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Innovative approaches and therapeutic perspectives for early-onset neurodevelopmental disorders: From bench to bedside

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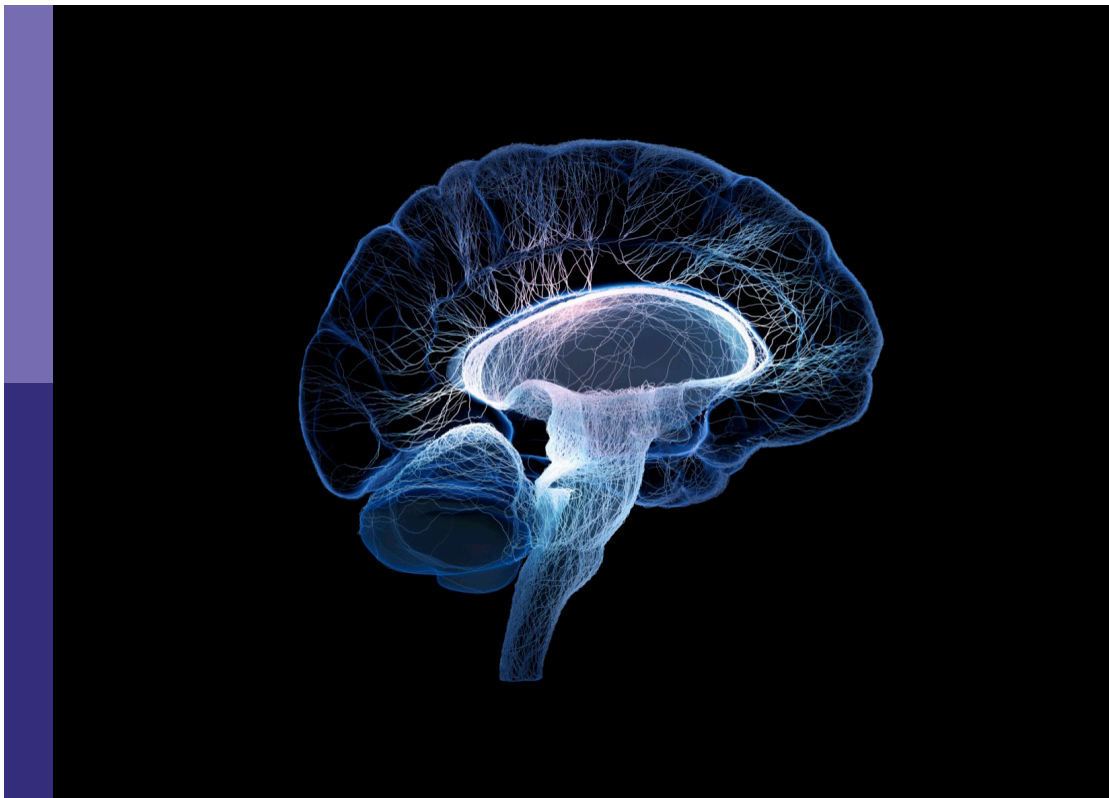

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Innovative approaches and therapeutic perspectives for early-onset neurodevelopmental disorders: From bench to bedside

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Editorial: Innovative approaches and therapeutic perspectives for early-onset neurodevelopmental disorders: from bench to bedside

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Editorial on the Research Topic
Innovative approaches and therapeutic perspectives for early-onset neurodevelopmental disorders: from bench to bedside

The topic "*Innovative approaches and therapeutic perspectives for early-onset neurodevelopmental disorders: from bench to bedside*" stands as a crucial exploration into the realm of neurodevelopmental disorders (NDDs). Organized into six insightful reviews and seven groundbreaking original research papers, this Research Topic is a witness of the dynamic and evolving nature of research in the field.

Palmieri et al. lead the charge by not only providing a comprehensive overview of the state-of-the-art treatment for Rett Syndrome (RTT) but also delving into the potential application of cutting-edge therapies utilizing molecular delivery through nanoparticles. This forward-thinking approach opens up new possibilities for the treatment of RTT, a severe form of NDD.

Similarly, Tempio et al. embarked on a journey to unravel the complexities of Fragile X Syndrome (FXS) by framing it as an interneuronopathy. Their proposal to reintroduce functional interneurons into the brains of FXS patients, based on recent findings regarding the isolation of FXS interneurons and alterations in the Meis2-expressing interneuronal class (Castagnola et al., 2020), adds a novel dimension to therapeutic exploration.

Bertocchi et al. focused their review on the challenges associated with generating and validating preclinical models of developmental and epileptic encephalopathies (DEEs). Their objective is to identify new molecular targets specific to these syndromes and to gain a better understanding of associated comorbidities, such as behavioral and cognitive deficits.

Desprez et al. contributed to the collective knowledge by updating information on dihydropyrimidinase-like (DPYSL) proteins. They shed light on the role of these proteins in synaptic processing during later stages of neurodevelopment and their potential contribution to the pathophysiology of autism spectrum disorders (ASD) and intellectual disability (ID).

Dobrigna et al. provided a comprehensive overview that navigates through the intricate molecular changes in group I p21-activated kinases (PAK1, 2, and 3) and their implications across a broad clinical spectrum of NDDs. The authors underscore the importance of understanding different PAK mutations for the development of personalized treatments.

The review by Li et al. takes a unique perspective by analyzing the effects of various physical activities in children with Attention Deficit Hyperactivity Disorder through a network meta-analysis. Their emphasis on tailoring physical activity based on individual symptom severity brings attention to the personalized nature of interventions.

Turning to original research articles, three delve into Down syndrome, the most common form of genetic intellectual Disability (ID).

Bonne et al. investigated an uncommon neurodevelopmental regression termed Down Syndrome Regression Disorder, distinct from ASD, with an unknown etiology. Symptomatic therapeutic interventions proved ineffective and poorly tolerated in the four analyzed patients (Bonne et al.). In contrast, etiological treatments, such as anti-inflammatory drugs and corticosteroids, resulted in partial or substantial recovery in all cases.

Thomazeau et al. endeavored to unravel the intricate synaptic underpinnings of prefrontal cortex (PFC) dysfunction in Down Syndrome (DS). Building on a prior study involving mBACtgDyrk1a mice, where synaptic plasticity deficits within the PFC were observed (Thomazeau et al., 2014), their focus shifted to another DS model – the Ts65Dn mice. These mice exhibit the overexpression of several genes, including Dyrk1a, a key gene in the pathophysiology of DS. In this study, Thomazeau et al. identified alterations in the intrinsic properties of PFC layer V/VI pyramidal neurons in Ts65Dn male mice. Notably, they discovered the absence of long-term depression, while synaptic or pharmacological long-term potentiation remained fully expressed (Thomazeau et al.).

Conan et al. employed a multifaceted approach, combining genetic and drug screenings utilizing a cellular model that overexpressed CYS4, the homolog of Cystathionine beta synthase (CBS) in Saccharomyces cerevisiae. Their goal was to gain further insights into the molecular mechanisms governing the regulation of CBS, a pivotal protein underlying DS pathology along with Dyrk1a (Panagaki et al., 2022). The study shed light on the significance of Akt/GSK3β and NF-κB pathways in regulating CBS activity and expression.

Shovlin et al. employed a unique approach to pinpoint molecular biomarkers and surrogate endpoints for RTT. They utilized RNA sequencing to assess differential gene expression in whole blood samples from participants in the phase I mecasermin trial. Mecasermin, a recombinant human IGF-1, had previously shown success in pre-clinical tests with RTT mouse models. The analyses identified gene expression profiles linked to the severe breathing phenotype and its improvement following mecasermin administration in RTT. This study led the authors to a significant conclusion, indicating the involvement of inflammatory/immune pathways and IGF-1 signaling in treatment response. Consequently, it steers future investigations toward a novel direction in understanding the pathophysiology of RTT.

In Magel2-knockout (KO) mice, a model of Schaaf-Yang Syndrome, the dysregulation of oxytocin receptors (OXTR) in the hippocampus of adult male mice is normalized through oxytocin (OXT) treatment at birth, resulting in the rescue of autistic-like behavior and cognition in adulthood (Bertoni et al., 2021). Gigliucci et al. analyzed both male and female Magel2-KO mouse brains at different life stages, concluding that OXTRs undergo region-specific modifications related to age, sex, and postnatal OXT treatment. These findings provide valuable insights for tailoring precisely-timed OXT-based therapeutic strategies in Schaaf-Yang Syndrome patients (Gigliucci et al.).

Bouquier et al. introduced a groundbreaking transgenic mouse line, the Shank3Venus/Venus knock-in mouse, enabling the monitoring of the endogenous expression of the major Shank3 isoform in the brain. Mutations in this isoform cause a form of ASD. The study revealed a developmental delay in the brain expression of the Venus-Shank3a isoform in Shank3Venus/ΔC mice compared to Shank3Venus/+ control mice (Bouquier et al.). This innovative approach serves as a powerful tool to study endogenous Shank3a expression under physiological conditions and in ASD, facilitating isoform-specific investigations of endogenous Shank3 proteins.

Prader-Willi disorder (PWS), a NDD characterized by growth delay, hypogonadism, narcolepsy, lack of satiety, compulsive eating, and mild to moderate cognitive impairment, was the focus of the study by Louveau et al.. They examined the response to topiramate in 24 patients affected by different genetic causes of PWS, including deletion or uniparental disomy (UPD) in a region of chromosome 15. The study revealed that topiramate was less effective and less tolerated in UPD cases compared to deletion cases. Interestingly, despite these differences, patients with deletions exhibited less severe clinical features compared to those with UPD (Louveau et al.). The study suggests the relevance of a pharmacogenomic-based approach for studying PWS.

In conclusion, this thematic Research Topic offers a sweeping overview of a rapidly evolving research field, encapsulating various NDDs. The articles collectively tackle crucial issues, including phenotypic complexity, the role of sex as a biological variant, challenges in developing validated models, complexities in drug development, and the imperative need for innovative delivery methods and cell-based treatments and the pharmacogenomics as new approach to unravel new pathophysiological elements. These insights not only contribute significantly to our current understanding but also lay the foundation for future, more targeted explorations within the expansive realm of NDDs.

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Conflict of interest

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ORIGINAL RESEARCH
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Molecular Signatures of Response to Mecasermin in Children With Rett Syndrome

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Rett syndrome (RTT) is a devastating neurodevelopmental disorder without effective treatments. Attempts at developing targetted therapies have been relatively unsuccessful, at least in part, because the genotypical and phenotypical variability of the disorder. Therefore, identification of biomarkers of response and patients' stratification are high priorities. Administration of Insulin-like Growth Factor 1 (IGF-1) and related compounds leads to significant reversal of RTT-like symptoms in preclinical mouse models. However, improvements in corresponding clinical trials have not been consistent. A 20-weeks phase I open label trial of mecasermin (recombinant human IGF-1) in children with RTT demonstrated significant improvements in breathing phenotypes. However, a subsequent randomised controlled phase II trial did not show significant improvements in primary outcomes although two secondary clinical endpoints showed positive changes. To identify molecular biomarkers of response and surrogate endpoints, we used RNA sequencing to measure differential gene expression in whole blood samples of participants in the abovementioned phase I mecasermin trial. When all participants (n = 9) were analysed, gene expression was unchanged during the study (baseline vs. end of treatment, T0–T3). However, when participants were subclassified in terms of breathing phenotype improvement, specifically by their plethysmography-based apnoea index, individuals with moderate-severe apnoea and breathing improvement (Responder group) displayed significantly different transcript profiles compared to the other participants in the study (Mecasermin Study Reference group, MSR). Many of the differentially expressed genes are involved in the regulation of cell cycle processes and immune responses, as well as in IGF-1 signalling and breathing regulation. While the Responder group showed limited gene expression changes in response to mecasermin, the MSR group displayed marked differences in the expression of genes associated with inflammatory processes (e.g., neutrophil activation, complement activation) throughout the trial. Our analyses revealed gene expression profiles associated with severe breathing phenotype and its improvement

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after mecasermin administration in RTT, and suggest that inflammatory/immune pathways and IGF-1 signalling contribute to treatment response. Overall, these data support the notion that transcript profiles have potential as biomarkers of response to IGF-1 and related compounds.

Keywords: Rett syndrome, mecasermin, insulin-like growth factor 1 (IGF1), methyl-CpG binding protein 2 (MECP2), biomarker

INTRODUCTION

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that affects predominantly females (∼1/9,000–1/10,000) (Kaufmann et al., 2016). The diagnosis of RTT is clinical, taking into account a phenotypic spectrum of severity. The four core diagnostic criteria that define classic/typical versus variant/atypical RTT are partial or complete loss of hand function, partial or complete loss of spoken language, impaired gait, and presence of repetitive hand movements termed hand stereotypies (Neul et al., 2010). All four criteria are required for the diagnosis of classic RTT, while atypical RTT is diagnosed when at least 2 of these 4 main criteria are present plus 5 of 11 supportive criteria (i.e., breathing disturbances, bruxism when awake, impaired sleep, tone abnormalities, peripheral vasomotor disturbances, scoliosis/kyphosis, growth retardation, small cold hands and feet, inappropriate laughing/screaming spells, diminished pain response, and intense eye communication) (Neul et al., 2010). These supportive criteria are also prevalent in classic RTT (Percy et al., 2010).

Rett syndrome is usually associated with a pathogenic mutation in the *methyl-CpG binding protein 2* (*MECP2)* gene, particularly in those individuals with the classic presentation (Neul et al., 2010). Genotype-phenotype correlations have led to identifying groups of *MECP2* mutations with different levels of severity (Cuddapah et al., 2014). *MECP2* encodes the methyl CpG-binding protein 2 (MeCP2), a chromatin binder and transcription regulator (Ip et al., 2018). Abnormal expression of MeCP2 results in impaired brain development and function associated with disruption in synaptic plasticity (Kaufmann et al., 2005; Asaka et al., 2006; Blackman et al., 2012). The discovery of mutations in *MECP2* (Amir et al., 1999) as the genetic abnormality associated with most cases of RTT, has led to the generation of mutant mouse models that replicate many features of the disorder (Chen et al., 2001; Guy et al., 2001; Nguyen et al., 2013; Ross et al., 2016). These mutant mouse models have become a valuable resource for the study of the molecular and cellular mechanisms underlying RTT, and for testing candidate treatments for the disorder (Katz et al., 2012). Management of RTT is mainly symptomatic (Kaufmann et al., 2016; Leonard et al., 2017); therefore, the discovery of disease-modifying therapies in models of RTT has become a priority in the field. Preclinical studies in mice have already identified several promising drugs, some of which have moved to clinical development (Tropea et al., 2009; Castro et al., 2014; Park et al., 2014; Kaufmann et al., 2019). One of the best studied candidate drugs is Insulin-like growth factor 1 (IGF-1).

Insulin-like growth factor 1 is a growth factor and signalling molecule that is involved in growth, maturation, and ageing. In the CNS, IGF-1 plays a role in developmental and mature brain synaptic plasticity (Dyer et al., 2016). IGF-1's role in neuronal development and function presents multiple similarities to that of BDNF; however, the latter signalling molecule has limited therapeutic potential because of its inability to cross the blood-brain barrier. More recent evidence suggests that IGF-1 signalling is implicated in metabolic, homeostatic processes, which underlie synaptic plasticity and are disrupted in RTT (De Felice et al., 2014; Banerjee et al., 2016; Gazit et al., 2016; Neul et al., 2020; Crivellari et al., 2021). IGF-1 is naturally cleaved by proteases into the small tripeptide Glycine-Proline-Glutamic acid (GPE) and the larger Des (1–3) IGF-1 peptide. GPE has neuroprotective properties through different modulatory processes to those of IGF-1 (Guan and Gluckman, 2009). Both full-length IGF-1 and GPE have been shown to ameliorate features of relevance to RTT in a genetic mouse model of the disorder (Tropea et al., 2009; Castro et al., 2014). These encouraging results have led to clinical trials in RTT using either a recombinant human form of IGF-1 (i.e., rhIGF-1, mecasermin) or a modified GPE (i.e., trofinetide). Both mecasermin and trofinetide have shown safety and tolerability and initial evidence of efficacy (Pini et al., 2012, Pini et al., 2016b; Khwaja et al., 2014; Glaze et al., 2017, 2019; O'Leary et al., 2018).

The first clinical trial of mecasermin, an open label phase I pharmacokinetic and exploratory efficacy study, demonstrated that mecasermin reached the CNS compartment following a non-linear kinetics with greater distribution in the peripheral compartment (Khwaja et al., 2014). In terms of efficacy, several parameters showed improvements during a 20-weeks open label extension (OLE) of the pharmacokinetic segment. Improvements in measures of anxiety and mood during the OLE were associated with reversal of right frontal alpha band asymmetry on EEG, a biomarker of these behavioural abnormalities. Cardiorespiratory measures showed that apnoea, a characteristic and severe breathing abnormality in RTT, also improved markedly. Since these assessments were carried out by plethysmography, an objective methodology, these were considered the most meaningful clinical findings of the study (Khwaja et al., 2014). A follow up larger randomised placebo-controlled phase II trial, did not replicate these findings. However, secondary endpoints measuring stereotypic behaviour and social communication demonstrated significant improvements (O'Leary et al., 2018). One of several possible explanations for the discrepancy between the two mecasermin studies is that severity of breathing abnormality, specifically a minimum apnoea index, was not part of the inclusion criteria.

This resulted in that only 14 out of 30 participants in the phase II trial presented at baseline an apnoea index high enough to demonstrate treatment efficacy.

The failure in consistently demonstrating mecasermin's efficacy in children with RTT, in conjunction with the continuous interest in trofinetide - now reporting encouraging results following adult and paediatric trials (Glaze et al., 2017, 2019) - emphasises the importance of further characterising the mechanisms underlying therapeutic responses to IGF-1-related compounds and the need for identifying biomarkers linked to clinical improvements. The present study aimed at delineating molecular profiles associated with therapeutic responses to mecasermin in children with RTT, on the basis of data from the mecasermin phase I trial. Because this trial included two periods of drug administration and their intervening washout period, it offered the possibility of evaluating the dynamics of gene expression in response to mecasermin. For this purpose, we analysed RNA profiles on whole blood samples and correlated them with apnoea responder status (Khwaja et al., 2014). We chose the latter as measure of efficacy because of its objective nature. A link between two sets of objective parameters would provide stronger evidence for molecular factors underlying clinical responses to mecasermin and, probably, also other IGF-1-related compounds. In terms of the use of RNA profiles as biomarkers, we contemplated both that RNA profiles could serve as predictors of response (i.e., baseline levels) or as surrogate endpoints (i.e., change in levels between baseline and end of treatment). Whole blood is particularly useful in this respect due to availability and accessibility of the tissue. *MECP2* is considered to be widely expressed in peripheral tissues, where it has even been shown to contribute to certain RTT symptoms, including hypoactivity, exercise fatigue, and bone abnormalities (Song et al., 2014; Ross et al., 2016). Moreover, a recent study of *Mecp2* mutant mice revealed that some genes differentially expressed in blood are also altered in brain (Sanfeliu et al., 2019). These factors indicate that whole blood is a particularly relevant sample source for the aims of our study.

We found that individuals with RTT, severe breathing abnormalities and positive response to mecasermin, as shown by an improved apnoea index, had molecular signatures of relevance to their phenotype and treatment that can be distinguished before drug administration and, to a lesser extent, at later timepoints. This finding could assist in the design and analysis of future trials with mecasermin and other IGF-1-related compounds.

MATERIALS AND METHODS

Outcome Measures and Definition of Responder

Multiple outcome measures were used for evaluating efficacy. They included a wide range of clinician- and caregiver-rated neurobehavioural assessments, several of them developed or adapted for RTT [Clinical Global Impression scales (CGI-S, CGI-I), Parent-Targetted Symptom Visual Analogue Scale (PTSVAS), Mullen Scales of Early Learning (MSEL), the Clinical Severity Scale (CSS), the Motor–Behavioural Assessment (MBA), the Rett Syndrome Behaviour Questionnaire (RSBQ), and the Anxiety, Depression, and Mood Scale (ADAMS)], and automated cardiorespiratory measures. The latter consisted of time synchronised chest respiratory inductive plethysmography and electrocardiography. While neurobehavioural assessments were performed only during the OLE, cardiorespiratory measures were collected throughout the trial. Using a wireless data-acquisition plethysmography device (BioRadio, Great Lakes Neurotechnologies, Independence, OH, United States), breathing abnormality profiles with a focus on breath holding were determined for all participants. Clinically significant apnoea, which was defined as apnoeic episodes >10 s in length, was present in five participants. Apnoea was graded as moderate–severe when these apnoeic episodes were >5 per hour, a pattern present in 4 participants at the beginning of the study, before the multiple ascending dose (MAD)/pharmacokinetics period (**Figure 1** and **Table 1**). All these four participants experienced a decrease in apnoea severity to mild (<5 episodes per hour) by the end of the OLE (**Table 1**). Details on outcome measures can be found in Khwaja et al. (2014). For the purpose of evaluating therapeutic responses, Apnoea Responder was defined as a participant who had a decrease in apnoea frequency $>50\%$ or a reduction to ≤ 5 apnoeic episodes per hour (the four participants mentioned above). By default, the five participants who did not fulfil the criteria for Responder (R) were assigned to the Mecasermin Study Reference (MSR) group.

Of the total 12 clinical trial participants, nine had a diagnosis of classic RTT (participants #1, #4, #5, #6, #7, #8, #9, #11, and #12) while three participants had a diagnosis of *MECP2* related disorder (MRD) (participants #2, #3, and #10). Participants with MRD are characterised by having pathogenic *MECP2* mutations, some of them also identified in individuals with RTT, but do not display a clinical presentation compatible with either typical or atypical RTT. They were included in the trial (Khwaja et al., 2014) in order to determine whether treatment with mecasermin could be effective in most phenotypes associated with *MECP2* mutations. However, since participants with MRD did not present a breathing phenotype and were not included in the OLE period, their samples were analysed in this molecular study only as pre-treatment reference data. *MECP2* mutations of each participant with classic RTT are described in **Table 2**. All RTT participants were included in both the MAD and OLE treatment periods, but only 1 MRD participant (participant #10) was included in the OLE phase (see **Figure 1**). Mutations in participants with RTT were classified according to their profile of severity as severe (R168X, R255X, deletions and insertions), intermediate (T158M) or mild (other point mutations), following the report by Cuddapah et al. (2014).

RNA Collection and Sequencing

RNA from whole blood samples was collected and extracted using PAXgene Blood RNA tubes (BD Biosciences, Radnor, PA, United States), and analysed by 3′-Digital Gene Expression (3′-DGE). Sequencing was performed at the Massachusetts Institute of Technology (MIT)'s BioMicro Center, an integrated genomics core facility. 3′-DGE was adapted from Soumillon et al. (2014)

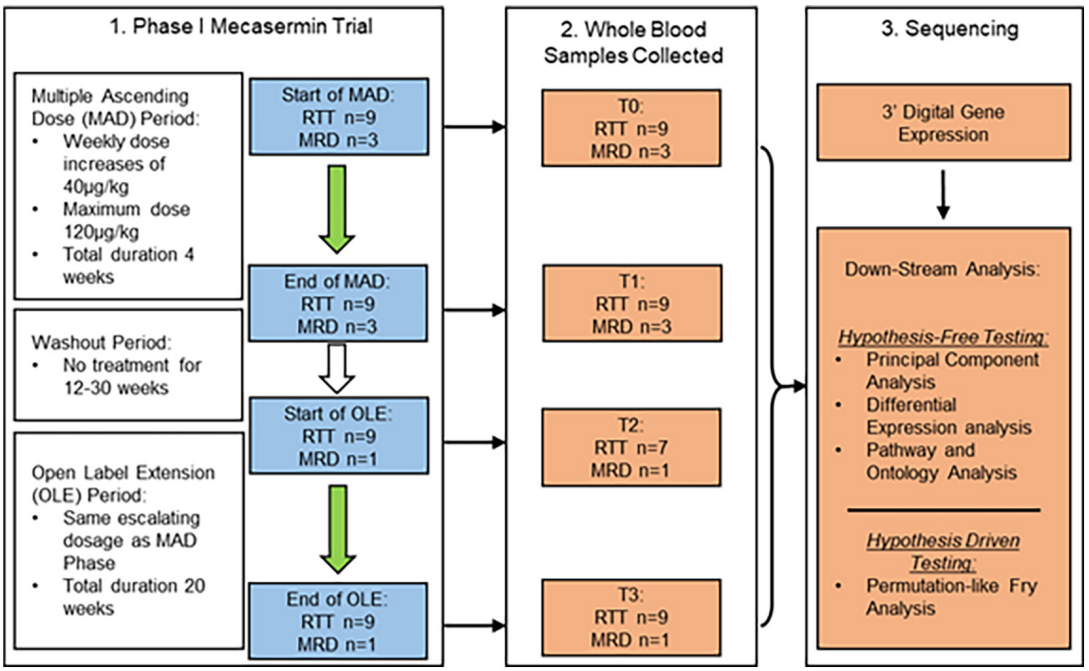

FIGURE 1 | Study design and dosing schedule of phase I mecasermin trial in RTT. All participants (n = 12) were included in the multiple ascending dose (MAD) period while only those with RTT (n = 9; n = 7 at T2) and one with MRD progressed onto the open label extension (OLE) component. Participants were administered mecasermin twice daily by subcutaneous injection. Time points when blood sampling was performed are denoted T0 (start of MAD), T1 (end of MAD), T2 (start of OLE), and T3 (end of OLE). Whole blood samples were used for 3′-Digital Gene Expression (3′-DGE) sequencing.

TABLE 1 | Apnoea index profiles of participants with classic Rett syndrome (RTT).

Participants ID	Breathing phenotype at baseline	Apnoea index Start of MAD	Apnoea index End of MAD	Apnoea index Start of OLE	Apnoea index End of OLE
#1 MSR	None	0	1	1	1
#4 MSR	BH, HV, and AE	0	0	2	0
#5 MSR	BH and HV	0	0	0	0
#6 MSR	AE	2	1	3	1
#7 R	BH and AE	8	2	3	2
#8 MSR	AE	0	0	0	0
#9 R	BH and AE	7	4	5	3
#11 R	BH, AE, and Cyanosis	60	30	22	18
#12 R	BH. AE, and Cyanosis	14	8	6	2

BH, breath holding; HV, hyperventilation; AE, air expulsion; R, responder group; MSR, Mecasermin Study Reference group.

using a tag-based transcriptome sequencing method, which provides cost-effective means of generating expression data for characterising major patterns in heterogeneous samples (Soumillon et al., 2014). The sequencing read data was then combined with barcode information in a FASTQ format and mapped onto the Hg19 reference sequence using BWA. Per-gene count quantification was conducted with the End Sequence Analysis Toolkit (ESAT) for downstream differential gene expression analysis (Derr et al., 2016). Raw data will be available at the public repository Gene Expression Omnibus (GSE198856). Samples from two participant (participant #5 and participant #12) were not collected at T2 (beginning of OLE); these missing data were not imputed in the analyses.

Differential Gene Expression Analyses

Differential gene expression was quantified using EdgeR, a popular software specifically designed for analysing sequencing data from small sample sizes (Robinson et al., 2009). EdgeR was operated on an R studio environment using R statistical programming language (Venables et al., 1995). EdgeR uses an empirical Bayes estimation, based on a negative binomial model,

TABLE 2 | Methyl-CpG binding protein 2 (*MECP2*) mutation profiles of participants with classic RTT.

Participant ID, Analysis group	Mutation (nucleotide nomenclature)	Mutation (amino acid nomenclature)	Mutation location (MeCP2 domain)	Mutation type	Mutation severity profile
#1, MSR	c.538C > T	R168X	ID, TRD	Non-sense	Severe
#4, MSR	c.790_808del119	–	TRD-NLS	Deletion	Severe
#5, MSR	Deletion of Exon 3 and 4 (min 6.0kb – max 7.1kb)	–	Multiple	Deletion	Severe
#6, MSR	c.1159_1273del114	–	C-Term	Frameshift, Insertion or Deletion	Mild
#7, R	c.763C > T	R255X	TRD	Non-sense	Severe
#8, MSR	c.763C > T	R255X	TRD	Non-sense	Severe
#9, R	c.473C > T	T158M	MBD	Missense	Intermediate
#11, R	Deletion Exon 1 and 2	–	Start codon	Deletion	ND
#12, R	c.965C > T	R322L	C-Term	Missense	Mild

MSR, Mecasermin Study Reference group; R, responder group; ID, interdomain; TRD, transcriptional repression domain; NLS, nuclear localisation signal; C-term, carboxy-terminus; MBD, methyl-binding domain; ND, not yet determined.

and a quasi-likelihood F test (QLFT) to determine differential expression. QLFT is the preferable choice for comparing gene expression on small samples, as it better reflects the uncertainty of estimating gene expression dispersion (i.e., variability), resulting in a lower error rate (Chen et al., 2017).

Using QLFT, we conducted two different types of analyses to examine the drug's effect on gene expression across the trial: (1) time-point and (2) responder status comparisons. Based on sampling, there were six possible time-point comparisons (T0–T1, T0–T2, T0–T3, T1–T2, T1–T3, and T2–T3), which were conducted on all RTT sample sets ($n = 9$ at T0, T1, and T3; $n = 7$ at T2). On the other hand, responder status comparisons contrasted R ($n = 4$ at T0, T1, and T3; $n = 3$ at T2) and MSR ($n = 5$ at T0, T1, and T3; $n = 4$ at T2) groups at each of time point.

Significant gene sets identified by these comparisons were used to conduct pathway analysis using Reactome[1] and Ontology analysis using Panther's Gene Ontology database (GO).[2] The gene sets were entered into these online tools filtering out any unidentified genes. Both Reactome and GO analysis use over-representation analysis to determine if a given gene set is over- or under-represented in a given pathway or ontology, with respect to a hypothetical random selection (Fabregat et al., 2018; The Gene Ontology Consortium, 2000, 2021). In both GO and Reactome analyses, significant pathways were considered those with Entities false discovery rate (FDR) values < 0.05, and the significant pathways were validated with Fry() to eliminate false positives caused by correlations between genes in the set. This function, uses operations (analogous to fractional permutations) on the gene sets to determine if a gene set was differentially expressed across randomly generated comparison sets (Wu et al., 2010; Chen et al., 2016; Muley et al., 2020; Grisaru-Tal et al., 2021). In summary, Fry(), by cross-checking the selected gene sets shuffling the data between the two compared groups, controls for false positives. This analysis was also employed to validate the pathways and ontologies that had been identified using the hypothesis-free methods. Only pathways validated with Fry() are reported in this study.

Using edgeR, two categories of analyses were performed:

(A) *Hypothesis-free testing*: We evaluated the differential expression of all the annotated genes expressed in all the participants (26,116 genes in total). This approach was used to detect changes in gene expression without any bias from previous studies or the literature. This type of analysis is, however, curtailed by the requirement of a high FDR due to the large number of tested genes. Hypothesis-free testing was conducted using the QLFT() function.

(B) *Hypothesis-driven testing*: We also used a hypothesis-driven (HD) approach for testing specific genes, based on previous research or the literature. Specifically, we tested five main classes of gene sets that have been associated to RTT pathophysiology: IGF-1 and BDNF pathways; metabolic homeostatic mechanisms, including mitochondria, protein ubiquitination, and chromatin mediated processes (Pecorelli et al., 2013); abnormal inflammatory responses (Maezawa and Jin, 2010; O'Driscoll et al., 2013; Lin et al., 2016; Zhao et al., 2017); pathways linked to the apnoea phenotype (e.g., monoamine metabolism) (Viemari et al., 2005; Toward et al., 2013; Vogelgesang et al., 2018); and autism spectrum disorder (ASD) iPSCs-IGF-1 induced genomic changes (Linker et al., 2020). To test whether these gene sets were differentially expressed across the time-point comparisons or the R vs. MSR comparisons, we used edgeR's Fry function. Gene sets analysed with Fry were further validated by permutation analyses (Bach et al., 2020), which take into consideration control pathways with the same size of the HD gene sets and use the distribution of the *p*-value of the controls and the HD gene sets to confirm statistical significance. Only HD gene-sets with *p*-values falling into the top 5 percentile of the *p*-value distribution are reported in this study.

[1]https://reactome.org/

[2]http://geneontology.org/

Statistical Analysis and Graphing Software

Principal component analysis (PCA) was conducted using the built-in prcomp() function in R studio, by analysing sample counts at T0, applying scaling and centring of data. For graphical representations, we used the ggplot2 package, also in R studio. All the tests were corrected for multiple testing by FDR, and differences were defined as significant with a p-value < 0.05. Analyses examining the relationship between PCs and variables of clinical significance were performed by the non-parametric Spearman rank correlation test.

RESULTS

Cohort and Trial Design

The study is regulated by Institutional Review Board protocol number 10-08-0403, and by MTA agreement #18081. The cohort in the phase I open label mecasermin trial (Khwaja et al., 2014) included 9 participants with classic RTT and 3 with *MECP2*-related disorders [MRD; non-RTT clinical presentations in individuals with *MECP2* mutations (Neul et al., 2010)]. Details about the cohort, including individual *MECP2* mutations in participants with RTT, can be found in **Table 2** and in the original publication on the trial by Khwaja et al. (2014). The study consisted of two different components: a 4-week multiple ascending dose (MAD) period and a 20-week open label extension (OLE) period. The MAD and OLE periods were separated by a variable interval of 12–30 weeks. The MAD period was focussed on assessment of safety and collection of serial pharmacokinetic data. The goal of the OLE period was to extend the evaluation of mecasermin safety and to conduct a preliminary assessment of the drug's efficacy. Both MAD and OLE periods began with a dose of 40 μg/kg, which was increased by 40 μg/kg per week to a maximum dose of 120 μg/kg. Whole blood samples were taken from the participants at four different time points denoted T0–T3 (T0 and T1, corresponding to the beginning and end of the MAD, respectively; and T2 and T3, corresponding to the beginning and end of the OLE, respectively). Of note, for participants #5 and #12 samples were not obtained at T2. At T2 and T3 (OLE period), in addition to the cardiorespiratory evaluations described below, multiple neurobehavioral assessments were also performed (details in Khwaja et al., 2014). A schematic of the dosing schedule and study design are shown in **Figure 1**.

Gene Expression Profiles in Participants With Rett Syndrome and *MECP2* Related Disorder

The first treatment period, the Multiple Ascending Dosage (MAD) period, intended to determine the pharmacokinetics of mecasermin. The second treatment period, the Open Label Extension (OLE), was an additional treatment segment intending to obtain additional information on safety and preliminary data on efficacy. The MAD period included, in addition to the nine participants with classic RTT, three girls with MRD. *MECP2* mutations identified in each participant with RTT are described in **Table 2**.

We found no significant differential gene expression, calculated with edgeR, when comparing MRD and RTT groups at T0. Because the T1, T2, and T3 time points had only one MRD sample, participants with MRD were not considered for further analysis. **Table 3** presents the changes in gene expression in the entire RTT cohort throughout the trial, by comparing the different sequential time points.

Gene Expression Profiles of Responder and Mecasermin Study Reference Groups at Baseline

All analyses described in this and the following sections, used only data from the participants with RTT. We used PCA to delineate the relationship between gene expression profiles at baseline (T0) in R (participants #7, #9, #11, and #12) versus MSR (participants #1, #4, #5, #6, and #8) groups. **Figure 2** shows a plot of the largest principal components (PC1 and PC2) at T0 which accounted, respectively, for 44.5 and 13.1% of the variance. R (n = 4, red) and MSR (n = 5, blue) groups were divided by PC1; R to the right and MSR to the left of 0 on the PC1 axis. Participants in the MSR group were relatively close to each other with exception of one sample (participant #6), varying mainly in PC2, while the R group was more dispersed. Interestingly, although that MSR participant did not meet our stringent criteria for responder, she had a mild apnoea phenotype with episodes of the same length as those in the R group and showed an improvement in her apnoea index at the end of the OLE. As reference, the PC profile of MRD participants is intermediate between the R and MSR groups. Thus, this gene expression variance analysis showed that R and MSR groups segregated from each other before treatment with mecasermin along PC1, which is consistent with their clinical profiles and outcome. Analyses examining the relationship between gene expression profiles, measured by PC1, and variables of clinical significance, found no significant relationship between PC1 and mutation severity category (Rho = 0.52, p = 0.15). In contrast, we found a strong correlation between PC1 and apnoea index at baseline. When considering all the participants in the study (n = 9), the

TABLE 3 | Differentially expressed genes in the entire RTT cohort throughout the trial.

Interval	Gene	Log2 Fold Change	p-value	FDR
T0–T1	–	–	–	–
T0–T2	–	–	–	–
T0–T3	–	–	–	–
T1–T2	*TMEM176B*	−2.53	1.54×10^{-11}	3.41×10^{-07}
	TMEM176A	−2.72	2.61×10^{-11}	3.41×10^{-07}
T1–T3	–	–	–	–
T2–T3	*ERVMER34-1*	2.35	3.22×10^{-09}	7.81×10^{-05}
	TMEM176B	2.07	5.98×10^{-09}	7.81×10^{-05}
	RRM2	2.06	3.42×10^{-06}	0.03
	CENPF	2.81	5.07×10^{-06}	0.03

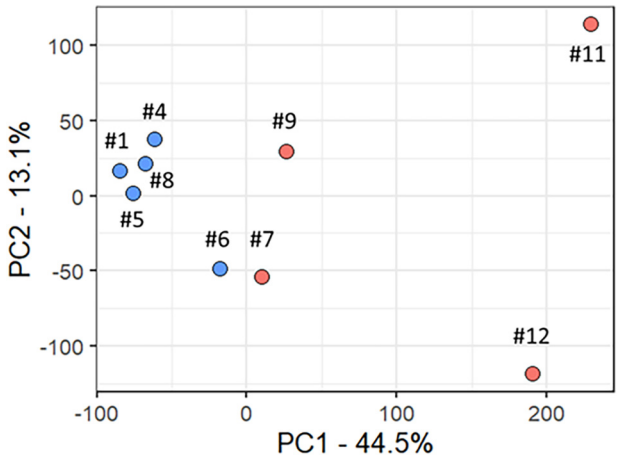

FIGURE 2 | Principal component analysis of baseline transcript profiles in participants with RTT. Scatter plot of Principal Components 1 and 2 (PC1 and PC2), which accounted, respectively, for 44.5 and 13.1% of total variance. The Responder group is shown in red and the Mecasermin Study Reference group in blue. Note that participant #6 did not meet all criteria for the Responder group; however, she also showed an improvement in her apnoea index at the end of the trial.

correlation was significant (Rho = 0.94, $p = 0.00016$). However, when considering only participants with apnoea index > 0 ($n = 5$), although the correlation was strong (Rho = 0.90), the test failed to reach significance ($p = 0.08$; **Supplementary Figure 1**).

Gene Expression Profiles of Responder and Mecasermin Study Reference Groups Throughout the Trial

In order to determine how the R and MSR groups reacted differentially to IGF-1 treatment, we performed gene expression analysis at different time-points throughout the trial. We compared gene expression between the two groups at T0, T1, T2, and T3. Such comparisons revealed a large number of changes (**Supplementary Table 1**) with the greatest difference in gene expression observed at T0, as presented in detail in the previous section, and relatively higher overall expression in the R group with respect to the MSR group (3,693 upregulated, 221 downregulated). Similar patterns, with relatively higher expression in R versus MSR groups, were also observed at T1 (113 upregulated, 34 downregulated) and T2 (66 upregulated, 16 downregulated). However, the direction of these differences was reversed at T3 (2 upregulated, 28 downregulated). Thus, the analyses revealed decreasing differential gene expression between the R and MSR groups, from baseline to end of treatment (T0–T3), confirming the existence of different "molecular" subcohorts at the beginning of the trial. The two groups, identified by their genomic profiles, appeared to respond differently to mecasermin treatment, with the MSR group showing more changes (**Table 4**).

In order to identify pathways differentially regulated in R versus MSR groups at different periods of the trial, the list of differentially expressed genes was input into Reactome (pathway database) and Gene Ontology (GO). Results were controlled for false positives using Fry() (EdgeR). GO found significantly enriched ontologies only at T0 (all upregulated in the R group). The top 50 validated GO gene sets are depicted in **Table 5**, while the full list is reported in **Supplementary File 2**. Validated pathways in Reatcome, at different time points, are shown below in **Table 6**.

Preliminary Assessment of Mechanisms Underlying Response to Mecasermin in Rett Syndrome: Hypothesis-Free Analysis

In order to ascertain mechanisms underlying the response to mecasermin in RTT, we examined differential gene expression in the entire RTT cohort ($n = 9$; $n = 7$ at T2) at different intervals (T0–T1, T0–T2, T0–T3, T1–T2, T1–T3, and T2–T3). There were no significant differences between T0 and T1 (MAD period), T0 and T2, T0 and T3 (entire trial), or T1 and T3. During the off-treatment period (T1–T2), two related genes *TMEM176A* and *TMEM176B* showed a reduction in levels (**Table 3**). In the interval corresponding to the OLE period (T2–T3), we found 4 differentially expressed genes (i.e., increased expression): *ERVMER34-1*, *RRM2*, *CENPF*, and the abovementioned *TMEM176B*. Thus, mecasermin treatment induced limited changes in gene expression that were mainly present during the OLE period (**Supplementary Material**), most likely due to the heterogeneity of the population. Therefore, we included separate comparisons of gene expression patterns across the different study intervals in the R ($n = 4$; $n = 3$ at T2) and MSR ($n = 5$; $n = 4$ at T2) groups.

The R group showed only two differentially expressed genes across all study intervals: *HLA-DRB5* and *SMCR5*. *HLA-DRB5* encodes the major histocompatibility complex class II DRβ5, *SMCR5* is the non-coding *Smith-Magenis Syndrome Chromosome Region Candidate 5* gene. In the R group, *HLA-DRB5* decreased significantly from T0 to T1 (MAD period) and from T0 to T2, but it increased significantly during the OLE (T2–T3). In contrast, in the MSR group *HLA-DRB5* expression decreased only during the OLE (T2–T3).

Many of the genes differentially expressed along the study in the MSR group have roles in the immune system. Among these immune function genes are the *TMEM176* genes, which are associated with maintenance of dendritic cell immaturity (Condamine et al., 2010). *TMEM176A* is differentially expressed in the MSR group between T1 and T2 ($p < 0.01$), while *TMEM176B* is differentially expressed between T1 and T2 ($p < 0.01$) and T2 and T3 ($p < 0.02$). Fold change levels are reported in **Supplementary Table 1**. The MSR group also showed increases in several defensin-α genes. Defensins are antimicrobial and cytotoxic peptides involved in host defence, which are stored in granules (azurophils). During phagocytosis, these granules fuse into phagocytic vacuoles and contribute to antimicrobial response (Ganz, 2003). The increases in defensin gene expression mainly represent changes during the OLE (T2 to T3), but also between T0 and T3 and T1 and T3 (all p-values < 0.001; for detailed fold changes see **Supplementary Table 1**).

TABLE 4 | Number of genes differentially expressed in the RTT cohort throughout the trial.

Interval	Number of genes (all RTT, *n* = 9; *n* = 7 at T2)	Number of genes (R, *n* = 4; *n* = 3 at T2)	Number of genes (MSR, *n* = 5; *n* = 4 at T2)
T0–T1	–	1 (0 ↑, 1 ↓)	13 (1 ↑, 12 ↓)
T0–T2	–	2 (0 ↑, 2 ↓)	24 (0 ↑, 24 ↓)
T0–T3	–	–	28 (16 ↑, 12 ↓)
T1–T2	2 (0 ↑, 2 ↓)	–	49 (5 ↑, 44 ↓)
T1–T3	–	–	27 (21 ↑, 6 ↓)
T2–T3	4 (4 ↑, 0 ↓)	1 (1 ↑, 0 ↓)	37 (36 ↑, 1 ↓)

↑, *upregulated*,↓, *downregulated*.

Overall, the differential gene expression analyses between different periods of the trial revealed a significant change only in the MSR group. At all intervals, we found several differentially expressed genes: 13 genes in T0–T1, 24 genes in T0–T2, 28 genes in T0–T3, 49 genes in T1–T2, 27 genes in T1–T3, and 37 genes in T2–T3. **Table 4** summarises interval comparisons in all three groups under analysis: all participants with RTT, R group, and MSR group. A full list of results is included in **Supplementary Table 1**.

All the differentially expressed genes identified in the entire cohort were also found in the MSR group, suggesting that the differences in the entire cohort were mainly driven by the former. Therefore, all subsequent analyses of differential gene expression were carried out separately in the R and MSR groups. We used the differentially expressed genes identified in these two groups to perform pathway and ontology analyses using Reactome and GO. The significant results from these analyses were then validated using the edgeR's Fry function. The validated results, all in the MSR group, are: Mitotic Cell Cycle Process (GO:1903047) upregulated at T3 versus T2, Non-sense mediated Decay (NMD) (R-HSA-927802), downregulated at T2 versus T1, and NMD enhanced by exon Junction Complex (R-HSA-975957) upregulated at T2 versus T1.

Preliminary Assessment of Mechanisms Underlying Response to Mecasermin in Rett Syndrome: Hypothesis-Driven Testing

We then tested if mechanisms previously associated to RTT pathogenesis were different between R and MSR groups, and if they were modulated by the administration of Mecasermin. We retrieved the corresponding gene sets in GO and then tested the hypothesis using permutation analyses in R (edgeR's Fry function). This analysis showed significant results in R versus MSR comparisons, at T0, T1, and T2.

All the results of hypothesis-driven testing are included in **Table 7**. The analysis reveals that most of the gene sets were differentially expressed between R and MSR groups at T0, except for the BDNF receptor signalling pathway. At T1, which corresponded to the end of the MAD period, the IGF-1 receptor signalling pathway was differentially expressed. At T2, a number of signalling pathways were differentially expressed including PI3K, BDNF receptor, dopamine receptor, and serotonin receptor. At T3, there were no significantly different gene sets when R and MSR groups were compared. Results of R vs. MSR group analyses are shown in **Table 7**.

The same sets of genes were then examined within each group (R and MSR) at different intervals. We found that in the R group only the IGF-1 receptor signalling pathway was differentially expressed from T1 to T2, while in the MSR group Response to Chronic IGF-1 treatment in ASD iPSCs was significantly different between T1 and T2 (**Table 7**).

In summary, both hypothesis-driven and hypothesis-free analyses demonstrated that the RTT cohort was not homogeneous at baseline, and in its molecular response to mecasermin treatment. **Figure 3** summarises the results of the gene expression analyses in relationship with changes in the apnoea index.

DISCUSSION

Molecular Biomarkers and Response to Mecasermin Treatment

The present study aimed at identifying RNA profiles associated with therapeutic responses to mecasermin in children with RTT. For this purpose, we used samples from a mecasermin phase I trial, which explored in a preliminary fashion clinical response to the compound in a study including two periods of drug administration. Of the two positive clinical endpoints, we selected breathing abnormalities because it included an objective measure: plethysmography. We defined as Responder to the drug an individual who had moderate to severe apnoea index at baseline, and significantly improved breath holding (i.e., apnoea index) based on plethysmographic evaluations. We then compared the gene expression profiles of Responders with the rest of the cohort, which we termed MSR group, throughout the trial. We also evaluated changes within each of the two groups. Although not definitive because of the lack of a non-responder group, our analyses showing differences between R and MSR groups in gene expression that included IGF-1- and breathing phenotype-related genes at baseline suggest that RNA profiles may be able to identify individuals with RTT more likely to respond to IGF-1-like compounds.

Comparisons between T0 and T1 (first drug administration), T1–T2 (washout drug-free period), T2–T3 (second drug

TABLE 5 | Top 50 GO gene sets differentially regulated in R and MSR groups throughout the trial.

