symptoms are reversible, and at which stage intervention is most effective.

Insights into the effective reversibility of DS phenotype and the potential to overcome secondary modifications were gained from the generation and characterization of a conditionally reversible DS mouse model, in which physiological expression of $Na_v1.1$ can be restored in the cell types that normally express it, thanks to the removal of a Stop cassette in one allele of the *Scn1a* gene[66]. This model allowed for the mimicry of an ideal gene therapy, enabling the assessment of many open questions in the field. When *Scn1a* was reactivated after symptom onset at P30, full protection from SUDEP, spontaneous and hyperthermia-induced seizures was achieved, along with an almost complete recovery of behavioral symptoms, including hyperactivity and deficits in social interaction. Cognitive impairment was also rescued, with the restoration of both working memory and spatial reference memory. These results demonstrated that DS symptoms are indeed reversible after symptom onset, supporting the potential of gene-targeted approaches as disease-modifying therapies. Of note, since the Cre recombinase responsible for inducing *Scn1a* gene reactivation is

supplied by an AAV vector that requires 5-8 days for expression *in vivo*, this approach induces gene reactivation at the very early stage of the chronic phase (around P35), when adaptive and compensatory rearrangements have likely just begun. In this study, the effect of the ideal gene therapy administered at P30 on secondary modifications at the gene expression level was evaluated by transcriptomic analysis at four months of age. This analysis revealed marked changes in gene expression in both the cerebral cortex and hippocampus of DS animals compared to control mice, with alterations in several Gene Ontology (GO) categories relevant to the DS phenotype, including sodium ion transport, regulation of synapses, cytoskeleton and extracellular matrix reorganization, suggestive of circuit rearrangement in these two brain areas. Surprisingly, most of the differential expressed genes (DEGs) were completely rescued in the cerebral cortex of 4-month-old DS mice in which the *Scn1a* gene had been re-expressed at P30, while a few remained deregulated in the hippocampus. Given the timing at which it was performed, this experiment leaves open the question of whether an optimal gene therapy delivered at P30 can only prevent the transcriptional alterations that would have occurred later on during the progression of the disease or can even revert some of them.

To address this question, given the lack of expression data for P30 DS mice, we decided to perform a comparative analysis with DEGs found at disease onset, specifically at P24[70] [Figure 1A]. Interestingly, some of the genes deregulated in the hippocampus of 4-month-old DS mice were already altered at disease onset compared to age-matched control mice [Figure 1B]. Specifically, 150 downregulated and 230 upregulated genes were shared between the two experiments [Figure 1B]. Among the upregulated GO categories, pathways associated with structural and functional circuit remodeling, such as extracellular matrix disassembly, dendrite development, and ion transport, were identified, along with the activation of protective anti-inflammatory processes, including response to hypoxia and oxidative stress, as well as transforming growth factor (TGF)-Beta signaling [Figure 1C]. Among the shared downregulated categories, pathways related to the inflammatory response and angiogenesis were found [Figure 1C]. Notably, we observed a common downregulation of genes involved in potassium ion transport, suggesting that shortly after disruption of sodium physiology, alterations in the second major determinant of neuronal excitability occur. While this could represent a homeostatic change attempting to counterbalance sodium current deficit, it could also signify a maladaptive event, depending on the cell type in which these modifications occur.

The observation that gene expression alterations were almost completely reversed in DS mice upon *Scn1a* reactivation at P30, suggests that not only symptoms but also some secondary pathogenetic mechanisms, such as transcriptional changes, might be reversible in DS.

Despite the rescue of both epilepsy and behavioral alterations, at the functional level, *Scn1a* gene reactivation at P30 did not induce a complete recovery of IN function. Specifically, in the CA1 region of the hippocampus, increased IN excitability was observed in DS mice upon $Na_v1.1$ restoration[66]. This observation may indicate that some secondary changes cannot be reverted; nevertheless, circuit function recovery was achieved, possibly by the establishment of a distinct but equally functional balance. While at this stage of the disease, the reversibility of secondary events might be explained by the high plasticity of neural circuits in juvenile mice[107-110], it would be of extreme relevance to evaluate whether comorbidities and secondary modifications can still be rescued by correcting *Scn1a* haploinsufficiency in a more advanced phase of the disease. Reactivation of *Scn1a* at P90 provided full protection from mortality and seizures, supporting a complete reversibility of the epileptic phenotype in the effective chronic phase (P90)[66]. However, no evidence of the effect on comorbidities and secondary modifications was provided in this study.