Comparison	ID	Gene ontology
RVMSR.T0	GO:0008152	Metabolic process
RVMSR.T0	GO:0051641	Cellular localisation
RVMSR.T0	GO:0022414	Reproductive process
RVMSR.T0	GO:0051252	Regulation of RNA metabolic process
RVMSR.T0	GO:0048870	Cell motility
RVMSR.T0	GO:0044237	Cellular metabolic process
RVMSR.T0	GO:0040011	Locomotion
RVMSR.T0	GO:0032446	Protein modification by small protein conjugation
RVMSR.T0	GO:0044260	Cellular macromolecule metabolic process
RVMSR.T0	GO:0098916	Anterograde trans-synaptic signalling
RVMSR.T0	GO:0050953	Sensory perception of light stimulus
RVMSR.T0	GO:0097746	Blood vessel diameter maintenance
RVMSR.T0	GO:0030198	Extracellular matrix organisation
RVMSR.T0	GO:0001704	Formation of primary germ layer
RVMSR.T0	GO:0031640	Killing of cells of another organism
RVMSR.T0	GO:0044419	biological process involved in interspecies interaction between organisms
RVMSR.T0	GO:0099536	Synaptic signalling
RVMSR.T0	GO:0007601	Visual perception
RVMSR.T0	GO:0007157	Heterophilic cell–cell adhesion via plasma membrane cell adhesion molecules
RVMSR.T0	GO:0051480	Regulation of cytosolic calcium ion concentration
RVMSR.T0	GO:0034329	Cell junction assembly
RVMSR.T0	GO:0035296	Regulation of tube diameter
RVMSR.T0	GO:0043062	Extracellular structure organisation
RVMSR.T0	GO:0050808	Synapse organisation
RVMSR.T0	GO:0099537	Trans-synaptic signalling
RVMSR.T0	GO:0061844	Antimicrobial humoural immune response mediated by antimicrobial peptide
RVMSR.T0	GO:0097485	Neuron projection guidance
RVMSR.T0	GO:0002376	Immune system process
RVMSR.T0	GO:0035150	Regulation of tube size
RVMSR.T0	GO:0007411	Axon guidance
RVMSR.T0	GO:0007155	Cell adhesion
RVMSR.T0	GO:0008015	Blood circulation
RVMSR.T0	GO:0048871	Multicellular organismal homeostasis
RVMSR.T0	GO:0003018	Vascular process in circulatory system
RVMSR.T0	GO:1903522	Regulation of blood circulation
RVMSR.T0	GO:0007409	Axonogenesis
RVMSR.T0	GO:0007268	Chemical synaptic transmission
RVMSR.T0	GO:0000902	Cell morphogenesis
RVMSR.T0	GO:0007369	Gastrulation
RVMSR.T0	GO:0001944	Vasculature development
RVMSR.T0	GO:0000904	Cell morphogenesis involved in differentiation
RVMSR.T0	GO:0098609	Cell–cell adhesion
RVMSR.T0	GO:1903034	Regulation of response to wounding
RVMSR.T0	GO:0003013	Circulatory system process
RVMSR.T0	GO:0048667	Cell morphogenesis involved in neuron differentiation
RVMSR.T0	GO:0048646	Anatomical structure formation involved in morphogenesis
RVMSR.T0	GO:0061564	Axon development
RVMSR.T0	GO:0072359	Circulatory system development
RVMSR.T0	GO:0001568	Blood vessel development
RVMSR.T0	GO:0048812	Neuron projection morphogenesis

All gene sets are upregulated in the R group with respect to the MSR group.
RVMSR, Responder group versus Mecasermin Study Reference group.

TABLE 6 | Validated differentially regulated Reactome pathways in R and MSR groups throughout the trial.

Comparison	ID	Reactome pathways	Direction	*p*-Value	FDR
RVMSR.T0	R-HSA-1474228	Degradation of the extracellular matrix	Up	0.000867	0.001536
RVMSR.T0	R-HSA-1474244	Extracellular matrix organisation	Up	0.001152	0.001536
RVMSR.T0	R-HSA-6805567	Keratinisation	Up	0.001545	0.001536
RVMSR.T0	R-HSA-1266738	Developmental Biology	Up	0.000997	0.001536
RVMSR.T2	R-HSA-1462054	Alpha-Defensins	Up	0.006901	0.001536
RVMSR.T2	R-HSA-1474228	Degradation of the extracellular matrix	Up	0.00809	0.001536
RVMSR.T2	R-HSA-1474244	Extracellular matrix organisation	Up	0.015394	0.001536
RVMSR.T2	R-HSA-8939242	RUNX1 regulates transcription of genes involved in differentiation of keratinocyte	Up	0.011898	0.001536

The full list of validated Reactome gene sets is included in ***Supplementary Table 2****.*
RVMSR, Responder group versus Mecasermin Study Reference group.

administration), and T0–T3 (entire trial), allowed examination of baseline gene expression and its changes in response to single and repeated mecasermin exposure. Comparisons at baseline between classic RTT and MRD groups, both included in the original study (Khwaja et al., 2014), revealed similar gene expression profiles. PC profiles placed MRD participants between the R and MSR RTT groups, as expected from patients with some RTT features but no apnoea as reported (Khwaja et al., 2014). Thus, molecular phenotypical profiles before mecasermin administration were in general correspondence with clinical phenotypes. Since the MRD group was not included in the OLE period, we focussed our gene expression analyses on the classic RTT cohort. These analyses demonstrated marked pre-treatment R versus MSR differences that diminished over time. Changes in the MSR group between T0 and T2 indicate that their gene expression profiles were modified by mecasermin treatment. The latter hypothesis is supported by the clinical changes reported by Pini et al. (2014) in a single-case study, where the authors conducted two periods of mecasermin administration (6 and 4 months, respectively) separated by a washout period of 2 years. Both administration cycles led to moderate decreases in impairments (e.g., hand wringing, bruxism, apnoea) and increases in abilities (e.g., reaching, pointing, gesturing). However, improvements were not maintained between treatment cycles and outcome profiles differed between cycles. These results support dynamic and partially compensatory responses after mecasermin administration, which could be reflected in gene expression profiles.

The main differences between R and MSR groups were observed at baseline (T0) while the main changes during the course of the trial occurred between the end of the MAD period and the beginning of the OLE period (T1–T2) and throughout the OLE (T2–T3), predominantly in the MSR group. Genes found to be differentially expressed regulate cell cycle processes and, in particular, immune responses (e.g., *TMEM176A*, *TMEM176B*). These were discrete changes of variable direction, suggesting both intrinsic RTT pathogenetic processes as well as the effects of the intervention under study.

Gene Expression Profiles of Responders to Mecasermin in Rett Syndrome

Analyses of baseline (T0) transcript profiles from R and MSR groups revealed a clear separation between groups, as evidenced by a single principal component accounting for almost half of the variance in overall gene expression. The differences represented relatively higher expression in the R group with respect to the MSR group (i.e., 3,693 upregulated, 221 downregulated), a pattern that continued but markedly decreased in magnitude throughout the trial (**Figure 3**). Relevance of these gene expression patterns is supported by the significant correlation between principal components and apnoea index at baseline. Thus, the profile and dynamics of the differential transcript profiles seems to reflect an IGF-1 signalling "favourable" status at baseline in the R group, which facilitated selective cellular responses to IGF-1 administration exemplified by the decrease in *HLA-DRB5* expression. On the other hand, mecasermin administration in the MSR group led to multiple presumably adaptive molecular changes throughout the study. These hypotheses are supported, first, by the unbiased, hypothesis-free analysis of pathways and mechanisms that revealed baseline differences in the expression of genes regulating vascular dynamics (i.e., vasoconstriction, vascular permeability), extracellular matrix, or inflammatory/immune responses. These functions are in line with the main phenotype targetted by mecasermin, namely breathing abnormalities, as well as with peripheral vasomotor disturbances commonly observed in individuals with RTT. The fact that the participant in the MSR group with a milder breathing phenotype and

TABLE 7 | Pathways evaluated in hypothesis-driven analysis.

Comparison	Gene ontology	ID	Direction	*p*-Value	FDR
RVMSR T0	BDNF receptor signalling pathway	GO.0031547	Up	0.0212	0.023388
RVMSR T0	Serotonin receptor signalling pathway	GO.0007210	Up	0.0016	0.005957
RVMSR T0	Dopamine receptor signalling pathway	GO.0007212	Up	0.0002	0.003271
RVMSR T0	Response to catecholamine	GO.0071869	Up	0.0018	0.005957
RVMSR T0	Linker et al. ASD_Chronic	ASD_Chronic	Up	0.0028	0.006034
RVMSR T0	MAPK cascade	GO.0000165	Up	0.0010	0.005957
RVMSR T0	Phosphatidylinositol 3-kinase signalling	GO.0014065	Up	0.0036	0.006208
RVMSR T0	Inflammatory response	GO.0006954	Up	0.0025	0.005957
RVMSR T0	Protein ubiquitination	GO.0016567	Up	0.0024	0.005957
RVMSR T0	Linker et al. Control_Acute	Control_Acute	Up	0.0040	0.006208
RVMSR T0	Reactive oxygen species metabolic process	GO.0072593	Up	0.0024	0.005957
RVMSR T0	Chromatin organization	GO.0006325	Up	0.0083	0.010816
RVMSR T0	Linker et al. ASD_Acute	ASD_Acute	Up	0.0220	0.023388
RVMSR T1	Insulin-like growth factor receptor signalling pathway	GO.0048009	Up	0.0005	0.008814
RVMSR T2	Phosphatidylinositol 3-kinase signalling	GO.0014065	Up	0.0004	0.003133
RVMSR T2	Response to catecholamine	GO.0071869	Up	0.0000	0.00052
RVMSR T2	BDNF receptor signalling pathway	GO.0031547	Up	0.0065	0.022009
RVMSR T2	Dopamine receptor signalling pathway	GO.0007212	Up	0.0043	0.02021
RVMSR T2	Serotonin receptor signalling pathway	GO.0007210	Up	0.0088	0.024885
RVMSR T2	Reactive oxygen species metabolic process	GO.0072593	Up	0.0048	0.02021
R T1T2	insulin-like growth factor receptor signalling pathway	GO.0048009	Up	0.001056	0.017946
MSR T1T2	Linker et al. ASD_Chronic	ASD_Chronic	Down	6.93E-09	1.18E-07

Gene sets were considered differentially expressed with a FDR < 0.05.
RVMSR, Responder group versus Mecasermin Study Reference group.

improvement had a gene expression profile similar to the R group, underscores the relationship between transcript patterns and clinical profiles and outcomes. Hypothesis-driven analyses discussed below provided additional support to the notion that the identified changes in gene expression were in response to IGF-1 administration.

Mechanisms Underlying Positive Response to Mecasermin in Rett Syndrome

To get additional insight into the mechanisms underlying positive response to mecasermin, we examined gene expression dynamics throughout the study using hypothesis-driven analyses. We selected pathways and mechanisms that have been implicated in the pathophysiology of RTT, such as MAPK and PI3K signalling (Tropea et al., 2009; Mellios et al., 2014), BDNF (Zhou et al., 2006), metabolic abnormalities in RTT such as mitochondrial dysfunction (Shulyakova et al., 2017; Shovlin and Tropea, 2018), and immunological function (Maezawa and Jin, 2010; Shovlin and Tropea, 2018). We also considered pathways associated to monoamine modulation (Viemari et al., 2005; Toward et al., 2013; Pini et al., 2016a; Vogelgesang et al., 2018) and IGF-1 signalling (Linker et al., 2020) since these are relevant to the breathing phenotype and its treatment with mecasermin. These analyses confirmed the involvement of inflammatory and immune responses, but also revealed differences in monoamine- and metabolism/homeostasis-related genes. Underscoring mecasermin's mechanism of action, differential gene expression dynamics between the R and MSR groups demonstrated distinct transcript profiles related to IGF-1 signalling. Indeed, the main differences between the R and MSR groups at T0, involved the BDNF cellular pathway as well as previously reported gene expression changes in response to IGF-1 treatment in ASD iPSCs. As in the hypothesis-free analyses, most differences between the R and MSR groups were found at T0. Nonetheless, IGF-1, BDNF, and apnoea-related genes were also differential at later time points suggesting

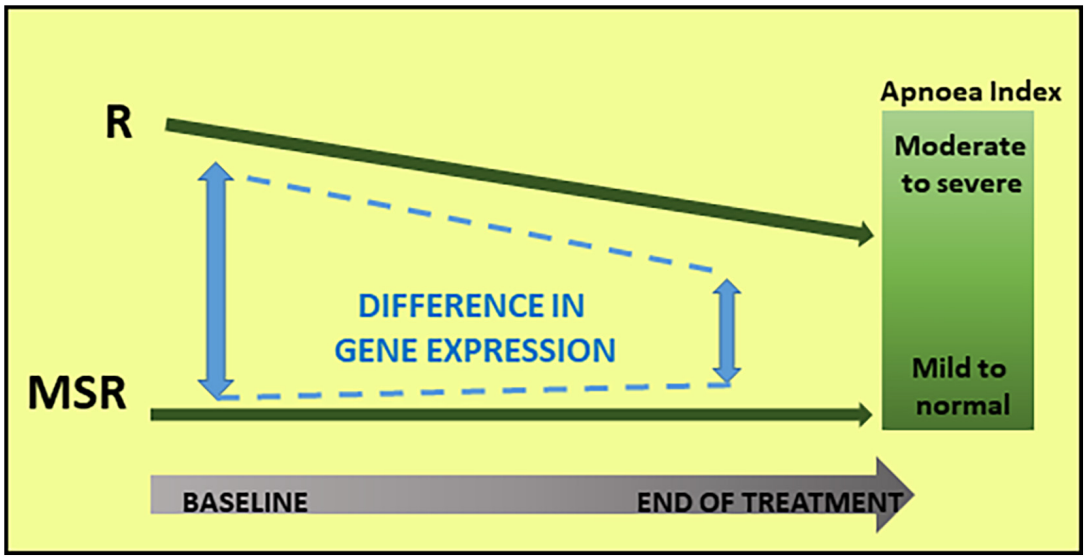

FIGURE 3 | Molecular and phenotypic changes in Responder (R) and Mecasermin Study Reference (MSR) groups throughout the study. Based on their breathing phenotype, the cohort was divided into two groups: Responder and MSR groups. The R group, including participants with moderate to severe breath holding phenotype, responded to mecasermin administration by decreasing their apnoea index. Because of their virtual lack of breath holding phenotype, those in the MSR group experienced minimal changes. In parallel to these changes in breathing phenotype in the R group, there was a decrease in group differences in transcript profiles (i.e., significant at baseline) throughout the study.

continuous action of IGF-1 on target pathways. This molecular dynamics resembles the effects of mecasermin on brain activity in participants with RTT, as previously reported by us (Keogh et al., 2020). The reason for the MSR group's greater changes in gene expression, in response to mecasermin, is unknown and deserves further examination since these molecular changes may disclose key events associated with response to IGF-1 and related compounds.

Potential of RNA Profiles as Molecular Signatures of Response to Mecasermin and Related Compounds

This preliminary study supports the potential of gene expression profiles as biomarkers in RTT drug trials. Although our data only revealed gene expression patterns in participants with severe breathing phenotype who also improved after mecasermin administration, and no treatment response comparison group (i.e., severe apnoea without improvement) was available, the nature and evolution of the expression profiles (i.e., correlated with apnoea index at baseline, higher expression of IGF-1 signalling and monoamine modulation genes) suggest that they were treatment related. This and the fact that other informative genes in this study are in line with our current knowledge of RTT pathogenesis (e.g., immune and metabolic mechanisms) underscore the relevance of the findings. Nonetheless, follow up investigations need to address response to treatment more directly. Ideally, studies with larger samples or other IGF-1 related drugs following the course of clinical responses will elucidate whether RNA profiles could become surrogate endpoints, and will provide additional validation of the reported results (i.e., qPCR).

Our findings encourage similar assessments for other drugs under preclinical and clinical investigation in RTT. While other aspects of study design, including dosage, length, and endpoints, continue to be critical for the successful outcome of drug trials in RTT, cohort selection for all candidate treatments for RTT could be improved by molecular profiling. Whether the present data will lead to a re-examination of the therapeutic potential of IGF-1 treatment will depend on follow up supportive studies. Nonetheless, ongoing RTT studies with trofinetide may benefit of the reported data.

Although the present study used an objective measure of clinical outcome, breathing patterns by plethysmography, we acknowledge several limitations. These included small sample size, wide age range, and the limited nature of the molecular investigations. Indeed, proteomics or metabolomics studies could provide additional insights into the molecular mechanisms associated with clinical outcomes. Another limitation is the use of whole blood for RNA analysis, which could be influenced by the individual's inflammatory/immunological status and its associated variability in cell types. Analyses of RNA expression in different cell types could have been more informative, but they were not feasible in the present study. Nonetheless, we consider the reported data the first step for identifying blood-based biomarkers in drug trials of IGF-1-related compounds in RTT. Future investigations will ideally assess the correlation between blood biomarkers with brain activity and other biomarkers, as well as with a wider range of clinical endpoints. Preclinical studies in animal models will be helpful for studying the correlation between changes in candidate biomarkers and other neurologic

parameters that are also measurable in humans, such as motor function and sensory processing (e.g., prepulse inhibition of the startle response) (Kaufmann et al., 2019).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession number GSE198856.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Boston Children's Hospital Institutional Review Board (IRB-10-08-0403). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SS analysed the data, prepared the figures and tables, and contributed to the preparation of the manuscript. CD performed some experiments and contributed to the discussion of the data. LS contributed to data collection. SB contributed to data analysis. MSa contributed to data collection, discussion of the results, and preparation of the manuscript. MSu contributed to the discussion of the data and the preparation of the manuscript. WK contributed to the planning of the experiment, collection of the data, and preparation of the manuscript. DT contributed to the planning of the experiment, data analysis, and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.868008/full#supplementary-material

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Conflict of Interest: WK was the Chief Scientific Officer of Anavex Life Sciences.

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The Shank3$^{Venus/Venus}$ knock in mouse enables isoform-specific functional studies of Shank3a

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Background: Shank3 is a scaffolding protein essential for the organization and function of the glutamatergic postsynapse. Monogenic mutations in *SHANK3* gene are among the leading genetic causes of Autism Spectrum Disorders (ASD). The multiplicity of Shank3 isoforms seems to generate as much functional diversity and yet, there are no tools to study endogenous Shank3 proteins in an isoform-specific manner.

Methods: In this study, we created a novel transgenic mouse line, the Shank3$^{Venus/Venus}$ knock in mouse, which allows to monitor the endogenous expression of the major Shank3 isoform in the brain, the full-length Shank3a isoform.

Results: We show that the endogenous Venus-Shank3a protein is localized in spines and is mainly expressed in the striatum, hippocampus and cortex of the developing and adult brain. We show that Shank3$^{Venus/+}$ and Shank3$^{Venus/Venus}$ mice have no behavioral deficiency. We further crossed Shank3$^{Venus/Venus}$ mice with Shank3$^{\Delta C/\Delta C}$ mice, a model of ASD, to track the Venus-tagged wild-type copy of Shank3a in physiological (Shank3$^{Venus/+}$) and pathological (Shank3$^{Venus/\Delta C}$) conditions. We report a developmental delay in brain expression of the Venus-Shank3a isoform in Shank3$^{Venus/\Delta C}$ mice, compared to Shank3$^{Venus/+}$ control mice.

Conclusion: Altogether, our results show that the Shank3$^{Venus/Venus}$ mouse line is a powerful tool to study endogenous Shank3a expression, in physiological conditions and in ASD.

KEYWORDS

Shank3, knock in, ASD model, isoform-specific tagging, Shank3ΔC, Shank3a

Introduction

Autism Spectrum Disorders (ASDs) affect about 1% of the human population. Patients with ASD have impairments in social communication and interaction and present repetitive and stereotyped behaviors (American Psychiatric Association, 2013). A significant proportion of ASD is due to genetic mutations, which has given hope for treatment (Kleijer et al., 2014). The discovery of monogenic forms of ASD offers a unique opportunity to explore the molecular and cellular mechanisms underlying ASD. The striking convergence of "ASD genes" onto glutamatergic signaling (Connor et al., 2014; Lee et al., 2016; Brown et al., 2018) supports the synaptic hypothesis as a leading cause of autism (Bourgeron, 2015), with the prominent role played by postsynaptic proteins linking group I mGlu receptors with AMPA and NMDA receptors (Connor et al., 2014). Heterozygous mutations in the *SHANK3* gene are among the leading monogenic causes of ASD and can lead to Phelan McDermid syndrome (PMS) (Durand et al., 2007; Jiang and Ehlers, 2013; Sala et al., 2015; Monteiro and Feng, 2017). PMS is characterized by a significant delay in expressive speech, intellectual disability, hypotonia, dysmorphic facial features, increased tolerance to pain, epilepsy, and autism-like behavior (Costales and Kolevzon, 2015).

The *SHANK3* gene is located on mouse chromosome 15E3 and human chromosome 22q13.3. There are at least 6 different Shank3 protein isoforms, Shank3a to Shank3f (**Figure 1A**), the full-length isoform (Shank3a) being the most abundant form in the brain (Wang et al., 2014). Shank3a is also the longest isoform and includes a Shank/ProSAP N-terminal domain (SPN), 6 ankyrin repeats (ANK), a Src Homology 3 domain (SH3), a PDZ (PSD-95/Dlg1/ZO-1) domain, a proline-rich region (Pro-rich), and a sterile alpha motif (SAM) domain at the C-terminus (Naisbitt et al., 1999; Tu et al., 1999). These protein-protein interaction domains multiply the possibility of interactions of Shank3a protein with dozens of synaptic proteins, making it a key component of the glutamatergic post-synaptic architecture. Unfortunately, due to the lack of specific antibodies or genetic tools, there is currently no possibility to study this Shank3a isoform exclusively.

In an attempt to study the physiological role of endogenous Shank3a, we generated a Shank3$^{Venus/Venus}$ knock in (KI) mouse. This KI strategy is adding a tag on the protein of interest while bypassing the need to overexpress the coding gene, thus providing information on the endogenous protein at physiological expression levels in an intact cellular context. In addition, only the major isoform, Shank3a, is tagged in this mouse line, allowing to focus on Shank3a expression and function amongst all other isoforms. Finally, this new mouse line can be crossed with Shank3 mutant mice to study the repercussion of such mutations on Venus-Shank3a wild type protein in heterozygous mouse models of ASD. This strategy, focusing on the physiological role of endogenous Shank3, complements the previous attempts to understand how alterations of the Shank3 protein can lead to the development of ASD (Yoo et al., 2013; Sala et al., 2015; Varghese et al., 2017).

Materials and methods

Generation of *Shank3*$^{Venus/Venus}$ KI mice at the Mouse Clinical Institute and genotyping

The targeting vector was constructed as follows. A 3.6 kb 3′ homology arm fragment encompassing exons 3–7 was amplified by PCR and subcloned in an Mouse Clinical Institute (MCI) proprietary vector. This vector bares a floxed Neomycin resistance cassette associated with a Cre auto-excision transgene that allows the excision of the whole cassette in the chimera's male germ line. A 1.5 kb fragment corresponding to the fusion of two PCRs amplicons (the Venus tag sequence and a 740-bps genomic fragment encompassing exon 2) was cloned in a second step using the endogenous SrfI site in 5′ and finally, a 3.6 kb (GC rich as covering the 5′ UTR of the gene) fragment corresponding to the 5′ homology arms was amplified by PCR and subcloned in step 2 plasmid to generate the final targeting construct. The linearized construct was electroporated in C57BL/6N mouse embryonic stem (ES) cells. After selection, targeted clones were identified by PCR using external primers and further confirmed by Southern blot with a Neo probe (5′ and 3′ digests) as well as a 3′ external probe. Three positive ES clones were injected into BALB/cN blastocysts. One gave germ line transmission. Resulting male chimeras were bred with wildtype C57BL/6N females. Germline transmission of the knock in allele with a direct excision of the floxed selection cassette was obtained. The allele nomenclature (according to Mouse Genome Informatics) is Shank3$^{tm1(Venus)Ics}$.

The primers used for genotyping have the following sequence: GGTACGGCGAGATCGCAAAGG and CTCTCTCC GCCGGGAACAG. The size of the PCR products is 862 bp for the KI allele and 133 bp for the WT allele.

Animal handling

All animal procedures were conducted in accordance with the European Communities Council Directive, supervised by the IGF institute's local Animal Welfare Unit (A34-172-41) and approved by the French Ministry of Research (agreement numbers: APAFIS#23357-2019112715218160 v4 and APAFIS#23476-2020010613546503 v4). The Shank3$^{\Delta C/\Delta C}$ mice (Jackson Laboratory, Bar Harbor, ME, USA, stock #018398) have a deletion of the Shank3 3′ terminal part, starting

just before exon 21. The resulting protein is truncated before the Homer-binding domain. Homozygous Shank3$^{Venus/Venus}$ mice were bred with homozygous Shank3$^{\Delta C/\Delta C}$ to obtain Shank3$^{Venus/\Delta C}$ mice (**Figure 5A**). Heterozygous Shank3$^{Venus/+}$ mice were crossed together to obtain Shank3$^{Venus/Venus}$ mice, Shank3$^{Venus/+}$ mice and Shank3$^{+/+}$ mice. Mice were grouped after weaning with respect to their sex.

Western blot analysis

Mice of both sexes were anesthetized with isoflurane. Brain structures were solubilized in a 10% SDS solution supplemented with protease inhibitors (Roche Diagnostics, Germany). Samples were sonicated and centrifuged. Concentration of solubilized proteins was quantified using BCA assay (Sigma-Aldrich, USA). All samples were then set at the same volume and concentration for the rest of the protocol and 30ug of proteins were loaded for each condition. Proteins were eluted in Laemmli sample buffer, resolved on a tris-acetate 3-8% gradient gel (BioRad, USA) enabling the detection of all expected bands at different molecular weights into the same gel (from Venus-Shank3a to GAPDH). Proteins were transferred onto a nitrocellulose membrane, which was cut in two pieces upper and lower than 75 kDa and then incubated with a mouse anti-GFP antibody and anti-GAPDH antibody for the upper and lower part, respectively. After this first immunostaining, we applied 0.5% azide-containing solution to the upper part of the membrane to quench the chemiluminescence and incubated the rabbit anti-Shank3 antibody for a second revelation of this upper part of the gel. Immunoblot detection was performed using the following primary antibodies: GFP (mouse monoclonal from Clontech, USA, reference Cat.#632381, dilution 1:500), GAPDH (rabbit polyclonal from Santa Cruz reference, dilution 1:25,000), Shank3 (rabbit polyclonal from Santa Cruz, USA, reference SC-30193, dilution 1:500). A Mann–Whitney test was used for experiments comparing 2 conditions and a non-parametric Kruskal–Wallis test with uncorrected Dunn's post-test for those comparing more than 2 conditions.

Hippocampal primary cell culture

Cultures were prepared from postnatal Shank3$^{Venus/Venus}$ mice as previously described (Moutin et al., 2020). Briefly, hippocampi were mechanically and enzymatically dissociated with papain (Sigma-Aldrich, USA) and hippocampal cells were seeded in Neurobasal-A medium (Gibco, USA) supplemented with B-27, Glutamax, L-glutamine, antibiotics and Fetal Bovine Serum (all media from Gibco, ThermoFisher Scientific, USA). After 2 days in culture, cytosine β-D-arabinofuranoside hydrochloride (Sigma-Aldrich, USA) was added to curb glia proliferation. The day after, 75% of the medium was replaced by BrainPhys medium (Stemcell Technologies, Canada) supplemented with B-27, Glutamax and antibiotics.

Immunocytochemistry

Shank3$^{Venus/Venus}$ hippocampal primary cultures were fixed at *Day In Vitro* 15 with 4% paraformaldehyde for 10 min and then permeabilized and blocked with a 3% BSA, 0.1% Triton X-100, PBS solution (blocking buffer) for 1 h at room temperature. Cultures were then incubated overnight at 4°C with primary antibodies against GFP (TP401, Biolabs, USA) to stain Venus, and against MAP2 (M4403, Sigma-Aldrich, USA) diluted in blocking buffer to final concentrations of 1:1,000. After washes, cells were incubated with secondary antibodies for 2 h at room temperature, washed, incubated with Hoechst 33258 (B2883, Sigma-Aldrich, USA) diluted in water for 5 min at room temperature, mounted on slides and observed under an Axio-Imager Z1 microscope equipped with appropriate epifluorescence and filters (Carl Zeiss, Germany).

Behavioral experiments

In order to characterize the psychomotor development of Shank3$^{Venus/Venus}$ mice, weight, flipping, cliff avoidance and walking tests were performed each day from P0 to P15 on both male and female pups. Self-grooming, Three chambers sociability test, Open field and Marble burying were performed on male and female mice aged 12–14 weeks.

Weight: The mice were weighed each day on a precision balance.

Flipping: Mice were placed on their backs. Those remaining on their backs for at least one minute were scored 0. Those that flipped onto their stomachs within 30 s to 1 min, 15–30 s, or 5–15 s were scored 1, 2, 3, respectively. Mice that flipped over in less than 5 s received a score of 4.

Cliff avoidance: Mice were placed on the edge of a Plexiglas platform over a 20 cm cliff with their nose and front legs over the edge. Mice that moved away from the edge within 30 s received the maximal score of 1, while mice that did not move got the score of 0.

Walking: Mice were observed for 1 min. Mice that remained immobile or moved in circles received a score of 0. Mice that walked but inconsistently and asymmetrically received a score of 1. Mice that walked symmetrically but slowly got a score of 2 while those that walked correctly and quickly received a maximum score of 3.

Open Field consisted of a square arena (50 cm × 50 cm). Mice were placed in the center of the arena and left to explore freely for 10 min. Total distance traveled, speed and time

spend in the center zone (defined as a 25 cm side square) were measured during 10 min by video tracking (Ethovision, Noldus, Netherlands).

Self-grooming: Mice were placed in a 20 cm Plexiglas square arena and video monitored. Time spend self-grooming was measured visually during 10 min.

Marble test: Mice were placed individually in a clean cage with 5 cm thick bedding together with 15 marbles evenly distributed on top of the bedding. After 30 min in the cage, mice were removed and we evaluated the level of marble burying according to the following score: 0 for a totally buried marble; 1 for a half-buried marble and 2 for a totally visible marble. We also evaluated the level of interaction with the marbles counting marbles that were untouched during the test.

Three chambers sociability test: Mice were tested individually in a three chambers test apparatus (rectangular 40 × 60 plexiglass, Panlab, Spain). The apparatus was cleaned with alcohol 30% between trials. Each of the two side chambers contained a cage (9 cm in diameter) in which social (stranger mouse) and non-social (object) stimuli could be confined. Testing consisted of three trials: trial 1 (habituation), the test mouse was placed in the middle chamber and then could freely explore the test box for 10 min with the cages empty. After which they returned to their home cage for 1 h before proceeding with the next trial. During trial 2 (social preference), an unknown mouse (stranger 1: social stimulus) was introduced in one of the cages in one side chamber, whereas an unknown object (object: non-social stimulus) was enclosed in the other cage in the opposite side chamber. Location of stranger 1 in the left or right-side chamber was balanced across subjects. The test mice were allowed to explore during 10 min. After 1 h in their home cage, mice were exposed to trial 3. During trial 3 (social novelty), the object present in trial 2 was replaced by a novel unfamiliar mouse (stranger 2: novel social stimulus). Again, the test mouse was allowed to explore during 10min. Behavior was video monitored during all trials (Ethovision, Noldus, Netherlands) to analyze general activity (distance traveled, speed). Social preference index was calculated as the ratio of time spent in the side chamber containing the social stimulus by the time spent in both side chamber during trial 2. Social novelty recognition index was calculated as the ratio of time spent in the side chamber containing the novel social stimulus by the time spent in both side chamber during trial 2.

Statistical analysis: For pups, we analyzed the evolution of mice proportion over time using Generalized Linear Model (GLM) (Ordinal Logit model, SPSS® IBM®) with score as dependent variable and genotype and days as covariates. We also used GLM to compare the evolution of weight over time with weight as dependent variable and genotype and days as covariate (Linear Scale Response model, SPSS® IBM®).

For the adults' tests, differences between the three genotypes groups were analyzed using One-way ANOVA or Kruskal–Wallis accordingly to their distribution (Graph Pad Prism 9.3.0).

Results

Generation of a *Shank3*$^{Venus/Venus}$ KI mouse line allowing the exclusive detection of Shank3a isoform

In mice, the *SHANK3* gene is composed of 22 exons (**Figure 1A**). Multiple intragenic promoters, at least six (see Monteiro and Feng, 2017 for review), lead to six major protein isoforms: Shank3a to Shank3f. We have generated the Shank3$^{Venus/Venus}$ KI mouse line by inserting the DNA coding for Venus fluorescent tag at the beginning of exon1 of *SHANK3* gene, just after the ATG start codon (**Figure 1B**). This strategy enables to tag exclusively the endogenous Shank3 variants that are produced by the first promoter: variants of the Shank3a isoform only, Shank3b to f being generated by promotors located downstream Venus DNA coding sequence. To check for the effective expression of this tagged version of Shank3, we performed Western blots on brain samples of adult Shank3$^{Venus/Venus}$ mice with two different primary antibodies. The first one was raised against Venus and the second one against the C-terminal part of Shank3 (Verpelli et al., 2011) (which recognizes Shank3a, c/d e, and f isoforms). Consistently, Western blot analysis of adult Shank3$^{Venus/Venus}$ mouse brains showed only one double band (**Figure 1C**), with an apparent molecular weight corresponding to the expected size of full-length Venus-Shank3a (theoretical Venus-Shank3a molecular weight: 212 kDa). As expected, the shorter isoforms lacking at least the N-terminal exon 1 and exon2 (Shank 3b to f) could not be detected using an anti-Venus antibody. These shorter bands are present when using the anti-Shank3 antibody labeling all Shank3 isoforms conserving the C-terminal part, excepted for Shank3f which molecular weight is around 10 kDa (Monteiro and Feng, 2017) and cannot be detected in our experimental conditions (**Figure 1C**). Furthermore, as already reported for Shank3c, Shank3d, and Shank3e, additional transcripts may be produced by alternative splicing of exons (exon 11, exon 12, exon 18, exon 21, and exon 22). For example, for Shank3c, alternative splicing gives rise to Shank3c1, Shank3c3, and Shank3c4 (Monteiro and Feng, 2017). We noticed that the Venus-Shank3 band was indeed a double band, which may be the result of alternative splicing. To complete the characterization of endogenous Venus-Shank3 expression, immunocytochemistry on hippocampal neuronal cultures from Shank3$^{Venus/Venus}$ mice shows that labeled endogenous Venus-Shank3 proteins are expressed in a punctate manner along dendrites (**Figure 1D**), as expected from the endogenous expression of Shank3 in dendritic spines (Tu et al., 1999).

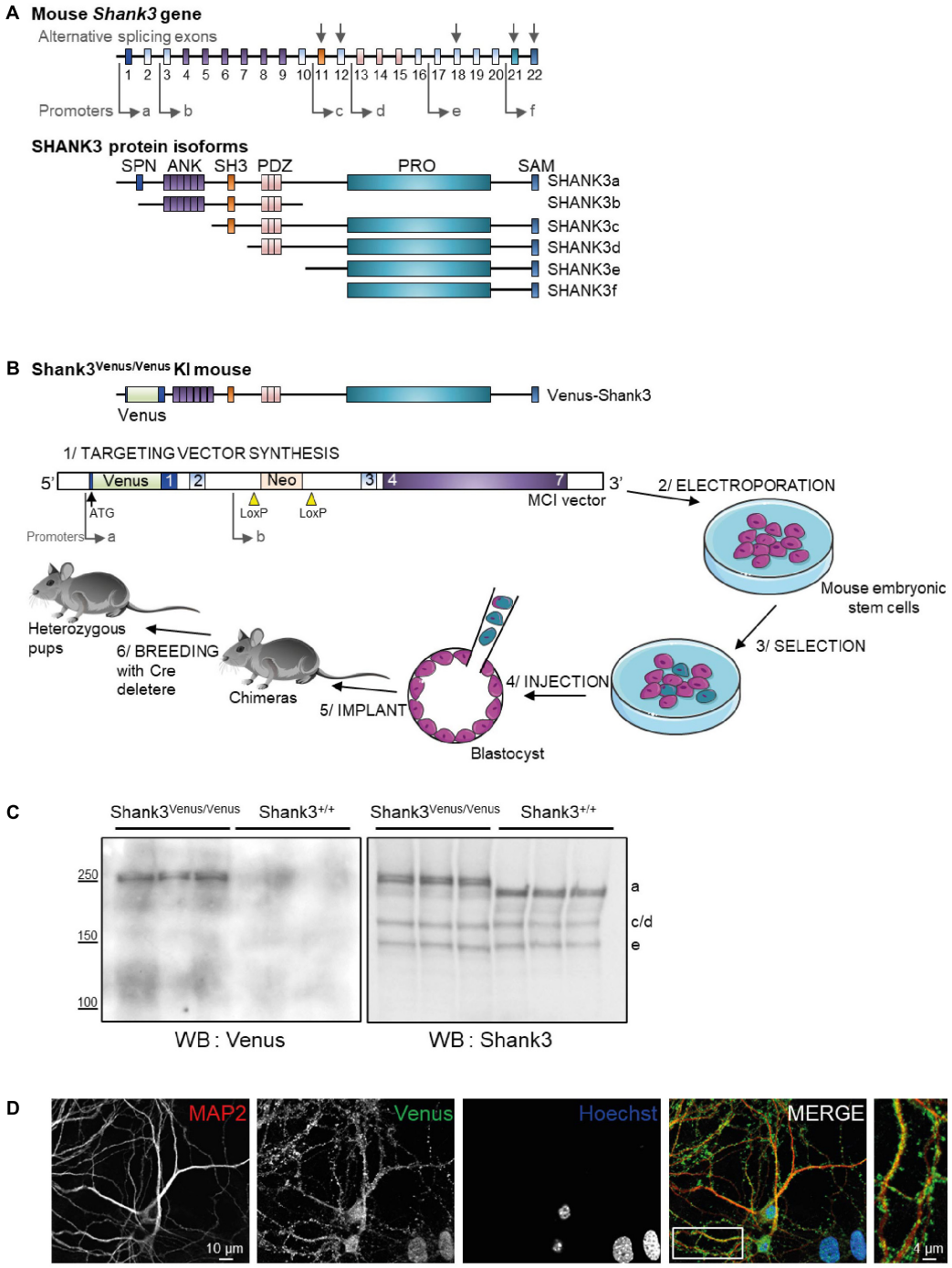

FIGURE 1
Generation of the Shank3$^{Venus/Venus}$ knock in mouse line and detection of Venus Shank3a isoform expression. **(A)** Schematic representation of the mouse Shank3 gene structure and protein domains of the different protein isoforms. Sites of intragenic promoters and alternative splicing exons are represented by arrows. SPN for Shank3/ProSAP N-terminal domain (1-75 aa); ANK for Ankyrin repeat domain (148-345 aa); SH3 for src Homology-3 domain (470-529 aa); PDZ for PSD-95/DLG/ZO-1 domain (570-664 aa); PRO for proline-rich domain (831-1417 aa); SAM (1667-1730 aa). **(B)** Schematic representation of the Venus-Shank3 protein domains and steps of Shank3$^{Venus/Venus}$ KI mouse line creation. Please note that the Venus coding sequence was inserted at the beginning of exon 1 (in frame with and just after the ATG), enabling the exclusive Venus-tagging of the Shank3 variants that are generated by the first promoter, Shank3a isoforms. **(C)** Total brain lysates from 3 different Shank3$^{Venus/Venus}$ and Shank3$^{+/+}$ adult mice were immunoblotted for Venus (left) and Shank3 (right) expressions. Expected molecular weight for untagged Shank3a, 3c/d and 3e isoforms are indicated on the right side of the blot (a, c/d, and e). **(D)** Immunocytochemistry images from *Day In Vitro* 15 Shank3$^{Venus/Venus}$ hippocampal primary cultures stained with antibodies against the dendritic marker MAP2 (red), Venus (green), and the nuclear marker Hoechst (blue).

Shank3$^{Venus/Venus}$ KI mice exhibit typical development and behavior

We evaluated Shank3$^{Venus/+}$ and Shank3$^{Venus/Venus}$ mice metabolic and sensorimotor development by tracking mice weight, sensorimotor reflexes (flipping and cliff avoidance tests), and walking abilities (**Figure 2**). We used mice from postnatal day 0 (P0) to 15 (P15) to detect possible early deficits. We found no significant difference in weight from birth until P15 between Shank3$^{Venus/+}$ and Shank3$^{+/+}$ littermates. Similarly, flipping, cliff avoidance, and walking scores were not significantly affected in Shank3$^{Venus/+}$ mice compared to WT mice. Because Shank3a mutations are associated with ASD like phenotypes, to rule-out any behavioral effect of the fusion of the Venus tag on Shank3a, we further characterized 12–14 weeks adult mice behaviors in tests known to reveal autistic-like phenotypes at this age. We evaluated mice social interests using the three chambers test. We found no difference between Shank3$^{+/+}$, Shank3$^{Venus/+}$, and Shank3$^{Venus/Venus}$ mice in social preference nor in social novelty, highlighting the absence of atypical social interest in these KI mice (**Figure 3A**). We assessed stereotyped behaviors and anxiety measuring self-grooming, locomotion in the open field and mice propensity to interact with marbles. The time spent grooming was not significantly different between Shank3$^{+/+}$, Shank3$^{Venus/+}$, and Shank3$^{Venus/Venus}$ mice (**Figure 3B**), suggesting the absence of stereotyped behavior and no atypical anxiety level in Venus-tagged Shank3a mice. The time spent in the center zone of an open field (**Figure 3C**) was not significantly different between genotypes either, suggesting the absence of atypical anxiety. We however noticed a slight but statistically significant decrease in burying score of Shank3$^{Venus/+}$ heterozygous mice compared to Shank3$^{+/+}$ in the marble test (**Figure 3D**) meaning that this genotype tends to burry more marbles. Yet, the propensity to interact with the marbles (number of untouched marbles) remained the same. As Marble burying test can be used as a proxy to measure anxiety, the outcome of this test reflects complex behavior combining anxiety together with novelty avoidance, stereotypical movements and general locomotion. That is why we performed supplemental behavioral tests for this genotype: Elevated Plus Maze test confirmed that anxiety-like behaviors of Shank3$^{Venus/+}$ adult mice were typical (**Supplementary Figure 1A**). Performance in the Cyclotron (**Supplementary Figure 1B**) and Rotarod (**Supplementary Figure 1C**) showed that motor activity, motor coordination and endurance were typical as well. Aforementioned absence of hyper self-grooming ruled out stereotypical movements' abnormalities. Altogether, these behavioral experiments show that Shank3$^{Venus/+}$ and Shank3$^{Venus/Venus}$ KI mice behave as WT mice. In future studies, Shank3$^{Venus/Venus}$ KI mice can then be crossed with Shank3 mutant mouse models of ASD to understand the consequences of a Shank3 ASD mutation on the molecular dynamics of Venus-Shank3a at synapses in heterozygous mice.

Shank3$^{Venus/Venus}$ KI mice exhibit typical expression of Shank3 isoforms in the brain

To compare the expression of the different Shank3 isoforms in Shank3$^{Venus/Venus}$ (**Figure 4A**) and wild type mice (**Figure 4B**), Western blotting analysis was conducted on adult brain samples using Shank3 antibody. The Shank3a isoform is highly expressed in the striatum, hippocampus and cortex (**Figures 4A',B'**) while the c/d isoforms predominate in the cerebellum (**Figures 4A",B"**). Shank3e is present but weakly expressed in all brain areas studied. Relative to the other variants, it is most highly expressed in the striatum. We did not find major differences in Shank3 variants expression profile between Shank3$^{Venus/Venus}$ mice and Shank3$^{+/+}$ littermates. The absence of signal using the anti-Venus antibody on Western blot from Shank3$^{+/+}$ mouse brain extracts (**Figure 4B**), which does not express Venus-tagged proteins, confirmed the specificity of endogenous Venus-Shank3a isoform detection by Venus tagging.

Overall, these experiments using Shank3$^{Venus/Venus}$ mice confirm a predominant expression of Shank3a in the striatum, hippocampus and cortex.

Crossing Shank3$^{Venus/Venus}$ KI mice with the Shank3$^{\Delta C/\Delta C}$ mouse model of ASD reveals altered expression of the wild type Shank3a isoform in ASD

The Shank3ΔC mouse line displays a mutation found in humans and is commonly used to investigate autistic-like phenotypes (Kouser et al., 2013; Duffney et al., 2015; Moutin et al., 2021). We crossed *Shank3*$^{Venus/Venus}$ mice with *Shank3*$^{\Delta C/\Delta C}$ or *Shank3*$^{+/+}$ mouse strains to compare heterozygous mice expressing either Venus-Shank3 and untagged wild type Shank3 (*Shank3*$^{Venus/+}$) or Venus-Shank3 and untagged Shank3ΔC (*Shank3*$^{Venus/\Delta C}$), **Figure 5A**. This allowed us to study Venus-Shank3a isoform expression in the context of ASD. In *Shank3*$^{Venus/+}$ mice, Venus-Shank3a was poorly expressed until postnatal day 7 in all studied brain regions (**Figure 5B**). At postnatal day 14, it considerably increased and reached a plateau at one month. These Venus-Shank3a expression kinetics were modified in *Shank3*$^{Venus/\Delta C}$ mice. Indeed, the expression of Venus-Shank3a appeared to be delayed in the striatum and hippocampus, until one month. To better characterize this developmental gap in Venus-Shank3a expression, we directly compared Venus-Shank3a expression between the two genotypes at the critical

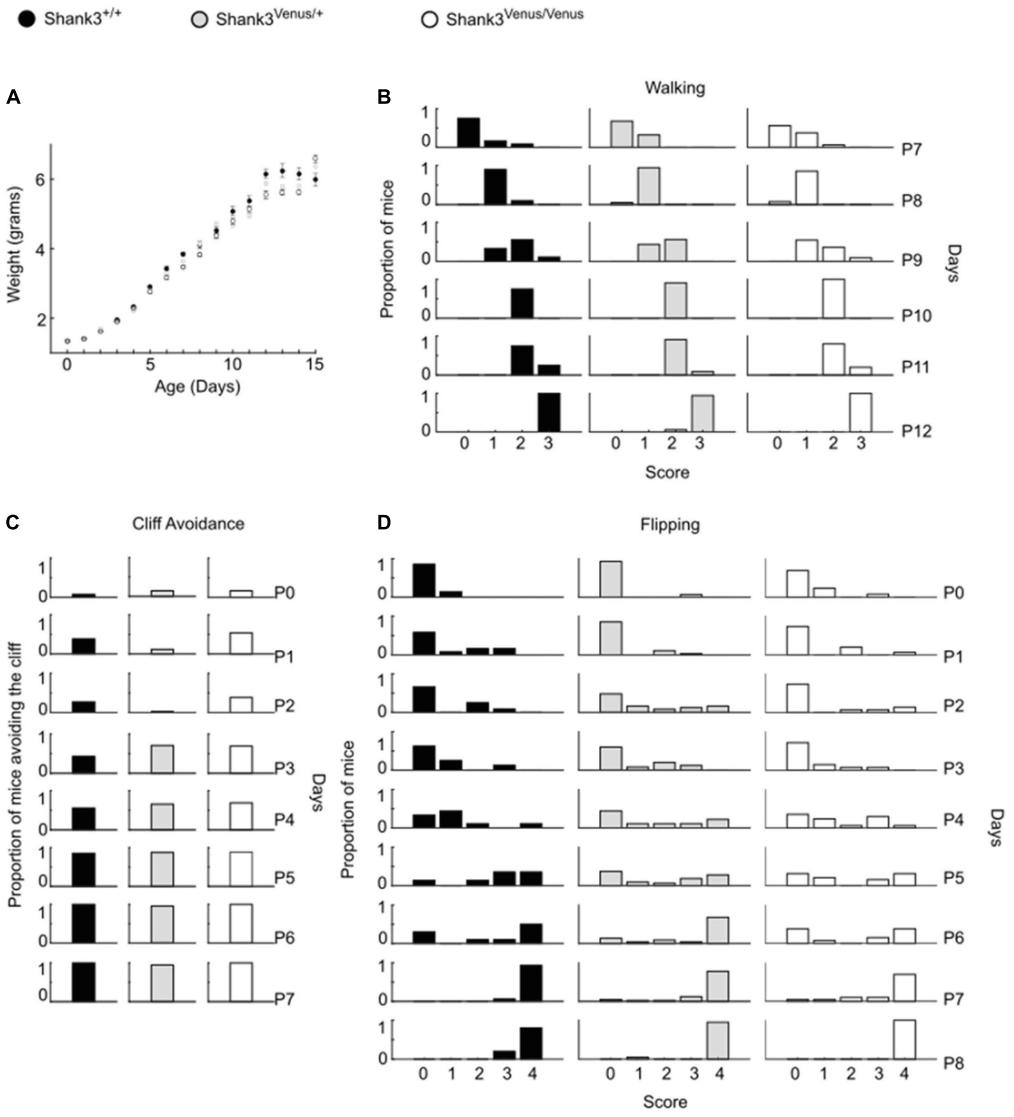

FIGURE 2
Psychomotor ability test battery in Shank3$^{Venus/Venus}$, Shank3$^{Venus/+}$ and Shank3$^{+/+}$ KI pups. All behavioral experiments were performed on at least 18 mice per group. **(A)** *Weight*. Pups were weighed from birth to P15. Data are mean ± SEM. **(B)** *Walking*. Mice were scored for walking from P7 to P12. Data are proportion of mice reaching the different score through days. GLM, genotype effect: p-value = 0.81, interaction genotype by time: p-value = 0.48. **(C)** *Cliff Avoidance*. Proportion of mice avoiding the cliff from birth to P7. GLM, p-value = 1.00. **(D)** *Flipping*. Mice were scored in their ability to flip over when put on their back from birth to P8. Data are proportion of mice reaching the different score through days. GLM, p-value = 0.64.

period of synaptogenesis in juvenile (P7–14) mice (Figure 5C). Venus-Shank3a expression in the juvenile group (P7–14) was normalized by Shank3a expression in the adult group (1–3M), in both *Shank3*$^{Venus/+}$ *and Shank3*$^{Venus/\Delta C}$ genotypes. We confirmed a significant decrease of Venus-Shank3a expression in juvenile mice when co-expressed with ShankΔ3C, in the striatal and hippocampal regions. This alteration was absent when analyzing the full brain and only a tendency in the cortex. Altogether, these experiments show that the *Shank3*$^{Venus/Venus}$ KI mouse line can be crossed with ASD Shank3-mutant mice to track the expression of Venus-Shank3a isoform. The first results obtained with *Shank3*$^{Venus/Venus}$ KI mouse line revealed a delay in Venus-Shank3a expression during the postnatal critical period of development (P14) in the *Shank3*ΔC mouse model of ASD.

Discussion

Shank3 is a key scaffolding protein that organizes glutamatergic postsynapse architecture and function (Boeckers et al., 2002; Monteiro and Feng, 2017). Many Shank3 isoforms

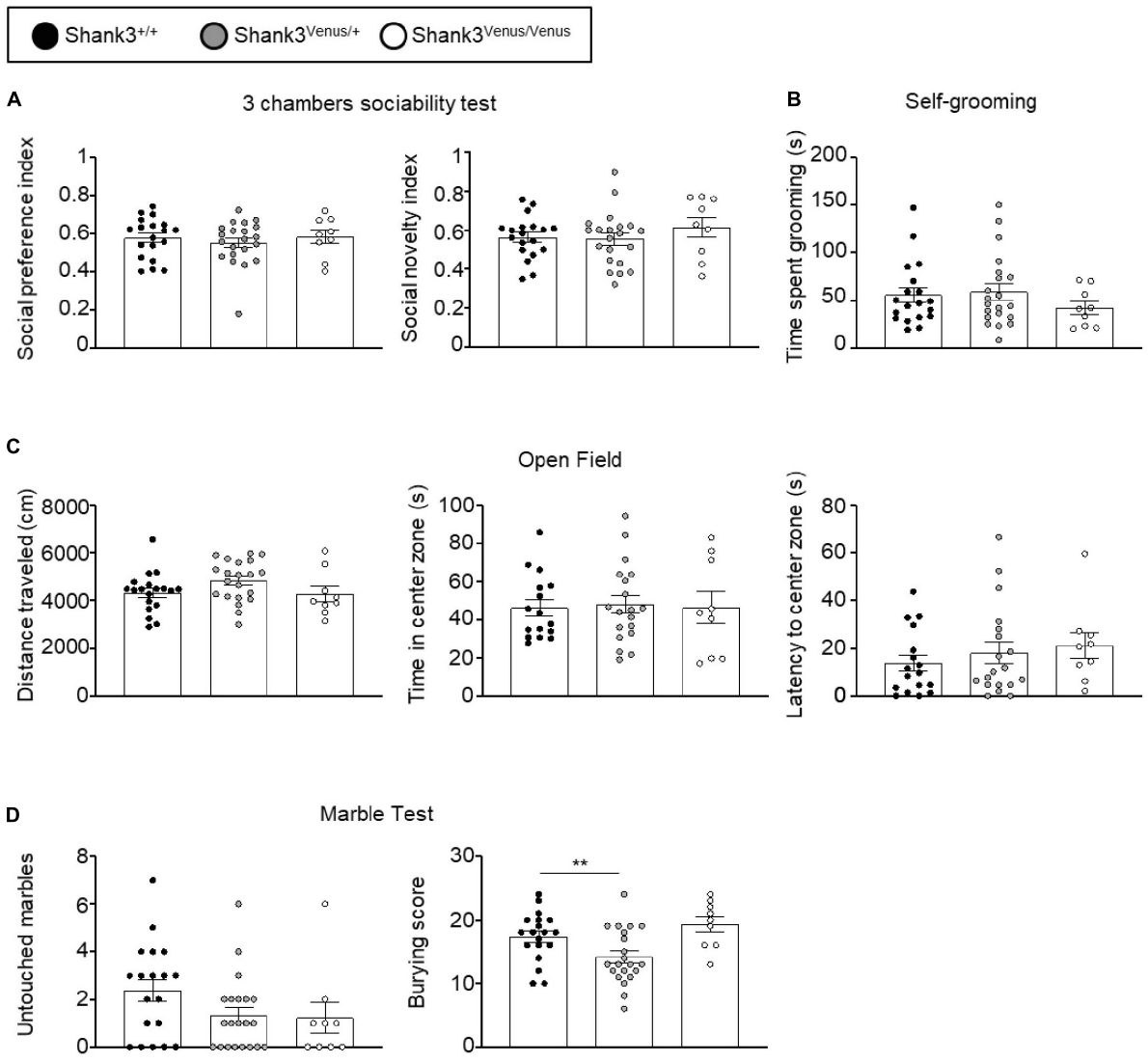

FIGURE 3
Behavioral test battery in Shank3$^{Venus/Venus}$, Shank3$^{Venus/+}$, and Shank3$^{+/+}$ KI adult mice. **(A)** Three chambers sociability test. Social preference index: time spent in social stimulus side chamber divided by time spent in both chambers. Social novelty index: time spent in the novel social stimulus side chamber divided by time spent in both chambers. One-way ANOVA, p-values = 0.71 and 0.51 for social preference and social novelty indexes respectively. **(B)** Self-grooming. Time spent by the mice grooming during a 10 min period. Kruskal–Wallis test, p-value = 0.48. **(C)** Open Field. Total distance traveled by the mice during the 10 min test. One-way ANOVA, p-values = 0.13. Time spent in the center zone. One-way ANOVA, p-values = 0.95; latency to the first entry into the center zone. Kruskal–Wallis, p-value = 0.38. **(D)** Marble burying. Number of marbles untouched. Kruskal–Wallis, p-value = 0.11. Score of marble burying. One-Way ANOVA, p-value = 0.004. Bars are mean ± SEM. from 19 Shank3$^{+/+}$, 21 Shank3$^{Venus/+}$, and 9 Shank3$^{Venus/Venus}$ except for Open field's time spent in the center zone and latency to the center zone where data are from 17 Shank3$^{+/+}$, 19 Shank3$^{Venus/+}$, and 9 Shank3$^{Venus/Venus}$.

are expressed in the brain, each one has its own unique specificity of brain area, cell type or even subcellular expression and each interacts with a specific plethora of proteins, strongly suggesting differential functions for each isoform (Wang et al., 2014; Monteiro and Feng, 2017). The longest and major Shank3 isoform has never been selectively studied due to the lack of specific antibodies or genetic tools enabling to isolate its functions from that of other isoforms. Here, we developed a transgenic *Shank3$^{Venus/Venus}$* KI mouse to specifically study this Shank3a isoform, in its intact cellular context, at endogenous expression levels. Moreover, as a proof of concept, we show that this mouse line can be crossed with Shank3 mutant mice to study the Venus-Shank3a wild type protein in heterozygous mice models of ASD.

Although studies with overexpressed proteins have been essential so far to highlight different Shank isoforms specific functions (Han et al., 2013; Eltokhi et al., 2021), genome editing is a powerful technique for studying gene expression and

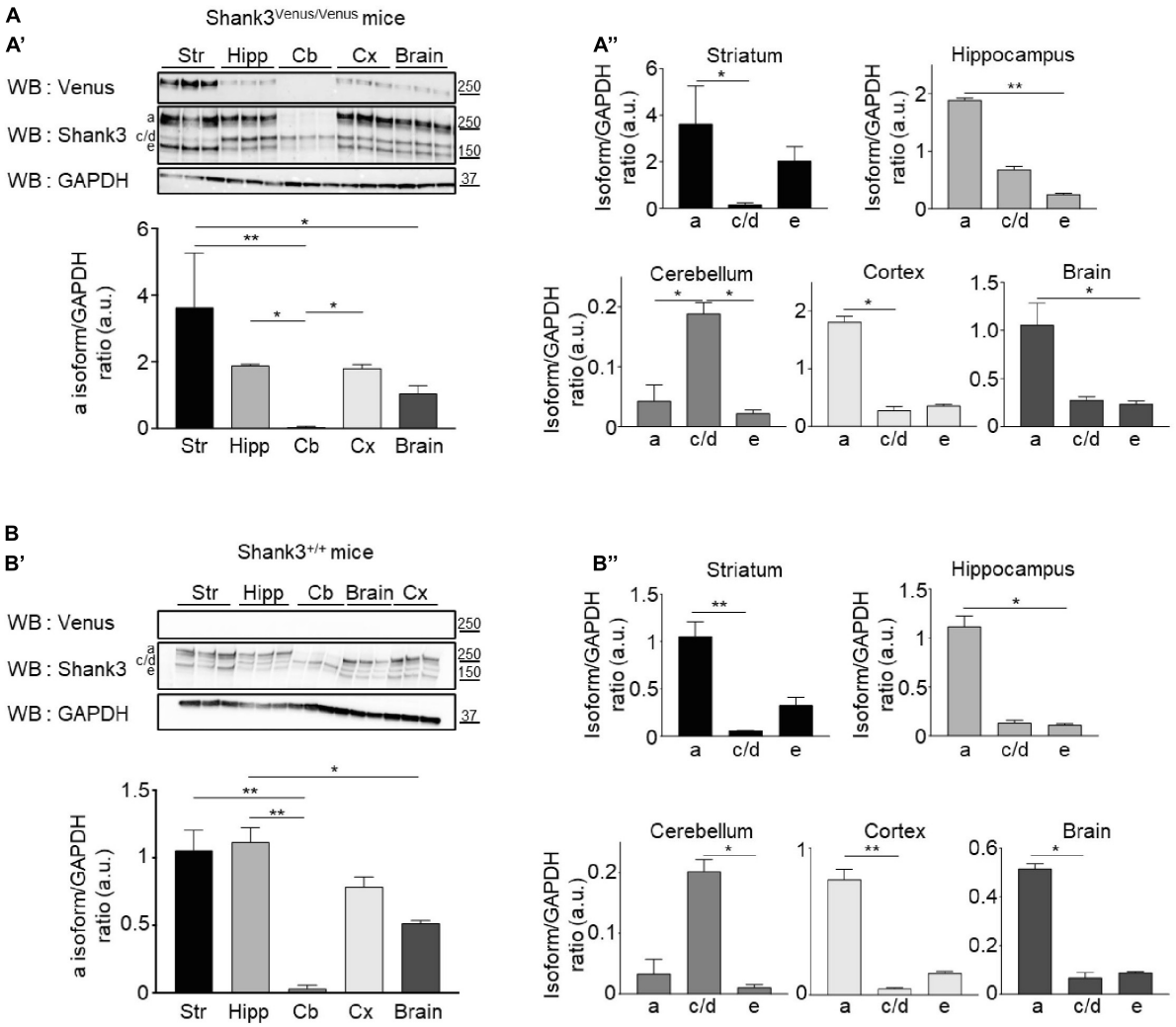

FIGURE 4
Shank3 protein isoforms expression in different brain areas. (**A**, Top **A'**) Total lysates of striatum (Str), hippocampus (Hipp), cerebellum (Cb), cortex (Cx), and full brain from 3 different Shank3$^{Venus/Venus}$ adult mice immunoblotted for Venus, Shank3 and GAPDH. (Bottom **A'**) Summary graph of the Shank3a/GAPDH ratios (quantified using Shank3 antibody). (**A''**) Graphs present the Shank3a, Shank3c/d and Shank3e over GAPDH ratios in the full brain or the different brain areas (quantified with Shank3 antibody). **(B)** Same as panel **(A)** but in 3 Shank3$^{+/+}$ adult mice. For panels **(A,B)** data are mean ± SEM, *indicates p-value < 0.05, **p < 0.01, non-parametric Kruskal–Wallis test with uncorrected Dunn's post-test.

functions in an intact cellular environment. The main advantage of this strategy precisely resides in its non-invasive nature, which circumvents protein overexpression frequently resulting from the use of mammalian-cell gene delivery vectors, such as viruses. Hence, tagging the endogenous protein minimizes experimental bias which may arise from aberrant protein expression or from deficient protein-protein interactions when the stoichiometry of functional complexes is altered. In agreement with these considerations, we found that the endogenous Venus-Shank3a protein in the KI mice displays similar developmental expression profile to the untagged endogenous Shank3a (Wang et al., 2014; Monteiro and Feng, 2017), it is correctly targeted to dendritic spines, the overall metabolic and sensorimotor development of the *Shank3*$^{Venus/Venus}$ KI mice is typical and adult *Shank3*$^{Venus/Venus}$ KI mice do not display autistic-like features. On the other hand, respecting endogenous expression levels can complicate the performance and analysis of experiments that rely on the fluorescence of a small number of Venus-tagged proteins at the single cell level. We by-passed the problem of weak fluorescence by amplifying the Venus fluorescence using fluorescent antibodies raised against Venus. Overall, we showed that the *Shank3*$^{Venus/Venus}$ KI mouse line provides a potential tool to elucidate the localization of Shank3a endogenous proteins in neurons by Venus tagging without altering Shank3 isoforms expression and preserving typical development and behavior of the mice. The morphology of *Shank3*$^{Venus/Venus}$ hippocampal neurons in culture is spiny,

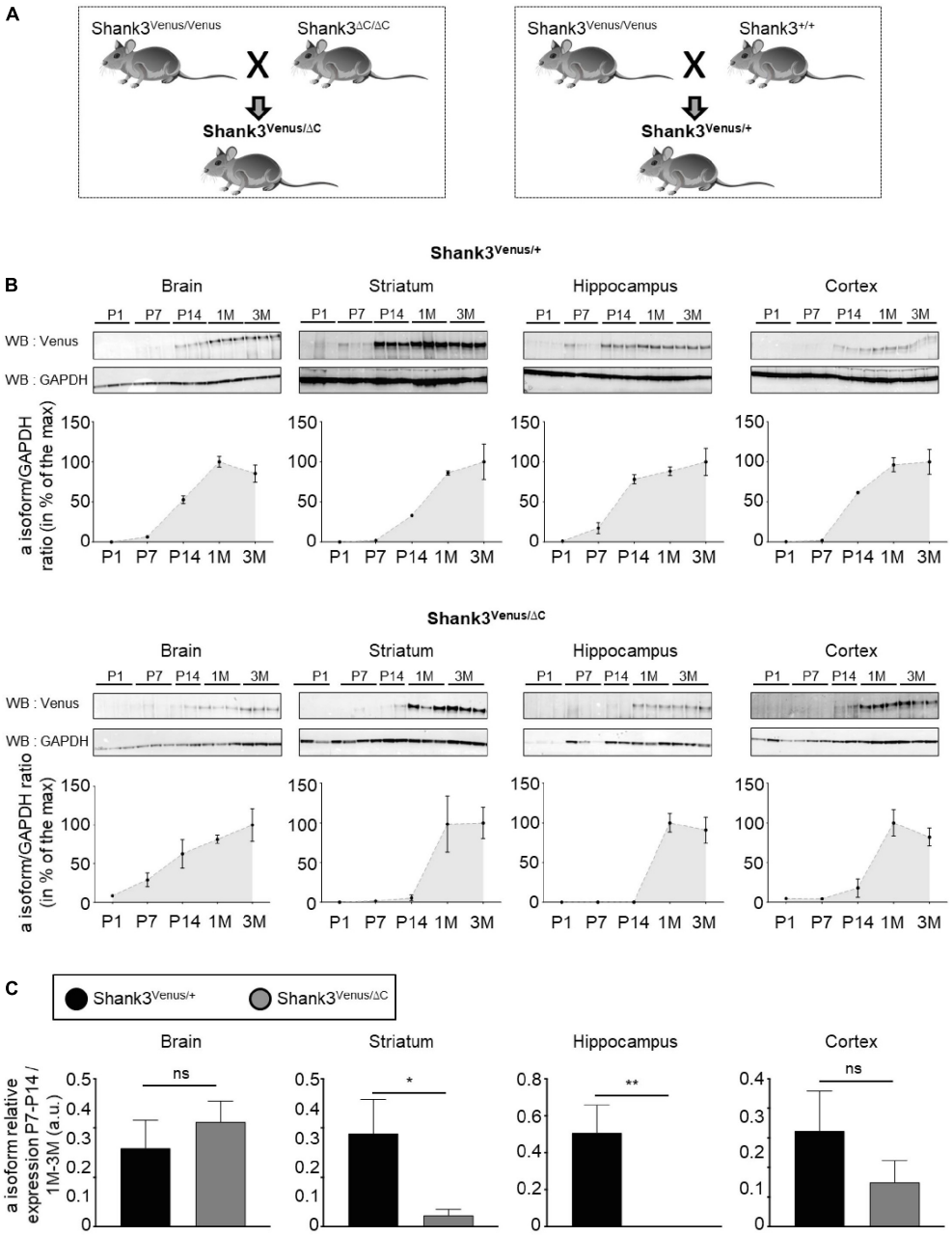

FIGURE 5
Expression of Shank3a isoform in ASD. **(A)** Mouse lines crossing scheme. **(B)** Total lysates extracted from full brain, striatum, hippocampus, and cortex of Shank3$^{Venus/+}$ (top) or Shank3$^{Venus/\Delta C}$ (bottom) mice at different developmental stages, from Postnatal day 1 (P1) to 3 months-old (3M), immunoblotted for Venus and GAPDH. All replicates are presented, each from a different mouse. Data are expressed as a percentage of the maximum Shank3a/GAPDH ratio average. **(C)** Shank3a/GAPDH ratio in juvenile (P7 and P14) mice, expressed as a percentage of expression in adult mice (1 and 3 months) quantified in different brain regions from Shank3$^{Venus/+}$ (black) and Shank3$^{Venus/\Delta C}$ (gray) mice. n = 6 samples from 6 different mice for each structure at each time point, excepted for Shank3$^{Venus/\Delta C}$ mice at P7–14 where n = 5. Data are mean ± SEM. ns: not significant; p-value: *<0.05; p-value: **<0.01 Mann–Whitney test.

with a punctiform distribution of Venus-Shank3 at synapses. These mice will represent a powerful tool, by circumventing the lack of isoform specific antibodies, to investigate molecular dysfunctions at the synaptic level in various shankopathies (by crossing this line with mice carrying Shank3 mutations).

In a first set of experiments, Western blot analysis of the transgene expression revealed that only the Shank3a isoform was tagged with Venus. This observation is coherent with the genome editing chosen strategy, which was to add the coding sequence of Venus in frame with Shank3 exon1.

Shank3 exon1 is absent from Shank3 b to f isoforms, each of them being produced by gene transcription starting at specific promoters located downstream of exon2. Hence as expected, only the isoform driven by the first promoter (upstream exon1) could display the Venus tag, i.e., the Venus-Shank3a isoform. However, downstream from the first transcription start site, *SHANK3* gene has alternative options of splicing, which could have resulted in the generation of a wide array of mRNA transcripts and protein isoforms. Alternative splicing of exon (exon 11, exon 12, exon 18, exon 21, and exon 22) have indeed already been shown to trigger the variety of additional transcripts for Shank3c, 3d, and 3e (Jiang and Ehlers, 2013). Our biochemical data revealed a unique band for Venus-Shank3 (**Figure 1C**), the Shank3a full-length, which appears, however, as a doublet. Hence, even if no alternative Shank3a isoform has been reported for so far (Monteiro and Feng, 2017), this doublet could indeed arise from alternative splicing. Alternatively, post-translational modifications, such as Shank3a phosphorylation (Wu et al., 2022), would similarly induce a slight shift in molecular weight. Future experiments may test these hypotheses, which are not mutually exclusive.

Consistent with previous reports (Lim et al., 1999; Wang et al., 2014; Lee et al., 2015; Mei et al., 2016), we found that the different Shank3 protein isoforms are differently expressed according to developmental stages and in a brain region specific manner, suggesting the existence of isoform specific functions. Systematic analysis of isoform-specific binding partners and isoform-specific brain expression patterns is an important step for future research. In that attempt, we used the herein generated Shank3$^{Venus/Venus}$ KI mouse line in which endogenous Shank3a full-length isoform is specifically tagged to follow its neurodevelopmental expression. Our data show that Venus-Shank3a is mainly expressed in the cortex, striatum and hippocampus at early developmental stage when synaptogenesis occurs (P14). This specific brain area expression persists in the adult brain with an additional delayed expression in the adult cortex. We found no expression of Venus-Shank3a in the cerebellum, or a marginal one.

Given the potential importance of the *SHANK3* gene in ASD (Betancur and Buxbaum, 2013; Carbonetto, 2013; Leblond et al., 2014; Sala et al., 2015), it is crucial to understand more about its physiological role at the synapse and how it is disrupted by mutations. In humans, *SHANK3* gene (22q13.3) deletion triggers neurodevelopmental disorders like Phelan–McDermid syndrome (PMS), characterized by autistic like behaviors, hypotonia and delayed or absent speech (Bonaglia et al., 2001; Phelan et al., 2001; Phelan, 2008; Phelan and McDermid, 2012). Shank3 protein truncation is thought to cause the core neurodevelopmental and behavioral deficits that are observed in patients. By definition, in this heterozygous condition only one of the two Shank3 alleles displays the mutation. This means that the core PMS symptoms could either be triggered by Shank3 haploinsufficiency (Phelan and McDermid, 2012), and/or by a dominant negative effect of the Shank3 mutant form (resulting from aberrant trafficking to the dendritic spines or lack of interaction with functional partners). The latter hypothesis is more frequently investigated (Yoo et al., 2013; Sala et al., 2015; Varghese et al., 2017). Our strategy, focusing on the expression and role of the non-mutated endogenous Venus-Shank3a when co-expressed with the truncated untagged form, Shank3ΔC, complements the previous attempts to understand how alteration of the Shank3 protein can lead to the development of ASD. Here we showed that Venus-Shank3a expression is delayed during brain development when co-expressed with Shank3ΔC. In particular, Venus-Shank3a expression is deficient around P7-14, a period during which Shank3 is essential because of its role in the synaptogenesis and neuronal wiring of the developing brain (Sala et al., 2001, 2015; Roussignol et al., 2005; Durand et al., 2012; Macgillavry et al., 2016; Sarowar and Grabrucker, 2016; Pagani et al., 2019). We observed this delayed expression in the cortex, striatum and hippocampus. Defect in neuronal connectivity within the limbic system could explain, at least in part, the cognitive deficits associated with ASD. Hence, ASD may also depend on the ability of the wild type Shank3 allele to be typically expressed and engaged into functional complexes at synapses. Similarly, the difference in impairment severity might be explained not only by the expression pattern of Shank3 mutated form but also by the extent to which the spared Shank3 protein can (or cannot) compensate for its loss.

To conclude, the Shank3$^{Venus/Venus}$ KI mouse line enables to track the Shank3a isoform in physiological and ASD conditions. This mouse line will be further useful to screen for differential Venus-Shank3a protein interactors in physiological and pathological conditions. Understanding Shank3 proteins function from an isoform specific perspective may also help to explain how different *SHANK3* gene mutations may result in distinct phenotypic consequences in different transgenic mice (Monteiro, 2018). Importantly, isoform-specific studies will help understanding the clinical conditions of patients with SHANK3 mutations, for whom genotype–phenotype stratification will help the design of specific pharmacological agents.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the French Ministry of Research (APAFIS#23357-2019112715218160 v4 APAFIS#23476-2020010613546503 v4). Written informed

consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EM and JP conceived research and wrote the manuscript. JP and FR designed the genome editing strategy. NB, SS, FR, FB, JP, and EM designed research experiments. NB, SS, and EM performed mice genotyping. VC and EM performed immunocytochemistry. A-LH-G performed tissue dissections. SS, EM, and FB performed behavioral experiments. VS, NB, and EM performed Western blots. JP supervised the project. All authors contributed to the preparation of the manuscript and approved it.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.1081010/full#supplementary-material

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New insights into the regulation of *Cystathionine beta synthase (CBS)*, an enzyme involved in intellectual deficiency in Down syndrome

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Down syndrome (DS), the most frequent chromosomic aberration, results from the presence of an extra copy of chromosome 21. The identification of genes which overexpression contributes to intellectual disability (ID) in DS is important to understand the pathophysiological mechanisms involved and develop new pharmacological therapies. In particular, gene dosage of *Dual specificity tyrosine phosphorylation Regulated Kinase 1A* (*DYRK1A*) and of *Cystathionine beta synthase* (*CBS*) are crucial for cognitive function. As these two enzymes have lately been the main targets for therapeutic research on ID, we sought to decipher the genetic relationship between them. We also used a combination of genetic and drug screenings using a cellular model overexpressing *CYS4*, the homolog of *CBS* in *Saccharomyces cerevisiae*, to get further insights into the molecular mechanisms involved in the regulation of CBS activity. We showed that overexpression of *YAK1*, the homolog of *DYRK1A* in yeast, increased *CYS4* activity whereas *GSK3*β was identified as a genetic suppressor of *CBS*. In addition, analysis of the signaling pathways targeted by the drugs identified through the yeast-based pharmacological screening, and confirmed using human HepG2 cells, emphasized the importance of Akt/GSK3β and NF-κB pathways into the regulation of CBS activity and expression. Taken together, these data provide further understanding into the regulation of CBS and in particular into the genetic relationship between *DYRK1A* and *CBS* through the Akt/GSK3β and NF-κB pathways, which should help develop more effective therapies to reduce cognitive deficits in people with DS.

KEYWORDS

***CBS*, *DYRK1A*, GSK3β, Akt, NF-κB, pharmacological inhibitor**

Introduction

Down syndrome (DS) is the most frequent chromosomic aberration, with a prevalence of one in 650–1,000 live births worldwide. This genetic condition results from the presence of an extra copy of chromosome 21, as first described by Lejeune et al. (1959). The triplication of this chromosome and of its ∼225 genes leads to a complex phenotype that includes particular craniofacial features, hypotonia, cardiac, and digestive defects, high incidence of leukemia, early onset of Alzheimer's disease and intellectual disability (ID). Although the detailed consequences of the overexpression of all these individual genes is difficult to assess, a few of them have been suggested to be of crucial importance in the development of certain phenotypic aspects (Antonarakis, 2017). Concerning ID, a few genes are considered as highly relevant candidates, among which the Amyloid Precursor Protein (*APP*) (Salehi et al., 2007), the Glutamate Receptor, Ionotropic, Kainate 1 (*GRIK1*) (Valbuena et al., 2019), the Regulator of CAlciNeurin 1 (*RCAN1*) (Dudilot et al., 2020) and the *Dual-specificity tyrosine phosphorylation-Regulated Kinase 1A* (*DYRK1A*) (Altafaj et al., 2013; García-Cerro et al., 2014). So far, DYRK1A has been the main target for therapeutic research, leading to the identification of compounds that inhibit its protein kinase activity and are able to improve cognition in mouse models for DS (Guedj et al., 2009; De la Torre et al., 2014, 2016; Kim H. et al., 2016; Nakano-Kobayashi et al., 2017; Neumann et al., 2018; Nguyen et al., 2018). However, their efficiency in DS patients is limited, showing the need to combine multiple therapies to improve cognitive deficits and more generally the quality of life of DS patients.

More recently, studies of transgenic mouse models have revealed that the triplication of *CBS* gene also contributes to cognitive phenotypes and that *CBS* and *DYRK1A* show epistatic interactions (Maréchal et al., 2019). *CBS* encodes a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the first reaction in the transsulfuration pathway. This pathway leads to the synthesis of cysteine and glutathione (GSH) at the expense of homocysteine and methionine (Jhee and Kruger, 2005). In the brain, CBS is also the major enzyme catalyzing the production of hydrogen sulfide (H_2S) from L-cysteine (Kimura, 2011) or from the condensation of homocysteine with cysteine (Chen et al., 2004). H_2S is now considered as a major gasotransmitter in the brain, which plays a role in synaptic transmission (Kamat et al., 2015) and its increased production resulting from CBS triplication has been suggested to contribute to the cognitive phenotype of DS patients (Kamoun, 2001; Kamoun et al., 2003; Szabo, 2020). For this reason, the identification of pharmacological inhibitors of CBS has been an important field of research in the last 10 years. Unfortunately, most of the screening methods used were *in vitro* and have only led to the identification of compounds with relatively low potency and limited selectivity (Asimakopoulou et al., 2013; Thorson et al., 2013, 2015; Zhou et al., 2013; Druzhyna et al., 2016), suggesting that CBS may be difficult to target pharmacologically. We recently developed a new screening method based on the budding yeast *Saccharomyces cerevisiae* which allows the identification of drugs or genes that interfere with the phenotypical consequences of *CYS4* (*CBS* homolog in yeast) overexpression. Using this method, we recently identified four molecules (disulfiram, chloroxine, clioquinol, and nitroxoline), all involved in metallic ion binding (Maréchal et al., 2019; Conan et al., 2022), which effect on CBS activity has been validated in different cellular models (Zuhra et al., 2020; Conan et al., 2022).

A genetic interaction between *CBS* and *DYRK1A* has been previously suggested in mouse (Tlili et al., 2013; Latour et al., 2015; Baloula et al., 2018; Maréchal et al., 2019) but the nature of this interaction was still undetermined. It was described as positive in certain studies and negative in others depending on the context or the organ (liver vs. brain). Here, we took advantage of our yeast-based model to explore the relationship between *CBS* and *DYRK1A* genes, which allowed us to confirm the positive regulation of Cbs activity by Dyrk1A. Next, we further investigated the molecular mechanisms involved in the regulation of CBS activity using a combined genetic and drug screening approach. Our results highlighted the importance of the Akt/GSK3β and the NF-κB pathways in the regulation of CBS activity and expression.

Materials and methods

Genetic and drug screening in *S. cerevisiae*

Saccharomyces cerevisiae strains used in this study are listed in **Supplementary Table 1** and were cultured as previously described (Maréchal et al., 2019). Cultures in exponential growth phase, obtained by diluting overnight cultures and incubation for 4–5 h to reach OD_{600}∼0.6–1, were used in all experiments. Subcloning of full-length cDNA of *CYS4* in expression vectors of the pRS42X series was performed as previously described (Maréchal et al., 2019) using primers listed in **Supplementary Table 2**. To obtain a sufficient level of methionine auxotrophy, in all the figures presented (except **Figure 1B**, in which 2 centromeric plasmids were used), *CYS4* overexpression was obtained through the transfection of two 2 μ vectors of the pRS42X series and the addition in the medium of serine (at a final concentration of 1.5 mM), a limiting substrate for Cys4p activity.

To test the genetic interaction between *YAK1* and *CYS4*, the coding sequence of *YAK1* was amplified from the genomic DNA of a W303 wild-type strain and subcloned into either a pRS416-*GPD* (centromeric plasmid) or a pRS426-*GPD* (2 μ) vector using

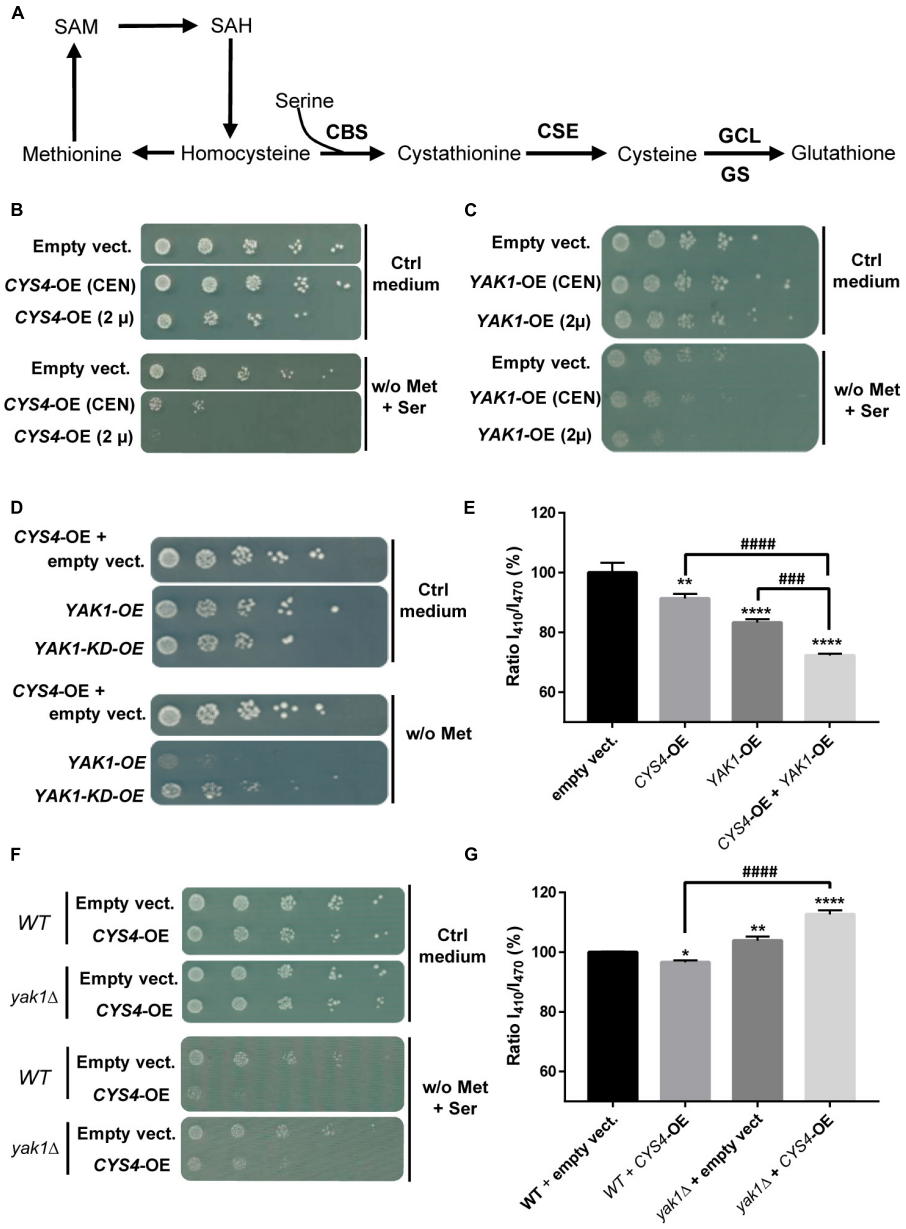

FIGURE 1
Interaction between *CYS4* and *YAK1*. **(A)** Simplified representation of the transsulfuration pathway. Yeast *CYS4* gene encodes the cystathionine beta synthase protein (CBS) which converts homocysteine and serine into cystathionine. The other enzymes of the transsulfuration pathway are CSE, GCL (γ-glutamylcysteine synthetase), and GS (glutathione synthetase). *CYS4/CBS* overexpression would favor cysteine and glutathione synthesis at the expense of homocysteine and methionine. **(B)** Methionine auxotrophy of *CYS4*-overexpressing (*CYS4*-OE) cells. Methionine auxotrophy, revealed by the absence of growth on medium lacking methionine, was assessed by spotting serial dilutions of wild-type yeast cells transformed with two centromeric (*CEN*) or 2 μ plasmids either empty or containing full length *CYS4*, which expression in yeast is driven by the strong GPD promoter. The presence of a centromere segment (*CEN*) in plasmids enhances their mitotic stability but results in a lower copy number than 2 μ plasmids, thus producing less proteins. **(C)** Methionine auxotrophy of *YAK1*-OE cells. Similarly to *CYS4*-OE, *YAK1*-OE leads to methionine auxotrophy using either a single centromeric plasmid (*CEN*) or a single 2 μ plasmid. **(D,E)** Additive effect of *YAK1*-OE and *CYS4*-OE. **(D)** *YAK1*-OE (obtained with one single 2 μ plasmid) enhances methionine auxotrophy of *CYS4*-OE cells (obtained with two 2 μ plasmids). This effect depends on the kinase activity of *YAK1* as a kinase dead form of *YAK1* (*YAK1*-KD) was not able to strengthen *CYS4*-OE induced phenotype. Note that we used in these experiments a methionine-free medium without serine supplementation to be able to see stronger methionine auxotrophy than the one caused by *CYS4*-OE. **(E)** *YAK1* and *CYS4* overexpression have additive effects on cytosolic acidification. **(F,G)** *YAK1* deletion prevents *CYS4*-OE induced phenotypes. **(F)** *YAK1* deletion mitigates *CYS4*-OE induced methionine auxotrophy. **(G)** Similarly, *CYS4*-OE is not able to induce acidification defects in cells deleted for *YAK1* suggesting that Yak1p is necessary for Cys4p activity. Note that *YAK1* deletion even increases cytosolic pH, suggesting a decrease in GSH synthesis and/or the presence of oxidative stress which may deplete intracellular glutathione. **(E,G)** One-way ANOVA with Tukey's *post-hoc* test. Comparison with DMSO: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Comparison between conditions: $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$.

primers listed in **Supplementary Table 2**. *YAK1* overexpression was obtained through the transfection of only one vector of the pRS42X series (2 μ plasmids).

For the genetic screening, a yeast genomic DNA library (Lista et al., 2017) constructed by inserting ∼4 kb genomic DNA fragments (obtained by *Sau3A* partial digestion) at the unique *Bam*HI site in the replicative 2 μ multicopy pFL44L vector containing *URA3*-marker, was transformed into a yeast strain overexpressing *CYS4* (with pRS423 and pRS424 plasmids). Transformants were selected on solid minimal medium lacking tryptophan, histidine, uracil, and methionine and supplemented with 1.5 mM of serine. Plasmids originated from the pFL44L-based library were extracted and purified with the Zymoprep kit (Zymo Research), amplified in *Escherichia coli* and then retransformed into the yeast strain overexpressing *CYS4* to confirm their ability to reverse methionine auxotrophy. The extremities of the confirmed clones were sequenced using primers listed in **Supplementary Table 2**. The coding sequences of genes obtained in the genetic screen were amplified from the pFL44L plasmids extracted from the library and subcloned into pRS426-*TEF* (2 μ) plasmids using primers listed in **Supplementary Table 2**, which introduced *Sma*I and *Xho*I restriction sites. Yeast deletion of *MCK1* in the W303 background was performed by standard one-step gene replacement with PCR-generated cassettes (Longtine et al., 1998) using primers listed in **Supplementary Table 2**.

The *YAK1*-KD (kinase dead, p.K398R) and *MCK1*-KD (p.K68R) mutants were created by site-directed mutagenesis (QuickChange Lightning, Agilent technologies, Santa Clara, CA, USA) according to the manufacturer instructions using primers listed in **Supplementary Table 2**.

Drug screening was performed as previously described (Maréchal et al., 2019; Conan et al., 2022) using compounds obtained from the NIH from different sets: 166 FDA-approved Oncology Drugs (at a concentration of 10 mM in DMSO), 1,584 compounds of the NCI Diversity Set VI (at a concentration of 10 mM in DMSO), 811 compounds of the Mechanistic Set VI (at a concentration of 1 mM in DMSO) and 390 compounds of the Natural Products Set V (at a concentration of 10 mM in DMSO).

Determination of yeast cytosolic pH was performed as previously described (Conan et al., 2022) using a pRS416-*ADH* plasmid containing a pH-sensitive ratiometric GFP variant named pHluorin (kindly obtained from S. Léon, IJM, Paris).

Cell culture and drug treatment

The human liver cancer cell line HepG2 was obtained from ATCC and was cultured in DMEM glutamax high glucose medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (Invitrogen), in a humidified incubator at 37°C and 5% CO_2 atmosphere.

All the molecules used in this study were either purchased from Merck or obtained from the NIH libraries and were resuspended in DMSO. For drug treatment, 20,000 HepG2 cells were plated in each well of a Greiner Bio-One black 96-well plate with transparent flat bottom in 100 μL of culture medium. The following day, cells were incubated for 24 or 48 h with selected drugs at indicated concentrations with a final concentration of 1% DMSO (v/v).

Measurement of H_2S production in live cells

For HepG2 transfection, 250,000 cells were seeded per well in 6 well plates 24 h before transfection. Cells were then transfected with either the pcDNA3 vector (Invitrogen, Waltham, MA, USA) alone as a negative control or with *GSK3*β or *DYRK1A* cDNA using JetOptimus transfection reagent (Polyplus transfection, Illkirch, France) following the manufacturer's instructions. The *GSK3*β plasmid was obtained from Addgene (#14754) and corresponds to a constitutively active enzyme (mutant p.S9A). Wild-type and mutant p.S324R and p.S311F *DYRK1A* plasmids were obtained from Courraud et al. (2021). These two mutations, located in the kinase domain of DYRK1A affect its kinase activity (Courraud et al., 2021). Fourty-eight or 72 h after transfection, H_2S production was assessed as followed.

Following a 24 h-treatment, cells were washed once with 1× PBS and incubated for 2 h in a saline buffer (139 mM NaCl, 0.56 mM $MgCl_2$, 10 mM Hepes, 2.7 mM KCl, 1 mM K_2HPO_4, 1.8 mM $CaCl_2$ pH7.4 supplemented with 10 mM glucose) containing 100 μM of 7-Azido-4-Methylcoumarin (AzMC) fluorescent probe (Sigma Aldrich), which selectively reacts with H_2S to form a fluorescent compound. Fluorescent AzMC signal acquisition (λ_{Ex} = 365 nm and λ_{Em} = 450 nm) was performed on a Flexstation 3 microplate reader using the SoftMax Pro 5.4.5 software (Molecular Device, San Josa, CA, USA). Values were expressed as a percent of the corresponding controls.

Cell viability assessment

The cytotoxicity of all tested compounds was examined using the Cell Counting Kit WST-8/CCK8 (Abcam). Briefly, following the measurement of H_2S levels, cells were washed once with 1× PBS and incubated for 2 h in the WST-8 reagent mixed in the culture medium according to the manufacturer's instructions. The absorbance signal acquisition (at 450 nm) was performed on the Flexstation 3 microplate reader with SoftMax Pro 5.4.5 software (Molecular Device, San Josa, CA, USA). Values were expressed as a percent of the corresponding controls.

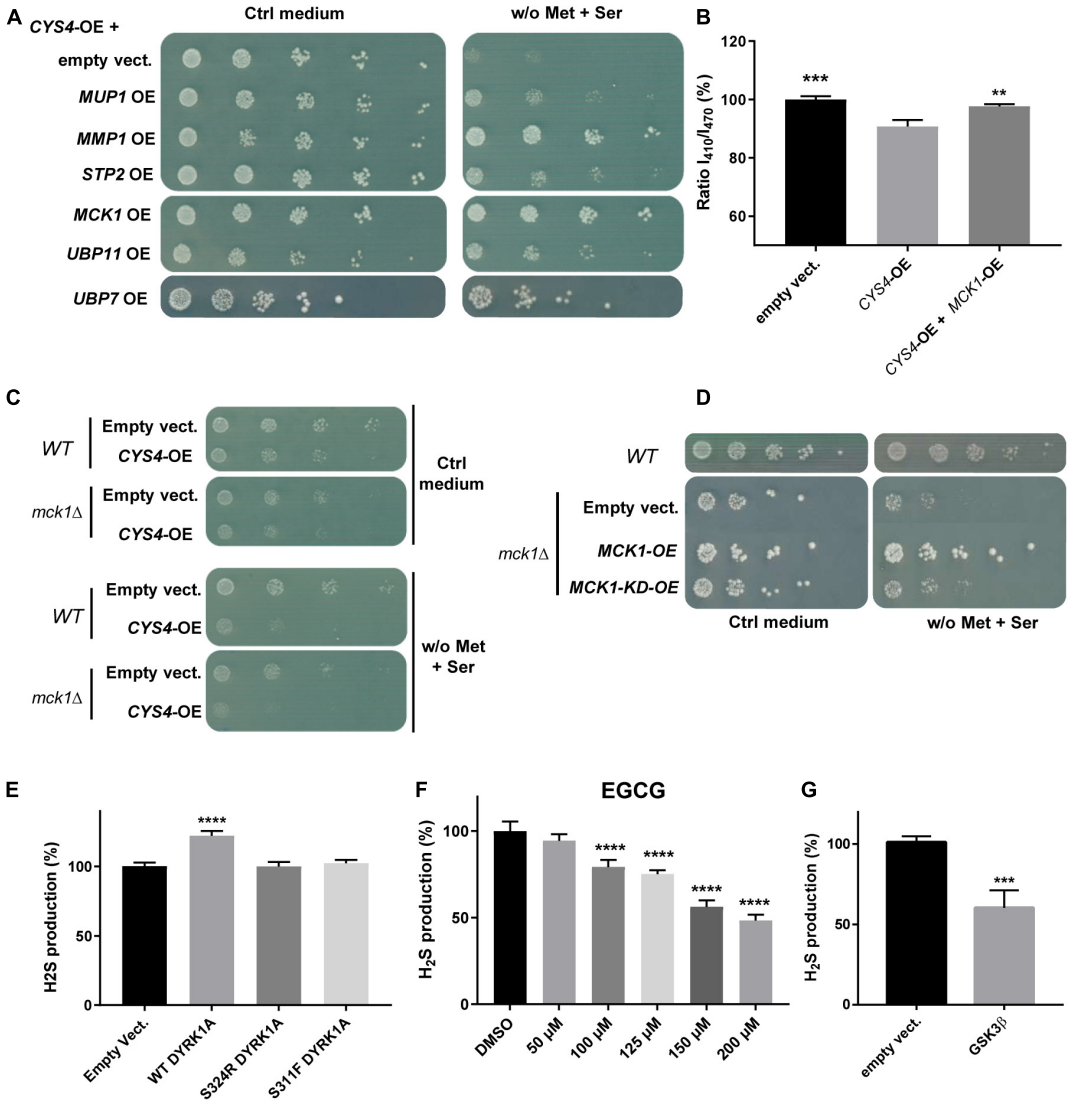

FIGURE 2
A genetic screening identified *MCK1* as a genetic repressor of *CYS4*-OE induced phenotypes. **(A,B)** *MCK1*-OE rescues the consequences of *CYS4*-OE. **(A)** Genes that were the most effective in rescuing the methionine auxotrophy induced by *CYS4*-OE include genes related to amino acid import (*MUP1*, *MMP2*, *STP2*), a kinase (*MCK1*), and two genes encoding deubiquitinating enzymes (*UBP7* and *UBP11*). **(B)** *MCK1*-OE restores normal cytosolic acidification of *CYS4*-OE cells. Comparison of each condition with *CYS4*-OE, one-way ANOVA with Dunnett's *post-hoc* test: $^{**}p < 0.01$; $^{***}p < 0.001$. **(C,D)** Deletion of *MCK1* causes slight methionine auxotrophy. **(C)** Deletion of *MCK1* strengthens the effect of *CYS4*-OE on methionine auxotrophy. **(D)** The m*ck1*Δ strain, similarly to *CYS4*-OE, has difficulty to grow on a methionine-free medium complemented with serine, and this methionine auxotrophy is rescued by the expression of wild-type *MCK1* but not of a kinase-dead form of Mck1p (*MCK1-KD*). **(E)** Effect of human *Dual specificity tyrosine phosphorylation Regulated Kinase 1A (DYRK1A)* overexpression on H_2S production in HepG2 cells. The expression of wild-type Dyrk1A induced increased H_2S expression 48 h after transfection, effect that was not visible using two mutants p.S324R and p.S311F which affect its kinase activity. **(F)** Inhibition of Dyrk1A activity decreased H_2S production. A 24 h-treatment with epigallocatechin gallate (EGCG), an inhibitor of Dyrk1A resulted in a dose-dependent decrease in H_2S production. **(G)** The expression of a constitutively active form of GSK3β (p.S9A) resulted in decreased H_2S production 72 h after transfection. **(E,F)** Comparison of each condition with empty vector or DMSO, one-way ANOVA with Dunnett's *post-hoc* test: $^{****}p < 0.0001$. **(G)** Student's *t*-test: $^{***}p < 0.001$.