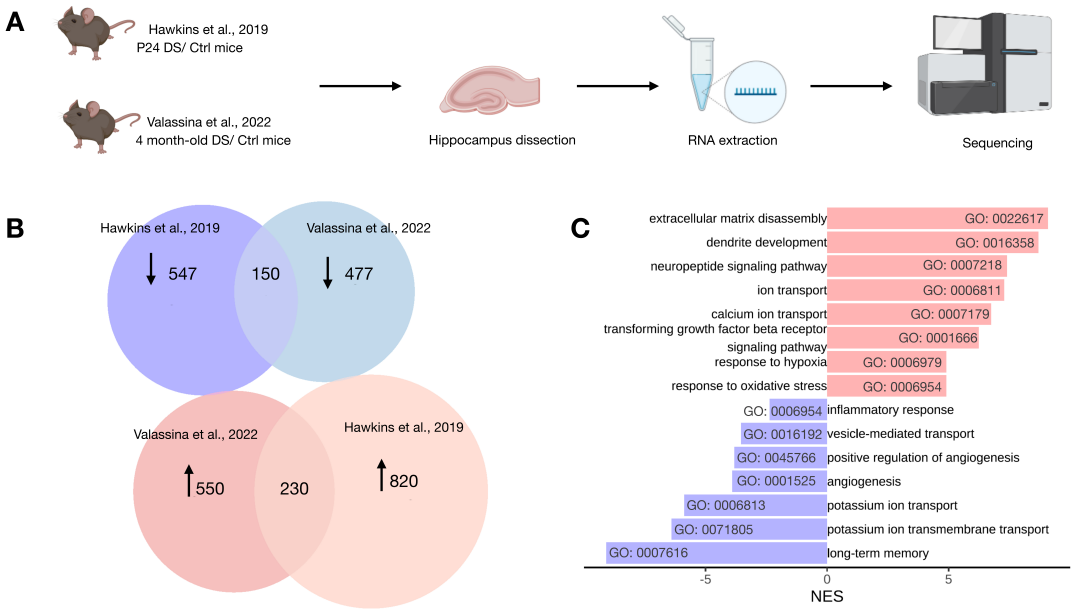

Figure 1. Comparative analysis of the transcriptomic alterations revealed by RNA-seq in DS mouse hippocampus at disease onset and chronic stage. (A) Transcriptomic data were obtained from two different studies that analyzed differential gene expression in P24 and 4-month-old DS mice, respectively, compared to age-matched WT littermates (Ctrl). After hippocampal dissection and RNA extraction, bulk RNA sequencing was performed to obtain the transcriptomic profile and identify differentially expressed genes (DEGs). (B) Venn diagrams showing overlap of downregulated genes (upper diagrams) and upregulated genes (lower diagrams) in DS *vs.* Ctrl hippocampi at P24 from Hawkins *et al.* 2019[70] and at 4 months from Valassina *et al.* 2022[66]. Comparative analysis of DEGs revealed 150 common downregulated genes and 230 shared upregulated genes. (C) Bar plot showing the most represented Gene Ontology (GO) categories among the shared deregulated genes. Pink bars are used for upregulated categories and light blue bars for downregulated ones. Colored bar length is set on the normalization enrichment score (NES) axis below.

In conclusion, our analysis supports the potential efficacy of gene-targeted therapies in rescuing both epilepsy and comorbidities in DS, including the secondary changes in gene expression, at least in juvenile mice, suggesting that upon implementation, gene therapies might achieve similar results.

Therefore, future studies must focus on better elucidating the mechanisms behind the therapeutic effect of *SCN1A*-targeted therapies and define their impact on brain circuit remodeling, as well as on the gene expression profile, which might be determinant to understand the extent of their curative potential. From this perspective, it would be beneficial to investigate potential synergies between gene-targeted therapies and current treatment approaches for DS, such as antiepileptic drugs and behavioral interventions. This could aid in optimizing treatment strategies and enhancing overall outcomes for DS patients.

DECLARATIONS

Authors' contributions

Conceived the idea and wrote the manuscript: Di Berardino C, Colasante G
Performed RNA-seq data analysis: Massimino L, Ungaro F

Availability of data and materials

This manuscript is a review, and all discussed data are from published material, or posters presented at the American Epilepsy Society (AES) Annual Meetings. All sources are quoted and listed in the Bibliography. Our comparative analysis of transcriptomic data is available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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