Western blot

HepG2 cells were treated for 24 or 48 h with selected drugs and were harvested in the following buffer: 150 mM NaCl, 1% Igepal, 50 mM Tris–HCl pH 7.4 with Protease inhibitor cocktail (Roche). Cell lysis was then performed by 6 cycles of vigorous vortexing and freeze-thawing. Protein amount in the supernatants was evaluated by classical Bradford method. Twenty micrograms of each sample were then loaded onto 10% NuPAGE Bis-Tris gels (precast NuPAGE, Invitrogen), and transferred onto 0.45 µm nitrocellulose membranes (Cytiva, Velizy-Villacoublay, France). Membranes were blocked during

1 h at room temperature in 1× PBS containing 0.1% Igepal and 5% milk and then incubated overnight at 4°C with the following primary antibodies: anti-CBS mouse monoclonal antibody (ab12476, Abcam, 1:1,000) and anti-α-tubulin mouse monoclonal antibody (T6793, Sigma-Aldrich, 1:10,000). The following day, membranes were washed with fresh 1× PBS with 0.1% Igepal and incubated for 45 min with gaot anti-mouse (ab6789, Abcam, 1:3,000) conjugated to horseradish peroxidase at a 1:3,000 dilution, and analyzed by enhanced chemiluminescence (ECL, Cytiva) using a Vilbert-Lourmat Photodocumentation Chemistart 5000 imager.

RT-qPCR

HepG2 cells were treated for 24 h and RNA was isolated using the NucleoSpin RNA mini kit (Macherey-Nagel) and reverse transcribed using RT^2 First Strand Kit (SA Biosciences) following the manufacturers' instructions. Real-Time PCR was performed on an *ABI* PRISM® 7300 Sequence Detection System using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen). Expression levels were normalized across samples using the GAPDH or β-actin housekeeping genes. Primers used are listed in **Supplementary Table 3**. Data analysis was done by the $2^{-\Delta\Delta Ct}$ method and relative expression was calculated using DMSO condition as the reference sample (expression = 1). Significant differences were assessed by the unpaired two-tailed *t*-test.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (San Diego, CA, USA). Presented results in each figure represent data obtained in at least 3 independent experiments.

Results

Yak1p promotes Cys4p activity through its kinase activity

We first investigated the relationship between CBS and DYRK1A using an original yeast-based assay that we recently set-up to identify CBS inhibitors (Maréchal et al., 2019; Conan et al., 2022), and which is based on the overexpression of *CYS4*, the homolog of *CBS* in *S. cerevisiae*. Located at a metabolic hub, CBS/Cys4p converts homocysteine and serine into cystathionine (**Figure 1A**). As previously shown (Maréchal et al., 2019; Conan et al., 2022), *CYS4*-overexpression (OE) in yeast favors cysteine and glutathione synthesis at the expense of methionine and homocysteine (**Figure 1A**), leading to a decreased ability of yeast cells to grow without external supply of methionine (**Figure 1B**). This effect can be enhanced by the addition in the medium of serine, a substrate of the reaction, making this phenotype a convenient read-out that can be easily monitored and used for drug or genetic screening.

Similarly to what was observed for *CYS4*-OE, the overexpression of *YAK1*, the homolog of *DYRK1A* in *S. cerevisiae*, induced by itself methionine auxotrophy in a dose-dependent manner on medium supplemented with serine (**Figure 1C**). In addition, simultaneous *YAK1*-OE and *CYS4*-OE showed additive effect on methionine auxotrophy (**Figure 1D**). Yak1p activity appeared to be mediated by its kinase activity as its effect was lost when a kinase dead (KD) form p.K398R of *YAK1* (Moriya et al., 2001) was used (**Figure 1D**). We previously identified cytosolic acidification as another phenotype specific to *CYS4*-OE (Conan et al., 2022). We show here that, similarly to *CYS4-OE*, *YAK1-OE* induced cytosolic acidification and that combined effect of *CYS4-OE* and *YAK1*-OE on cytosolic pH was additive (**Figure 1E**). On the opposite, *YAK1* deletion partially rescued methionine auxotrophy due to *CYS4-OE* (**Figure 1F**). Similarly, *yak1*Δ cells showed increased cytosolic pH, suggestive of an absence of Cys4p activity as shown by Conan et al. (2022). Finally, *CYS4*-OE in a *yak1*Δ strain was unable to induce cytoplasmic acidification defects and even further accentuated cytosolic pH basification (**Figure 1G**). Taken together, these results suggest that Yak1p promotes Cys4p activity through its kinase activity and that, in the absence of Yak1p, Cys4p activity is reduced.

Identification of *MCK1*, the yeast homolog of *GSK3*, as a genetic suppressor of *CYS4*-OE phenotypes

To get better insights into the cellular mechanisms involved in *CYS4*-OE induced phenotypes, we sought to identify genetic suppressors. Amongst the genes having the capacity to save the methionine auxotrophy of *CYS4*-OE cells, six genes had a strong effect: *MUP1* (a methionine permease), *MMP1* (a S-methylmethionine permease), *STP2* (a transcription factor that activates the transcription of amino acid permease genes), *MCK1* (one of the four genes that encode glycogen synthase kinase 3 (GSK3) homologs in yeast), and *UBP7* and *UBP11*, two ubiquitin specific proteases involved in endocytosis and in the sorting of internalized receptors (Tardiff et al., 2013; Weinberg and Drubin, 2014; **Figure 2A**). The overexpression of *MUP1*, *MMP1*, and *STP2* may act in bringing up traces of methionine or related amino acids present in the medium to rescue the methionine auxotrophy induced by *CYS4*-OE. However, as the overexpression of *MUP1* and *MMP1* appears to also decrease cytosolic acidification of *CYS4*-OE cells (**Supplementary Figure 1A**), their action may also involve other molecular mechanisms. Similarly, *MCK1* (**Figure 2B**), *UBP11* and *UBP7* (**Supplementary Figure 1B**)

were also able to counteract the effects of *CYS4*-OE on cytosolic acidification.

Then, due to the important role of *MCK1/GSK3* in cell signaling, we focused our attention on this genetic repressor of *CYS4*-OE induced phenotypes. As shown in **Figures 2A, B** *MCK1*-OE counteracted both methionine auxotrophy and cytosolic acidification phenotypes induced by *CYS4*-OE. On the opposite, *MCK1* deletion appeared to slightly enhance the methionine auxotrophy of *CYS4*-OE cells (**Figure 2C**), suggesting that in the absence of Mck1p, Cys4p activity may be promoted. This hypothesis is further strengthened by the fact that a *mck1*Δ strain (without *CYS4* overexpression) shows slight methionine auxotrophy, which is rescued by the expression of wild-type *MCK1* but not by a KD form of *MCK1* (p.K68R, Lim et al., 1993; **Figure 2D**). Taken together, these results thus show that Cys4p activity is reduced in the presence of Mck1p but increased in the absence of Mck1p, suggesting that Mck1p inhibits directly or not Cys4p activity, and that Mck1p kinase activity is required.

The genetic interactions between *CYS4* and *YAK1*, and between *CYS4* and *MCK1*, are conserved in mammalian cells

Then, to check whether the regulation of Cys4p by Yak1p is conserved in mammalian cells, we tested the effect of the expression of *DYRK1A* in the human hepatoma HepG2 cell line. We chose this cell line because CBS is known to be highly expressed and active in the liver. In addition, this cell line is commonly used to study CBS-mediated hydrogen sulfide (H_2S) production (Wang et al., 2018), as cystathionine γ lyase (CSE), the other enzyme involved in H_2S production, is expressed at very low levels in HepG2 cells. H_2S production, measured using the 7-AzMC fluorogenic probe as previously described (Conan et al., 2022), can be thus considered in HepG2 as a read-out for CBS activity. As shown in **Figures 2E, D** YRK1A expression in HepG2 cells induced a significant increase in H_2S production without affecting cell proliferation and/or viability (**Supplementary Figure 1C**), whereas two mutant forms of *DYRK1A* (p.S324R and p.S311F) that had lost their kinase activity (Courraud et al., 2021) had no effect on H_2S production (**Figure 2E**). Conversely, a 24 h treatment with epigallocatechin gallate (EGCG), an inhibitor of DYRK1A, decreased H_2S production in a dose-dependent manner (**Figure 2F** and **Supplementary Figure 1D**). Similarly, the expression of a constitutively activated form of *GSK3*β (p.S9A mutant) in HepG2 cells induced a significant decrease of H_2S production (**Figure 2G**) without affecting cell proliferation and/or viability (**Supplementary Figure 1E**). Taken together, these results confirm genetic interactions between *CBS* and *DYRK1A* and between *CBS* and *GSK3*β, which are conserved between yeast and human.

Several small molecules identified in a drug screening appear to converge on metal ion chelation and/or inhibition of NF-κB and Akt/GSK3β pro-survival signaling pathways

To get further insights into the molecular mechanisms involved in the regulation of CBS activity, we screened a set of 2,932 small molecules from the National Cancer Institute, consisting in diverse chemical scaffolds, including natural products and approved oncology drugs. Several compounds were able to restore cell growth of *CYS4*-OE cells on medium without methionine (**Supplementary Table 4**). Remarkably, the vast majority of the molecules identified in this screen have been described to form complexes with metal ions or to either inhibit NF-κB and/or the Akt/GSK3β pathway (**Table 1**). Interestingly, six of these compounds have the property to form complexes with Cu(II) (**Table 1**), supporting our previous findings that copper chelation efficiently decreases CBS activity both in yeast and mammalian cells (Conan et al., 2022). Similarly, zinc pyrithione, a zinc ionophore (Ding and Lind, 2009) that we previously described (Conan et al., 2022), was also found active in the drug screening (**Table 1** and **Supplementary Table 4**).

Among the other compounds identified, eight of them are known to inhibit pro-survival pathways (**Table 1** and **Supplementary Table 4**). Among these compounds, 9-methylstreptimidone, an inhibitor of NF-κB (Wang et al., 2006; Ishikawa et al., 2009) and possibly of the Akt/GSK3β pathway (Brassesco et al., 2012; Koide et al., 2015), showed a strong capacity to rescue yeast growth on a medium without methionine (**Supplementary Table 4**). Similarly, 8α-hydroxy verrucarin A and chrysomycin A, both inhibitors of NF-κB and of the Akt/GSK3β pathway (Deeb et al., 2016; Liu et al., 2016), also counteracted the growth defect phenotype induced by Cys4p overexpression (**Supplementary Table 4**). In order to check whether these drugs were also able to inhibit CBS activity in mammalian cells, we tested some of the most active compounds on H_2S production in HepG2 cells. As shown in **Figure 3**, inhibitors of NF-κB and/or Akt/GSK3β pathway such as 9-methylstreptimidone (**Figure 3A**), 8α-hydroxy verrucarin A (**Figure 3C**) and chrysomycin A (which has been identified twice in the screening) (**Figure 3D**) were able to decrease H_2S production (upper panel), although 8α-hydroxy verrucarin A and chrysomycin A also affected cell viability (lower panel). Similarly, two members of the anthracycline antibiotic family, doxorubicin and daunorubicin hydrochloride decreased H_2S production in HepG2 cells (**Figure 3B**, upper panels) although they induced cell toxicity at higher concentrations (**Figure 3B**, lower panels). Finally, several small molecules with moderate

TABLE 1 The molecules identified in the screen share common properties such as metal cation binding or target pro-survival pathways.

Compound	Metal-binding properties	Effect on NF-κB pathway	Effect on Akt/GSK3β pathway	References
9-Methylstreptimidone	None	Inhibitor	possible inhibitor	Wang et al., 2006; Ishikawa et al., 2009; Brassesco et al., 2012; Koide et al., 2015
4-(2-Thiazolylazo)-resorcinol	Cu(II)			Stanley and Cheney, 1966
1-(2-Thiazolylazo)-2-naphtol	Cu(II)			Pease and Williams, 1959
Zinc pyrithione	Zn(II)	Inhibitor		Kim et al., 1999; Ding and Lind, 2009
N,N-dimethyldaunomycin hydrochloride	Cu(II)	Inhibitor		Malatesta et al., 1987; Boland et al., 1997; Ho et al., 2005; Jabłońska-Trypuæ et al., 2017
Doxorubicin dihydrochloride				
Daunorubicin hydrochloride				
γ-thujaplicin	Cu(II), Zn(II)	Inhibitor	Inhibitor	MacLean and Gardner, 1956; Miyamoto et al., 1998; Byeon et al., 2008; Huang et al., 2015; Wu et al., 2020
Verrucarin A,10-epoxide		Inhibitor	Inhibitor	Deeb et al., 2016; Liu et al., 2016
8α-Hydroxy-verrucarin A				
Monoacetyl verrucarin A epoxide				
Chrysomycin A		Inhibitor	Inhibitor	Liu et al., 2021, 2022
Naphtoquinones	Cu(II)	Inhibitor		Brandelli et al., 2004; Golan-Goldhirsh and Gopas, 2014

activity were obtained in the screening, including several derivatives of naphtoquinones, which are also inhibitors of the NF-κB pathway (**Supplementary Table 4**). However, as their activity was moderate in rescuing cell growth in yeast, they were not tested on H_2S production in HepG2 cells.

To further confirm the hypothesis of a regulation of CBS activity by the Akt/GSK3β and/or NF-κB pathways, we assessed the impact of inhibitors of these pathway (9-methylstreptimidone, 8α-hydroxy verrucarin A, chrysomycin and daunorubicin) on the mRNA levels of *CBS*, *CSE* and *NAD(P)H quinone oxidoreductase 1* (*NQO1*), three genes known to be regulated by these pathways (Wang et al., 2014; Mutter et al., 2015; Ozaki et al., 2018). We also evaluated the effect of nitroxolin (NHX), a copper chelator that we previously identified as a candidate inhibitor for CBS (Conan et al., 2022) and which has been reported to reduce Akt phosphorylation and decrease GSK3β phosphorylation (Xu et al., 2019; Veschi et al., 2020). As shown in **Figures 4A–C**, all the compounds that reduced H_2S production in HepG2 cells (**Figure 3**) also decreased mRNA levels of *CBS*, *CSE*, and *NQO1* genes, after 24 h of treatment. Accordingly, the levels of CBS protein were also reduced after 48 h but not after 24 h of treatment (**Figure 4D**). Altogether, these data suggest that the Akt/GSK3β and NF-κB pathways are involved both in the regulation of CBS activity (as assessed by the decreased H_2S production 24 h after treatment), but also in the regulation of CBS expression, effect which is observed at the mRNA level 24 h after treatment and at the protein level 48 h after treatment.

Discussion

DYRK1A positively regulates CBS activity

Several studies have previously suggested a genetic interaction between *CBS* and *DYRK1A* but the nature of this interaction was still undetermined. Indeed, most of these data have been obtained in mouse models and it is sometimes difficult to assess in a complete organism whether the effects observed are direct or indirect consequences of *DYRK1A* or

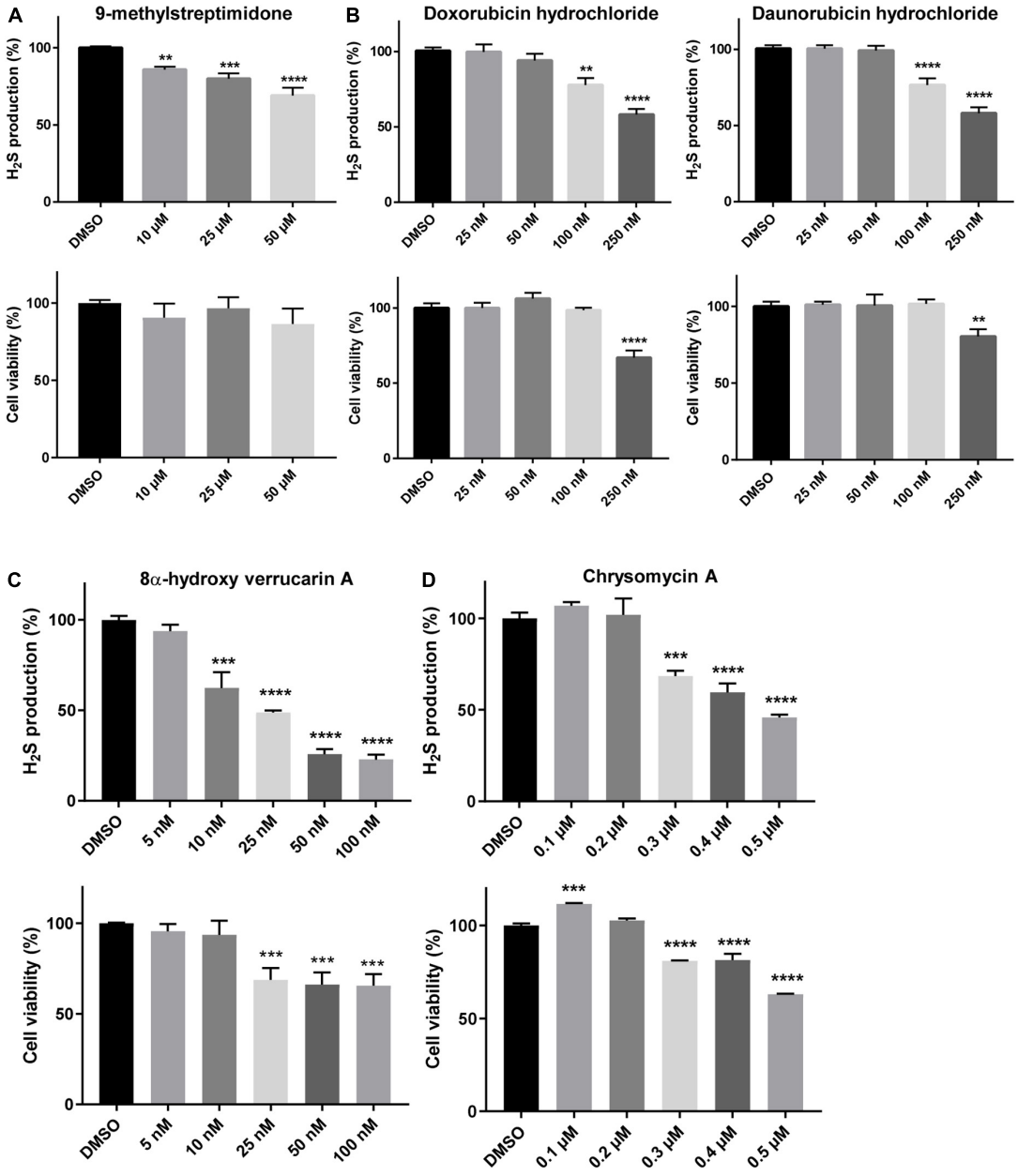

FIGURE 3

Molecules reducing the phenotypes induced by *CYS4*-OE decrease H_2S production. The level of H_2S production and cell viability were assessed using the Azido-4-Methylcoumarin (AzMC) probe and the WST-8 assay, respectively. A 24 h-treatment of HepG2 cells with 9-methylstreptimidone **(A)**, doxorubicin and daunorubicin hydrochloride **(B)**, 8α-hydroxy verrucarin A **(C)**, and chrysomycin A **(D)**, resulted in decreased H_2S production (upper panels) without decreasing cell viability, except at the highest concentrations tested (lower panels). Comparison of each condition with DMSO, one-way ANOVA with Dunnett's *post-hoc* test: $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$.

CBS deregulation. Here our data obtained in simple cellular models suggest that DYRK1A positively regulates CBS activity and that this relationship is conserved between yeast and human. This observation is consistent with previous findings obtained in mouse. For example, the triplication of both *DYRK1A* and *CBS* results in additive effects on hyperactivity and locomotion compared to mice having a triplication of either gene (Maréchal et al., 2019). Similarly, Delabar et al. (2014) have described increased CBS activity in the liver of *Dyrk1A*-overexpressing mice. This group also showed that forced expression of *Dyrk1A* (using an adenoviral construct) in the liver of *CBS*+/− mice induced increased CBS activity both in the liver (Tlili et al., 2013; Latour et al., 2015) and the brain (Baloula et al., 2018). The pathways involved in this

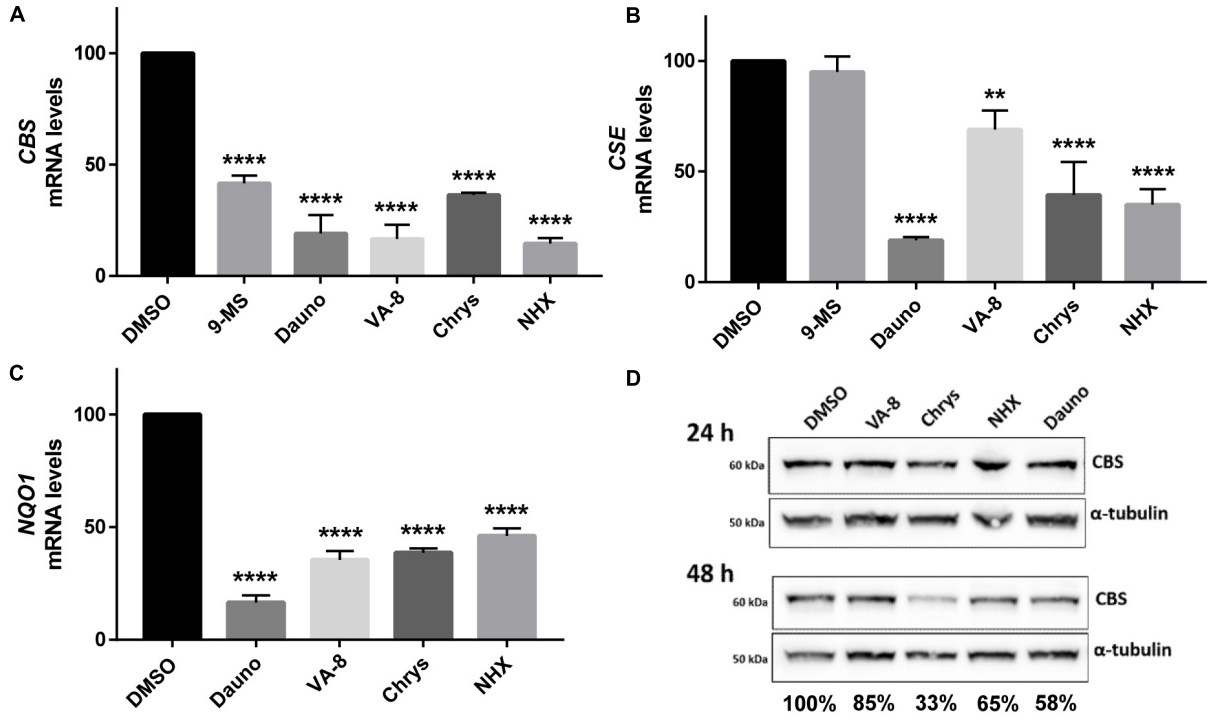

FIGURE 4
Effect of molecules identified in the pharmacological screening on *Cystathionine beta synthase (CBS)*, *cystathionine γ lyase (CSE)* and *NQO1* mRNA and *CBS* protein levels. **(A–C)** The level of *CBS* **(A)**, *CSE* **(B)**, and *NQO1* mRNAs: three genes involved in the defense against oxidative stress and which are regulated by the NF-κB and the Akt/GSK3β pathways, was assessed by RT-qPCR 24 h after treatment with 30 μM of 9-methylstreptimidone, 150 nM of daunorubicin hydrochloride, 10 nM of 8α-hydroxy-verrucarin A (VA-8), 200 nM of chrysomycin (Chrys) and 50 μM of NHX in HepG2 cells. **(D)** Similarly, the level of CBS protein was assessed by western-blot 24 and 48 h after treatment with the same drugs. Ratios of CBS/α-tubulin signals are indicated below each lane for 48 h fater treatment. **(A–C)** Comparison of each condition with DMSO, one-way ANOVA with Dunnett's *post-hoc* test: $^{**}p < 0.01$; $^{****}p < 0.0001$. Note that there is no error bar for DMSO because the different values we obtained were expressed as percentages compared to the DMSO condition which was set up at 100%.

regulation have been explored: decreased homocysteine levels, resulting from *Dyrk1A* overexpression, induced increased phosphorylation of the serine threonine kinase Akt (on Serine 473) (Guedj et al., 2012; Abekhoukh et al., 2013), which is then activated to promote cell survival by inhibiting apoptosis. On the contrary, mice or rats with hyperhomocysteinemia (resulting from a high-methionine diet) have decrease Dyrk1A protein levels both in the liver and the brain (Hamelet et al., 2009; Rabaneda et al., 2016) and consequently decreased phosphorylation of Akt (Liu et al., 2010, 2011; **Figure 5**). Our data, obtained in a simple model organism, thus confirm previous observations suggesting a link between DYRK1A, homocysteine levels and Akt phosphorylation in one hand and between DYRK1A expression and CBS activity on the other hand.

GSK3β negatively regulates CBS activity

The transcription factor NF-E2-related factor 2 (Nrf2) encodes a protein which has a short half-time of only 30 min and which stability is controlled through the regulation of its turnover by the ubiquitin-proteasome system. It controls the expression of over 100 genes, including *CBS* (Hourihan et al., 2013; Liu et al., 2020), *CSE* (Jamaluddin et al., 2022), and *NQO1* (Mutter et al., 2015), three genes that play an important role in cell response to oxidative stress. The homolog system to Nrf2 in yeast is Yap1p, which also confers protection against oxidative stress and regulates the expression of yeast homologs of *CBS* and *CSE* (Orumets et al., 2012). The kinase GSK3β has been shown to prevent the transcription of Nrf2 targets by phosphorylating two serine residues in Nrf2, leading to its nuclear exclusion and degradation (Salazar et al., 2006; Rada et al., 2011). All these data thus point toward a mechanism of regulation of CBS expression at the transcriptional level involving the Nrf2 pathway. This is consistent with our results showing that (i) the expression of Mck1p, the yeast homolog of GSK3β, antagonizes the effect of *CYS4*-OE in yeast (**Figures 2A–B**) and (ii) the expression of a constitutively active form of GSK3β decreased H_2S production in HepG2 cells (**Figure 2G**). Interestingly, both kinase DYRK1A and Akt have convergent effects by directly inactivating GSK3β by phosphorylation at Thr356 (for DYRK1A) (Song et al., 2015)

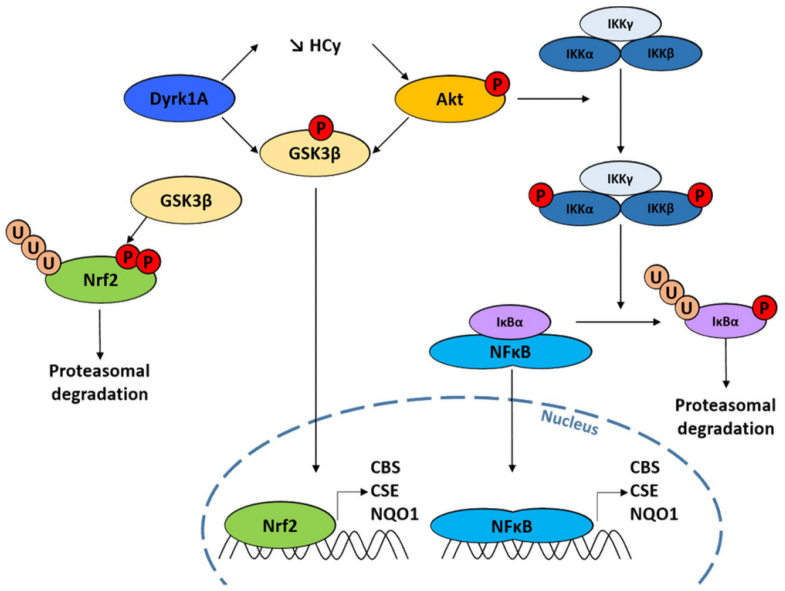

FIGURE 5
Scheme illustrating the genetic connection between Dual specificity tyrosine phosphorylation Regulated Kinase 1A (DYRK1A), GSK3β and Cystathionine beta synthase (CBS) through the Akt and NF-κB pathways.

and on serine 9 (for Akt) (Tlili et al., 2013; Latour et al., 2015; **Figure 5**).

Role of NF-κB and Akt/GSK3β pathways in the regulation of the expression and activity of CBS

The compounds identified in our screening also confirm the importance not only of the Akt/GSK3β pathway but also of NF-κB, which is often activated by Akt/GSK3β (Ozes et al., 1999), in the regulation of CBS activity and expression. NF-κB is a key regulator of genes involved in the response to inflammation and stress. Ordinarily, NF-κB is sequestered in the cytoplasm through a direct interaction with a member of the IκB family of inhibitor proteins such as IκBα. Diverse range of stimuli, including oxidative stress, lead to the activation of the IKK complex (which contains two IκB kinases, IKKα and IKKβ). Phosphorylation of IκBα by the IKK complex triggers its recognition by an E3 ligase complex which leads to its polyubiquitination and subsequent degradation by the proteasome. The liberated NF-κB dimer then translocates to the nucleus, where it binds specific DNA sequences, inducing the transcription of genes such as *CBS* (Li et al., 2012; Zhang et al., 2013), CSE (Wang et al., 2014; Ozaki et al., 2018), and *NQO1* (Dinkova-Kostova and Talalay, 2010). Supporting these observations, we observed that several of the compounds identified in our screen and known to inhibit Akt and/or NF-κB pathways (9-methylstreptimidone, 8α-hydroxy verrucarin A, chrysomycin. . .) induced a significant decrease in *CBS*, *CSE*, and *NQO1* mRNA levels (**Figure 4**). Similar results were obtained for NHX (**Figure 4**), a metal chelator that we previously identified as a candidate inhibitor for CBS (Conan et al., 2022), and which has been reported to reduce both Akt and GSK3β phosphorylation (Xu et al., 2019; Veschi et al., 2020). However, it is important to note that we cannot completely rule out possible additional effects on CBS at the post-transcriptional level. Indeed, several post-translational modifications, such as phosphorylation, glutathionylation or sumoylation, have been reported to play a role in the regulation of CBS enzymatic activity. In addition, CBS protein levels have been reported to be increased following Akt activation, without affecting *CBS* mRNA expression (Zhu et al., 2022), which suggests that these pathways may also target CBS enzymatic activity in addition to its expression. This is also suggested by the fact that H_2S production was decreased 24 h after treatment with 9-methylstreptimidone, 8α-hydroxy verrucarin A, chrysomycin and daunorubicin, whereas the level of CBS protein was not reduced before 48 h of treatment. This suggests that these pathways act at the level of both CBS enzymatic activity and expression level.

Concerning the identification of *MUP1*, *MMP1*, *STP2*, *UBP7*, and *UBP11* genes in the genetic screen, all five suggest a common role in overexpressing amino acid permeases at the plasma membrane, possibly to capture the low traces of methionine present in the medium and due to possible incomplete purification of other amino acids added in the medium. Indeed, Ubp7p and Ubp11p have been shown

to deubiquinylate permeases ubiquitinylated by Rsp5p (the homolog of Nedd4L in yeast), preventing their endocytosis (Tardiff et al., 2013; Weinberg and Drubin, 2014). However, the fact that these genes also mitigate the cytosolic acidification of *CYS4*-OE cells suggest a possible other molecular mechanism. A possible mechanism to explain these results could be that an import of extracellular leucine or methionine would activate the Target Of Rapamycin (TOR) signaling pathway (Takahara et al., 2020; Vellai, 2021), which in turn inhibits the retrograde response, the pathway which regulates the response to oxidative stress and which is the equivalent of the NF-κB pathway in yeast (Johnson and Johnson, 2014).

Role of metal chelation in the regulation of CBS activity

Several compounds identified in our drug screening point toward an important role of metal ion chelation in the regulation of CBS activity, as previously reported (Maréchal et al., 2019; Conan et al., 2022). Indeed, we previously showed that decreasing intracellular copper levels decreased the effects of *CBS* overexpression in yeast and H_2S production in HepG2 cells (Conan et al., 2022). Here, the identification of several compounds involved in metal chelation [4-(2-Thiazolylazo)-resorcinol, 1-(2-Thiazolylazo)-2-naphtol, γ-thujaplicin, doxorubicin and daunorubicin hydrochloride, naphtoquinones. . .] confirm the importance of this process to decrease CBS activity. Several studies have shown that exposure of HepG2 cells or neuronal-like SH-SY5Y cells to copper (and to a lesser extent Zinc) induce phosphorylation of Akt and GSK3β (Ostrakhovitch et al., 2002; Walter et al., 2006; Barthel et al., 2007; Hickey et al., 2011) and that copper chelation reduces the levels of activated Akt and thus of inactivated GSK3β (Guo et al., 2021). Accordingly, we previously reported that several copper chelators such as D-penicillamine, trientine and several members of the 8-hydroxyquinoline family [clioquinol, chloroxine, NHX, and PBT2 (2-(dimethylamino) methyl-5,7-dichloro-8-hydroxyquinoline)] efficiently decreased CBS activity in several cell lines (Conan et al., 2022). Although further investigation is needed, the data obtained here with NHX (**Figures 4A, D**) suggest that the mechanism of action of this compound involves, at least in part, decreased CBS activity through the NF-κB and Akt/GSK3β pathways.

Disulfiram (DSF) is another compound that we previously identified in a similar drug screening (Maréchal et al., 2019). Its mechanism of action has not been fully deciphered. It has been suggested that DSF could inhibit CBS by its ionophore activity, increasing intracellular copper levels, as Cu-DSF was found more active than DSF on its own (Zuhra et al., 2020; **Supplementary Figures 2A, B**), which seemed in disagreement with our finding that increasing copper levels increased CBS activity (Conan et al., 2022). The relationship we here describe between the Akt and NF-κB pathways and CBS regulation could bring an explanation to this apparent discrepancy. Indeed, several groups have shown in various cellular models that DSF, on its own or combined to copper, decreases Akt phosphorylation (Kim J. Y. et al., 2016; Park et al., 2018; Nasrollahzadeh et al., 2021; Zha et al., 2021) as well as the NF-κB pathway (Wang et al., 2003; Guo et al., 2010; Yip et al., 2011). These data are in agreement with the observation that DSF on its own decreases H_2S production, although with a limited effect, but has an increased effect (as well as increased toxicity) when combined to copper (**Supplementary Figures 2A, B**). Accordingly, we observed decreased *CBS* (as well as *CSE* and *NQO1*) mRNA levels in HepG2 cells treated with DSF-Cu (**Supplementary Figure 2D**). On the contrary, incubation with Bathocuproine disulphonate (BCS), a copper chelator totally abolished the effect of DSF (**Supplementary Figure 2C**).

Extrapolation of these findings to the context of Down syndrome

Our results, supported by others in mouse models (Tlili et al., 2013; Delabar et al., 2014; Latour et al., 2015; Baloula et al., 2018; Maréchal et al., 2019), suggest that the overexpression of *DYRK1A* and *CBS* have additive effects and that CBS expression and/or activity is increased following DYRK1A overexpression. On the contrary, Dyrk1A inhibition results in decreased CBS expression and/or activity as shown by H_2S production in HepG2 cells treated with EGCG. This suggests that therapeutic research focusing on DYRK1A inhibition should, at least in part, also take care of the problem of CBS triplication, at the condition that these inhibitors are specific of DYRK1A and do not inhibit GSK3β as well, as our data suggest that GSK3β activation is needed to reduce Nrf2 activity and thus CBS expression.

Several studies have shown that the expression and/or activity of CBS is tightly regulated and strongly depends on the redox state of the cell (Mosharov et al., 2000; Banerjee and Zou, 2005), meaning that the triplication of this gene would not necessarily mean an overexpression. However, CBS overexpression has been extensively reported in patients with DS (Kamoun et al., 2003; Ichinohe et al., 2005; Panagaki et al., 2019). Accordingly, increased oxidative stress, confirmed in several studies in these patients, as well as a hyperactivation of the PI3K/Akt/GSK3β pathway have been reported in the brain of DS patients (Perluigi et al., 2014). All these data suggest that any molecule decreasing the level of oxidative stress, inhibiting NF-κB pathway and/or activating GSK3β activity may result in decreasing CBS expression. Although several preclinical studies and clinical trials aimed at reducing oxidative stress using various anti-oxidant molecules have been performed (Rueda Revilla and Martínez-Cué, 2020), none of them has looked at

the level of CBS expression and/or activity. This would be worth further investigating.

However, the regulation of CBS expression and/or activity also depends on other genes of chromosome 21. We showed here that *DYRK1A* overexpression increases CBS activity. Several other genes present on chromosome 21, including *SOD1* and *APP* are directly or indirectly involved in mitochondrial function, contributing to oxidative stress (Izzo et al., 2018) and may thus on their own or in combination with others have an impact on CBS expression and/or activity. On the contrary, *RCAN1*, also present on chromosome 21, encodes an inhibitor of the NF-κB pathway and its overexpression may then be expected to decrease CBS activity and/or expression.

Taken together, our data provide further insights into the regulation of CBS activity and into the relationship with other genes important for brain development and functioning such as *DYRK1A* and GSK3β. Although further studies are still needed to fully understand the different contributions of these molecular actors into the pathophysiology of DS, the hope is that they will lead to a better understanding of the molecular mechanisms underlying the pathology of DS, and thus to the development of more effective therapy that will bring amelioration or prevention of cognitive deficits in people with DS.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

PC, AL, FB, CV, and GF: conceptualization. PC, AL, NC, FB, OM, CV, and GF: formal analysis. GF: funding acquisition and project administration. PC, AL, NC, CR, LC, and JM: investigation. PC, AL, FB, OM, CV, and GF: methodology. OM, CV, and GF: resources. OM, FB, CV, and GF: supervision. PC, AL, NC, CR, LC, CV, and GF: validation and visualization. PC, AL, and GF: writing—original draft preparation. PC, CV, and GF: writing—review and editing. All authors commented on the different versions of the manuscript, and read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.1110163/full#supplementary-material

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Prader–Willi syndrome: Symptoms and topiramate response in light of genetics

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Introduction: Prader–Willi Syndrome (PWS) is a rare genetic condition, which affects one in 25,000 births and results in various phenotypes. It leads to a wide range of metabolic and endocrine disorders including growth delay, hypogonadism, narcolepsy, lack of satiety and compulsive eating, associated with mild to moderate cognitive impairment. Prognosis is especially determined by the complications of obesity (diabetes, cardiorespiratory diseases) and by severe behavioral disorders marked by impulsivity and compulsion. This heterogeneous clinical picture may lead to mis- or delayed diagnosis of comorbidities. Moreover, when diagnosis is made, treatment remains limited, with high interindividual differences in drug response. This may be due to the underlying genetic variability of the syndrome, which can involve several different genetic mutations, notably deletion or uniparental disomy (UPD) in a region of chromosome 15. Here, we propose to determine whether subjects with PWS differ for clinical phenotype and treatment response depending on the underlying genetic anomaly.

Methods: We retrospectively included all 24 PWS patients who were referred to the Reference Center for Rare Psychiatric Disorders (GHU Paris Psychiatrie and Neurosciences) between November 2018 and July 2022, with either deletion (N = 8) or disomy (N = 16). The following socio-demographic and clinical characteristics were recorded: age, sex, psychiatric and non-psychiatric symptoms, the type of genetic defect, medication and treatment response to topiramate, which was evaluated in terms of eating compulsions and impulsive behaviors. We compared topiramate treatment doses and responses between PWS with deletion and those with disomy. Non-parametric tests were used with random permutations for p-value and bootstrap 95% confidence interval computations.

Results: First, we found that disomy was associated with a more severe clinical phenotype than deletion. Second, we observed that topiramate was less effective and less tolerated in disomy, compared to deletion.

Discussion: These results suggest that a pharmacogenomic-based approach may be relevant for the treatment of compulsions in PWS, thus highlighting the importance of personalized medicine for such complex heterogeneous disorders.

KEYWORDS

Prader–Willi, topiramate, treatment, genetics, deletion, disomy, personalized medicine

Introduction

Prader–Willi syndrome (PWS) is a rare genetic disease, whose prevalence is estimated at 1/15,000−30,000 worldwide. Typical features may involve a narrow forehead, almond-shaped eyes, thin upper lip and drooping corners of the mouth, as well as very small feet and hands (Cassidy et al., 2012). Their developmental trajectory is marked by severe hypotonia and feeding deficits starting in the neonatal period, and followed by a period of hyperphagia and food obsession, which often leads to severe obesity from childhood to adulthood (Gunay-Aygun et al., 2001; Poitou et al., 2023). It is further complicated by a wide range of endocrine dysfunctions (Tauber and Diene, 2021). Growth hormone deficiency contributes to statural growth delay, an excess of fat mass, and an insufficiency of lean mass, resulting in a decrease in energy expenditure. Hypogonadism may lead to incomplete pubertal development (Noordam et al., 2021). Hypothalamic disturbances may cause aberrant temperature control, while orexin deficits may lead to phenotypes ranging from daytime sleepiness to narcoleptic phenotypes (Colmers and Wevrick, 2013; Kim and Choi, 2013; Beauloye et al., 2015; Grugni et al., 2016; Correa-da-Silva et al., 2021). Insatiable hunger and hyperphagia may be caused by a reduced number of oxytocin neurons in the hypothalamic paraventricular nucleus (Tauber and Hoybye, 2021). Conversely, ghrelin, a hormone secreted by the stomach to stimulate appetite, may be overexpressed (Tauber and Hoybye, 2021). In the absence of a strict diet, weight gain can be very rapid and accounts for much of the morbidity and mortality of these patients (Kim and Choi, 2013; Saunders et al., 2018). Moreover, this food addiction behavior is the main obstacle to autonomy and socialization in patients with PWS, because of clastic crises in connection with dietary frustrations (Salles et al., 2021).

From a neurodevelopmental perspective, learning difficulties, social skills deficits, and severe behavioral problems are important determinants of the functional outcome (Sinnema et al., 2011; Butler et al., 2019). Intellectual deficit is rarely major and is extremely variable from one individual to another. People with PWS may have a generalized anxiety disorder of the obsessive-compulsive type (OCD), including dermatillomania–where scratching lesions can lead to *Staphylococcal aureus* infection, or manual extraction of feces, representing a risk of infectious and gastroenterological complications (Dykens and Shah, 2003; Sinnema et al., 2011; Shriki-Tal et al., 2017; Whittington and Holland, 2018; Guinovart et al., 2019; Tarsimi et al., 2021). The prevalence of psychotic episodes is also increased. Patients may present dysthymic disorders such as a depressive episode, particularly when they are aware of their pathology and its genetic aspect. There are rare reports of bipolar disorder associated with PWS, but this may also be related to the behavioral and hormonal dysfunction of PWS (Bellman et al., 2021). Clinical diagnosis is often challenging for psychiatrists, with overlaps between comorbidities: anxiety disorder, mood disorder, psychotic disorder, personality disorder, autism spectrum disorder, eating disorder, or OCD.

To date, there is no consensus on drug treatment for PWS. However, several studies suggested that topiramate may lead to significant clinical improvement, particularly in cases of compulsive overeating, dermatillomania, and frustration intolerance (Shapira et al., 2002; Smathers et al., 2003; East and Maroney, 2018). Topiramate is an antiepileptic drug classically used to treat generalized and partial epilepsy, migraine headaches, and bipolar disorder, because of its mood stabilizing effect. In PWS, dosages are variable (between 50 and 500 mg/day) and depend mainly on its efficacy and tolerability. The only randomized control trial to date found a significant hyporexigenic action (Consoli et al., 2019). Physiologically, it would seem that topiramate regulates the satiety loop and compulsive behaviors, explaining its effect on appetite and binge eating (Smith et al., 2016; Saunders et al., 2018; Khalil et al., 2019).

Overall, there is great variability both in tolerance and efficacy of topiramate treatment, several studies showing beneficial effects of this treatment, while others warn on side effects (Smith et al., 2016; Consoli et al., 2019; Khalil et al., 2019; Steinhoff et al., 2021). This heterogeneous clinical and treatment response profile may stem from an underlying genetic heterogeneity (Shapira et al., 2002, 2004; Smathers et al., 2003; East and Maroney, 2018; Consoli et al., 2019). Thus, in 60% of cases, there is a 15q11-q13 deletion on the paternal chromosome, while in 40% of cases a maternal uniparental disomy is found, whereby the child has inherited two maternal chromosomes 15. Rarely, imprinting anomalies or translocations are found. Therefore, in this study, we proposed to explore whether the type of genetic anomaly could explain differences in clinical presentation and response to treatment, focusing on topiramate. In a retrospective approach, we tested the difference between the two most frequent anomalies, deletion and disomy, in terms of symptomatology, efficacy, and tolerance of topiramate.

Materials and methods

Population

In this monocentric retrospective descriptive observational study, we included all patients having a diagnosis of PWS with genetic confirmation of either disomy or deletion, with no age limit, seen between November 2018 and July 2022 at the reference center for rare diseases with psychiatric expression ("Centre de Références Maladies Rares," CRMR, GHU Paris Psychiatry and Neurosciences). Most patients were referred to the adult CRMR, from the French reference

center for PWS and children CRMR of Pitié-Salpêtrière hospital, as part of a transition from pediatric to adult care.

Clinical assessment

In a retrospective reading of medical records, the presence of the following clinical information was recorded: age, sex, body mass index (BMI), aggressiveness, anxiety, psychosis (defined by the presence of hallucinations or delusions), depression (according to DSM-5 criteria for major depressive disorder), dermatillomania (according to DSM-5 criteria for excoriating skin disorder). Treatment characteristics were also noted: the use of antidepressant, antipsychotic, or antiepileptic medication, as well as topiramate use, its dosage, and its tolerance and efficacy. The treatment was considered effective when the patient and the family described a stable clinical condition with improvement of the disabling symptoms, impulse control, or weight loss. Tolerability was assessed by the presence or absence of side-effects (hyperammonemia, confusion, increased aggressiveness, sudden weight loss).

Statistical analysis

Quantitative variables (age, BMI, topiramate dosage) were compared between subjects with deletion and those with disomy with a non-parametric Mann–Whitney–Wilcoxon U test. Categorical variables (presence or absence of symptoms, topiramate tolerance and efficacy) were compared between subjects with deletion and those with disomy with a Chi2-test for proportions. In light of the small number of subjects, we used random permutations to compute a non-parametric *p*-value for each comparison. A conservative significance threshold was set at 0.004 after Bonferroni correction for 12 comparisons, but results below or equal to a nominal significance of 0.05 were also considered in this exploratory study. Lastly, we used a bootstrap simulation to estimate how the difference in variable proportions may vary, providing a non-parametric 95% confidence interval for each distribution. A T-test was used to compare the bootstrapped distributions. Analysis was carried out on Python with SciPy.

Results

Clinical and therapeutic characteristics of the population are presented in Table 1. There were no differences in age, sex, and BMI between deletion and disomy. In this psychiatric setting, the initial symptoms at first consultation were behavioral disorders with auto- and hetero-aggressiveness.

All patients presented with aggressiveness and anxiety. Characteristic depressive episodes of moderate to severe intensity, associated or not with suicidal thoughts, were found significatively higher in subjects with a disomy (100%, 16/16) than in subjects with a deletion (37.5%, 3/8), with a *p*-value of 0.002. Among subjects with disomy, 43.8% (7/16) presented persecutory statements, of intuitive or interpretative mechanism, with partial or total adhesion,

TABLE 1 Clinical and treatment response characteristics.

	Mutation		Statistics	
	Deletion	Disomy	U/Chi2	*P*-value
Population description				
Number of subjects	$N = 8$	$N = 16$	–	–
Age (years)	27 ± 6	27 ± 9	29.5	0.56
Sex (F/M)	3/5	5/11	0.00	1.00
BMI (kg/m^2)	35 ± 12	41 ± 7	16.5	0.66
Psychiatric symptomatology				
Aggressiveness	100% (8/8)	100% (16/16)	–	–
Anxiety	100% (8/8)	100% (16/16)	–	–
Psychosis	0% (0/8)	43.75% (7/16)	3.10	0.05
Depression	37.5% (3/8)	100% (16/16)	9.10	0.002
Dermatillomania	37.5% (3/8)	56.25% (9/16)	0.20	0.67
General medication				
Antidepressant use	75% (6/8)	62.5% (10/16)	0.02	0.66
Antipsychotic use	62.5% (5/8)	62.5% (10/16)	0.00	1.00
Antiepileptic use	75% (6/8)	75% (12/16)	0.00	1.00
Topiramate treatment				
Topiramate use	100% (8/8)	56.25% (9/16)	3.05	0.06
Dose (mg/kg)	1.25 ± 0.73	0.97 ± 0.42	27.0	0.83
Tolerance	100% (8/8)	55.55% (5/9)	2.51	0.08
Efficacy	100% (8/8)	33.33% (3/9)	5.58	0.01

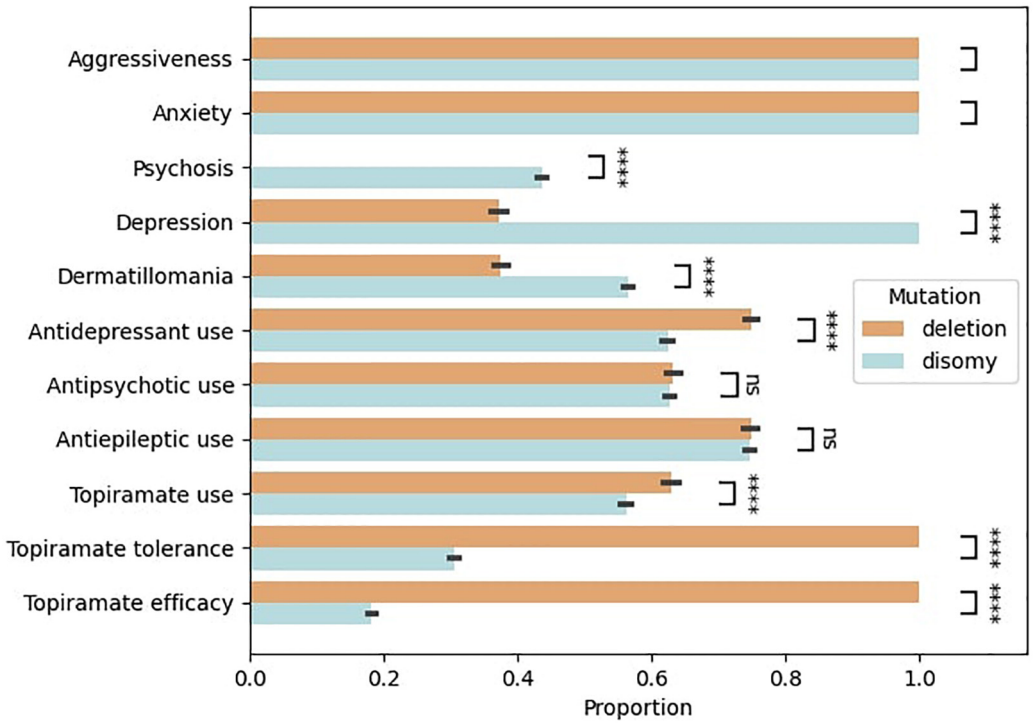

FIGURE 1
Clinical profile of patients with Prader–Willi syndrome depending on the genetic mutation (deletion versus disomy). Bootstrap simulation based on the 24 patients of the cohort. A *T*-test was used to compare the bootstrapped distributions. **** Simulated p-value < 0.0001.

while none of the patients with a deletion had psychotic symptoms ($p = 0.05$). All subjects had eating disorders of the bulimic type with a compulsive mechanism. Among compulsive behaviors, dermatillomania, mainly on the forearms, thorax and neck, with infected scratch marks in some cases, was found in 37.5% (3/8) of patients with deletion and in 56.3% (9/16) of patients with disomy ($p = 0.67$). Two subjects with disomy reported compulsions to remove feces resulting in lesions of the anal margin.

There was no difference between the two groups regarding antidepressant, antipsychotic, or antiepileptic use. In cases when topiramate was introduced, it was with an initial dosage of 25 mg/day, increased by 25 mg/day every 7 days, after a weekly clinical assessment for efficacy and tolerance. The maximum dosage used was 200 mg/day, with no difference in dosage per kg between groups. There was a tendency to a smaller proportion of subjects receiving topiramate in the disomy group ($p = 0.06$), as well as a tendency of lower efficacy ($p = 0.01$), and tolerance ($p = 0.08$) in this group compared to the deletion group. The main side effects of treatment were hyperammonemia, clinically observed as confusion and temporo-spatial disorientation with increased behavioral disturbances, and biologically confirmed by plasma assay. This poor clinical tolerance of topiramate led to its discontinuation. Thus, none of the patients with disomy were completely stabilized clinically, with persistent behavioral disorders such as intolerance to frustration and aggressiveness (albeit less than at first assessment).

The bootstrap simulation of the variation in proportion of symptoms and treatment response between groups is shown in Figure 1. It suggests that patients with disomy tend to have more severe psychiatric symptomatology in terms of psychosis, depression, and dermatillomania (simulated p-value < 0.0001). Likewise, patients with disomy tend to receive more antidepressants (simulated p-value < 0.0001). There tends to be more use of topiramate in subjects with deletion than disomy, and less tolerance and efficacy in subjects with disomy than deletion (simulated p-value < 0.0001).

Discussion

In this retrospective analysis of subjects with Prader-Willi syndrome, we studied differences in clinical presentation and treatment response between subjects with 15q11 deletion and those with a uniparental maternal disomy. Our current results suggest a clinical profile dependent on the genetic mutation. Disomy seems to lead to a more pronounced psychiatric symptomatology, with more psychotic and dysthymic disorders than in the deletion group. Disomy may also be associated with less tolerance (hyperammonemia, drowsiness, depressive syndrome) and lower response of impulsive behaviors to topiramate treatment, whereas topiramate seems more effective and better tolerated in subjects with a deletion. This genotype-dependent difference therefore requires a cautious monitoring that may benefit from a more personalized approach. In the disomy group, antidepressant treatments were less prescribed while depression was more frequently reported than in the deletion group. This could be explained by the fact that patients with disomy are more at risk of pharmaco-induced manic episodes (Sinnema et al., 2011). It also explains the general use of antiepileptic treatment (75% of patients), including topiramate,

which provides thymic coverage. It should be noted that among the antipsychotic treatments prescribed in this cohort, aripiprazole represented a treatment of choice. Indeed, a recent study highlighted the benefit of aripiprazole treatment in clastic seizures (Deest et al., 2022). Moreover, it has the added advantage of having less metabolic side effects (weight gain), making it the antipsychotic treatment of choice in this condition (Gupta et al., 2021; Sobiś et al., 2022).

However, the size of the cohort is small and does not allow definitive conclusions. To increase the size of the cohort, we aim at extending collaborations between CRMRs in a multicentric study. Importantly, it should be noted that all patients were referred to the CRMR because of severe psychiatric symptoms. This referral constitutes a selection bias that may explain the higher proportion of disomy and lower tolerance of treatment. Interestingly, while in the literature, disomy accounts for only 25—30% of the genetic anomalies found in PWS, here it represented 66% of the population. This tentatively supports the idea of a more severe psychiatric expression in this subgroup, in line with previous reports suggesting a higher risk of psychotic disorders in disomies (Aman et al., 2018; Butler et al., 2019). Of note, we excluded one patient with an imprinting mutation from the analysis. Clinically, his profile was more similar to patients with disomy. The patient had suicidal thoughts and psychotic symptoms. He was treated with an antiepileptic and an antidepressant.

This specific recruitment of patients with severe behavioral difficulties may also explain the observed intolerance to topiramate in a number of subjects, which contrasts with the recent randomized trial of topiramate, reporting a good overall tolerance (Consoli et al., 2019). Another limit of our analysis is its retrospective design that did not allow us to report specific levels of hyperammonemia in relation to topiramate dosage. The results presented here are therefore preliminary and invite further study. The prospective collection of new data will allow the study to gain in power.

From a cognitive perspective, there is no clear difference between disomy and deletion. No difference in overall intelligence quotient (IQ) was reported between these groups. Performance IQ was higher in those with a deletion, while verbal IQ was higher in those with a disomy, who also were reported to have poorer visual acuity and stereoscopic vision (Postel-Vinay et al., 2006). This lack of difference in cognitive profiles may be due to higher heterogeneity inside each group. In subjects with disomy, cognitive function seems more preserved in the case of uniparental disomy, heterodisomy being more favorable than isodisomy (Zhang et al., 2022). In subjects with deletion, phenotype varies according to the length of the deletion. Of the two main deletion types, the long and the short forms, depending on the break points on chromosome 15, the longer form is accompanied by a more marked clinical picture (Butler et al., 2004; Milner et al., 2005; Varela et al., 2005). Unfortunately, due to the retrospective nature of the study, we did not manage to obtain specific deletion types with known break points for each patient. This heterogeneity at the level of the genetic anomaly calls for more specific genotype-based phenotyping (hetero- versus iso-disomy, and long versus short form of deletion).

In conclusion, Prader–Willi syndrome is highly heterogeneous both at clinical and genetic levels and may benefit from a genetically-based phenotyping to identify specific profiles. Should our results be replicated in a larger cohort, it would suggest that the type of mutation (disomy or deletion) could be a genetic marker of response to topiramate treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CL, BC, and AI contributed to study design. CL and M-CT contributed to data gathering. CL and AI contributed to data analysis. CL, M-CT, AC, CP, MO-K, BC, and AI contributed to data interpretation and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: Prader-Willi syndrome: Symptoms and topiramate response in light of genetics

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Prader–Willi, topiramate, treatment, genetics, deletion, disomy, personalized medicine

A corrigendum on
Prader-Willi syndrome: Symptoms and topiramate response in light of genetics

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In the published article, an author name was incorrectly written as Turtuluci. The correct spelling is Turtulici.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Down syndrome regression disorder, a case series: Clinical characterization and therapeutic approaches

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Down syndrome (DS) is one of the most frequent genetic disorders and represents the first cause of intellectual disability of genetic origin. While the majority of patients with DS follow a harmonious evolution, an unusual neurodevelopmental regression may occur, distinct from that described in the context of autism spectrum disorders, called down syndrome regression disorder (DSRD). Based on four patients, two males and two females, with age range between 20 and 24, treated at the Reference Center for Rare Psychiatric Disorders of the GHU Paris Psychiatry and Neurosciences [Pôle hospitalo-universitaire d'Évaluation Prévention et Innovation Thérapeutique (PEPIT)], we describe this syndrome, discuss its etiologies and propose therapeutic strategies. DSRD often occurs in late adolescence. There is a sudden onset of language disorders, loss of autonomy and daily living skills, as well as behavioral symptoms such as depression, psychosis, or catatonia. These symptoms are non-specific and lead to an overlap with other diagnostic categories, thus complicating diagnosis. The etiologies of the syndrome are not clearly identified but certain predispositions of patients with trisomy 21 have suggested an underlying immune-mediated mechanism. Symptomatic therapeutic approaches (serotonergic antidepressants, atypical antipsychotics, benzodiazepines) were not effective, and generally associated with poor tolerance. Etiological treatments, including anti-inflammatory drugs and corticosteroids, led to partial or good recovery in the four cases. Early recognition of regressive symptoms and rapid implementation of adapted treatments are required to improve the quality of life of patients and their families.

KEYWORDS

down syndrome, trisomy 21, catatonia, inflammation, corticosteroids, personalized medicine

Introduction

Down syndrome (DS), has an incidence of 1 per 800 births worldwide (Bull, 2020). In 1959, Lejeune, Gauthier, and Turpin described the association between DS and a third copy of chromosome 21, reporting for the first time in a disease a link between genotype and phenotype (Lejeune et al., 1959). Management of patients with DS significantly improved, with a constant increase in life expectancy, gaining more than 25 years (Antonarakis et al., 2020). Classical morphological features include facial dysmorphia, flattened occiput, microcephaly, single palmar crease, clinodactyly of the 5th finger, ligament and skin hyperlaxity, and overall muscle hypotonia. From a neurodevelopmental perspective, DST21 represents the leading cause of intellectual disability of genetic origin, and is often associated with autism spectrum and attention-deficit hyperactivity disorders, with high interindividual phenotypic variability and frequent co-occurrence of other psychiatric disorders (psychotic and mood disorders) (Antonarakis et al., 2020). Medical complications include cardio-pulmonary, hearing, oncologic, musculoskeletal, and notably, autoimmune disorders (Bull, 2020). In particular, thyroid-related disorders are estimated at 24% in DS children and 50% in adults (Pierce et al., 2017).

Although a majority of patients with DS follow a harmonious evolution, an adolescent behavioral regression may occur, making it distinct from those described earlier on in autism spectrum disorders. It occurs around 20 years old (18 years for girls and 21 years for boys), compared with around 5 years for DS patients with ASD and 20 months for autistic patients without DS (Worley et al., 2015). This entity, called down syndrome regression disorder (DSRD), is increasingly reported in the medical literature. There is a female predominance in DSRD, 64% being female as compared to the male predominance in ASD associated or not with DS (70–80%) (Castillo et al., 2008; Mircher et al., 2017). Regression occurs regardless of the level of intellectual disability and is not correlated with patients' cognitive performance (Mircher et al., 2017). It is characterized by the acute onset, in adolescence, of a loss of previously acquired social and communicative skills, a loss of autonomy in activities of daily living (dressing, toileting, eating, continence), and disruption of executive functions (memory, attention, processing speed) and motor skills (appearance of abnormal movements, stereotypies, perseveration, extrapyramidal symptoms, catatonic syndrome) (Rosso et al., 2020).

While the exact prevalence of DSRD is unknown, more than 40 cases have been described in the literature (Miles et al., 2019; Santoro et al., 2020), leading to a better recognition of this condition that may not be especially rare. A recent international expert consensus established a nomenclature, clinical diagnostic criteria, and first-line complementary tests (Santoro et al., 2022b). DSRD is now used instead of Down Syndrome Disintegrative Disorder (DSDD) and Unexplained Regression in Down Syndrome (URDS). Diagnostic criteria were classified in 8 dimensions, of which three make the diagnosis possible, and six probable. They include: (1) an altered mental status or behavioral dysregulation, (2) a cognitive decline, (3) a developmental regression with or without new autistic features, (4) new focal neurologic deficits on examination or seizures, (5) insomnia or circadian rhythm disruption, (6) language deficits, (7) movement disorders (excluding tics), and (8) psychiatric symptoms.

Symptom onset needs to be sudden, over a period of less than 12 weeks in a previously healthy individual with DS, in the absence of alternative causes (Santoro et al., 2022b). Some symptoms are more frequently observed than others.

(i) Approximately 90% of patients with DSRD showed language regression, with symptoms ranging from dysfluency (38%) to mutism (52%) (Mircher et al., 2017). A change in voice (sometimes barely audible whispering) and a slowing of verbal fluency are often observed.

(ii) Psychiatric syndromes such as mood disorders are reported in 42% of cases (apathy, abulia, sadness of mood), followed by social withdrawal (34%) and anxiety disorders (16%). In addition, 14% of psychotic symptoms, including delusions, hallucinations, soliloquies, and unmotivated laughter are found in patients with DSRD. Behavioral disorders are frequent, with aggressive behavior occurring in 42% of patients (Mircher et al., 2017).

(iii) A marked psychomotor slowing down, sometimes as part of a catatonic syndrome, is often described. The clinical picture frequently includes sudden-onset abnormal movements such as stereotypies, tics and grimaces, or daytime bruxism. In the literature, a catatonic presentation is predominant in these patients at the time of diagnosis of DSRD. The catatonic syndrome, as a mode of revelation of DSRD, may concern 37% of patients (Mircher et al., 2017).

(iv) Sleep disorders are frequent, including inversions of the nycthemeral rhythm and insomnia. Sudden onset insomnia has been described in about 40% of cases of DSRD (Worley et al., 2015). This inaugural sleep disorder appears to be a characteristic feature of DSRD, often reported by parents. Finally, anorexia nervosa is a comorbidity affecting about 12% of patients with DSRD (Rosso et al., 2020).

Regarding the natural course of DSRD, an acute phase of six months is followed by a chronic phase of variable duration, after which previously acquired skills may not be fully recovered. Thus, 58% of people with DSRD experienced partial or total recovery, 7% of patients experienced further deterioration, while 35% of patients stabilized. However, in patients with stabilized DSRD, full recovery to the premorbid baseline appears to be uncommon (Rosso et al., 2020).

Based on these consensual diagnostic criteria, we reported here a series of four clinical situations of probable DSRD, and we described the therapeutic measures proposed. While the previously reported cases underwent invasive therapy, including electroconvulsive therapy or intravenous immunoglobulin, we propose to use less aggressive drugs, including corticosteroid or non-steroid anti-inflammatory drugs, with satisfying outcomes.

Methods

Between December 2021 and December 2022, we included four patients with DS who presented criteria for DSRD and were seen at the reference center for rare diseases with psychiatric expression (CRMR, PEPIT department, GHU Paris Psychiatry and Neurosciences). Diagnosis of DSRD was done by two expert psychiatrists (PE and BC) according to the Expert Consensus

(Santoro et al., 2022b) and after reasonable exclusion of alternative causes of regression, including primary psychiatric disorders. All patients were referred from the Jérôme Lejeune Institute in Paris, where patients were regularly followed. The Jérôme Lejeune institute is an expert center specialized in healthcare and research for individuals with DS. All patients and their families gave their consent for publication of this case series. Clinical presentations, the main workup, and the therapeutic challenges of the four cases are summarized in the **Table 1** below.

Case series

Case 1

A 20-year-old woman with DS was referred to our tertiary center for a sudden onset of visual and auditory hallucinations. Her previous medical history was unremarkable. She had a yearly medical check-up for her DS. She lived with her mother and had an adapted schooling. At age 20, in less than 24 h; she presented severe behavioral symptoms: auditory hallucinations with soliloquy, fits of anger, unmotivated laughter, a fixed gaze, and insomnia. There was a psychomotor regression, with loss of graphic abilities, perseverations (doubling of letters at the end of words), tics, repetitive and abnormal eye and mouth movements, as well as partial mutism. During interviews, the patient's speech was poor and undetailed, but she explained seeing dragons and having pain in her ears. Her mother also reported a loss of appetite with weight loss, a loss in sphincter control, a dramatic decrease in autonomy in terms of daily living skills (dressing) and praxis difficulties, particularly losing the ability to use a knife. In this context, the family consulted the emergencies several times, which referred them to the outpatient psychiatric consultation associated with their place of residence. A treatment with risperidone 2 mg/day was introduced. Despite an improvement in psychotic symptoms, it had to be reduced to risperidone 1 mg/day with the addition of tropatepine 5 mg, due to a worsening of the psychomotor regression and occurrence of lingual dyskinesias. It allowed a quick symptomatic improvement, and treatment cessation was proposed three months later. However, the patient soon after presented with an episode of diarrhea and vomiting with prostration, followed by the reappearance of behavioral problems. An electroencephalogram (EEG) showed a well-spatialized background pattern, with a few acute right temporo-occipital slow irregularities possibly related to artifacts and no epileptic abnormalities. The brain magnetic resonance imaging (MRI) was also normal. Upon referral to our center, the diagnostic hypothesis of DSRD related to Pediatric Acute-onset Neuropsychiatric Syndrome (PANS) was considered. A biological workup was ordered including: blood count, serum protein electrophoresis, thyroid hormonology, thyroid ANA and anti-soluble antibody assays. Only hypergammaglobulinemia was found at 16 g/L. In this context and in the hypothesis of a PANS, a treatment with a non-steroidal anti-inflammatory drug was started - naproxen 275 mg twice a day (10 mg/kg). This treatment improved the language problems and the psychomotor slowing down after 1 week, but the initial cognitive abilities have not yet been completely recovered. Naproxen cessation was followed each time by a relapse, leading to prolonged use. A total of 1 year after starting naproxen, the patient remains stable.

Case 2

A 24-year-old woman with DS was referred to our center for anxiety attacks, incoherent speech, swallowing problems without hyporexia or general alteration. There were also attention problems, a loss of enthusiasm, and a marked psychomotor slowdown. While she was autonomous in daily life, she became dependent on assistance for washing and dressing. Episodes of encopresis occurred. Her previous difficulties in speech and language increased, making it difficult for her to communicate verbally, and isolating her from conversations with friends. She was very affected by this and presented ideas of persecution related to her social exclusion. Her medical history included Graves' disease discovered at 17, which was treated with Neo-Mercazole without thyroidectomy; the resulting mild hypothyroidism was treated with levothyroxine. She also suffered from astigmatism, myopia, and a keratoconus surgically treated at 18, as well as hyperuricemia. The psychiatric symptomatology suddenly appeared at age 22 without specific trigger event. From age 23, soliloquies and verbigerations appeared, in particular repetitions of sentences in a whispered voice referring to past events (e.g., an argument between neighbors, a potential previous aggression). The family described staring, a social withdrawal, an additional loss of autonomy, as well as sleep disorders. The already present throat clearing worsened and obsessive-compulsive disorders (OCDs) (hand and face washing rituals and drinking rituals) increased. Motor tics (ocular, digital) and verbal tics evocative of Gilles de la Tourette's syndrome appeared as well as cries, first occasionally, and then progressively every day and at night. At 24, following a consultation with a psychiatrist, treatment with valproic acid was introduced but had no effect on the symptoms. The MRI and EEG were normal. Upon referral to our center, the biological workup revealed highly positive anti- thyroperoxidase (TPO) (>13,000 U/mL), anti-thyroglobulin and anti-thyroid receptor antibodies, leading to a suspicion of steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT). She was hospitalized in internal medicine for intravenous corticosteroid boluses over 3 days, followed by an oral corticosteroid therapy at 60 mg/day for 1 month before tapering. Psychomotor function improved with a clear recovery in autonomy, associated with a decrease in anti TPO levels. However the patient was more agitated leading to the prescription of symptomatics low dose antipsychotics. During our last consultation, the parents described their daughter as more lively, having resumed certain games and routines of interaction with them, and considered her condition to be close to that prior to the regression. The corticosteroid therapy is currently at 30 mg/day and 13-fold the anti-TPO antibody.

Case 3

A 24-year-old man with T21 was referred to our center for anxiety and psychomotor regression that started 4 years ago. His medical history was notable for Hirschsprung's disease, which required digestive surgery 3 days after birth due to an occlusive

TABLE 1 Description of the four cases: age, sex, clinical criteria and score for DSRD according to the expert consensus, paraclinical workup, treatments (+: presence; —: absence).

	Case 1	Case 2	Case 3	Case 4
Age	20	24	24	22
Sex	F	F	M	M
(1) Altered mental status or behavioral dysregulation	+ (disorientation, anorexia, inappropriate laughter)	+ (hyporexia, confusion)	+ (disorientation)	+ (disorientation)
(2) Cognitive decline	+ (apathy)	+ (avolition)	+ (acute memory impairment)	+ (apathy)
(3) Developmental regression with or without new autistic features	+ (loss of previously developmental acquired milestones such as drawing and writing)	+ (inability to perform activities of daily living, social withdrawal)	+ (loss of previously developmental acquired milestones such as enuresis, inability to perform activities of daily living, motor stereotypy)	+ (Loss of previously developmental acquired milestones such writing)
(4) New focal neurologic deficits on examination and/or seizure	–	–	–	–
(5) Insomnia or circadian rhythm disruption	+ (insomnia)	+ (insomnia, wake up during the night)	+ (irregular sleep cycles at polysomnography)	+ (insomnia)
(6) Language deficits	+ (partial mutism)	+ (whispered speech, verbigerations)	+ (mutism)	+ (major stammering)
(7) Movement disorder	+ (slowliness)	–	+(catatonia)	+ (catatonia)
(8) Psychiatric symptoms	+ (hallucinations)	+ (persecutive delusion)	+ (anxiety and depression)	+ (anxiety)
Total score	7 (probable DSRD)	6 (probable DSRD)	7 (probable DSRD)	6 (probable DSRD)
Duration before adequate treatment	9 months	22 months	4 years	6 years
Rapidity of onset	<24 h	2 weeks	4 weeks	4 weeks
MRI	Normal	Normal	Normal	Normal
EEG	Normal	Normal	Normal	Normal
Biological workup for inflammation/auto-immunity	Hypergammaglobulinemia No auto-antibodies (antinuclear, anti-thyroid) Normal level of C-reactive protein	Thyroperoxidase antibodies (>13,000 IU/ml) and anti-TRAK (30.6 UI/ml)	Anti-thyroglobulin antibodies (193 IU/ml)	Anti-thyroglobulin antibodies (9.6 IU/ml)
Anti-inflammatory treatment	Good response to naproxen 275 mg x 2/d	Good response to 3 days of IV corticoids followed by oral corticotherapy (prednisone 1 mg/kg/d) with progressive decrease during 8 months	Partial response to naproxen 550 mg x 2/d	No efficacy of naproxen 27 5 mg x 2/d Partial response to oral corticotherapy (prednisone 1 mg/kg/d)
Other treatments	Risperidone 1 mg/d had adverse reactions (worsening of psychomotor regression and lingual dyskinesias) and was stopped	Low doses of aripiprazole (1–2 mg/d) were efficient on screaming but increased apathy Previous challenge with valproic acid was unsuccessful.	ISRS (fluoxetine, sertraline) were initially efficient but then resistance occurs Lormetazepam 2 mg/d not efficient and not tolerated (sleepiness)	Lorazepam (1–2 mg/d) and ISRS (fluoxetine, sertraline) partially effective. Fluoxetine was associated with HyperStudio

syndrome and regular hospitalizations until the age of seven. He also underwent a tonsillectomy in childhood, had thyroiditis with calcifications and obstructive sleep apnea syndrome. There was a language and motor delay (he walked at 24 months). The patient attended ordinary kindergarten, then followed an adapted schooling, before integrating a care home. Until his twenties, the patient was fairly autonomous, able to take public transport alone, and despite his speech difficulties, had no comprehension difficulties. At 21, without any particular event being reported, he progressively developed a loss of spatial orientation, difficulties in his relationships with his peers, and an important susceptibility. He complained of low mood and presented a social withdrawal, a loss of autonomy in public transport due to panic attacks, and subthreshold persecutory delusions. He therefore required permanent help in his journeys. The parents also reported secondary enuresis. In this context, he was diagnosed with major depressive disorder and underwent psychotherapy sessions associated with fluoxetine which initially improved his mood. At 22 years, his relatives described a further loss of autonomy and apathy, the appearance of memory problems, soliloquies, emotional lability, and motor stereotypies. The introduction of sertraline led to a transient behavioral improvement (3 months) but without a return to baseline. Polysomnography found a very disturbed sleep, with apneas and irregular sleep cycles. Continuous positive airway pressure led to improvement in asthenia and memory problems, but he was still no longer autonomous for daily living activities such as washing and dressing. Upon first consultation at the CRMR, the interview was marked by apathy and akinetic mutism. There was no cerebellar syndrome, motor stereotypies, obvious sadness, or psychotic elements. The parents did not report any loss of appetite. On the other hand, the clinical picture was dominated by catatonic elements with maintenance of attitudes, staring, grimacing, negativism, slight grasping, and some extrapyramidal symptoms (stiffness, loss of arm swing). Brain MRI and EEG were normal. The biological workup, done in search of autoimmune and inflammatory syndromes, found

an elevated anti-thyroglobulin antibody level (193 IU/ml- three times above the norm). In this context, we progressively introduced lorazepam up to 4 mg/day, which allowed transient improvement of slowness and distractibility. However, because lorazepam was not well tolerated, leading to fatigue, it required an adjustment of the dosage to 2 mg/day then 1 mg/day. The patient remained very slow and mute with graphic perseverations. After unsuccessful lorazepam treatment, we introduced NSAIDs (Naproxen 550 mg x2/d). This strategy improved his speech and writing abilities, as well as his motricity, but only when orders were given sequentially. He remained frozen when he had to self-motivate for actions.

Case 4

A 22-year-old man with DS was referred to our center for a behavioral regression that started 6 years ago. He presented a history of congenital heart disease with a normal control ultrasound at age 10, hypothyroidism treated with levothyroxine, keratoconus treated with cross-linking at 17, a cataract treated with surgery, and obstructive sleep apnea syndrome treated with continuous positive airway pressure. His mother and sister both had auto-immune thyroiditis. He followed psychomotricity sessions during childhood and orthoptic reeducation for a convergent strabismus. He pronounced his first words at 1 year, started speech therapy at two and a half years, and had a slight stutter. He walked at 36 months. His parents described a child who was always very calm, with difficulty expressing his emotions and pain. He helped with shopping and household chores, was autonomous for daily life gestures (toilet, dressing), and was well oriented in time and space. The patient continued his schooling with individual support in a regular environment during kindergarten and primary school, then in an adapted secondary school. Since childhood, he was very ritualized, presented with motor stereotypies and difficulties in social interactions, especially to maintain the gaze, which suggested autism spectrum disorder. An OCD of alignment, tidying and washing was also reported. Soliloquies were described, but without delusions or verbigerations. At age 16, those close to him reported a behavioral regression with psychomotor slowing, passivity and apragmatism. He could remain still in his bed for hours. Soliloquies and stereotypies increased, echolalia, grimaces, and stiffness appeared. The stammering became more pronounced and hindered communication. No particular event seemed to have occurred that could explain this clinical picture. At age 19, his writing was disrupted. The patient repeatedly went over his writing in a stereotyped way until it became illegible. He also had throat clearing tics, potomania, and sleep disorders with delayed sleep onset, bruxism, and nightly awakenings to eat or drink. In view of a suspicion of polyuro-polydipsia syndrome, a pituitary MRI was performed but came back normal. No agitation, auto-, hetero- aggressive behavior, or delusions were observed. The patient was cooperative during his interactions with his family but his psychomotor slowing was very pronounced. Sometimes maladaptive reactions occurred, such as undirected screams and vocalizations. We first received the patient when he was 21. Clinical examination revealed poor contact with gaze avoidance. He often stared at the ground, with no spontaneous speech, significant latency in answering questions, throat clearing before speaking, and a major stammering that impeded speech intelligibility. There was a global psychomotor slowdown and catatonic symptoms with a frozen attitude, stiffness of the upper limbs and maintenance of a sitting posture with the head and chest bent forward. The Bush-Francis scale score was at 23/69. Brain MRI, EEG, and standard biological workup were normal. Treatment with lorazepam 1 mg led to slight improvement of language and writing. Given the combination of autistic, anxious, and catatonic symptoms, treatment with fluoxetine 20 mg/day was started and then increased to 30 mg/day. In the following months, symptom improvement was noticed with a richer and more fluid speech, and more adapted answers to questions. The patient was more involved in the exchange and gained autonomy. Six months later, we switched fluoxetine 30 mg/day to sertraline 50 mg/day because of hypersudation, but sertraline was less effective. The delayed sleep onset was treated with extended-release melatonin. An autoimmune biological workup showed anti-thyroglobulin and anti-TSH receptor antibodies slightly above the norm. A trial treatment with naproxen showed no efficiency. Oral corticosteroid therapy led to significant but transient improvement in speech difficulties, psychomotor slowing and social anxiety. At our last consultation, the patient partially recovered but still presented slowness and perseveration in oral and written language.

Discussion

We presented four clinical vignettes illustrating that the symptoms presented by patients with DSRD were multiple and non-specific. All cases exhibited altered mental status, cognitive decline (especially apathy), developmental regression with loss of previously acquired milestones, circadian rhythm disruption, and language deficits. We did not find focal neurological deficits nor seizures but these symptoms may have led to orientation toward a neurologist rather than our psychiatric outpatient unit. Indeed, each patient reported psychiatric symptoms ranging from anxious to psychotic phenomena, although they did not meet full criteria for a psychiatric disorder (subthreshold level of symptoms, fluctuating presentations). In all cases, the response to psychotropic drugs was generally poor, with side-effects, suggesting that the etiology of DSRD should be identified in order to implement specific treatments.

While it is not an etiological diagnosis, catatonia seems to be a syndrome that is often associated with the regression of DS patients (Ghaziuddin et al., 2015). Thus, it could be a relatively common but insufficiently recognized cause of reversible decline in adolescents with DS. Moreover, some hypotheses postulate that DS may be a risk factor for catatonia, independent of DSRD and in addition to those already known such as ASD, mental retardation, and schizophrenia (Ghaziuddin et al., 2015; Miles et al., 2019). Indeed, DSRD could meet the diagnostic criteria for unspecified catatonia not otherwise specified (NOS) according to the DSM-5 criteria (American Psychiatric Association, 2013). Conversely, in our clinical experience, it seems that a diagnosis of catatonia NOS in a patient with DS is in favor of diagnosis of DSRD.

All patients presented with positive autoimmune work-ups, three with autoantibodies directed against the thyroid and one with hypergammaglobulinemia. They responded, at least partially or transiently, to corticosteroid and anti-inflammatory treatments. The hypothesis of an autoimmune or inflammatory etiology in

some DSRD therefore seems plausible. Two previously described entities may shed light on this clinical picture. First, PANS has clinical similarities with DSRD as it includes the sudden onset of OCD or restrictive eating disorder, associated with other signs such as abnormal movements, behavioral regression, mood disorder, or anxiety disorder. The etiology of PANS is believed to be an immune response to viral or bacterial infections resulting in the production of antibodies directed to brain structures, which may be responsible for the neuropsychiatric symptomatology. Secondly, SREAT is another rare cause of cognitive decline that may also manifest as catatonia associated with elevated levels of antithyroid antibodies, and may thus have a similar presentation to DSRD. It differs, however, in that convulsions, hallucinations, and ataxia are quite common but not mandatory. It is likely that therapeutic intervention, with anti-inflammatory or corticosteroids, is necessary from the very first signs, in order to prevent the development of the chronic phase of the disorder and allow an optimal recovery.

In line with an inflammatory or autoimmune hypothesis, neuroimaging studies using magnetic resonance spectroscopy based on a glial biomarker demonstrated a higher inflammatory glial component in DS carriers than in control individuals (Hithersay et al., 2017). Many genes involved in microglial activation are also localized on chromosome 21 and thus overexpressed in trisomy (Fortea et al., 2021). Similarly, four of the six interferon receptors are located on chromosome 21, which confers a predisposition in patients with DS to chronic inflammatory reactions and the occurrence of autoimmune pathologies. Recent research revealed the presence of autoantibodies, such as antinuclear antibodies, anti-striated muscle antibodies, anti-TPO antibodies and anti-tissue transglutaminase antibodies, at high levels in some individuals with DSRD (Cardinale et al., 2019; Hart et al., 2021). DS is generally associated with elevated serum levels of proinflammatory cytokines and increased consumption of complement proteins. Autoimmune diseases and in particular Hashimoto's thyroiditis are common in DS (about 60% of cases) (Miles et al., 2019). In patients with DS, the prevalence of thyroid abnormalities, and in particular hypothyroidism and Hashimoto's thyroiditis, is 50%, a rate that increases with age (Bull, 2020). Interestingly, patients with DSRD were seropositive for anti-TPO antibodies at a significantly higher rate (91%) than patients of the same age with DS without regression (23%), which may suggest an autoimmune etiology such as SREAT (Worley et al., 2015; Cardinale et al., 2019). Moreover, it is possible that patients with DS may have brains more vulnerable to auto-antibodies compared to the general population, explaining why, in case 4, the patient had more severe consequences than his mother and sister who also exhibit auto-immunity.

These results suggest the involvement of an immune-mediated process in a subgroup of DSRD. Previously reported therapeutic strategies using intravenous immunoglobulins in particular showed significant efficacy in reducing the symptoms of DSRD and support this etiological hypothesis (Cardinale et al., 2019; Hart et al., 2021; Santoro et al., 2022a). Given the clinical response, their use has been proposed in cases of severe and rapid regression, even in the absence of documented autoimmunity (Hart et al., 2021).

There are several limitations that need to be acknowledged. This study is a cases series with a limited number of patients, it is retrospective, and unblinded. Due to this retrospective aspect, we did not perform any specific neuropsychological evaluation, which would allow a better characterization of the treatment effect on cognition. Therefore, a prospective longitudinal approach with quantifiable and standardized outcome measures will be necessary to confirm our tentative observations.

Overall, by highlighting four cases of DSRD, we hope to extend the panel of medical interventions to less invasive strategies. Shortening the delay before adequate treatment might be associated with a better response to NSAIDs or corticosteroids, decreasing the need for more aggressive treatments. Thus, DSRD should be detected early to improve the longitudinal outcome of DS. Awareness of this possible evolution in DS patients, as well as future identification of triggering factors (e.g., stressful events, onset of an auto-immune disorder, infection. . .) could shorten the use of appropriate therapies.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SB, PE, and BC contributed to the study design. SB contributed to the data gathering. SB, AI, CM, MC, CL, AL, CD-A, CC, AD, AC, M-OK, PE, and BC contributed to the data analysis and interpretation. SB, AI, PE, and BC contributed to the manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The molecular basis of p21-activated kinase-associated neurodevelopmental disorders: From genotype to phenotype

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Although the identification of numerous genes involved in neurodevelopmental disorders (NDDs) has reshaped our understanding of their etiology, there are still major obstacles in the way of developing therapeutic solutions for intellectual disability (ID) and other NDDs. These include extensive clinical and genetic heterogeneity, rarity of recurrent pathogenic variants, and comorbidity with other psychiatric traits. Moreover, a large intragenic mutational landscape is at play in some NDDs, leading to a broad range of clinical symptoms. Such diversity of symptoms is due to the different effects DNA variations have on protein functions and their impacts on downstream biological processes. The type of functional alterations, such as loss or gain of function, and interference with signaling pathways, has yet to be correlated with clinical symptoms for most genes. This review aims at discussing our current understanding of how the molecular changes of group I *p21-activated kinases* (*PAK1*, *2* and *3*), which are essential actors of brain development and function; contribute to a broad clinical spectrum of NDDs. Identifying differences in *PAK* structure, regulation and spatio-temporal expression may help understanding the specific functions of each group I *PAK*. Deciphering how each variation type affects these parameters will help uncover the mechanisms underlying mutation pathogenicity. This is a prerequisite for the development of personalized therapeutic approaches.

KEYWORDS

intellectual disability, autism spectrum disorder (ASD), neurodevelopmental disorders, genotype/phenotype correlation, p21-activated kinase, mutations

Introduction

The 5th edition of the Diagnostic and Statistical Manual of mental disorders (Crocq and Guelfi, 2015) defines neurodevelopmental disorders (NDDs) as a broad class of brain diseases characterized by a spectrum of early clinical manifestations with developmental delay and cognitive/social impairments representing the most recurrent phenotypes. A non-exhaustive list of NDDs includes intellectual disability (ID), Autism Spectrum Disorders

(ASD), Attention-Deficit/Hyperactivity Disorder (ADHD), developmental epilepsies and motor disorders such as cerebral palsy. Schizophrenia (SCZ) and Bipolar disorders (BP) could also be considered as NDDs (Crocq and Guelfi, 2015; Michetti et al., 2022). These psychiatric and neurological disorders participate in a network of neuropsychiatric diseases that share etiology and clinical commonalities related to their complex nosology (i.e., classification of diseases) (Vissers et al., 2016; Owen and O'Donovan, 2017; Glasson et al., 2020; Morris-Rosendahl and Crocq, 2020; Leblond et al., 2021; Vilela et al., 2022). Next-generation sequencing (NGS) has helped identify pathogenic variants in many genes for a large proportion of patients presenting ID and highlights the genetic complexity in NDDs (de Ligt et al., 2012). For instance, more than 1,000 genes have been associated with ID risk and more than 400 with ASD (Willsey et al., 2017; OMIM, 2022). The large number of genes involved makes the identification of pathogenic variants by clinicians and geneticists extremely complex (Piton et al., 2013; Wright et al., 2015; Chiurazzi and Pirozzi, 2016). Hence, distinguishing deleterious variants from neutral polymorphisms is still a challenge for many single nucleotide variations (SNVs), even if they are located in coding sequences. Moreover, different mutations in the same gene can lead to a broad spectrum of clinical symptoms, which makes the etiological characterization of NDDs difficult. An ongoing challenge is to understand how various genetic changes can cause different NDDs. The many approaches developed based on robust algorithms and systematically updated databases open the possibility of elaborating frameworks of analysis for the relationship between genetics and clinics (Gussow et al., 2016; Geisheker et al., 2017; Sanders et al., 2019).

The genes involved in NDDs regulate central signaling pathways that often share a limited number of functions such as chromatin regulation, proliferation and differentiation of neural stem cells, and synaptic plasticity (Ernst, 2016; Forrest et al., 2018; Moretto et al., 2018; Liaci et al., 2021). This convergence of signaling pathways in NDDs can help identify common therapeutic targets. Thus, uncovering the way signaling pathways are disrupted remains a priority to better understand the pathology and define new therapeutic approaches. The RAC1/CDC42 pathway is central to brain development and function since it regulates actin cytoskeleton dynamics, which is essential to cell migration, synaptic plasticity, axon guidance and neurogenesis (Nadif Kasri and Van Aelst, 2008; van Bokhoven, 2011; Penzes and Cahill, 2012; Gentile et al., 2022). The switch between cytosolic, GDP-loaded inactive-GTPases and membrane-bound, GTP-loaded active forms usually regulates this pathway. Two large families control these states: the Guanine Exchange Factor (GEF) and the GTPase-Activating Proteins (GAP). Variations in *RAC1*, *CDC42* as well as those in several GEF such as the *PIX* guanine exchange factors, and in several GAP such as *Oligophrenin*, and other genes connected to this RHO GTPase pathway are responsible for NDDs, epilepsy and are often associated with brain structural anomalies (**Figure 1A**; Frangiskakis et al., 1996; Billuart et al., 1998; Kutsche et al., 2000; Reijnders et al., 2017; Martinelli et al., 2018; Tastet et al., 2019; Barbosa et al., 2020; Halder et al., 2022). Genetic data also helped identify pathogenic variations in *Lim Kinases* and *cofilin* involved in neuropsychiatric conditions, confirming the role of the RAC/CDC42/PAKs/LimK/Cofilin pathway in NDDs (Frangiskakis et al., 1996; Tastet et al., 2019; Halder et al., 2022). Cofilin activity is regulated by LIM kinase-dependent phosphorylation, Slingshot-dependent dephosphorylation, and other interactions with protein partners. Cofilin controls dendritic spine dynamics and synaptic expression of glutamate receptors in the post-synaptic compartment, as well as vesicle exocytosis and neurotransmitter release in the presynaptic compartment (Ben Zablah et al., 2020).

p21-activated kinases (PAKs) mediate several functions downstream of RAC1 and CDC42. They regulate actin cytoskeleton dynamics via Lim kinase activation and phosphorylation-dependent cofilin inhibition (Bokoch, 2003; Boda et al., 2010; Ben Zablah et al., 2020; Zhang K. et al., 2022). PAKs are divided in two groups according to their sequence identity, their structural properties, and regulatory mechanisms (Eswaran et al., 2008). Group I *PAKs* includes *PAK1*, *2* and *3*; group II includes *PAK4, 5* and *6* which have not been associated with NDDs yet (Jaffer and Chernoff, 2002; Bokoch, 2003). Several reviews describe the biological functions of PAKs in cell signaling and neuronal pathophysiology (Bokoch, 2003; Kreis and Barnier, 2009; Koth et al., 2014; Kumar and Li, 2016; Civiero and Greggio, 2018; Wang and Guo, 2022; Zhang K. et al., 2022). Functions of group I PAKs are similar in a broad way because of their high sequence identity, their common modes of regulation as well as the substrates they share. Recent data from human genetics highlight their implication in NDDs. Pathogenic variations of *PAK1*, *PAK2* and *PAK3* are responsible for ID and/or psychiatric disorders, and are often associated with brain anomalies (Allen et al., 1998; Harms et al., 2018; Wang et al., 2018). However, the clinical spectrum associated with mutations in each *PAK* genes is different (**Figure 1B**). This review aims to provide an overview of the different clinical cases associated with *PAKs* thus far, to reflect on the effects of mutations on PAK functions, and how *PAK* expression may be involved in the symptomatology of *PAK*-associated disorders.

History of case reports and clinical data

PAK3: From non-syndromic XLID to a complex clinical pattern

We briefly describe here the history of the identification of *PAK* genetic variants and the implementation of concepts that have accompanied these discoveries over the past 25 years (**Table 1**). The autosomal location of the two *PAK1* and *PAK2* genes explains the delayed timing of the discovery of their genetic involvement in NDDs, compared to the X-linked *PAK3* gene. The first evidence of a genetic factor in ID comes from studies of its transmission to men in large families and the analysis of the segregation of chromosomal markers. Because of the male-to-female ratio of patients affected with ID, the X chromosome has been linked to this pathology early on (Lehrke, 1972; Chelly and Mandel, 2001; Gécz et al., 2009; Lubs et al., 2012). Variant identification was carried out by analyzing karyotypes and segregation of markers, thus requiring cross-referencing of data from several patients. Then, the advent of NGS has made it possible to identify variations in the DNA of isolated patients. The first pathogenic mutation in the *PAK3* gene was discovered in a family (MRX30) described in 1996. The region involved was mapped between Xq21.3 and

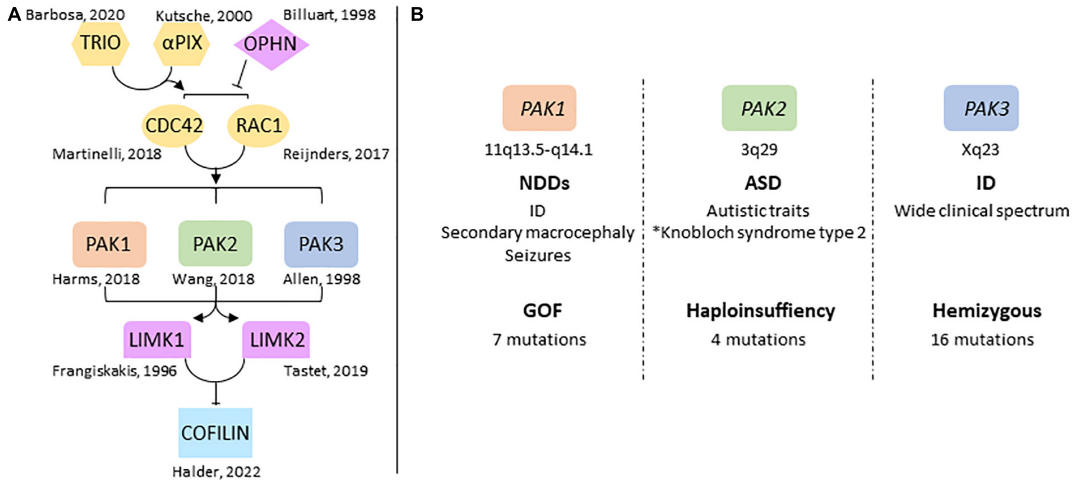


FIGURE 1
Overview of group 1-PAKs in NDDs **(A)** genes of the RAC1/CDC42 pathway are involved in NDDs. The identification of pathogenic variations in several genes highlights the central role the dysregulation of this pathway plays in NDDs, notably via its control of actin cytoskeleton dynamics through cofilin. **(B)** Chromosomal location of PAK genes and conditions generally associated with PAK variations. PAKs are associated with very different mutational and clinical landscapes. *Antonarakis et al. (2021).

Xq24 (Donnelly et al., 1996) and the variant was later identified as p.(Arg419*), and corresponds to a nonsense mutation in the sequence encoding the kinase domain of the *PAK3* gene (Allen et al., 1998). The second variant identified, p.(Arg67Cys), was the first missense mutation found within the *PAK3* gene (des Portes et al., 1997; Bienvenu et al., 2000). The MRX47 family bearing this mutation was previously described in 1997 (des Portes et al., 1997). This mutation localized in the regulatory domain modifies PAK3 affinity for GTPases (Kreis et al., 2007). The p.(Ala365Glu) substitution within a highly conserved region of the kinase domain was detected in 19 males in a family spread over five generations. Patients bearing this *PAK3* variant were diagnosed with mild non-syndromic X-linked ID with a few individuals also presenting neuropsychiatric problems such as aggressiveness, antisocial behavior, psychosis, depression or epilepsy (Gedeon et al., 2003). In 2007, a p.(Trp446Ser) mutation, located in the kinase domain and affecting 12 patients from a four-generation family was published (Peippo et al., 2007). It was the first description of learning difficulties in four carrier females that displayed borderline to mild ID (Peippo et al., 2007). Two males had drooling, otherwise all four males presented inarticulate speech, short attention span, anxiety, restlessness and aggressiveness and one of them had a small head. These data indicate that *PAK3* variations can be associated with psychiatric traits and brain anomalies, thus being responsible for a more complex clinical description than non-syndromic ID (**Figure 2**). Indeed, microcephaly is now often associated with *PAK3* variations. Notably, in 2008, two brothers bearing the first frameshift variant due to a four-bases insertion at the end of the sixth exon p.(Gly92Valfs*35), located after the second coding exon were described with ID, aggressiveness and microcephaly (**Figure 3**; Rejeb et al., 2008). This clinical report, combined with the previous literature, prompted these authors to suggest that *PAK3*-associated disorders should be considered as a distinguishable ID syndrome. In 2014, the p.(Lys389Asn) mutation associated with severe ID was described in two boys that displayed microcephaly, corpus callosum agenesis (CCA) and cerebellar hypoplasia and one fetus that displayed CCA and septum pellucidum agenesis (Magini et al., 2014). The two patients later died from epileptic seizures at age 6 and 8. The *in vitro* and *in vivo* analysis of the functional consequences of this variant indicated a large effect on cell signaling and on craniofacial structures. A less severe case was described soon after, the p.(Arg493Cys), responsible for borderline ID, epilepsy and hemiplegic cerebral palsy (McMichael et al., 2015). During the same period, the p.(Val294Met) was identified in patients displaying moderate ID, ADHD and microcephaly (Muthusamy et al., 2017). Soon after, a missense mutation, p.(Tyr427His), described in monozygotic twins, was associated with macrocephaly in addition to developmental delay, ID and autistic features, such as sensory processing issues (Hertecant et al., 2017). It was the first time macrocephaly was associated with *PAK3* mutations. More recently, a patient bearing a p.(Ser527Gly) mutation, located in the kinase domain, was described with severe ID, epilepsy, and self-injurious behavior (Horvath et al., 2018). Since 2018, almost only severe ID was described, such as the p.(Val326Leu) variation in a boy, the p.(Trp428Arg) variation in two siblings or the p.(Gly424Arg) variation in a familial case (Iida et al., 2019; Duarte et al., 2020; Nagy et al., 2020). The latter case displayed global developmental delay, stereotypies, and was later diagnosed with secondary microcephaly in addition to ID and corpus callosum dysgenesis. The same pathogenic variant was found retrospectively in the fetus from a previous pregnancy terminated after detection of CCA and hydrocephaly (Duarte et al., 2020). It was not until 2021 that female cases were reported as probands. Indeed, some pathogenic variations are now described in girls. The first variation responsible for such a case is the p.(Lys389Thr), which was also the second described substitution affecting the Lys389 residue. The patient bearing this mutation displayed ID, secondary microcephaly and ADHD (Pascolini et al., 2021). The published p.(Pro229Ser) variation indexed in *PAK3cb*, which corresponds to a p.(Pro193Ser) variation in the *PAK3a* isoform (**Figure 3B**), is associated with ID, microcephaly and combined immunodeficiency syndrome in the proband girl

TABLE 1 Historical discoveries of *PAK* variations in NDDs.

Mutation	*PAK1*	*PAK2*	*PAK3*	References
p.(Arg419*)			1st mutation, mild ID, ADHD, microcephaly	Allen et al., 1998
p.(Arg67Cys)			1st missense mutation, moderate to severe ID	Bienvenu et al., 2000
p.(Ala365Glu)			Mild ID, variable psychiatric presentations	Gedeon et al., 2003
p.(Trp446Ser)			Mild to moderate ID, ADHD, agressiveness	Peippo et al., 2007
p.(Gly92Valfs*35)			1st Frame-shift, mild to moderate ID, agressiveness, microcephaly	Rejeb et al., 2008
p.(Lys389Asn)			Severe ID, ASD, epilepsy, microcephaly, CCA, cerebellar hypoplasia	Magini et al., 2014
p.(Arg493Cys)			Borderline ID, epilepsy, hemiplegic cerebral palsy	McMichael et al., 2015
Xq23 deletion, PAK3 exons (4-15)del			Mild ID, developmental delay, ASD	Cartwright et al., 2017
p.(Val294Met)			Moderate ID, ADHD, microcephaly	Muthusamy et al., 2017
p.(Tyr427His)			Severe ID, ASD, macrocephaly	Hertecant et al., 2017
p.(Ser527Gly)			Moderate ID, ASD, seizures, ventriculomegaly	Horvath et al., 2018
p.(Asp311Asn) p.(Arg479*) p.(Arg524Cys)		Haploinsufficiency, ASD		Wang et al., 2018
p.(Tyr131Cys) p.(Tyr429Cys)	2 dominantly-acting Gain Of Function (GOF), ID, epilepsy, macrocephaly			Harms et al., 2018
p.(Val326Leu)			Mild to moderate ID, ASD, microcephaly	Nagy et al., 2020
p.(Trp428Arg)			Severe ID, ASD, microcephaly, CCA	Iida et al., 2019
p.(Ser110Thr) p.(Pro121Ser) p.(Ser133Pro) p.(Leu470Arg)	Moderate to severe ID, seizures, macrocephaly, ventriculomegaly			Horn et al., 2019
p.(Pro121Leu)	ID, ASD, epilepsy, macrocephaly			Kernohan et al., 2019
p.(Gly424Arg)			Severe ID, ASD, microcephaly, CCA	Duarte et al., 2020
p.(Lys389Thr)			1st female case, ADHD, microcephaly	Pascolini et al., 2021
p.(Pro193Ser)			Female patient, mild ID, ASD, Microcephaly	Almutairi et al., 2021
p.(Glu435Lys)		Knobloch type 2 syndrome		Antonarakis et al., 2021

Historical contexts of variant discoveries in *PAK1* and *PAK2* are very different from that of *PAK3*: more recent, concerning fewer patients, and associated with more homogeneous clinical spectra. Peptide numbering corresponds to Human *PAKs*, and the Human *PAK3a* splice variant in the case of *PAK3* variations.

(Almutairi et al., 2021). To summarize, the clinical picture associated with *PAK3* mutations was initially characterized as non-syndromic ID but the identification of additional *PAK3* mutations has unveiled the comorbidity of ID with many psychiatric traits as well as morphological abnormalities of the brain. *PAK3* mutations can lead to ID of varying severity and may be associated with autistic traits, ADHD, paranoid psychosis, aggressiveness, self-harm, and other neurological signs such as epilepsy and motor disorders.

PAK1 in developmental delay and macrocephaly

The identification of deleterious variations in the two other *PAK* genes is recent. The first mutation in the *PAK1* gene, p.(Arg500His), was reported in a meta-analysis of more than 2,000 patients with ID, but *PAK1* was not considered as a gene responsible for ID at that time (Lelieveld et al., 2016). In 2018, two p.(Tyr131Cys) and a p.(Tyr429Cys) mutations were identified in two unrelated patients displaying macrocephaly, ID, seizures, speech impairment and attention deficits (Harms et al., 2018). In 2019, other *PAK1* variants including p.(Ser133Pro), p.(Ser110Thr), p.(Pro121Ser) and p.(Leu470Arg) were reported in subjects with ID, macrocephaly and seizures in three boys and one girl, respectively, (Horn et al., 2019; Ohori et al., 2020). They are localized either in the regulatory domain or in the kinase domain such that their translated variants are predicted to suppress the auto-inhibitory mechanism, thereby directly activating or indirectly leading to PAK1 activation. In 2019, a second occurrence affecting the Pro121 residue was reported. This p.(Pro121Leu)

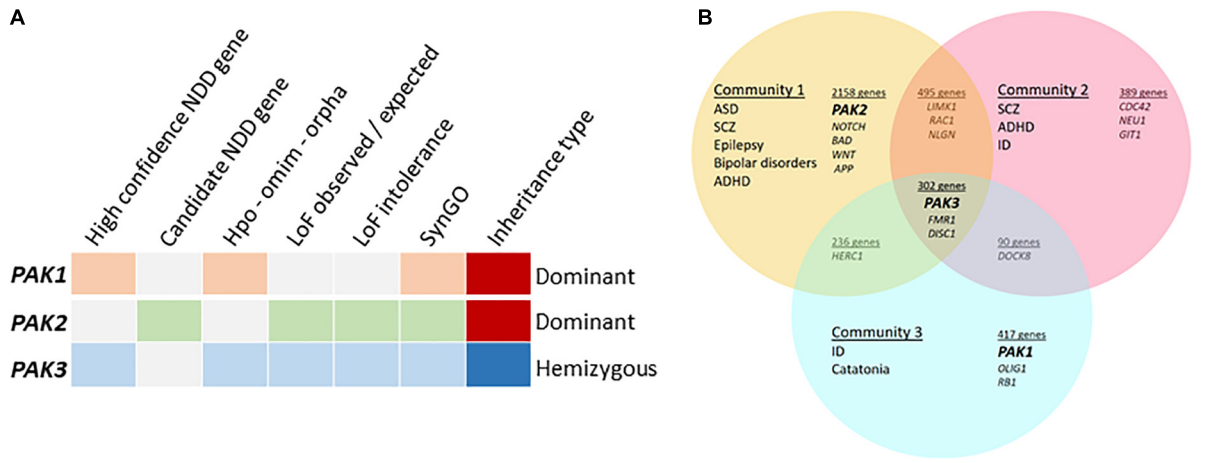


FIGURE 2
Meta-analyses of group I PAK-associated NDDs **(A)** PAKs in different databases, categories highlighted when PAK1 (orange), PAK2 (green) or PAK3 (blue) are detected. The high confidence NDD gene category was created by GeneTrek (Leblond et al., 2021) using data-mining from SFARI, SPARK, SysID, DDG2P, and DBD databases. The candidate NDD gene category includes candidate risk genes from the same databases (except SPARK). HPO-OMIM-Orpha: other databases. Loss of function (LOF) observed or expected: LOF can be expected from genes in this category. LOF intolerance corresponds to genes for which LOF leads to pathogenic effects. Genes from SYNGO database encode synaptic proteins. **(B)** *PAK* genes are associated with different community of diseases identified by Vilela et al. (2022).

mutation is responsible for an autosomal dominant disorder with severe regressive ASD, ID and epilepsy (Kernohan et al., 2019).

PAK2 variants are associated with ASD

The *PAK2* gene is localized at the end of the long arm of chromosome 3 and belongs to the 3q29 locus that is relatively frequently rearranged in patients with NDDs. The 3q29 duplication syndrome is associated with ID, microcephaly, cerebral palsy, and epilepsy. The minimal duplicated interval involves 22 annotated genes, including *PAK2* and *DLG1* (Lisi et al., 2008; Fernández-Jaén et al., 2014; Wen et al., 2022). Likewise, deletion of the 3q29 locus is associated with ASD (Sanders et al., 2015). Clinical studies reported ASD in patients bearing *PAK2* missense mutations or with *PAK2* deletions, causing *PAK2* haploinsufficiency (Wang et al., 2018). One missense p.(Glu435Lys) variation was recently identified by exome sequencing in two siblings displaying a Knobloch-like syndrome designated as KNO2 with ID, autistic behavior, retinal degeneration, and encephalocele (Antonarakis et al., 2021).

Despite the descriptions above, non-cognitive recurring symptoms have also been identified for several patients bearing *PAK1* or *PAK3* mutations. Some traits such as general hypotonia, drooling, nystagmus and strabismus, speech difficulties, and tremor, that may be due to neurological and/or neuromuscular defects, are described for patients bearing *PAK1* or *PAK3* variations. There are also some non-neurological symptoms associated with *PAK* variants. Interestingly, skin anomalies, such as café-au-lait spots, were described for the majority of patients bearing *PAK1* mutations and associated with some *PAK3* variations (Magini et al., 2014; Hertecant et al., 2017; Harms et al., 2018; Pascolini et al., 2021). These anomalies may be due to defects in the activation of the Ras-MAP kinase pathway, leading to RASopathy (Magini et al., 2014). Facial dysmorphic features are also commonly associated with *PAK1* and *PAK3* variations (deeply set eyes, large ears, hair alopecia, bushy eyebrows, nose shape, palate, etc.). A gross analysis suggests that the more severe the cognitive defects, the more non-neurological symptoms are present. This is probably due to the more damaging PAK defects in these cases. It should be noted that there is still no thorough clinical description of patients bearing *PAK2* variants, except for the KNO2 syndrome.

Clinical overview of *PAK* variants

The description above indicates that variations are associated with different conditions depending on each *PAK* gene: *PAK1* pathogenic variants are associated with developmental delay, ID, ASD and macrocephaly, *PAK2* variants with ASD only and *PAK3* variants with ID, often along with ASD, ADHD, epilepsy, and, in numerous cases, microcephaly. The pathophysiological landscape associated with each *PAK* may also be observed from genetic database mining or from meta-analysis data in web sites such as GeneTrek, which surveys the association of human genes with NDDs (**Figure 2A**; Leblond et al., 2021). This could also be illustrated by the analysis of disease communities based on comorbidities and clinical characteristics in a disease network approach to explore the shared genetics between ASD, ID, ADHD, SCZ, BP, and epilepsy (**Figure 2B**; Vilela et al., 2022). As expected, the three *PAK* genes segregate with different disease communities. *PAK1* is found within the community mostly defined by ID, *PAK2* is found in the community mostly characterized by ASD, though the comorbidities associated with ASD make this community highly heterogeneous. Indeed, around 70% of individuals with ASD display at least one co-occurring NDD or form of epilepsy (Rosen et al., 2018). Furthermore, even though *PAK2* variations are mostly associated with ASD, the number of cases described at present is not sufficient to get a precise idea of the extent of clinical consequences. For example, the *PAK2* mutation associated with KNO2 syndrome (Antonarakis et al., 2021) already differs from previous descriptions

of *PAK2* clinical cases that were exclusively associated with ASD. For *PAK3*, there is a large clinical variability so that *PAK3* is found in all three communities (**Figure 2B**). However, the exhaustive list of clinical traits associated with *PAK3* pathogenic variations is difficult to establish because of heterogeneous clinical descriptions of the patients, such as the relative importance given to certain traits compared to others or the context of medical practices. The absence of mutation hot spots precludes the description of a precise *PAK3*-associated syndrome and prevents ruling out effects other mutations or the environment might have on *PAK3*-associated NDDs (Lelieveld et al., 2016).

The meta-analyses previously mentioned (Leblond et al., 2021; Vilela et al., 2022) highlight the complex relationship between *PAK* variations and the extent of neuropsychiatric symptoms. Several reasons might explain the differences observed in the clinical symptoms associated with each *PAK*: the paucity of described variations, especially for *PAK1* (7) and *PAK2* (4), and the different functions of each *PAK* isoform. Furthermore, the scarcity of biochemical characterization of PAK variants limits our understanding of these differences. To summarize, the study of *PAK*-associated disorders is currently incomplete due to several hindrances. Yet, the improvement of variant detection methods, the homogenization of clinical analyses and the collaborative approaches between clinicians and researchers will support a more thorough approach.

PAK genetic context governs the expression of patient conditions

An important difference between *PAKs* is that the variations in *PAK1* and *PAK2* genes are transmitted according to an autosomal and dominant inheritance pattern, while those in *PAK3* are X-linked and hemizygous. Both *PAK1* and *PAK2* are autosomal genes, respectively, located on chromosome 11 and 3. Pathogenic variations in *PAK1* are monoallelic *de novo* mutations displaying dominant functional effects (Harms et al., 2018; Horn et al., 2019; Kernohan et al., 2019). The missense *PAK2* variations are *de novo* or inherited (Zhang K. et al., 2022). The newly described *PAK2* mutation, responsible for the KNO2 syndrome, is monoallelic and detected in both siblings, indicating a likely germ-line mosaicism in one of the parents (Antonarakis et al., 2021). Whether this variant is responsible for haploinsufficiency is still unknown. For *PAK3*, most variants are inherited and only two of them, p.(Lys389Thr) and p.(Ser527Gly) occurred *de novo* (Horvath et al., 2018; Pascolini et al., 2021).

Variants in genes localized on the X chromosome form a special class because of the unique mode of allele expression: in males, the mutated allele is expressed in all cells whereas there is a mosaic expression of only one of the two alleles in female cells. In this instance the genetic approaches for patient cohorts with X-linked disorders is particular (Piton et al., 2013; Leitão et al., 2022). Until 2014, *PAK3* mutations were identified in large families over several generations and were found to induce less severe symptoms than in most recent cases, whom mutations are responsible for severe ID associated with other psychiatric traits. This change in case reports is probably due to a bias induced by the evolution of diagnostic methods, such as NGS, and to the fact that PAK3 is now more investigated because it is recognized as a gene involved in NDDs. The women bearing *PAK3* mutations were either unaffected female carriers or mothers presenting borderline to moderate ID (Peippo et al., 2007; Rejeb et al., 2008). Although skewed X-inactivation has been observed in several women, this could not be correlated with clinical characteristics. Unfortunately, there are no further data on more recently identified female probands (Almutairi et al., 2021; Pascolini et al., 2021). Despite the difference between men and women in *PAK*-associated disease transmission, no evidence suggests that differences in PAK functions exist between the two sexes.

Another observation is that each *PAK* displays a unique mutational pattern (**Figure 3B**). The landscape of genetic abnormalities of the three *PAK* genes is also particular insofar as current published data are mainly made up of missense mutations. For the three genes combined, there are twenty-three missense mutations, two nonsense mutations and one splice site mutation inducing a frameshift, as well as one multi-exonic deletion (Cartwright et al., 2017). There are only few recurrences. One double occurrence is on the *PAK1* proline 121 residue (Horn et al., 2019; Kernohan et al., 2019). For *PAK3*, two identical mutations p.(Ser527Gly) have been published as well as two variants affecting the same lysine 389 residue (Magini et al., 2014; Tzschach et al., 2015; Horvath et al., 2018; Pascolini et al., 2021). Interestingly, two mutations affecting homologous residues in the PAK1 and PAK3 paralogs, the p.(Tyr429Cys) and p.(Tyr427His), respectively, lead to similar clinical phenotypes associating ID, ASD and macrocephaly (Hertecant et al., 2017; Harms et al., 2018). Of note, the low number of described cases may explain the low number of recurrences and the absence of mutation hotspots.

PAK mutations affect kinase functions, mechanisms of regulation and partner interactions

Nonsense mutations, monogenic deletion, and splice site mutations inducing a frameshift are qualified as Likely Gene Disrupting variations (LGD) that are responsible for loss of function or loss of protein (**Figure 3B**). These mutations could lead to truncated forms lacking kinase activity and exerting dominant-negative effects or signaling interference, but this possibility has not been thoroughly explored yet. However, mRNAs bearing premature termination codon can be degraded by the translation control system, named Nonsense-Mediated mRNA decay (Supek et al., 2021). This would lead to the total absence of defective proteins, responsible for a complete loss of function. *PAK3* LGD mutations are responsible for relatively mild to moderate severity of the disease. The *PAK2* gene is considered intolerant to loss-of-function (**Figure 2A**), and the *PAK2* nonsense mutation is also associated with haploinsufficiency. Furthermore, heterozygous LGD mutations in ASD-related genes are frequently predicted to cause haploinsufficiency (Parenti et al., 2020).

Other mutations are missense mutations and their variations could be associated with Loss Of Function (LOF) or Gain Of Function (GOF). In the case of kinases, these denominations correspond to complete loss of kinase activity or high constitutive kinase activity, respectively. This classification as LOF and GOF may be used to qualify the functional defects of the PAK1, PAK2 and some PAK3 variants. However, it does not cover the full

range of functional alterations observed for other PAK3 variants. Indeed, PAKs are multifunctional proteins, with a kinase activity but also scaffolding functions, thus leading to kinase-dependent and kinase-independent roles. The best described of these kinase-independent functions is the recruitment of PIXs, which makes it possible to assemble a GEF/GTPase/effector complex, thus ensuring specificity and efficiency of the GEF/GTPase/PAK module (Obermeier et al., 1998). In other words, interactions with certain partners such as α/βPIX, Nck1/2 and Grb2 promote the formation of signaling complexes associated with membrane recruitments, and mutations in these interaction domains could significantly affect PAK signaling. Finally, pathogenic variations have only been identified in the coding sequences of group *I PAK*s. Given the importance of non-coding region variations in the etiology of NDDs, it would be advisable to look more closely for these kinds of changes (Takata, 2019).

PAK1 to 3 share an overall structure composed of a carboxy-terminal catalytic domain and an amino-terminal regulatory region (**Figure 3A**; Bokoch, 2003). Notwithstanding, PAK amino-acid sequences diverge in specific areas and those primary sequence differences, that are conserved among vertebrates, may support specific functions. The regulatory region consists of two overlapping domains, one is the p21-binding domain (PBD), and the other one is an auto-inhibitory domain (AID). The kinase domain comprises two lobes: the small lobe in N-ter is essentially composed of β-sheets while the larger lobe in C-ter is mainly constituted of α-helixes, in which several residues can interact with the auto-inhibitory domain (Lei et al., 2000; Parrini et al., 2002). In resting conditions, when PAKs are inactive, they form dimer comprised of two molecules in an asymmetric, antiparallel, manner, one monomer adopting an active conformation, and the other one an inactive (Wang et al., 2011). This *trans-inhibited* dimer is activated by several mechanisms that modify its conformation, induce a multi-step dissociation, and trigger several events of *cis-* and *trans-phosphorylation*. It is, however, possible that there was a bias in some crystallographic analyses, since the kinase-dead variant of PAK1 retains a very low, but sufficient, residual activity that allows its autophosphorylation (Wang et al., 2011). The crystallization carried out in the presence of phosphatases suppress this residual phosphorylation and generates inactive proteins in a monomeric conformation. Furthermore, the first step in the activation process corresponds to the transient formation of dimers (Sorrell et al., 2019).

Almost all pathogenic mutations currently identified are located in two domains corresponding to the kinase domain and the PBD/AID regulatory domain (**Figure 3B**), a fact that is probably due to the strong structural constraint observed in the catalytic and regulatory domains of kinases. In contrast, non-pathogenic single nucleotide polymorphisms (SNPs) extracted from databases are mainly distributed in sequences coding for non-functional, more flexible, zones. Note also the seemingly total penetrance of *PAK1* and *PAK2* mutations, as well as *PAK3* mutations in boys.

In addition to the slight structural differences between the three group I PAKs, the *PAK3* gene also shows specificity as it can encode four splice variants (Rousseau et al., 2003; Kreis et al., 2007). PAK3a is devoid of any insertion between the position 92 and 93 of the amino acid sequence. PAK3b contains an insertion of 15 amino acids at this position. PAK3c displays an insertion of 21 amino acids, located between the coding exon 2 and exon b. Finally, PAK3cb (also called PAK3d) exhibits both b and c alternative exons (**Figure 3B**). To date, a gross characterization of the expression of PAK3 splice isoforms has been conducted using semi quantitative RT-PCR, since the splice variant-specific antibodies available at present cannot work for immunofluorescent detection. All spliced isoforms have been detected in the mouse adult brain and observed as early as E15. Western blot analysis of the 544aa-PAK3a and 559aa-PAK3b proves that these isoforms are equally expressed in the adult mouse brain. However, there is a significantly lower expression of the two 565aa-PAK3c and 580aa-PAK3cb. These alternative exons change drastically the biochemical properties of PAK3. For example: the PAK3b AID cannot inhibit PAK3a (Rousseau et al., 2003). Furthermore, PAK3b, c and cb display high constitutive kinase activity, and thus do not require GTPase activation. The difference in regulatory mechanisms and expression levels between PAK3a, PAK3b, PAK3c, and PAK3cb should be taken into account when estimating mutation pathogenicity. For example, one *PAK3* mutation was identified in the alternatively spliced exon b (**Figure 3B**) in a patient reported in a cohort of cerebral palsy (McMichael et al., 2015), even though new mining from a polymorphism database suggests that this mutation actually corresponds to a neutral polymorphism.

Variations in functional domains

The PBD directly interacts with active GTP-loaded RAC1 and CDC42 (Lei et al., 2000). At first glance, the classical mechanism of activation by GTPases appears to be similar for the three PAKs, considering the similarity of the protein structures involved. The CDC42 and RAC1 interactive binding domain (CRIB) is a consensus sequence found in downstream effectors of RAC1 and CDC42 that constitutes the core of the PBD and is necessary for PAK activation and its subcellular location. Other PBD residues are also heavily involved in PAK selective interactions with GTPases (Knaus et al., 1998; Sun et al., 2019). Only one mutation affects this domain: the missense *PAK3* p.(Arg67Cys) mutation that is located at the limit of the PBD. PAK3 normally displays a higher affinity for CDC42 than for RAC1 according to *in vitro* assays, but the ID-associated p.(Arg67Cys) variant has more affinity for RAC1 than for CDC42, which changes its dynamics of activation and explains its property to induce immature dendritic spines in hippocampal cells (Kreis et al., 2007). GTPases of the Rac/Cdc42 family, that also interact with PAKs and may be involved in the pathophysiology of PAK variants, are still insufficiently explored in the context of *PAK*-associated disorders. Especially when taking into consideration that pathogenic variations in *RAC3* were recently identified in patients with developmental delay, brain anomalies, and facial dysmorphic features (Scala et al., 2022). Like *RAC1*, *RAC3* is ubiquitously expressed in the brain and evidence suggests that *RAC1* and *RAC3* share functions in neuron survival (Katayama et al., 2023). In double *Rac1/3*-KO mice, which display severe neuronal loss in the postnatal cerebral cortex, PAK is downregulated and the over-expression of constitutively active PAK1 rescues neuron survival and differentiation. All other GTPases of the Rac/Cdc42 family interact with PAKs but with no indication of their involvement in NDDs, as currently indicated by Decipher or ClinVar databases.

The AID is made of three highly conserved subdomains: a dimerization interface (DI) that contributes to the stabilization of PAK dimers, the inhibitory switch (IS) that partially overlaps the GTPase binding region, and a kinase inhibitor domain (KI) carboxy-terminal to the IS. PAK1 crystallographic structure highlights its ability to form dimers (Lei et al., 2000; Parrini et al., 2002). However, PAK3 forms preferentially heterodimers with PAK1 rather than homodimers, *in vitro* and *in vivo* (Combeau et al., 2012). Dimers are maintained inactive via the DI and the IS (**Figure 4**). The IS is folded against the catalytic region which favors the positioning of the KI domain into the catalytic cleft. The KI domain of each PAK within the dimer interacts with the catalytic site of the other PAK, thus inducing a *trans-inhibition* (Lei et al., 2000; Parrini et al., 2002). In their dimeric form, the CRIB domain in the PBD of each PAK is exposed at the surface, allowing PAK binding to an active small G protein.

PAK1 mutations are mainly located in the regulatory domain (5/7). It is expected that mutations affecting the AID impair inhibitory interactions and induce constitutive activation (Tu and Wigler, 1999). To date, five *PAK1* mutations affecting the Ser110, Pro121, Tyr131 and Ser133 residues were thus identified in the AID and associated with ASD, ID and macrocephaly (Harms et al., 2018; Horn et al., 2019; Kernohan et al., 2019). Indirect experimental data strongly suggest that these PAK1 variants display constitutive or higher basal kinase activity (Harms et al., 2018). Moreover, the inhibitory domain of a given isoform acts on all three paralogs, and heterodimer formation facilitates crosstalk between PAK signaling. Consequently, mutations in the AID domain should lead to kinase activation of the variant and possibly heterodimerization defects, thus altering the kinase activity of other isoforms (Combeau et al., 2012).

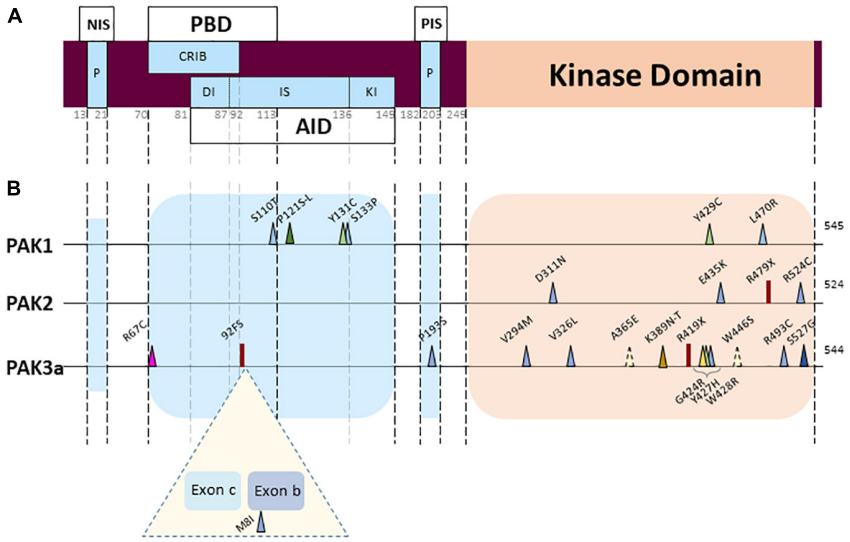
In contrast, a greater proportion of missense mutations are located in the kinase domain of PAK2 (3/3) and *PAK3* (11/14). Several *PAK* mutations are located in the kinase domain: eleven are inactivating mutations and four are activating. Indeed, mutations in the kinase domain, such as the ones mostly found in *PAK2* and *PAK3*, usually disrupt kinase activity. This was particularly well described in the biochemical characterization of several *PAK3* mutations *in vitro* (Allen et al., 1998; Kreis et al., 2007; Magini et al., 2014; Duarte et al., 2020). *PAK2* mutations, also located in the kinase domain, are probably associated with loss of function. Structural data, computational analysis, and mutation compilations already established in the field of cancer may help analyzing the effect that kinase variants have on kinase activity, but biochemical characterization is often necessary (Dixit and Verkhivker, 2014).

Proline-rich sequences are involved in regulated interactions with important partners. Among the other conserved features, several highly conserved proline-rich regions found in the amino-terminal tail serve as binding sites for SH3 domains. Two sites are involved in binding adaptor proteins NCK1/2 and GRB2 which is involved in axonal guidance and synaptic transmission through PAK recruitment at the membrane. Isoform particularity is that the autophosphorylation of the PAK1 Serine 21 residue negatively regulates PAK/NCK interactions (Zhao et al., 2000). Interestingly, PAK3 was shown to preferentially bind NCK2/GRB4 compared to NCK1 and this interaction is not regulated by autophosphorylation (Thévenot et al., 2011). The absence of mutations identified in the NCK-binding domain is probably due to the current low sampling of mutations. The second proline-rich sequence is not completely conserved between the different PAK proteins and GRB2 interaction was only demonstrated for PAK1 (Puto et al., 2003). This pathway is probably impacted by mutation of the non-conventional kinesin KIF26A that inhibits GRB2, as observed in patients with severe brain malformations (Qian et al., 2022).

The central proline-rich sequence located between the AID and the kinase domain corresponds to an atypical SH3 binding domain that strongly binds the PAK interacting exchange factor (PIX/COOL) (Bagrodia et al., 1998; Manser et al., 1998). There are two PIX isoforms in mammals: the ubiquitously expressed αPIX, encoded by the *ARHGEF6* gene (Manser et al., 1998) and βPIX encoded by *ARHGEF7* and more specific to the brain (Koh et al., 2001). The numerous splice variants of PIX form different transient complexes with PAKs. These PIX-PAK complexes are regulated by phosphorylation. PIXs are also important in the recruitment of PAKs to the membrane and their activation. This complex located at the membrane enables the activation of GTPases and selectively favors PAK activation. The particularities of the different PAK isoforms in these molecular mechanisms are not known (Zhang, 2005; Zhou et al., 2016). α/βPIX and GIT1/2 form larger complexes that interact with numerous proteins such as Paxillin and Shank, to coordinate their activity in cells and at synapses (Hashimoto et al., 2001; Parker et al., 2013; López-Colomé et al., 2017; Zhu et al., 2020). Disruption of the *ARHGEF6* gene leads to severe ID, dysmorphic features and sensorineural hearing loss (Kutsche et al., 2000). The mutation located near the PIX-binding sequence in *PAK3* was recently described in a girl with ID, microcephaly and immunological disease but without the biochemical analysis of the variant (Almutairi et al., 2021). The other proline-rich domains identified are not yet associated with partners and functions: indeed, PAK1 contains five proline-rich domains, while PAK2 has two and PAK3 has four. Whether all these proline-rich sequences bind the same partners with all three PAK isoforms is uncertain and has yet to be experimentally demonstrated.

The differences in peptide sequences also influence phosphosites. At the membrane, Rho-GTPase interactions induce a conformational change via the first event of *trans-phosphorylation* (threonine 423 for PAK1, 402 for PAK2 and 421 for PAK3). This restores some activity at the catalytic domain, which triggers *cis-phosphorylation* of sites, thus reinforcing kinase activity and completing the dissociation of dimers as well as maintaining monomers in an active conformation (Lei et al., 2000; Parrini et al., 2002; Pirruccello et al., 2006). No mutation affecting phosphosites has been described so far. Whether some *PAK* mutations can alter autophosphorylation or phosphorylation via other regulatory kinases has yet to be demonstrated. The caspase cleavage site, specific to PAK2, plays an important role in signaling but is not yet associated with NDDs (Jakobi et al., 2003).

PAK interactions with lipids, such as membrane lipids like PIP2, also participate in the opening of the kinase and its activation (Parrini et al., 2009; Malecka et al., 2013). PAKs are also enriched in dendritic spines (Zhang, 2005; Hayashi et al., 2007). As early as 2004, it was shown that PAKs copurify with synaptic markers (Hayashi et al., 2004). This analysis highlighted the colocalization of activated PAK with phospho-Cofilin and PSD95 upon neuronal activation in cortical neuron cultures and in organotypic hippocampal slices (Hayashi et al., 2004; Chen et al., 2007). Biochemical fractionation followed by Western blot analysis revealed the presence of active PAKs in the post-synaptic density (PSD) fractions of mouse brains, whereas inactive PAKs



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NDD-associated SNP in *PAK* genes					Kinase activity				other signaling defects
Domain	*PAK1*	*PAK2*	*PAK3*	Author	N	D	A	U	
PDB			R67C	Kreis	**N**				**GTPase**
			92FS	Rejeb		**D**			
AID	S110T			Horn			A		
	P121S			Horn			A		
	P121L			Kernohan			A		
	Y131C			Harms			**A**		
	S133P			Horn			A		
Pix			P193S	Almutairi				U	PIX
Kinase	D311N		V294M	Muthusamy		D			
				Wang		D			
			V326L	Nagy		D			
			A365E	Gedeon		**D**			
			C371Y	Qian		D			
			K389N	Magini		**D**			**MAPK**
			K389T	Pascolini		D			
			R419X	Allen		**D**			
			G424R	Duarte		**D**			**PIX**
	Y429C		Y427H	Harms			**A**		
			W428R	Iiida		D			
			W446S	Peippo		D			
		E435K		Antonarakis		D			
	L470R			Horn					
		R479X		Wang		D			
		R493C		Mc Michael					
		R524C		Wang		D			
			G527S	Horvath		D			

FIGURE 3
PAK general structure and localization of variants **(A)** Domain structure of PAKs. NIS, Nck interacting sequences; P, praline-rich sequences; PBD, p21 binding domain; AID, auto-inhibitory domain; DI, Dimerization segment; IS, Inhibitory switch domain; KI, kinase inhibitory segment; PIS, PIX interacting sequences. Numbering of the general structure is based on PAK 1. **(B)** Location of missense and LGD (likely gene disruptive) variations. Darker colors represent reccurrences. Both PAK3 alternative exons are represented (Exon b: 15 amino-acids; exon c: 21 amino-acids). **(C)** Validated or hypothetical functional alterations of *PAK* variants. N: normal kinase activity; D: defective; A: activated; U: unknown. Bold: experimentally validated; normal: hypothetical.

copurified mainly with presynaptic markers such as synaptophysin (Hayashi et al., 2004). However, fractionation experiments using adult mouse cortex suggest that PAK1 strongly localizes in the presynaptic fraction while PAK3 (especially PAK3a and PAK3b) co-purifies more with post-synaptic densities (Combeau et al., 2012). This suggests they may play different roles depending on their location and association with distinct synaptic elements.

PAK expression patterns and mutation pathogenicity

Group I *PAK*s share many structural and regulatory similarities, but their temporal and spatial expressions diverge. Globally, *PAK1* expression is high in muscles, in the spleen, and the brain except in the dentate gyrus (BrainSpan, 2022 *Atlas of the Developing Human*

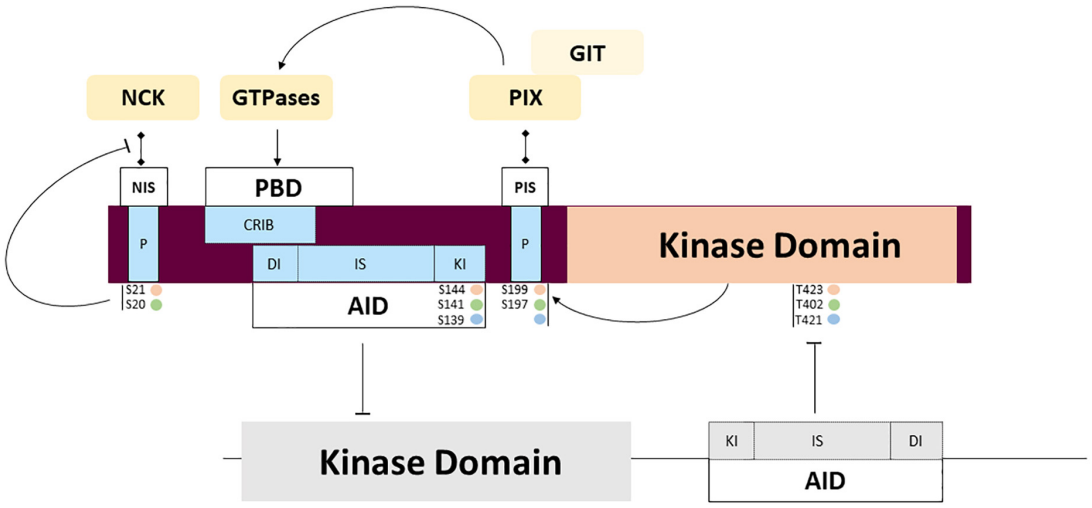

FIGURE 4
PAK regulation through partner interactions and intramolecular signaling. Complex mechanisms of regulation are triggered by interaction with activators and adaptors that may regulate activity and subcellular location. Several auto phosphorylation sites also participate in the regulation of interaction with the partners mentioned above, and the transitory location and activation of PAKs. Phosphorylation sites corresponding to PAKI (orange), PAK2 (green) and PAK3 (blue).

Brain) while *PAK2* expression is ubiquitous (Manser et al., 1995; Teo et al., 1995; Whale et al., 2011). *PAK3* is mostly expressed in the brain, with a particularly strong expression in the hippocampus. At the cellular level in the brain, *PAK1* is found only in neural lineage cells, *PAK2* is expressed in every cell types and *PAK3* can be found in neurons as well as oligodendrocyte progenitor cells (**Figure 5A**; Zhang et al., 2014; Maglorius Renkilaraj et al., 2017; Magne et al., 2021; Wang and Guo, 2022). However, some differences were noticed in *PAK* expression between mouse and human brain (**Figure 5A**; Civiero and Greggio, 2018; Zhang K. et al., 2022). Such differences should be taken into account when experiments are conducted on animal models and for extrapolation to humans.

PAK expression also differs along human lifespan (**Figure 5B**; BrainSpan, 2022: *Atlas of the Developing Human Brain*; Miller et al., 2014). The differences in pattern of expression may indicate that the different *PAKs* do not have the same role over the course of human brain development. *PAK* expression also presents variations depending on the brain region, which might reflect region-dependent specificities in addition to their concomitant functions. *PAK1* expression increases after birth in several regions such as the cerebellar and frontal cortices, but remains at a stable level in the striatum. *PAK2* expression profile appears to be similar in all brain regions (**Figure 5B**). Steadily decreasing after birth (Iossifov et al., 2014; Wang et al., 2018), *PAK3* expression is consistent throughout human brain development in the hippocampus, striatum and the frontal cortex but decreases after birth in the cerebellar cortex.

PAK expression is altered in several NDDs, suggesting that the decrease in PAK may be partly involved in cognitive or developmental symptoms. Therefore, the post-mortem meta-analysis of differentially expressed genes in the brain of ASD patients shows that neural *PAK1* expression is reduced in this pathology (Rahman et al., 2020). Hearing loss is correlated with ASD in children and this symptom may be due to the dysregulation of *PAK1* expression. Indeed, *PAK1* is highly expressed in the postnatal mouse cochlea, but the study of the *Pak1*-knockout mouse model showed that PAK1 deficiency downregulates cofilin phosphorylation and the expression of βII-spectrin, decreases the hair synapse density in the cochlea, and finally leads to hair cell apoptosis and severe hearing loss (Cheng et al., 2021).

Another example is *PAK3* downregulation in a model displaying iron deficiency-dependent cognitive alterations (Schachtschneider et al., 2016). *PAK3* is also one of the differentially expressed genes in BP and its expression is also regulated by valproate, a histone deacetylase inhibitor used to treat BP (Sinha et al., 2021). *PAK1* and *PAK3* mRNA levels are significantly reduced in the post-mortem brain of subjects affected with depression (Fuchsova et al., 2016). Transcriptomic analysis and genome-wide association in schizophrenia and bipolar disorder identify *PAK1* and *PAK3* as the differentially expressed genes along with several other genes in the RAC1/CDC42 pathway, highlighting the role of this pathway in these psychiatric disorders (Zhao et al., 2015).

Thus, it is important to consider *PAK* regulation of expression and the way it might change in the context of NDDs. Transcriptional and post-transcriptional regulations of each *PAK* present differences that may also change the way mutations affect brain development and functions. *PAK1* expression is activated via FOXO and E2F2 transcription factors and is inhibited by Rb (de la Torre-Ubieta et al., 2010; Sosa-García et al., 2015). *PAK2* transcriptional regulation is still an understudied subject, however, *PAK2* was found as differentially methylated in children with ASD (Jasoliya et al., 2022). *PAK3* expression is inhibited by Notch, ZEB1, SP8/9 and DLX1/2 transcription factors, highlighting its role in the proneural pathway (Souopgui, 2002; Cobos et al., 2007; Liu et al., 2019; Shen et al., 2019; Wei et al., 2019). *PAK3* transcription is also activated via Neurogenin and cJUN/AP-1 (Parker et al., 2013; Piccand et al., 2014). Several microRNAs (miRNAs) were identified in the regulation of *PAK* expression.

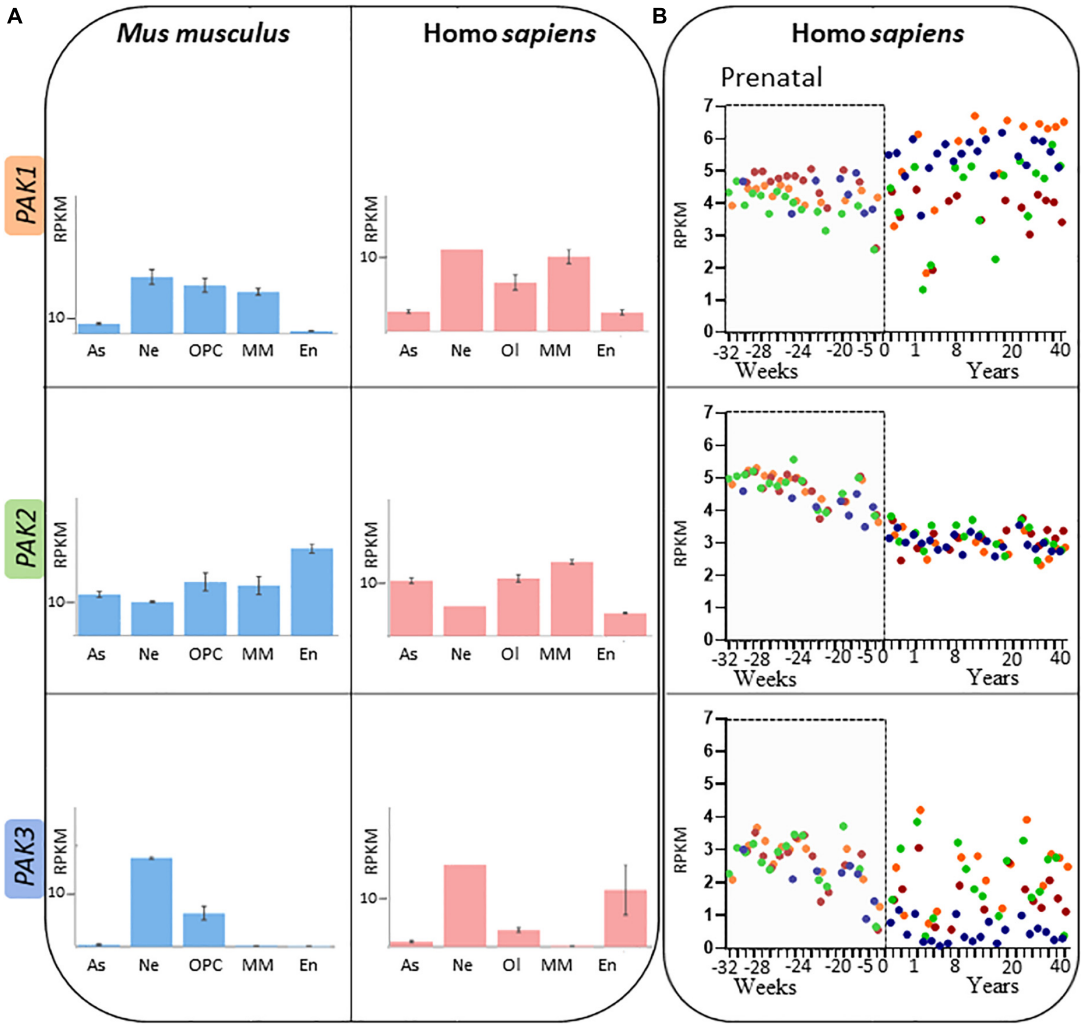

FIGURE 5
Spatio-temporal expression patterns of *PAK* isoforms **(A)** *PAK* expression depending on brain cell types, expressed in RPKM (reads per kilobase of exon per million reads mapped) in adult mouse (blue) and human (pink). Astrocytes (As), neurons (Ne), Oligodendrocyte precursor cells (OPC) or Oligodendrocyte (Ol), microglia/macrophages (MM) and endothelial cells (En). Data mining from brainrnaseq.org. **(B)** *PAK* expression throughout human lifespan in neurons of the cerebellar cortex (blue), hippocampus (green), striatum (brown), and frontal cortex (orange). Data mining from brainspan.org.

miRNAs are gene regulators frequently involved in neurological disorders (Kim and Pak, 2020; Yao et al., 2020). At least seven *PAK1*-targeting miRNAs regulate *PAK1* transcription: miR-7, 34b, 96, 140-5p, 145, 485-5p, and 494 (Yue et al., 2016; Yao et al., 2020; Zhao et al., 2022). More than 19 microRNAs target directly *PAK2* expression, such as miR4779 (Koo and Kwon, 2018), miR-7-5p (Li et al., 2019) and miR455-3p (Hu et al., 2019). Several miRNA such as miR-134-5p, miR-1252, miR-125b-5p, miR193b-3p, miRNA-495, miRNA542 and miRNA133 were shown to be involved in *PAK3* downregulation, mainly in cancer (Chiurazzi and Pirozzi, 2016; Zhang et al., 2020; Tan et al., 2021). Interestingly, expression of the miR-495 is also involved in schizophrenia (Santarelli et al., 2019). Alternative polyadenylation (APA) are post-transcriptional events that regulate gene expression and that play a key role in cell proliferation and differentiation, and in neuronal functions. APA events may produce different protein isoforms. *PAK3* exhibits bimodality of distal APA usage in a type of GABAergic interneurons, the chandelier cells. These interneurons have specific spatial and temporal origins, target the axon initial segment of pyramidal neurons of the hippocampus and are implicated in brain disorders, including schizophrenia, epilepsy, and ASD (Yang et al., 2022).

To our knowledge, no data exists concerning the role of PAKs in other brain cells, except for the oligodendrocyte lineage (Maglorius Renkilaraj et al., 2017). Interestingly, MRI analysis of white matter reveals hypomyelination in patients bearing *PAK1* pathogenic variations whereas partial agenesis of corpus callosum and thin fiber tracts have been observed in some patients with *PAK3* mutations (6/16). *In vitro* experiments demonstrated that *PAK3* deletion impairs the differentiation of oligodendrocyte precursors, supporting a developmental and cell-autonomous role for *PAK3* during the first steps of myelination. These fragmentary data suggest that defects induced by *PAK* variations in oligodendrocytes or oligodendrocyte precursors may also account for some phenotypical traits observed in affected patients.

To summarize, these different PAK isoforms may be involved in one of the several crucial cellular mechanism that shape the brain, through proliferation and apoptosis, cell type specification, migration, arborization, and synaptic genesis. Nonetheless, experimental evidence attributing unique and specific functions to one isoform is currently lacking, making it difficult to link spatiotemporal expression characteristics to clinical phenotypes.

The specific roles of PAKs isoforms

Among group I PAK isoforms, PAK1 is the most studied and is considered as representative of group I PAKs in general. Thus, there are currently more than sixty identified substrates of PAK1. These are involved in different major cellular processes such as proliferation, survival, motility and regulation of the actin cytoskeleton (Kanumuri et al., 2020; Liu et al., 2021). Such exhaustive list of specific substrates remains partial and cannot currently be compiled for the other isoforms (Ye and Field, 2012; Kumar and Li, 2016). Among the PAK-activated pathways, mentions should be made of the MAP kinase ERK pathways, the alteration of which leads to RASopathies, the Akt-PI3Kinase-mTor and Wnt pathways involved in ASD, the NF-κB pathway involved in apoptosis, among others (Yao et al., 2020; Caracci et al., 2021; Sharma and Mehan, 2021; Tartaglia et al., 2022). Many functions of the three PAK isoforms probably derive from their role in the regulation of actin cytoskeleton dynamics. It remains to be seen whether some of these functions are supported by only one of the three isoforms, which has not always been demonstrated experimentally. We can cite, for example, the following data. *PAK1* is involved in the proliferation and migration of neural progenitors in young mice (Pan et al., 2015) and dendritic maturation in the hippocampus (Dagliyan et al., 2017), which is essential to the regulation of postnatal cortical development. The abnormal increase in actin polymerization described in a mouse model of functional demyelination was caused by an over activation of PAK1 (Hu et al., 2016). *PAK2* probably plays an essential role during the first developmental stages as it regulates neuronal migration in the fetal brain and is, as such, essential for brain development. Interestingly, among *PAKs*, only *PAK2* suppression leads to the death of mouse embryos (Meng et al., 2005; Asrar et al., 2009; Wang et al., 2018). *PAK3* does not play the same role in cell cycle and cell division as *PAK1* and *PAK2*. While *PAK1* and *PAK2* have been characterized as oncogenes, *PAK3* is instead a probable tumor suppressor (Magne et al., 2021). In *xenopus laevis*, *PAK3* induces cell cycle arrest of precursors and their differentiation into neuroblasts during primitive neurogenesis (Souopgui, 2002). Furthermore, *PAK3* is also involved in axonal and dendritic arborization of immature interneurons and favors their tangential migration toward the cortex in mice (Cobos et al., 2007; Dai et al., 2014).

However, several studies have deciphered and attributed isoform-specific functions independent of actin. For example, PAK1 takes on actin-independent functions such as positive regulation of tonic GABA transmission via the downregulation of tonic endocannabinoïd (eCBs) secretion (Xia et al., 2018). The phosphorylation of CtBP1 by PAK1 is also a key step in synaptic vesicle retrieval in cultured hippocampal slices (Ivanova et al., 2020). Indeed, recordings of synaptic currents from CA1 neurons in *Pak1*-KO mice showed a decreased GABA signaling. This was also the case with acute PAK1 inhibition in hippocampal slices. Interestingly, the maturation of GABA function plays a major role in postnatal brain development (Deidda et al., 2014; Peerboom and Wierenga, 2021). PAK2 displays actin-independent pro-apoptotic functions, since PAK2, but not PAK1 nor PAK3, is cleaved by caspase 3, 8 and 10 in response to Fas signaling, which leads to PAK2-dependent apoptosis (Rudel and Bokoch, 1997; Fischer et al., 2006). PAK2 activated by RAC and CDC42 promotes cell survival while the caspase/PAK2 pathway is indeed pro-apoptotic (Marlin et al., 2011). Finally, one of PAK3 specific functions is the controls of AMPA receptor (AMPAR) trafficking via GluA1 subunit phosphorylation, upregulating the number of AMPAR at the membrane (Hussain et al., 2015).

Several data illustrate the link between PAK signaling and NDDs. PAKs were shown to regulate several proteins involved in NDDs, such as LIMK and cofilin (**Figure 1**). PAKs family members also interact with other downstream proteins that are dysregulated in NDDs or whose variations are causative factors in NDDs. For example, it was demonstrated that PAK1 binding to ERK2 facilitates ERK2 signaling, or activates their upstream activators RAF1 and MEK, which are involved in RASopathies (Sundberg-Smith et al., 2005; Motta et al., 2020; Liu et al., 2021). PAK1 is also required for the activation of NFκB pathway (Frost et al., 2000) via its modulation of the PPARγ/NFκB signaling (Dammann et al., 2015). The expression of NFκB pro-inflammatory transcription factor is dysregulated in the brain of ASD patients and animal models of ASD. This dysregulation could be a pathogenic mechanism of neuro-inflammation involved in ASD (Young et al., 2011; Honarmand Tamizkar et al., 2021). Furthermore, PAK1, but not PAK2 nor PAK3, interacts with FRX family proteins involved in Fragile X syndrome, notably by phosphorylating FRX1 at Ser-420 (Say et al., 2010; Majumder et al., 2020). PAK2 modulates Wnt and Hedgehog signaling (Sementino et al., 2022). These proteins are essential to brain development and are involved in ASD and brain structural anomalies (Mulligan and Cheyette, 2017; Memi et al., 2018). Variations in the GluA1 subunit of AMPA receptors, one of PAK3-specific targets, are associated with moderate to severe ID and sometimes epilepsy, ASD, ADHD and movement disorders (Ismail et al., 2022).

PAK dysregulation in neurodegenerative diseases: Cause or consequence?

Group I *PAK* dysfunctions and dysregulations, that are the root causes in several NDDs, are also involved in neurodegenerative diseases (Hayashi et al., 2004; Kreis et al., 2008; Ma et al., 2012). Numerous observations describe dysfunctions and abnormal decreases in the expression or activity of PAK during the evolution of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases, as well as some ataxias and prion diseases. These dysfunctions are often concomitant with dendritic spine abnormalities, emphasizing the question of whether PAK alteration is a cause or a consequence of synaptic

deterioration. We can summarize the data as follows. In post-mortem brains of Alzheimer's disease (AD) patients, PAK proteins are markedly reduced and their phosphorylated forms present abnormal localization, which is also correlated with abnormal cofilin activation and aggregates in neurites (Ma et al., 2008; Arsenault et al., 2013; Bories et al., 2017; Lauterborn et al., 2020). Concordant results were observed in the Tg2576 mouse model of AD which display peptide Aβ-containing plaques, but not apoptosis or microfibrillary tangles (Zhao et al., 2006). Interestingly, PAK inhibition in the brains of older mice triggers neuronal damage and cognitive deficits similar to those of the Tg2576 mice, clearly indicating a role of PAK in the Aβ pathology. It was shown in primary neuron cultures that interaction of PAK3 dominant-negative variant with the amyloid precursor protein (APP) blocks APP-mediated neuronal apoptosis in familial AD (McPhie et al., 2003). The C-terminal peptide cleaved from the APP at Asp664 may be responsible for these abnormal PAK activation (Nguyen et al., 2008). The inhibition of cofilin dephosphorylation in the Familial Autosomal dominant (FAD) mouse model of AD that express several pathogenic mutations in APP and Presinilin1 genes, decreases Aβ plaques, cellular defects and improves cognitive function, confirming the link between Aβ pathologies and actin cytoskeleton (Deng et al., 2016).

PAK1-mediated oligomerization of Huntingtin (HTT), which is independent of PAK1 kinase activity, is associated with Huntington's disease (Luo et al., 2008). Interestingly, αPIX also enhances HTT aggregation via PAK1 (Eriguchi et al., 2010). Furthermore, HTT negatively regulates PAK2 apoptotic functions by preventing PAK2 cleavage via caspases: this protective function is retained by mutated *HTT*. These studies suggest that *PAK1* and *PAK2* have completely different associations with Huntington disease, PAK1 increases the toxicity of mutated *HTT* while PAK2 possibly has protective effects (Luo and Rubinsztein, 2009). PAK1 also regulates the expression of Ataxin-1 that is involved in spinocerebellar ataxia type I, suggesting that PAK1 inhibition may be a therapeutic approach in this orphan neurodegenerative disease (Bondar et al., 2018).

Synaptic activity requires a significant energy supply, provided mainly by the mitochondria. The routing of presynaptic mitochondria is under the control of a recently identified signaling pathway, involving AMP-activated protein kinase, which is mediated by PAKs via their kinase activity (Kong et al., 2016). This pathway regulates the recruitment of mitochondria and their anchoring to the vicinity of presynaptic terminals via myosin VI phosphorylation (Li et al., 2020). Defects in energy metabolism are also features of neurodegenerative diseases and could involve this AMPK/PAK/MYOVI pathway. It is interesting to note that the hippocampus, a key brain region for memory formation, is one of the cerebral structures most sensitive to hypoxia. However, the involvement of PAKs in this respect has not been documented (Schmidt-Kastner, 2015). PAK1 and PAK3 are also down-expressed in the post-mortem brains of Parkinson patients. In conclusion, alterations in PAK expression and activity are also involved in neurodegenerative pathologies, associated with excessive apoptosis, energy deficit, and dendritic spine shrinkage.

In summary, these numerous observations describe PAK down-expression, dysfunction, mislocation, and dysregulation throughout the progression of neurodegenerative diseases. PAKs are at the center of three signaling pathways involved in neurodegeneration, namely the regulation of dendritic spine dynamics, apoptosis and neuronal energy homeostasis. However, signaling pathways that link together synaptic defects, energy balance dysregulations, and abnormal neuronal death are complex and the role of PAK defects in these diseases remains unclear. A seminal experiment in mice showed that PAK inhibition causes cofilin anomalies and memory impairments similar to AD defects, strongly suggesting a causal role of PAK dysfunctions in AD-associated cognitive deficits (Zhao et al., 2006). Are PAK dysfunctions a cause in the emergence of neurodegenerative diseases in the patients with NDDs? At the moment, the absence of published prospective clinical follow up of patients with *PAK*-associated conditions prevents us from knowing the incidence of neurogenerative diseases on these patients. This has not been addressed by experimental approaches either. While the role of PAKs is well documented in worsening actin-dependent synaptic dysfunctions in AD, their role in apoptosis and energy failure during neurodegenerative disease progression is still elusive. Moreover, although several isoform specificities have been observed, the role of each PAK in these pathologies is still unclear. Nevertheless, recent data suggest that the activation of the acetylcholine muscarinic receptor improves aversive memory in striatal and accumbens nuclei via a Protein kinase C/Rac1/PAK pathway and may thus be a new therapeutic approach, at least for this type of behavioral symptoms (Yamahashi et al., 2022).

Experiments in PAKs studies: Finding the right model

These recent and scarce data open the way toward a new understanding in the etiology of many cerebral pathologies. Yet, there is still much to uncover in the field of *PAK*-associated disorders. A deeper exploration of each *PAK* specificity, a more thorough investigation of the molecular mechanisms underlying *PAK* mutation pathogenicity and adapted therapeutic strategies are needed to improve diagnosis and treatment in *PAK*-related disorders. After the discovery of *PAK*s in 1994 (Manser et al., 1995) and after establishing their role in the regulation of cytoskeleton dynamics (Sells et al., 1997, 1999), studies conducted in cell and neuronal cultures helped characterizing PAKs regulation (Manser et al., 1998; Banerjee et al., 2002; Brown et al., 2002) and functions in migration (Causeret et al., 2009), neurite outgrowth (Daniels et al., 1998), spine morphogenesis, synapse formation and plasticity (Boda et al., 2004; Hayashi et al., 2007; Kreis et al., 2008; Dubos et al., 2012). Other studies have been conducted in animal models, widely used to understand mechanistic aspects of neurodevelopmental pathogenesis. Such models can be of translational significance or bring insight into fundamental mechanisms involved in NDDs (Fallah and Eubanks, 2020; Mariano et al., 2020; Rotaru et al., 2020). Even though some *PAK* studies have been conducted in non-mammalian models (Santiago-Medina et al., 2013; Magini et al., 2014), most are carried out in mouse models. Thus, *Pak1*-knockout mice have been generated and display MAPK signaling defects (Arias-Romero and Chernoff, 2008) and, accordingly, present slight immune

deficiencies (Allen et al., 2009). LTP deficits and a decrease in NMDA activity-induced cofilin phosphorylation have also been identified in *Pak1*-KO mice (Asrar et al., 2009). The fact that the phenotype of *Pak1* and *Pak3* KO mice are relatively mild, while the double *Pak1/3* KO generates substantial cognitive loss, brain morphological abnormalities, as well as functional and structural synaptic defects suggests that these two isoforms have redundant functions allowing functional complementation (Meng et al., 2005; Asrar et al., 2009; Huang et al., 2011). Nevertheless, all *PAK1* variations generate GOF that cannot be compensated by other isoforms.

Pak2-knockout mice are not viable (Arias-Romero and Chernoff, 2008). However, $Pak2^{+/-}$ mice have been studied and display ASD-related behaviors and reduced spine density, defective LTP and impaired actin polymerization (Wang et al., 2018), which is concordant with the effects of *Pak2* haploinsufficiency in humans. The high PAK2 expression during embryonic development, the lethality of its gene invalidation and its role in the establishment of cerebral vascularization, are in agreement with the fact that bi-allelic loss have not been identified in patients. However, it is surprising that no variant with gain of function has not been identified yet.

The deficits in late-LTP and CREB activity in the brain of *Pak3*-KO mice indicate a more important role of PAK3 in the actin-independent regulation of plasticity-associated genes (Meng et al., 2005). Interestingly, double *Pak1/Pak3*-knockout mice display hyperactivity, anxiety, learning and memory deficits, secondary microcephaly, reduced synaptic density but increased size of individual synapses and increased basal synaptic transmission, disrupted LTP and LTD, decreased cofilin regulation and reduced CREB activity (Huang et al., 2011). PAK1 displays a higher expression during development than in adulthood, in contrast to PAK3, suggesting that PAK3 has more developmental functions.

Thus, the global inhibition of PAKs in post-mitotic neurons of mice forebrain via the expression of the PAK kinase activity-inhibiting AID (*dnPak*) cause an increase in dendritic spine size, enhanced synaptic transmission and LTP as well as LTD deficits in the cortex (Hayashi et al., 2004). Consequently, mice expressing *dnPAK* displayed memory deficits. One can also wonder whether *Pak3*-AID only blocks kinase-activity-dependent functions, leaving PAK signaling at least partially functional. The AID of PAK3 was also used to post-natally block PAK activity in regions associated with social functions, which impaired social memory retrieval (Leung et al., 2018; Zhou and Jia, 2021). These models shed light on the roles of PAKs and how PAK disruption might affect brain functions. However, they only represent the loss of PAK proteins (in the case of *Pak*-knockouts) or loss of PAK kinase function (in the case of PAK inhibition via dn*Pak*) while *PAK1* mutations are usually gain of functions. There are also differences in *PAK3* nonsense mutations, that might lead to a complete loss of protein, and *PAK3* missense mutations that would have more variable effects.

New models, such as the knock-in mouse line which expresses the *Pak3*-Arg67Cys mutation, responsible for a moderate to severe ID in humans are expected to mimic more closely the precise biochemical alterations caused by PAK mutations. Thus the effects of a PAK3 variant displaying functional kinase activity, but with changes in partner interactions *in vitro*, has been studied and furthers our understanding pathogenic mechanisms underlying *PAK3* pathogenicity *in vivo*. The PAK3-Arg67Cys variant impacts newly generated neurons circuit integration in the adult mouse hippocampus, thus disturbing complex memory tasks performance such as pattern separation (Castillon et al., 2020). Through the study of animal models bearing *Pak* mutations, directly linking behavioral symptoms and brain dysfunctions to molecular mechanisms is now achievable. However, as shown in **Figure 5**, gene expression might not be the same between animal models and humans. Thus, patient-derived cell models are important to complement animal studies. PAK1 variants have been proposed to display high kinase activity in patient fibroblasts (Harms et al., 2018). A similar study was conducted in keratinocytes, which have a higher level of *PAK3* expression than fibroblasts (Magini et al., 2014). Obviously, the development of iPSCs from patient fibroblasts and models of embryonic bodies would help put together a more elaborated analysis of *PAK* mutation-dependent cell abnormalities. For example, the first data obtained for Down syndrome, the most frequent cause of ID, confirms the hyperactivation of the DsCam/PAK1 module in patient-derived iPSCs and highlight the possibility to target PAKs in this disease (Tang et al., 2021).

PAKs as therapeutic targets in brain diseases

Researchers are confronted with the contradiction of wanting, on one hand, to target a molecule in a strategy applicable to many psychiatric and neurodevelopmental disorders, and on the other hand, to develop approaches applicable to specific cases, with the perspective of personalized medicine. Cofilin which is a focal point of the Rho GTPase pathway, is a therapeutic target that has already been validated to correct behavioral and synaptic plasticity defects in several NDD models (Shaw and Bamburg, 2017). PAKs are also points of convergence and therefore potential therapeutic targets. Indeed, since the phenotypes associated with some NDDs appear to be due to exacerbations of the Rac/Cdc42/PAK pathway activity, PAK inhibition may be an interesting therapeutic approach. Several PAK inhibitors were developed and display high affinity and selectivity against group I-PAKs (Semenova and Chernoff, 2017). First evidence of this concept is that the expression of an autoinhibitory domain of PAK3, which targets all three group I PAKs improves the cellular and behavioral phenotype of Fmr1 KO mice, a model of Fragile X syndrome (Hayashi et al., 2007). Interestingly the bioavailable and brain–permeant catalytic inhibitor of group I PAK, FRAX486, rescues the spine defects, seizures, stereotypical behavior, and hyperactivity displayed by *Frm1*-KO mice (Dolan et al., 2013). FRAX486 also rescues neurobehavioral alterations in a mouse model of CDKL5 disorders, probably via the regulation of the complex between the main CDKL5 substrate ARHGEF2 and PAK1 (Zenke et al., 2004; Fuchs et al., 2022). PAK over-activation was also observed in neurons cultured from *Disc1*-KO mice, a mouse model of schizophrenia. DISC1 downregulates Kalirin7, a potent RAC1-activating GEF, upon NMDAR activation (Hayashi-Takagi et al., 2014). PAK inhibition using FRAX486 prevents DISC1 RNAi-induced spine deterioration (prophylactic effect) and reverses already existing spine deterioration triggered by DISC1 RNAi (treatment effect) in neuronal cultures. It also partially recues spine morphology in adult mice (Hayashi-Takagi et al., 2014). In the case of neurofibromatosis

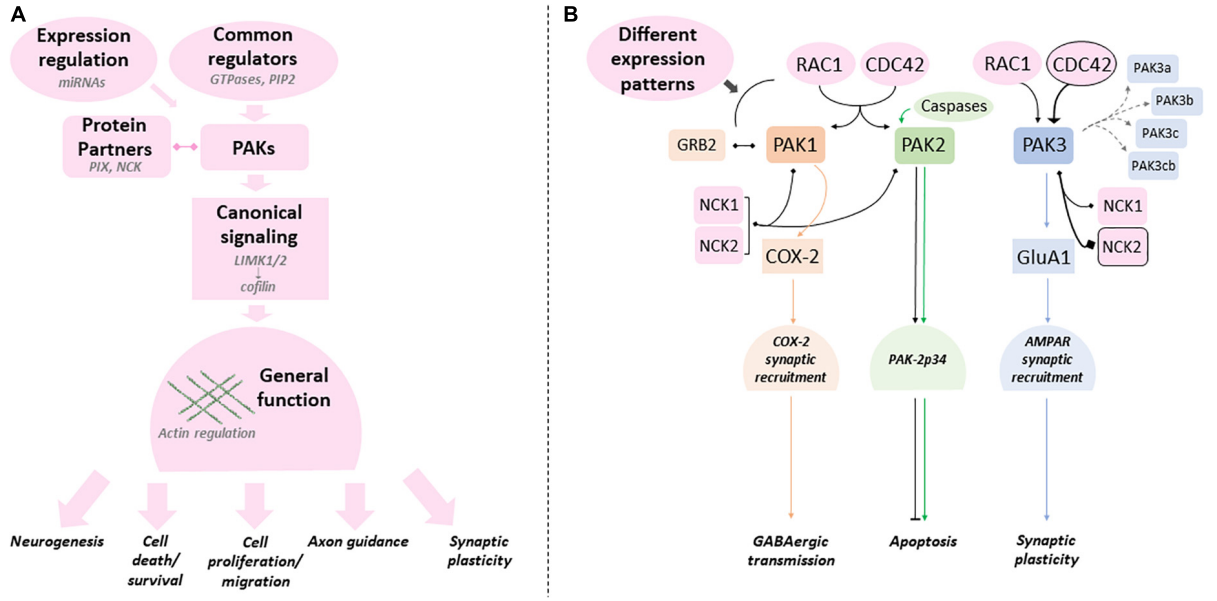

FIGURE 6
Canonical and specific PAK-dependent processes **(A)** Group I PAKs share several regulatory mechanisms, protein partners and substrates (canonical processes in pink - examples in light gray). *PAK* mutations can affect any of these processes. **(B)** Several studies emphasize the differences between PAKs (canonical processes in pink can still present quantitative differences). PAKl (orange), PAK2 (green) and PAK3 (blue) were shown to have their own specificities (colors matching the corresponding PAK). Protein partners and regulators that are favored by PAK are emphasized with a black frame. Mutations can have different effects on these specific processes and explain the variations found between PAKs and intragenic variability.

type 1 (NF1) there is also cognitive and learning disabilities that are associated to MAP Kinase activation and dendritic spine regulation. PAK1 gene invalidation or pharmacological inhibition of PAK by intracerebral injection of the IPA3 inhibitor restores social interaction and memory deficits (Molosh et al., 2014). PAK1 activity is increased in a mouse model of hereditary demyelinating neuropathy of demyelination, in which disease progression is associated with an increase of actin polymerization at the nerve myelin junction (Hu et al., 2016). Pharmacological inhibition of PAK using PF3758309 normalized levels of F-actin, completely prevented the progression of the myelin junction disruption, and restored nerve conduction.

Several PAK inhibitors target the ATP binding site of the catalytic domain via a competitive mechanism (FRAX compounds, PF3758309), but the high identity of peptide sequences within the catalytic domain prevents poor PAK-isoform specificity of inhibitors toward the three PAK isoforms. Another inhibitor, the IPA-3 compound, is a brain-permeant allosteric inhibitor that binds to the PDB/AID, impairs GTPase binding, and thus prevents group I-PAK activation but has no effect on already activated kinases (Deacon et al., 2008). However, recently, a compound (NVS-PAK1-1) that limits the PAK access to ATP was characterized as the first PAK1 specific inhibitor, especially compared to PAK2 (Semenova and Chernoff, 2017). It would be interesting to test to what extent these PAK inhibitors could constitute therapeutic approaches in cases where mutations confer kinase activity over-activation (6/23). However, for the majority of variants (15/23), mutations in the *PAK* genes lead to the loss of kinase activity (**Figure 3C**). In these situations, it would probably be more appropriate to target a downstream molecule, such as cofilin.

Conclusion and perspectives

PAK mutations are rare events but their genetic traceability as monogenic defects, their total penetrance, the expanding collection of human mutations and curated phenotypes, make the study of these genes ideal to discover the biological mechanisms that go awry in common human NDDs. The exploration of fundamental and novel biological processes can greatly benefit from the study of rare genetic disorders (Lee et al., 2020). Group I PAKs occupy a central place in neuron physiology by being RAC1/CDC42 downstream effectors as well as the convergence point of many other pathways that control cell fate, motility, cell proliferation and apoptosis. Furthermore, an increasing number of experiments also indicate that PAKs are heavily involved in synaptic transmission and plasticity. Nevertheless, clinical data and fundamental experiments in controlled paradigms strongly suggest that these kinases present both redundant and isoform-specific functions essential for the development and processes of the brain and other organs (**Figure 6**).

In summary, *PAK1* mutations are dominant gain of function, *PAK2* mutations generate haploinsufficiency, whereas *PAK3* pathogenic variations have various functional consequences. The genotype/phenotype correlation is relatively easy to comprehend for the first two genes, associated with developmental delay and macrocephaly or with ASD, respectively. However, this relation remains to be established for PAK3. The main goal is to identify the molecular and cellular processes at the foundation of the differences between each isoform genotype/phenotype correlation. Why *PAK1* mutations are GOF and *PAK2* mutations are LOF is elusive, and there is still a gap preventing the full comprehension of the link between isoform-specific functions

and the phenotypes associated with mutations in each *PAK* gene. More specifically, the biochemical alterations caused by different *PAK* mutations are still understudied. It is necessary to obtain a deeper understanding of the expression, localization, regulation, substrates and partners of each *PAK* isoform in the more global context of PAK interactome in order to gauge the impact of mutations on these processes. As listed above, several publications already demonstrate the role of these kinases in several neurological disorders such as Fragile X, neurofibromatosis, Down syndrome and several rare diseases caused by synaptic gene mutations, but also in neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's and some ataxias. With very few exceptions, it does not currently seem possible to decipher whether the three PAKs underlie the same dysfunctions in some or all of these neurological diseases. Understanding this is a challenge for future translational studies. It is perhaps also interesting to note that PAKs are associated with COVID pathologies, in particular *PAK1* in the virus infection process and *PAK3* in certain immunological signatures of patients (Yu et al., 2021; Zhang K. et al., 2022). This could be linked to the neurological damage associated with the long forms of COVID-19 (Monje and Iwasaki, 2022). Many experimental approaches already brought a proof of the concept that group I *PAK*s are interesting therapeutic targets in NDDs and neuropsychiatric disorders. The joint effort in the fields of cancerology, neurodegenerative disorders, rare and neurodevelopmental diseases will greatly advance our knowledge on *PAK* functions, dysfunctions and adapted therapeutic strategies in the view of personalized treatments. One of the remaining weaknesses in the study of the genotype/phenotype relationship in *PAK*-associated diseases is the rarity of reported cases. However, the efforts of clinicians and geneticists to implement clinical databases provide access to more genetic and clinical data, thus allowing the genotype/phenotype correlation to be established more precisely. Clinician and researcher collaborations, improved experimental models, precise biochemical characterization of variants and conceptual frameworks in the context of convergent neurosciences could be the missing stone to fully comprehend the biological mechanisms underlying mutation pathogenicity and to successfully translate research findings into clinical practices (Willsey et al., 2017; Srivastava et al., 2018).

Author contributions

MD and J-VB conceptualized the review and wrote it. AV and AT supervised the clinical data. SP-G and VR followed the successive steps of writing with their advice and corrections. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Oxytocin receptors in the Magel2 mouse model of autism: Specific region, age, sex and oxytocin treatment effects

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The neurohormone oxytocin (OXT) has been implicated in the regulation of social behavior and is intensively investigated as a potential therapeutic treatment in neurodevelopmental disorders characterized by social deficits. In the *Magel2*-knockout (KO) mouse, a model of Schaaf-Yang Syndrome, an early postnatal administration of OXT rescued autistic-like behavior and cognition at adulthood, making this model relevant for understanding the actions of OXT in (re)programming postnatal brain development. The oxytocin receptor (OXTR), the main brain target of OXT, was dysregulated in the hippocampus of *Magel2*-KO adult males, and normalized upon OXT treatment at birth. Here we have analyzed male and female *Magel2*-KO brains at postnatal day 8 (P8) and at postnatal day 90 (P90), investigating age, genotype and OXT treatment effects on OXTR levels in several regions of the brain. We found that, at P8, male and female *Magel2*-KOs displayed a widespread, substantial, down-regulation of OXTR levels compared to wild type (WT) animals. Most intriguingly, the postnatal OXT treatment did not affect *Magel2*-KO OXTR levels at P8 and, consistently, did not rescue the ultrasonic vocalization deficits observed at this age. On the contrary, the postnatal OXT treatment reduced OXTR levels at P90 in male *Magel2*-KO in a region-specific way, restoring normal OXTR levels in regions where the *Magel2*-KO OXTR was upregulated (central amygdala, hippocampus and piriform cortex). Interestingly, *Magel2*-KO females, previously shown to lack the social deficits observed in *Magel2*-KO males, were characterized by a different trend in receptor expression compared to males; as a result, the dimorphic expression of OXTR observed in WT animals, with higher OXTR expression observed in females, was abolished in *Magel2*-KO mice. In conclusion, our data indicate that in *Magel2*-KO mice, OXTRs undergo region-specific modifications related to age, sex and postnatal OXT treatment. These results are instrumental to design precisely-timed OXT-based therapeutic strategies that, by acting at specific brain regions, could modify the outcome of social deficits in Schaaf-Yang Syndrome patients.

KEYWORDS

neurodevelopmental disorders (NDD), Schaaf-Yang Syndrome, Prader-Willi Syndrome (PWS), postnatal oxytocin treatment, oxytocin receptor expression

Introduction

Oxytocin (OXT) is a small neuropeptide released by the hypothalamus into the bloodstream to control lactation and parturition and in the brain to control several aspects of behavior, such as emotional and social processing (Jurek and Neumann, 2018).

The action of OXT within the brain is mediated by OXT binding to a specific receptor, the oxytocin receptor (OXTR) (Busnelli and Chini, 2018). In some conditions, for example in the presence of supraphysiological OXT concentrations, OXT can also activate the highly related vasopressin 1a and 1b receptors (V1aR and V1bR) (Chini et al., 2017). OXTR is a G-protein coupled receptor expressed in several areas of the brain, and one of its most compelling features is the extreme variability in its regional distribution within the brain, which has been shown to be linked to a number of factors, including the species, sex and developmental age, as well as several environmental influences.

A highly variable regional distribution of OXTR is observed in mammals, even between closely-related species (Walum and Young, 2018), originally described in the prairie and montane voles, where different OXTR distributions relate to striking differences in social behavior (Insel and Shapiro, 1992; Young and Wang, 2004). Region-specific sex differences have also been reported in different species including mice (Hammock and Levitt, 2013; Sharma et al., 2019; Newmaster et al., 2020). Age is another well-established factor influencing OXTR levels, and the OXTR distribution in the brain undergoes dynamic changes in expression through the postnatal development, with peak expression at early infancy in rodents and humans (Tribollet et al., 1989; Hammock and Levitt, 2013; Vaidyanathan and Hammock, 2017; Prounis et al., 2018; Newmaster et al., 2020).

One relevant issue in the field is how OXT and OXTR levels in the early postnatal life influence the development of social abilities during infancy, adolescence and adult life and, most importantly, how these levels are modulated. A large body of literature, pioneered by Karen Bales and co-authors, has clearly shown that early-life experience has long-term effects on the OXT system, including the expression of OXTR (Bales and Perkeybile, 2012; Veenema, 2012; Perkeybile et al., 2019; Lapp et al., 2020). Mechanistically, it has been shown that environmental factors, particularly during early infancy, can epigenetically modify the OXTR gene and influence its expression levels at adulthood (Carter et al., 2020; Onaka and Takayanagi, 2021).

Early modulation of OXTR levels is of particular importance when considering neurodevelopmental disorders, many of which are characterized by deficits in social abilities and social cognition e.g. autism spectrum disorders (ASD) and schizophrenia. Several mouse models of neurodevelopmental disorders present abnormalities in OXT release and/or OXTR distribution (Meziane et al., 2015; Wagner and Harony-Nicolas, 2018; Borie et al., 2021), providing a strong rationale for the use of OXT as a possible therapeutic agent.

The *Magel2*-knockout (*Magel2*-KO) mouse has proven to be extremely useful to study the role of OXT and OXTR in a mouse model presenting neurodevelopmental impairments (Fountain and Schaaf, 2015; Meziane et al., 2015; Muscatelli et al., 2018). These mice lack *Magel2*, a gene contained in the Prader-Willi Syndrome (PWS) locus, an imprinted chromosomal region also known as "PWS paternal-only expressed region" (Butler et al., 2019). In humans, the specific lack of expression of the *MAGEL2* gene, causes a Prader Willi-like disease identified as Schaaf-Yang Syndrome (SYS; OMIM 615547) (Schaaf et al., 2013). In addition to many pathological PWS phenotypic traits, such as neonatal hypotonia, hypogonadism and feeding problems, this syndrome also presents a higher prevalence of autism spectrum disorders (up to 75% of affected individuals) (Schaaf and Marbach, 1993, updated in 2021).

Magel2-deficient mice recapitulate autistic-like symptoms and other defects observed in SYS patients. In particular, neonate *Magel2*-KO mice show feeding defects due to an altered onset of suckling activity, leading to neonatal growth retardation and a high mortality rate (approx. 50%) (Schaller et al., 2010); feeding defects are accompanied by low rates of separation-induced vocalizations and altered spectral features (Bosque Ortiz et al., 2022). The surviving adult *Magel2*-KO mice have been extensively investigated for alterations in sensory-motor, cognitive and social abilities. No differences were found between WT and *Magel2*-KO female mice, while altered spatial learning and social recognition memory were found in male *Magel2*-KO mice (Meziane et al., 2015; Bertoni et al., 2021). A reduction in mature OXT, with the accumulation of intermediate forms of the peptide, was reported in the neonate *Magel2*-KO hypothalamus, suggesting an impaired processing of the prohormone (Meziane et al., 2015); in contrast, a significant increase (2-fold) of mature OXT was found in the hypothalamic-hypophyseal system of adult *Magel2*-KO mice, accompanied by an increased number of OXT-expressing neurons (Meziane et al., 2015), representing a possible compensatory mechanism to overcome the strong suppression of the electrophysiological activity observed in OXT-expressing neurons (Ates et al., 2019). Most importantly, an early postnatal OXT treatment was demonstrated to rescue neonatal lethality and to prevent the appearance of social and learning deficits in adult *Magel2*-KO mice (Meziane et al., 2015; Bertoni et al., 2021), providing strong preclinical evidence for pilot studies of OXT treatment in PWS and SYS infants, such as that conducted in PWS babies showing encouraging positive results (Tauber et al., 2017).

More recently, a detailed investigation of the molecular bases of social memory impairments in *Magel2*-KO males revealed specific alterations in hippocampal circuitry and functions (Bertoni et al., 2021). In particular, *Magel2*-KO adult mice display an increased OXTR expression in the dorsal CA2/CA3 (dCA2/CA3) and in the Dentate gyrus (DG), but not in the ventral vCA2/CA3 region (vCA2/CA3) of the hippocampus. Moreover, postnatal OXT normalized OXTR in DG, but not in the dCA2/CA3 region (Bertoni et al., 2021). These findings strongly suggest that region specific alterations in receptor expression are present in *Magel2*-KO mice, and that postnatal OXT treatment could modulate OXTR in specific brain regions.

In the present work we extended the regional mapping of OXTR in male and female *Magel2*-KO brains, with or without treatment of OXT during the first week of postnatal life. As OXTRs are strongly regulated in mice in the first three weeks after birth (Hammock and Levitt, 2013; Newmaster et al., 2020), we investigated if the OXT treatment received in the first week of life had short and/or long term impact on regional OXTR levels. Understanding the specific sites within the brain where OXT exerts its rescue action is a fundamental step to strengthening the rationale for its use in PWS/SYS and further neurodevelopmental disorders.

We also evaluated the sexual dimorphism of OXTR distribution in *Magel2*-KO mice and looked for sex-specific modulation of OXTR by postnatal OXT treatment. Autism-related disorders are characterized by a strong sex bias, with a male to female ratio among affected individual close to 4:1 (Ferri et al., 2018). Understanding the molecular basis of sex differences could contribute to understanding the fundamental mechanisms of the biology of autism itself.

Materials and methods

Animals

Magel2 $^{tm1.1Mus\ +/+}$ (referred to as WT) and *Magel2* $^{tm1.1Mus\ -/-}$ (referred to as *Magel2*-KO) mice (Bertoni et al., 2021) were maintained on a C57BL/6J genetic background and housed in standard conditions, with *ad libitum* access to food and water. Mice were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (N.R.C., 2011) and the European Communities Council Directive of September 22 2010 (2010/63/EU, 74). All the experimental procedures were approved by the French Ministry of Agriculture with the accreditation no. B13-055-19. The protocol included 6 experimental groups (Group 1-6) for brain autoradiography, each composed of 3 males and 3 females, for a total of 36 animals and 3 experimental groups (Group 7-9) for ultrasonic vocalization (USVs) analysis, each composed of males and females, for a total of 100 animals, 53 males and 47 females.

Group 1: WT mice treated with saline and sacrificed at P8; Group 2: WT mice treated with saline and sacrificed at P90; Group 3: *Magel2*-KO mice treated with saline and sacrificed at P8; Group 4: *Magel2*-KO mice treated with saline and sacrificed at P90; Group 5: *Magel2*-KO mice treated with OXT and sacrificed at P8; Group 6: *Magel2*-KO *mice* treated with OXT and sacrificed at P90; Group 7: WT mice treated with saline and tested for USVs at P8; Group 8: *Magel2*-KO mice treated with saline and tested for USVs at P8; Group 9: *Magel2*-KO mice treated with OXT and tested for USVs at P8. The day of birth was considered postnatal day 0 (P0).

A *post-hoc* power calculations was performed on the autoradiography datasets recently published (Bertoni et al., 2021). Such analyses was run with the G*Power 3.1 software (RRID:SCR_013726, University of Düsseldorf).[1] The effect size f was derived from the available datasets (1.83 for DG and 1.66 for dCA2/CA3), and transferred into a *post-hoc* ANOVA (Fixed effects, omnibus, one-way) test with set number of groups = 3, total sample size = 9 and alpha error = 0.05. We then calculated the effective power achieved in Bertoni et al. (2021) for the DG and the dCA2/CA3, which were 0.97 and 0.93, respectively, thus indicating that 3 mice/group are sufficient for this type of analysis.

Treatments

Three to five hours after delivery, pups were subcutaneously injected with saline or OXT (Phoenix Pharmaceuticals. Inc., Strasbourg, France; Catalog No.051-01) dissolved in isotonic saline at a final concentration of 0.2 μg/μl (20 μl/injection). Four administrations of OXT or saline (indicated as Vehicle and abbreviated to Veh) were given subcutaneously every 2 days (at P0, P2, P4, and P6) to male and female *Magel2*-KO mice immediately after birth (**Figure 1A**). The dose and administration timings were selected on the basis of previous work showing that this administration protocol prevented social and learning deficits in adult *Magel2*-KO mice (Bertoni et al., 2021).

Brain collection and slice preparation

At P8 or P90, mice were sacrificed, the brains quickly extracted, flash-frozen by dipping in cold (-25°C) isopentane (Sigma Aldrich) and stored at −80°C until processing. 14 um coronal sections were collected using a Frigocut-2700 (Reichert-Jung) cryostat and collected on gelatin/chromium potassium sulfate-coated Superfrost slides.

Receptor autoradiography and ROI analysis

Quantification of OXTR was performed by receptor autoradiography according to the protocol originally developed by E. Tribollet (Tribollet et al., 1989). Brain sections were lightly fixed by dipping the slides for 5 min in a solution of 0.2% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4), then rinsed twice in 50 mM Tris–HCl buffer (pH 7.4) supplemented with 0.1% bovine serum albumin. Incubation was carried out for 2 hr under gentle agitation at room temperature in a humid chamber (kindly donated by E. Tribollet) by covering each slide with 400 μl incubation medium (50 mM Tris–HCl, 0.1 mM bacitracin, 5 mM $MgCl_2$, 0.1% bovine serum albumin) containing 0.02 nM final concentration of radioiodinated OXTR antagonist [^{125}I]d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr^9-NH_2]OVT (^{125}I-OVTA), Perkin Elmer, MA, USA (Elands et al., 1988a,b). The specific activity of radioligand was 2200 Ci/mmol; 1Ci = 37GBq. Non-specific binding was evaluated by incubating adjacent sections with incubation medium containing 0.02 nM ^{125}I-OVTA and 2 μM OXT. Incubation was followed by two 5 min washes in ice-cold incubation medium and a quick rinse in distilled water. The slides were rapidly dried under a stream of cool air. Once dry, the slides were placed in an X-ray cassette in contact with Biomax MR Films (Carestream, USA, #891–2560). After 5 days of exposure each film was developed and scanned for image analysis.

Regions of Interest (ROIs) were designed using the Franklin and Paxinos' Mouse Brain Atlas (Franklin and Paxinos, 2007) as reference. Regions analyzed include: the piriform cortex (Pir), the medial preoptic area (MPOA), the lateral septum (LS), the ventromedial nucleus of the hypothalamus (VMH), the amygdala in its basolateral (BLA), medial (MeA), and central (CeA) subdivisions, the hippocampus in its dorsal (dCA2/CA3), ventral (vCA2/CA3) and Dentate Gyrus (DG) regions and the paraventricular nucleus of the thalamus (PVT) (**Figure 1B**). Densitometric gray level were measured using NIH ImageJ software. To obtain single ROI values, the gray level of the film

1 http://www.gpower.hhu.de

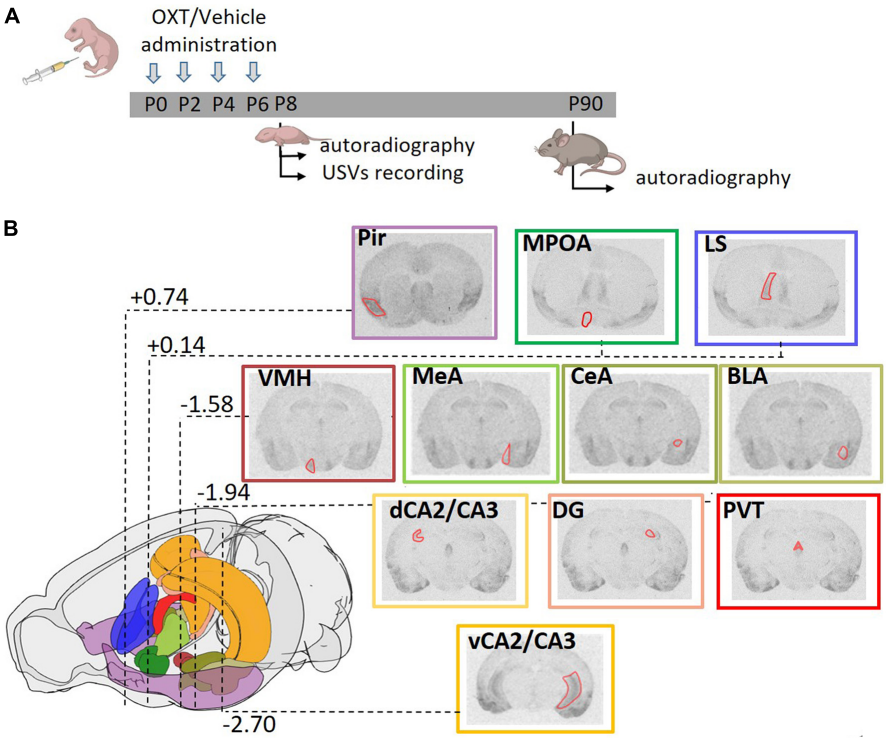

FIGURE 1
Experimental strategy. **(A)** Schematic diagram representing the treatment regime administered to the mice and the paradigm of the analysis performed. Mice were subcutaneously injected with OXT or vehicle (single injection/day) in the first week of life at P0, P2, P4, and P6. Brain autoradiography was performed at P8 or P90. A separate group of animals at P8 was tested for ultrasonic vocalizations (USVs). **(B)** Schematic representation of the mouse brain showing the stereotactic coordinates of the coronal planes in which OXTR were analyzed. Distance from bregma, reported in millimeters according to Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 2007), are highlighted for the most representative section. A color code was used to identify the different areas analyzed: violet: Pir, piriform cortex; emerald green: MPOA, medial preoptic area; blue: LS, lateral septum; brown: VMH, ventral medial nucleus of the hypothalamus; three values of olive green for MeA, medial amygdala; CeA, central amygdala; BLA, basolateral amygdala; orange: dCA2/CA3, dorsal and vCA2/CA3, ventral field CA2 and CA3 of the hippocampus; pink: DG, dentate gyrus; red: PVT, paraventricular thalamic nucleus. Within each representative autoradiographic section, whose border color follows the color code reported above, the ROI used for acquisition of the data is depicted in red.

(corresponding to background) was subtracted to the gray level of a ROI on a slice incubated with ^{125}I-OVTA (corresponding to total binding) and to the gray level of a ROI on an adjacent slice incubated with ^{125}I-OVTA + OXT (corresponding to non-specific binding); the obtained densitometric gray level values of total and non-specific binding were then converted to nCi/mg tissue equivalent using an autoradiographic iodinated ^{125}I-microscales (Amersham) on films exposed for 5 days (kindly provided by E. Tribollet). The specific binding value of each ROI was finally obtained by substracting non-specific binding to total binding. For each animal, at least 3 ROIs for each region were acquired (adjacent slices and/or left and right hemisphere areas on the same slice for bilateral regions).

Ultrasonic vocalization (USVs)

Briefly, the mother and litter were left to habituate to the testing room for 30 min, then P8 pups were separated from the mother and gently transferred to a new cage on a heating pad (37°C). After 5 min, each pup was transferred in an anechoic box (54 × 57 × 41 cm; Coulbourn instruments, PA, USA) and USVs were immediately recorded for 300 s by an ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16/CMPA, Avisoft bioacustic, Germany) sensitive to frequencies of 10-250 kHz. Recordings were done using Avisoft recorder software (version 4.2) with a sampling rate of 250 kHz in 16-bit format. Data were analyzed for the total number of calls using Avisoft SASLab software.

Statistical analysis

All graphs have been created and datasets analyzed with GraphPad Prism ver. 8.0.2 (GraphPad Software, Inc.). Molecular data were analyzed by analysis of variance (ANOVA). In particular, for the combined analysis of age, sex and genotype, for each brain area a three-way ANOVA was applied, followed by Tukey's multiple comparisons *post-hoc* test. Similarly, for the analysis by region of the combination of age, sex and OXT treatment, three-way ANOVA was applied, followed by Tukey's multiple comparisons *post-hoc* test. For treatment efficacy, two-way ANOVA followed by Tukey's multiple comparisons *post-hoc* test was applied, to account and correct for multiple testing. Adjusted p-values of $p < 0.05$ were deemed significant. USVs data were analyzed by One-way ANOVA followed by Tukey's multiple comparisons *post-hoc* test.

Significance was set at p-values of $p < 0.05$. Bars in the graphs display values as mean ± SEM.

The complete analysis of the all statistical data included in Figures 2-5 is reported in the Supplementary Tables 1-4.

Results

We have previously found a dysregulation of OXTRs in the hippocampus of male *Magel2*-KO mice that was normalized at adulthood by postnatal OXT treatment (Bertoni et al., 2021). Here, as outlined in Figure 1A, we designed a new study which also included female mice and the analysis of other brain regions relevant for OXT actions (reported in Figure 1B). *Magel2*-KO mice were treated during the 1st postnatal week and OXTR brain expression was analyzed at an early postnatal developmental stage (P8) and in adults (P90) (Figures 1A, B).

The possible interaction between sex, genotype and age in determining OXTR brain expression variations was analyzed in male and female WT and *Magel2*-KO mice at P8 and P90. A three-way ANOVA analysis was performed on OXTR levels measured in WT and *Magel2*-KO brains postnatally treated with saline. All statistical data of time x sex x genotype analysis are reported in the Supplementary Table 1.

As shown in Figure 2, we found that, at P8, OXTR expression in *Magel2*-KO brains was significantly reduced in all regions analyzed as compared to control WT animals, in both males and females; in only two regions, the trend toward a reduction did not reach a statistically significant value (male dCA2/CA3 hippocampus $p = 0.052$; males VMH $p = 0.26$ and females VMH $p = 0.079$). At this developmental stage, male and female WT animals expressed similar levels of OXTR in all areas, and, similarly, no sexually dimorphic areas for OXTR expression were found at P8 in *Magel2*-KO animals.

At P90, as compared to WT, adult *Magel2*-KO males displayed up-regulated OXTR levels in the amygdala (CeA and BLA) and in the hippocampus (dCA2/CA3 and DG). In contrast, a down-regulation of OXTR levels was observed in *Magel2*-KO females in the Pir, MPOA, LS, MeA and vCA2/CA3. At this age, in WT animals, a significant sexual dimorphic OXTR expression, with higher levels in females, was observed in all regions investigated with the only exception of the PVT. In *Magel2*-KO mice, male/female differences were only present in the Pir (where higher OXTR levels were measured in males) and in the VMH (where higher OXTR levels were observed in females); in all the other regions investigated *Magel2*-KO mice lost the sexual dimorphism in OXTR levels.

Finally, the analysis of the OXTR expression by genotype at the two different ages (P8 and P90) indicated a widespread age-dependent reduction of OXTR levels in WT animals, particularly in males. The only regions in which OXTR remained elevated in adult males were the hippocampus (dCA2/CA3 and vCA2/CA3) and the VMH. Notably, these are the same regions in which a substantial up-regulation was observed at P90 in WT female brains. In contrast, in *Magel2*-KO male mice, OXTR levels at P90 did not decrease as compared to the levels observed at P8, but remained stable (LS, MeA, CeA, BLA, VMH, and PVT) or, in some cases, increased (Pir, vCA2/CA3, DG and dCA2/CA3); the only region in which a reduction was observed was the MPOA; a similar trend was also observed in the *Magel2*-KO female mice. These results indicate a substantial alteration in the postnatal developmental patterns of OXTR expression in the *Magel2*-KO mice that presented low levels of OXTR at P8 and failed to reach physiological OXTR levels at P90.

We then investigated if postnatal OXT treatment could normalize the altered OXTR levels observed in *Magel2*-KO mice. Figure 3 reports the results of a three-way ANOVA analysis of OXTR binding levels in male and female *Magel2*-KO brains at P8 and P90 treated with OXT or Veh. All statistical data of time x treatment x sex analysis are reported in Supplementary Table 2.

With respect to OXT treatment, no differences were observed at P8 in any of the investigated areas in *Magel2*-KO males and females, with the only exception of a down-regulation of OXTR in male MPOA and an up-regulation of OXTR in female PVT. These results indicate that the postnatal administration of OXT (with the administration schedule used here) had no widespread short-term effects on OXTR levels in the *Magel2*-KO brain at this particular developmental stage.

In adult *Magel2*-KO mice, OXT treatment down-regulated OXTR levels in selected regions in males (Pir, CeA, dCA2/CA3, DG) and females (dCA2/CA3, VMH, and PVT), with no significant effects in the other areas.

Not finding any acute effect of OXT on OXTR levels in *Magel2*-KO pups at P8, we decided to assess if this lack of effect was also observed on a behavioral test that can be performed at this early postnatal developmental stage. Mother separation-induced USVs in pups are used as measure of early communication behavior in rodents and *Magel2*-KO pups have been previously reported to exhibit low rates of separation-induced vocalization with altered spectral features (Bosque Ortiz et al., 2022). We thus assessed the effect of an early OXT treatment on USVs in vehicle-treated *Magel2*-KO and vehicle-treated WT mice at P8 (Figure 4A) and observed lower rates of separation-induced USVs in both male and female *Magel2*-KO (KO-Veh) pups than in WT mice (Figures 4B, C), confirming the results previously obtained (Bosque Ortiz et al., 2022). Our data showed no effect of the perinatal OXT treatment on USV calls in either male or female *Magel2*-KO pups (Figures 4B, C), indicating that this impaired behavior cannot be acutely rescued by postnatal OXT treatment (Supplementary Table 3).

Finally, it was important to assess if the postnatal treatment in *Magel2*-KO had successfully restored OXTR levels similar to those observed in WT mice. In Figure 5, we report a multiple comparison analysis between WT mice treated with vehicle, *Magel2*-KO animals treated with vehicle and *Magel2*-KO treated with OXT. All statistical data analyses are reported in Supplementary Tables 4A, B. Representative film images of the various brain areas displaying main effects of treatment, genotype and sex are reported in Supplementary Figure 1.

Our data indicate that in *Magel2*-KO males, the postnatal OXT treatment reduced OXTR levels in all areas in which the receptor was abnormally upregulated (CeA, MeA, BLA, dCA2/CA3, DG) as well as in the Pir and VMH (Figure 5A and Supplementary Figure 1).

In *Magel2*-KO females, which display similar (MPOA, CeA, BLA, dCA2/CA3, DG, and PVT) or reduced (Pir, LS, MeA, vCA2/CA3, and VMH) OXTR levels compared to WT, the OXT treatment either had no effect (Pir, MPOA, LS, MeA, CeA, BLA, dCA2/CA3, and DG) or induced a further

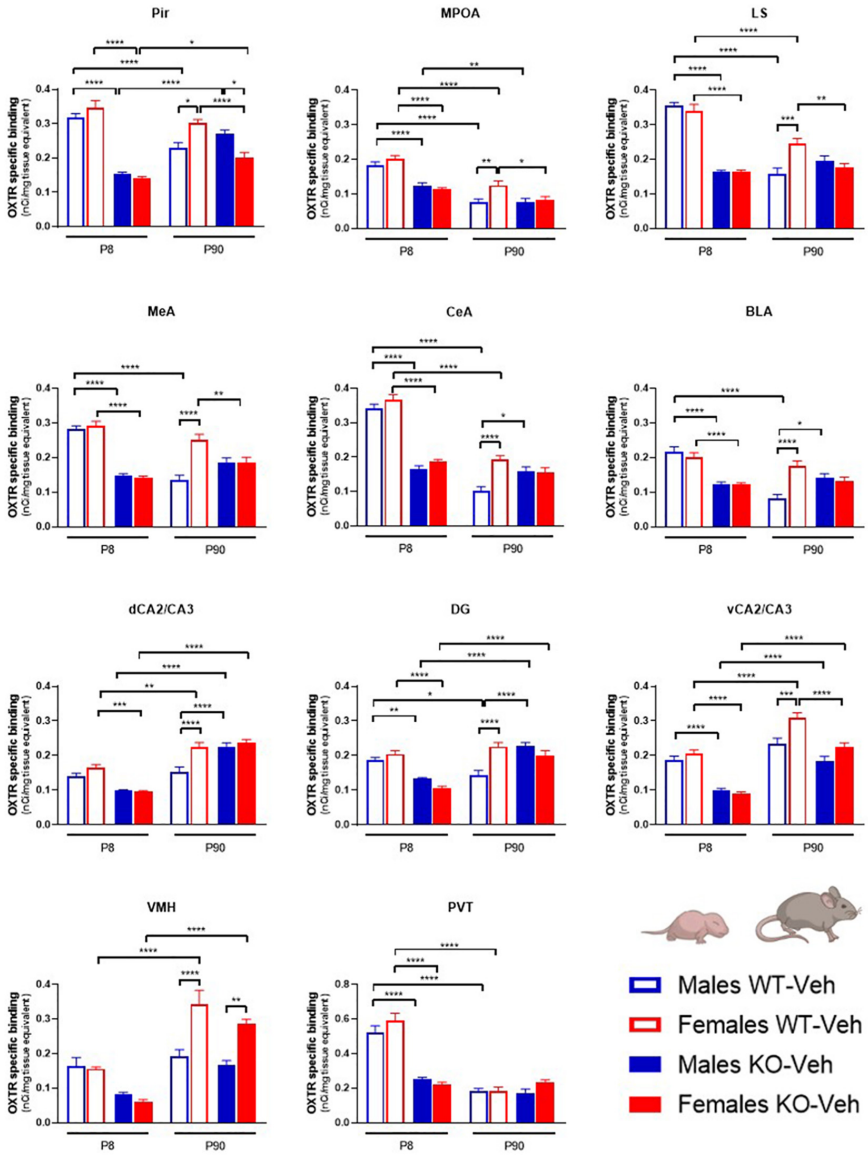

FIGURE 2
Contributions of age, sex and Magel2-KO genotype on physiological brain regional OXTR expression levels. Bar graphs of OXTR levels quantified by [^{125}I]-OVTA binding in P8 and P90, male and female, WT and *Magel2-KO* mice. Each histogram represents data expressed as mean + SEM of multiple datapoints collected from three animals. Unfilled bars are used for vehicle (Veh) treated WT, filled bars are used for vehicle (Veh) treated *Magel2-KO*; blue is used for males of both genotypes and ages, red for females of both genotypes and ages. Data were analyzed by three-way ANOVA, followed by a Tukey's multiple comparisons *post-hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. When a comparison was approaching statistical significance, the corresponding p-value was reported on the appropriate graph. Datasets and detailed statistical analyses are reported in the Supplementary Table 1.

down-regulation of OXTR levels (VMH and PVT) below those observed in WT animals.

In CeA, dCA2/CA3, PVT as compared to those observed in WT females, the final effect of the OXT was a down-regulation of OXTR levels below those observed in WT animals (Figure 5B).

Discussion

Our data indicate that highly variable region-specific patterns of OXTR expression related to age, sex and postnatal OXT treatment characterize male and female *Magel2*-KO mice.

OXTR levels at P8 in WT and *Magel2*-KO mice

The most striking result at P8 in *Magel2*-KO mice is the significant reduction in OXTR levels in all the regions analyzed when compared to WT animals, indicating a major defect in OXTR expression trajectories at this very early postnatal developmental age. Equally relevant are the findings that this reduction is observed in male and female animals and that no short-term effect of OXT treatment is evident, again, in either males or females.

A transient, developmental remodeling of OXTR expression has been previously reported in the male mouse brain

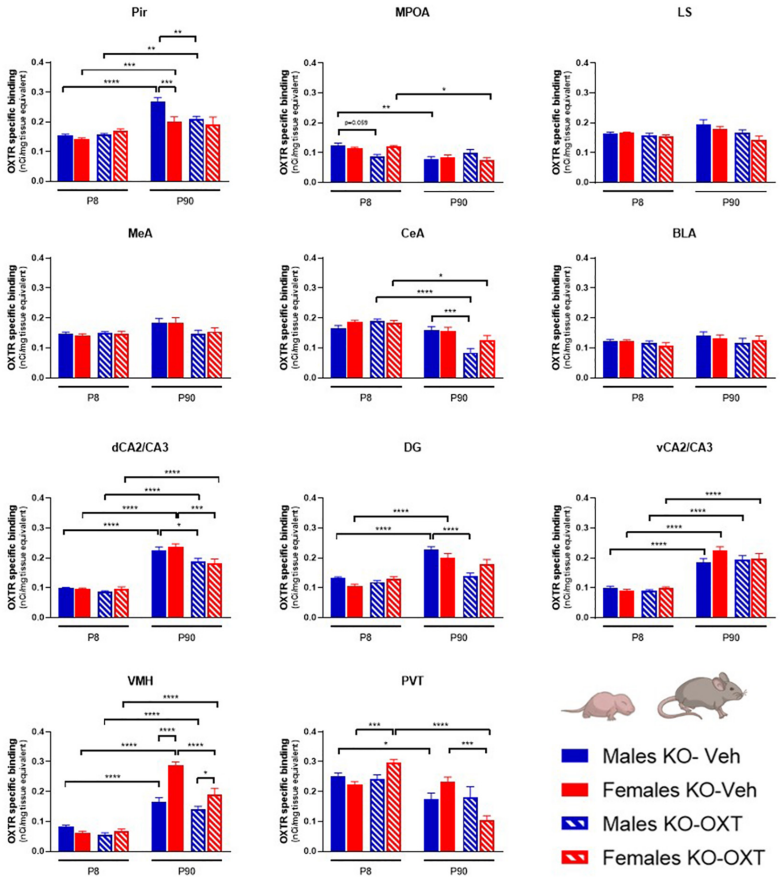

FIGURE 3
Long-lasting effects of a postnatal OXT on brain regional OXTR expression levels in Magel2-KO mice. Bar graphs of OXTR levels quantified by [^{125}I]-OVTA binding in P8 and P90, male and female, Vehicle or OXT-treated *Magel2-KO* mice. Each histogram represents data expressed as mean + SEM of multiple datapoints collected from three animals. Filled bars are used for vehicle (Veh) treated *Magel2-KO,* striped bars are used for oxytocin (OXT) treated *Magel2-KO*; blue is used for males of both treatment groups and ages, red for females of both treatment groups and ages. Data were analyzed by Three-way ANOVA, followed by a Tukey's multiple comparisons *post-hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. When a comparison was approaching statistical significance, the corresponding p-value was reported on the appropriate graph. Datasets and detailed statistical analyses are reported in the **Supplementary Table 2**.

(Hammock and Levitt, 2013), revealing a peak of OXTR expression in the 2nd postnatal week, which parallels maximal synaptogenesis and experience-dependent plasticity. A more recent study mapped the pattern of postnatal OXTR expression through the brain and confirmed a progressive increase in OXTR expression, which reached a peak in the 3rd postnatal week of life and decreased toward young adulthood (Newmaster et al., 2020). This general developmental profile is observed in a number of rodent species, despite regional differences that possibly subtend species-specific behavioral and/or developmental features: in rats, OXTRs were higher in juveniles than in adults in regions associated with reward and socio-spatial memory, while the opposite was found in regions of the social decision-making network (Smith et al., 2017). Our data confirm a generalized decreased OXTR binding in adult WT animals; in contrast, in the majority of the areas analyzed, P8 *Magel2*-KO mice display receptor levels similar to the low levels observed in the WT adults.

A key question concerns the mechanism(s) responsible for these low OXTR levels in *Magel2*-KO animals at this early developmental age. Our current data indicate that the reduction of OXTR levels affects several regions of the brain, at least all the ones analyzed for this work, therefore we favor the hypothesis of a mechanism based on a global impairment of OXTR expression at the protein and/or mRNA levels. As reduced OXT release throughout the brain could result in a general reduction in OXTR levels (Kenkel et al., 2019), one possibility is that a reduced neonatal OXT release could be responsible for a generalized down-regulation of OXTR. Indeed, reduced OXT production in the *Magel2*-KO neonate brain has been previously reported (Meziane et al., 2015). *Magel2* belongs to the *MAGE* family of ubiquitin ligase regulators, and has been shown to be involved in ubiquitination, actin regulation and endosomal sorting processes; in particular, it might regulate the quantity of secretory granules and bioactive neuropeptide production (Chen et al., 2020). *Magel2* is highly expressed in the hypothalamus during development, and, in the adult brain, high levels of *Magel2* mRNA are found in the neurons of suprachiasmatic (SCN), paraventricular (PVN), and supraoptic (SON) nuclei of the hypothalamus, strongly supporting an implication in OXT storage and release. Other mechanisms could contribute to variations in OXTR levels independently from OXT release: for instance, in the hippocampus, *Magel2* and OXTR mRNAs are co-expressed, as shown by RNAScope experiments

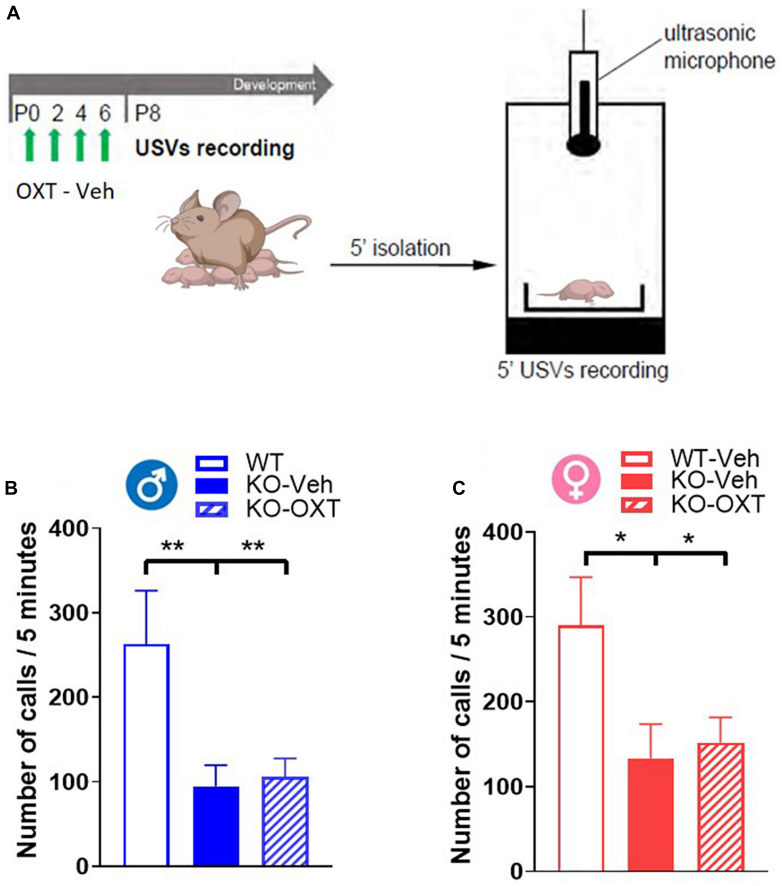

FIGURE 4
Ultrasonic vocalization calls (USVs) in P8 male and female WT and Magel2-KO pups treated with vehicle or OXT during the first week of life. **(A)** Schematic drawing of the protocol used to record separation-induced USVs in P8 mice. Pups were treated with vehicle (Veh) or oxytocin (OXT) from P0 to P6. Number of total calls, measured during 5 min isolation after pup separation in males **(B)** and females **(C)**. Each histogram represents data expressed as mean + SEM of 11-24 mice. Unfilled bars are used for vehicle (Veh) treated WT; filled bars are used for vehicle (Veh) treated *Magel2*-KO; striped bars correspond to oxytocin (OXT) treated Magel2-KO; blue bar is used for males of both genotypes, red bar for females of both genotypes. Histograms indicate the mean + SEM of the different groups analyzed by one-way ANOVA followed by a Tukey's multiple comparisons *post-hoc* test. $*p < 0.05$, $**p < 0.01$. Values and statistics are reported in **Supplementary Table 3**.

(Bertoni et al., 2021), and a *Magel2* deficiency could directly down-regulate the quantity of OXTRs at the cell membrane by affecting the intracellular recycling of membrane proteins.

Quite surprisingly, our data show that postnatal administration of OXT has no short-term effects on OXTR levels in the *Magel2*-KO neonate brain and, in parallel, on the low rate of USV calls that characterize *Magel2*-KO pups. These findings suggest that the effects of OXT on the adult brain depend on long lasting regulation of critical neuronal functions, in agreement with the proposal that life-long OXTR expression is, to some extent, determined by early life OXT exposure, a hypothesis known as "hormonal imprinting" effect (Carter, 2003; Kenkel et al., 2019). Neuronal and astrocytic differentiation/maturation, as well as microglia neurodevelopmental effects, continue in mice until the end of the first month of life, therefore trajectories of neuronal network plasticity and connectivity can be modulated by postnatal OXT with effects extending into adulthood, as elegantly demonstrated in the developing sensory cortices subjected to sensory deprivation (Zheng et al., 2014). In the early postnatal period OXT has been shown to play a role in the establishment of a correct excitation/inhibition (E/I) balance via modulation of the timing of the GABA switch (Leonzino et al., 2016; Ben-Ari, 2018), a decisive time point in neurodevelopmental trajectories that regulates proliferation, migration, differentiation and plasticity of developing neurons and whose perturbation is linked to a number of disorders (Virtanen et al., 2021). Consistently, in *Magel2*-KO hippocampal neurons, OXT early-postnatal treatment has been shown to rescue neurite outgrowth impairment (Reichova et al., 2021) as well as GABA polarity (Bertoni et al., 2021). At the cellular level, examples of OXT effects in neurodevelopmental processes include the regulation of cell fate in neuronal progenitors (Palanisamy et al., 2018), microglia protection against perinatal brain damage (Mairesse et al., 2019), neuron (Ripamonti et al., 2017), and astrocyte differentiation (Alanazi et al., 2020). Autoradiography measures the total level of OXTR in a selected area, but lack the resolution to discriminate binding levels in different cellular populations (i.e., neurons, microglia, astrocytes) and we cannot reveal how the receptor levels varied in these sub-populations in the different areas analyzed. Astrocytes have recently been shown to play a crucial role in mediating positive reinforcement effects of OXT in the central amygdala of mice and rats (Wahis et al., 2021; Baudon et al., 2022) and a reduction in the

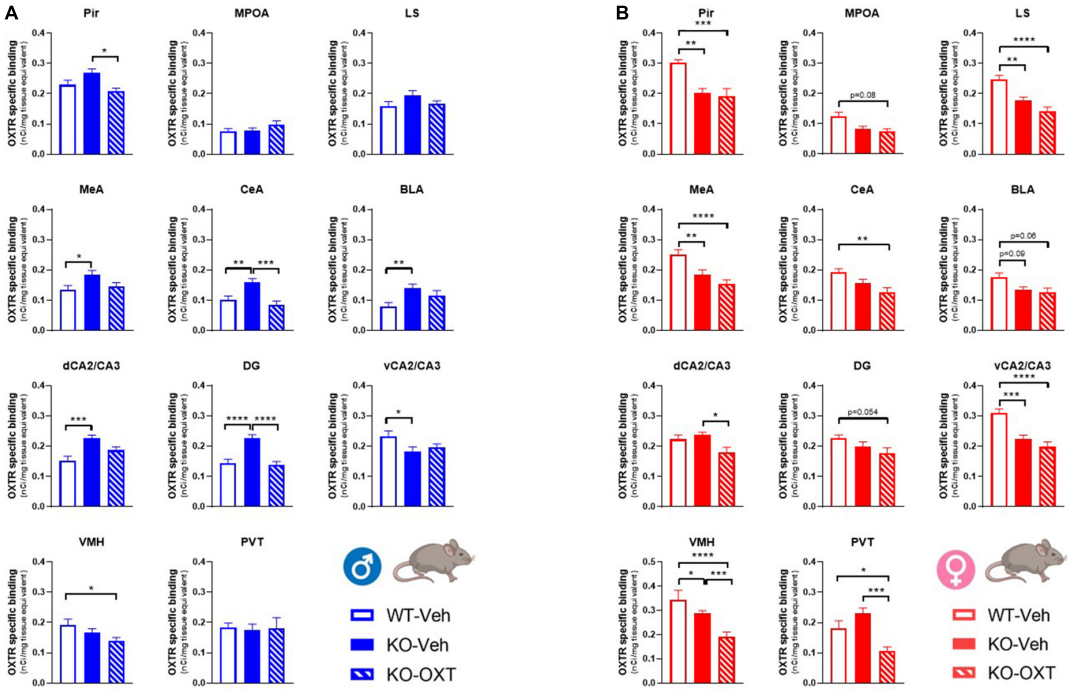

FIGURE 5
Long term effects of the postnatal OXT treatment on brain OXTR expression levels in P90 male and female WT and Magel2-KO mice. Bar graphs of OXTR levels quantified by [^{125}I]-OVTA binding in adult male **(A)** and female **(B)** mice. Each histogram represents data expressed as mean + SEM of multiple datapoints collected from 3 animals. Unfilled bars are used for vehicle (Veh) treated WT, filled bars are used for vehicle (Veh) treated *Magel2*-KO, striped bars are used for oxytocin (OXT) treated *Magel2*-KO. Data were analyzed by two-way ANOVA, followed by a Tukey's multiple comparisons *post-hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. When a comparison was approaching statistical significance, the corresponding *p*-value was reported on the appropriate graph. Datasets and detailed statistical analyses are reported in the **Supplementary Tables 4A, B**.

number of OXTR-expressing astrocytes was found in specific brain regions in another transgenic mouse model of PWS, the Magel2$^{tm1.1.Stw\ Mus}$ mouse (Althammer et al., 2022). Variations of OXTR levels in astrocytes in early postnatal brain development could crucially contribute to OXT signaling deregulation and life-long effects.

OXTR levels at P90 in WT and *Magel2*-KO mice

Adult *Magel2*-KO male mice presented a significant up-regulation of OXTR binding levels in the amygdala and hippocampus, indicating an impaired developmental pattern of receptor expression in selected brain regions. Of relevance is our finding that OXT treatment normalized adult OXTR levels in all these regions, consistent with the hypothesis that molecular region-specific effects underlie the behavioral rescue observed in *Magel2* animals treated with OXT at birth (Bertoni et al., 2021). Region-specific OXTR knock-down/rescue experiments will be necessary to show a causal link between OXTR levels in these regions and the expression of specific *Magel2*-KO behavioral phenotypes. Indeed, in mice with a CA2/CA3 region specific OXTR knock-down, the neonatal administration of OXT did not rescue social alterations (Pan et al., 2022) while, in *Dysbindin*-KO mice, a mouse model of schizophrenia with altered OXTR levels in the CeA, the manipulation of OXTR within this region rescued emotional recognition deficits (Ferretti et al., 2019).

An intriguing finding is that in *Magel2*-KO mice, the brain regions responding to postnatal OXT treatment are mainly those displaying dysregulated OXTRs at adulthood. One possible explanation is the existence of a temporal window in which these regions are particularly sensitive to OXT. During the postnatal life, neuronal circuits are undergoing maturation at different times, and under the influence of different external and internal factors. Within these factors, OXT is believed to play a modulatory and integratory role on sensory inputs, allowing to shape brain circuits and connectivity (Grinevich and Stoop, 2018; Muscatelli et al., 2022; Pan et al., 2022). It has been demonstrated that, in mouse pups, sensory experience influences OXT production and that OXT shapes neuronal circuitry by modulating spontaneous and evoked activity (Zheng et al., 2014). In *Magel2*-KO pups, characterized by a deficient production of hypothalamic OXT (Meziane et al., 2015), the neuropeptide may be unable to play its role of mediator of early sensory functions and the postnatal supplementation of OXT may restore this function. Interestingly, in *Magel2*-KO mice, we found a modulation OXT-mediated OXTR in the piriform cortex and amygdala, two structures belonging to a neural circuit that is involved in postnatal learning of the odor-preferences cues crucial to develop social behavior in the early postnatal life (Moriceau and Sullivan, 2005; Oruro et al., 2020).

In the hippocampus we observed a long-lasting effect of OXT treatment, consistent with what we have previously published

(Bertoni et al., 2021). We confirm here the OXTR up-regulation in the DG of *Magel2*-KO males and extend the findings of a dysregulation of OXTR also to dCA2/CA3 and vCA2/CA3, indicating a generalized hippocampal involvement. However, while in the DG and dCA2/CA3 we found an up-regulation of OXTR, in the vCA2/CA3 we observed a trend toward receptor down-regulation (statistically significant difference—p = 0.029 - in the multiple comparisons in **Figure 5A**—and close to significance—p = 0.081 - in the three-way ANOVA setting in **Figure 2**). Interestingly, the effect of OXT was selective for the DG and dCA2/CA3, further supporting the idea that the action of OXT is observed only in regions were an up-regulation will manifest in the adult age.

Surprisingly, in *Magel2*-KO mice, we did not find any OXTR dysregulation in a number of regions in which OXTRs are known to play important roles in social and non-social behaviors, such as the LS, MPOA, VMH, and PVT. In particular, in the LS, OXTRs have been shown to be involved in the regulation of different social behaviors including social fear and social preference (Menon et al., 2018); in the MPOA, OXTRs are involved in the modulation of parental (Tsuneoka et al., 2022) and sexual behavior (Menard et al., 2022); OXTRs in the VMH also play an important role in the regulation of sexual behavior (Menard et al., 2022). Lastly, the PVT is a key hub for the control of food seeking and intake and an OXT infusion in this area can attenuate the hypophagia induced by stress and anxiety, with no effects on food intake in normal conditions (Barrett et al., 2021). The lack of an OXTR dysregulation in these regions suggests that they are not the target of OXT in the first postnatal week, when the deficit of OXT production is observed in *Magel2*-KO mice, but will respond to the peptide later on, when the OXT production in the *Magel2*-KO hypothalamus is restored (Meziane et al., 2015). If this is the case, these regions will not suffer from the lack of the peptide in the neonatal period, nor will they be affected from the external OXT administration in the first postnatal week. This is consistent with a delayed modulatory effect of OXT on different brain circuits and functions, and with a lack of OXT neonatal effects on specific circuits/behaviors emerging/consolidating after the first week of life.

In adult *Magel2*-KO females, a completely different trend in OXTR binding levels was found, and an unexpected reduction in OXTR levels was observed in several areas of the brain (Pir, MPOA, LS, MeA, and vCA2/CA3). The functional impact of this receptor down-regulation is at present unclear, given that *Magel2*-KO females did not show impairments in social interactions and learning tests (Meziane et al., 2015). Among these regions, MPOA is an essential component of the neuronal network that regulates parental care and, in rat females, OXT increases sexual receptivity and facilitates maternal behavior (Jurek and Neumann, 2018). Deficits in maternal behavior has been recently reported in *Magel2*-KO females (Bosque Ortiz et al., 2022; Da Prato et al., 2022), however, both these works showed that the delayed maternal pup retrieval was related to reduced USV calls by *Magel2*-deficient pups; assessing studies are needed to evidence deficits in sexual/parental behavior that may be correlated to the MPOA OXTR alterations observed in *Magel2-KO* females.

Even more surprisingly, we found a further down-regulation of OXTR binding sites in OXT-treated adult females in the VMH, PVT, and dCA2/CA3. This may represent a compensatory mechanism to protect these regions from an overload of OXT in the first postnatal week, to which only specific females' regions would be sensitive. It will be interesting to assess if this compensatory OXTR down-regulation protects the female brain from behavioral alterations by pharmacological/genetic manipulation of OXTR in these areas of Magel2-*KO* females.

Sexual dimorphism in WT and *Magel2*-KO mice

The OXT/OXTR system, together with the highly related vasopressin system, have been long known to be sexually dimorphic (de Vries, 2008; Carter et al., 2020). For example, in humans, following a social stress test, a single dose of intranasal OXT increases distress and anger in women, but reduces distress in men (Kubzansky et al., 2012). In mice, activation of OXT interneurons in the medial prefrontal cortex (mPFC) is anxiolytic in males and prosocial in females (Nakajima et al., 2014; Li et al., 2016), while in California mice (*Peromyscus californicus*), a rodent species in which females regularly engage in territorial aggression similarly to males, activation of OXT neurons in the bed nucleus of the stria terminalis (BNST) has sex-specific effects on social approach, social avoidance and social vigilance (Duque-Wilckens et al., 2018; Williams et al., 2020). In the rat, sex-specific differences in OXTR distribution have been extensively investigated (Smith et al., 2017; Bredewold and Veenema, 2018), but only few studies are present in mice.

At P8, we did not observe sex-dependent differences in binding levels of OXTR in WT or *Magel2*-KO animals, suggesting that male/female differences in OXTR brain levels arise later in the postnatal life, possibly as a consequence of sexual hormonal influences (Ivell and Walther, 1999; Champagne et al., 2001). At this early postnatal developmental stage, OXT seems to exert general and crucial effects, with an impact on survival, such as those on suckling activity, which is impaired in both male and female *Magel2*-KO pups (Schaller et al., 2010; Meziane et al., 2015).

Adult WT mice presented sex-specific differences in OXTR expression in almost all regions analyzed, with OXTR levels always higher in females, in comparison to another study in which multiple brain regions of male and female mice were analyzed and a significant sexual dimorphism was found only in two hypothalamic regions, the ventral premammillary nucleus and the anteroventral periventricular nucleus (Newmaster et al., 2020), however, in the latter study the OXTR gene expression was determined in a transgenic *knock in* line by measuring a cytoplasmic Venus-GFP reporter that may not exactly match the level of membrane OXTR binding sites. A study in which OXTRs were evaluated with an anti-OXTR antibody, reported increased levels of OXTR in CeA, CA2, LS, Pir, and VMH of virgin females as compared to males (Mitre et al., 2016), in agreement with our current findings.

Most importantly, the sexual dimorphism was greatly impaired in adult *Magel2*-KO mice which only presented a sex-dependent difference in OXTR levels in the VMH and in the Pir, the latter region presenting a higher expression of OXTR levels in males. Further studies are needed to assess the role played by OXTR in regulating *Magel2* specific behaviors in these regions.

Our present findings confirm sex-based differences in OXTR levels, highlighting the need for an extensive investigation of the

specific role of OXT and OXTR in the female mouse brain, in particular in preclinical models of neurodevelopmental disease characterized by a well-recognized sex-bias.

Conclusion

The present study underscores the relevance of long-lasting sex and region-specific effects of postnatal OXT treatment on OXTR levels in the *Magel2*-KO mouse model of the PWS/SYS. Understanding the cellular and molecular mechanisms of this specific OXTR regulation represents a crucial step toward developing effective OXT-based therapeutic strategies.

Limitations of this study

A limitation of the present study is the absence of WT-OXT treated groups in our experimental design, as investigating the role of OXT treatment in WT animals was outside the main scope of this work. This study could also have been extended to include control groups of "handled only" mice (i.e., manipulated pups not receiving any injection) as it is well established that pup handling can influence OXTR levels in the brain (Bales and Perkeybile, 2012). In our experimental conditions, vehicle and OXT-treated pups were similarly handled, most likely leveling handling contribution equally across the groups. Particular attention was placed on WT mice used as controls, which all received postnatal vehicle injections, representing, in our opinion, the most appropriate controls to selectively highlight postnatal OXT-induced long-lasting effects on OXTR. The "non-handled" extra control groups would have contributed to build a wider and deeper knowledge of the physiological developmental changes in OXTR expression and distribution in the brain. However, the main aim of this study was to investigate the effect of early postnatal OXT treatment in male and female *Magel2*-KO mice in order to advance the translational research on OXT treatment for Schaaf-Yang Syndrome and Prader-Willi patients. Pilot clinical studies of OXT treatment in PWS and SYS infants have provided encouraging positive results (Tauber et al., 2017) and strong preclinical evidence on the effects of OXT treatment in *Magel2*-deficient animal models on biochemical and molecular parameters, including OXTR levels in the brain, is urgently needed.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by French Ministry of Agriculture with the accreditation no. B13-055-19.

Author contributions

FSa and AB performed the experiments and analyzed the data. VG analyzed the data, prepared the figures, and contributed to the preparation of the manuscript. MB analyzed the data, prepared the figures, and contributed to the writing of the manuscript. CP analyzed the data. FSc generated the mice and collected brains. FM contributed to the conception of the project. FSc and AB supervised the project. BC contributed to the conception of the project, supervised the planning and execution of the experiments, collected and analyzed the data, and prepared the manuscript. All authors supervised the manuscript and approved the submitted version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1026939/full#supplementary-material

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Effects of different physical activity interventions on children with attention-deficit/ hyperactivity disorder: A network meta-analysis of randomized controlled trials

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Background: Previous studies have shown that physical activity interventions positively affect core symptoms and executive functioning in children with attention-deficit/hyperactivity disorder (ADHD). However, comparisons between different physical activity interventions still need to be made. This study is the first to analyze the effects of 10 different types of physical activity on children with ADHD through a network meta-analysis.

Methods: PubMed, Embase, Web of Science, and Cochrane Library databases were searched for randomized controlled trials on the effects of physical activity interventions on children with ADHD. The search time frame was from database creation to October 2022. Two investigators independently performed literature screening, extraction, and quality assessment. Network meta-analysis was performed with Stata 15.1.

Results: A total of 31 studies were included, and the results indicated that perceptual-motor training was the most effective in terms of motor ability and working memory (SUCRA = 82.7 and 73.3%, respectively). For attention problems and cognitive flexibility, aquatic exercise was the most effective (SUCRA = 80.9 and 86.6%, respectively). For social problems, horsemanship was the most effective (SUCRA = 79.4%). For inhibition switching, cognitive-motor training was the most effective (SUCRA = 83.5%).

Conclusion: Our study revealed that aquatic exercise and perceptual-motor training had a superior overall performance. However, the effects of various physical activity interventions on different indicators in children with ADHD can vary depending on the individual and the intervention's validity. To ensure an appropriate physical activity intervention is selected, it is important to assess the severity of symptoms exhibited by children with ADHD beforehand.

KEYWORDS

physical activity, children, ADHD, neurodevelopmental disorders, network meta-analysis

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder that affects approximately 7.2% of children worldwide (Thomas et al., 2015; Thapar et al., 2017). Its primary characteristics are inattention, impulsivity, and hyperactivity (American Psychiatric Association [APA], 2013), and can be divided into three distinct subtypes: inattentive, hyperactive-impulsive, and combined inattentive and hyperactive-impulsive (American Psychiatric Association [APA], 2013). It has been shown that children with ADHD often develop diverse problems, including sleep disturbances, distractibility, motor deficits, decreased social skills, and decreased academic performance (Kim et al., 2011; Konicarova et al., 2014; Schneider et al., 2016; Yu et al., 2019). These issues are also persistent, frequently remaining when patients reach puberty and adulthood (Edition, 2013). Therefore, it is highly detrimental to the development of pediatric patients and hurts their physical and mental health, academic growth, and socialization process.

Given this, the treatment of pediatric patients with ADHD is of utmost importance. The most commonly used treatment modality is medication, such as methylphenidate (MPH) (Barkley and Poillion, 1994; Welsch et al., 2021), but it may cause side effects such as headache, stomach pain, and decreased appetite (De Sousa and Kalra, 2012). Meanwhile, in the past two decades, non-pharmacological interventions for ADHD have been rapidly developed and used (Cortese et al., 2022), such as physical activity interventions, neurofeedback interventions, and cognitive interventions (Jensen and Kenny, 2004; Sánchez-López et al., 2015; Sani et al., 2022), due to concerns about the side effects and long-term effects of pharmacological treatments (Coghill, 2019). Physical activity interventions, in particular, have gained traction due to their lower cost, ease of implementation, capacity to improve physical fitness, and additional benefits (Cornelius et al., 2017).

Previous research has uncovered a strong link between physical activity and various functions in individuals with ADHD. Barnard-Brak et al. (2011) utilized data from the Early Childhood Longitudinal Study, Kindergarten cohort (ECLS-K) to demonstrate that structured physical activity was associated with a decrease in ADHD symptoms over time. This may be due to the stimulation of the catecholamine system, which is known to be impaired in individuals with ADHD (Barnard-Brak et al., 2011).

A recent study conducted by Fard et al. (2022) investigated the impact of physical activity on the physical and mental health of children and adolescents with ADHD, with self-esteem as a moderating factor. The results indicated that physical activity and health levels are integral components of well-being for this population and that self-esteem could be a potential mediator for the connection between physical activity and health outcomes (Fard et al., 2022). Some previous meta-analyzes have also shown evidence of better efficacy of physical activity in patients with ADHD. Cerrillo-Urbina et al. (2015) explored the impact of physical activity on core symptoms of attention, impulsivity, anxiety, and executive functioning in patients with ADHD. The results showed that physical activity was more effective than non-physical activity, particularly aerobic exercise (Cerrillo-Urbina et al., 2015). Zang (2019) assessed the effects of physical activity interventions compared to non-physical activity interventions in children with ADHD. The findings indicated that physical activity interventions had a significant positive effect on anxiety and depression, aggressive behavior, thinking, and social problems in children with ADHD (Zang, 2019). Lambez et al. (2020) performed a meta-analysis evaluating the effects of non-pharmacological treatments for ADHD on cognitive functioning. The interventions studied included neurofeedback, cognitive behavioral therapy, cognitive training, and physical exercise. Physical exercise was found to have the greatest mean effect size, particularly for inhibition (Lambez et al., 2020). Seiffer et al. (2022) studied the efficacy of moderate to vigorous exercise (MVPA) on children with attention deficit hyperactivity disorder (ADHD), focusing on the intensity component of physical activity. The study indicated that MVPA was the most effective treatment for ADHD and that it might be used as an alternative (Seiffer et al., 2022). Collectively, these findings suggest that physical exercise may be an effective treatment option for ADHD patients.

However, previous meta-analyzes have largely compared physical and non-physical activity, without examined the potential distinctions between different types of physical activity interventions. The types of physical activity are diverse and include many types of aquatic exercise, ball games, mind-body exercise, and high-intensity interval training. Therefore, what specific types of physical activity provide the most significant benefit to pediatric patients with ADHD? Through a network meta-analysis of randomized controlled trial studies of physical activity in pediatric patients with ADHD, this study provides valuable information for selecting the best physical activity for treating pediatric patients with ADHD.

2. Materials and methods

2.1. Protocol and registration

The meta-analysis was conducted using the Cochrane Handbook for Systematic Reviews of Interventions, and the findings were reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA) statement (Higgins et al., 2019; Page et al., 2021). This network meta-analysis was prospectively registered in PROSPERO (CRD 42022363255).

2.2. Data sources and search strategy

We conducted a comprehensive search of four databases (PubMed, Web of Science, Embase, and the Cochrane Library) to identify relevant studies. Search strings included physical activity interventions, age ranges, and outcomes related to patients with ADHD. The search was performed up to October 2022. **Supplementary Appendix A** shows the detailed search strings for this search.

2.3. Study selection

Following guidelines, two authors (DW and DL) independently assessed the search results and vetted the publications retrieved

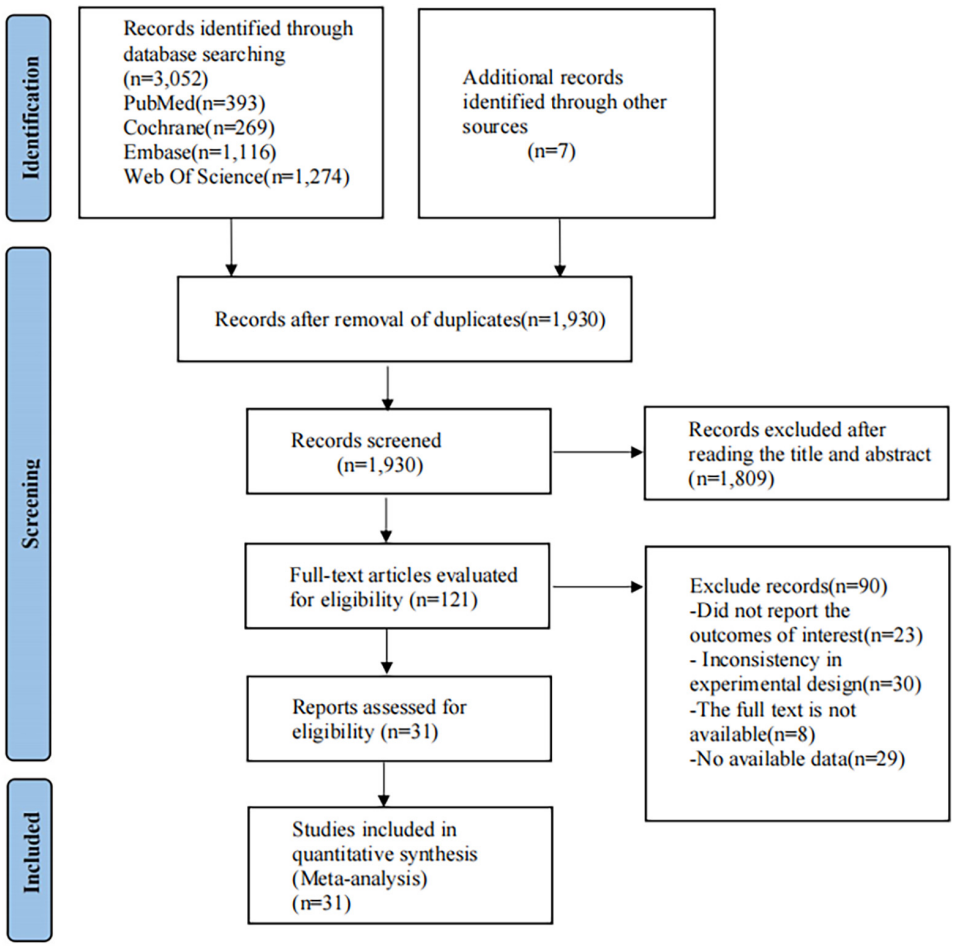

FIGURE 1
Preferred Reporting Items for Systematic Reviews and Meta-Analyzes flow diagram of the study process.

from databases and reference lists. The titles and abstracts of the research were first used to determine their relevance. Then, relevant full-text studies were retrieved and evaluated for inclusion. Any disagreements were resolved through discussion and consensus.

2.4. Inclusion and exclusion criteria

This systematic review employed specified inclusion criteria. Each study met the following criteria:

(1) Only randomized controlled trials were included, and observational and cross-sectional studies were excluded.
(2) The range of age participants in the sample must be 18 years or less.
(3) The physical activity intervention had to contain a sports or physical activity component. Studies without physical activity intervention were excluded.
(4) The study must report data on indicators of motor skills, attention problems, social problems, cognitive flexibility, inhibitory switching, and working memory in children with ADHD before and after the intervention. Studies that do not report on these indicators must be excluded.
(5) Presented original data.
(6) We only analyzed papers written in English and excluded papers written in other languages.

2.5. Data extraction

The data were extracted to a standardized Excel spreadsheet. Two authors collected the required data separately from the included studies. Disagreements encountered during the process were resolved through discussion with the group. The following data were extracted from the final study: author, year, country, subject characteristics, intervention characteristics, and ADHD-related outcome indicators.

2.6. Quality assessment

The risk of bias was assessed using the Cochrane System Risk of Bias Assessment tool *via* Review Manager 5.4 software, which evaluates the studies' quality on seven indicators: 1. Random sequence generation; 2. Allocation concealment; 3. Blinding of

TABLE 1 Summary table of included reviews.

Study	Country	Sample size		Gender (M/F)	Mean age (year)		Intervention						Outcome	ADHD diagnostics
		EG	CG		EG	CG	EG			CG				
							Intervention content	Intervention time, frequency, period	Tape	Intervention content	Interven-tion time, frequency, period	Tape		
Oh et al. (2018)	Korea	17	17	31/3	8.30	8.00	Horsemanship practice	60 min, 2 weekly, 12 weeks	HMS	Pharmacotherapy	Consistent with EG	PCT	A1;A2	DSM-4
Meßler et al. (2018)	Germany	14	14	28/0	11	11	High intensity interval training	25 min, 3 weekly, 3 weeks	TAE	Low-to-moderate intensity ball games	60 min, 3 weekly 3 weeks	CMT	A1;A2;A3	DSM-4
Sabzi et al. (2021)	Iran	23	23	23/23	9.60	9.50	Water treadmill exercise	30 min, 3 weekly, 8 weeks	AE	NI	NI	NI	A3	DSM-5
Mansson et al. (2019)	Denmark	64	64	109/19	11.39	11.63	Target-shooting sport	20–45 min, 1 weekly, 24 weeks	CMT	NI	NI	NI	A5	DSM-4
Sani et al. (2022)	Iran	20	20	29/11	7.50	7.78	Perceptual-motor training	40–45 min, 3 weekly, 7 weeks	PMT	Neurofeedback training	Consistent with EG	NFT	A1;A5	DSM–5
Yazd et al. (2015)	Iran	12	12	20/4	8.7	8.00	Perceptual-motor training	NR min, 3 weekly, 6 years	PMT	Pharmacotherapy	NR min 3 weekly 6 years	PCT	A1	DSM-4
Ahn et al. (2021)	Korea	8	7	12/3	7.50	7.14	Horsemanship practice	40 min, 2 weekly, 16 weeks	HMS	NI	NI	NI	A2;A3	DSM-5
Smith et al. (2020)	America	42	38	53/27	7.6	7.20	Computerized cognitive training + physical exercises	120 min, 4 weekly, 15 weeks	CMT	Pharmacotherapy	NR	PCT	A5;A6	DSM-4
Da Silva et al. (2020)	Brazil	10	10	14/6	12	12.00	Swimming training	45 min, 2 weekly, 8 weeks	AE	NI	NI	NI	A1;A2;A4	DSM-4
Milligan et al. (2019)	Canada	48	38	72/14	13.10	12.82	Mindfulness martial arts	90 min, 1 weekly, 20 weeks	MBE	Mental health treatments+ educational interventions	NR	CI	A2;A4;A5;A6	DSM-4

(Continued)

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TABLE 1 (Continued)

Study	Country	Sample size		Gender (M/F)	Mean age (year)		Intervention						Outcome	ADHD diagnostics
		EG	CG		EG	CG	EG			CG				
							Intervention content	Intervention time, frequency, period	Tape	Intervention content	Interven-tion time, frequency, period	Tape		
Chang et al. (2012)	China	20	20	37/3	10.42	10.45	Aerobic exercise	30 min, 1 weekly, 1 week	AAE	Watch video	Consistent with EG	CI	A4;A5	DSM-4
Chang et al. (2014)	China	14	13	23/4	8.19	8.78	Water exercise	90 min, 2 weekly, 8 weeks	AE	NI	NI	NI	A1;A4;A5	DSM-4
Kang et al. (2011)	Korea	15	13	28/0	8.4	8.6	Sports therapy	90 min, 2 weekly, 6 weeks	CE	Education for behavior	NR	CI	A2;A5;A6	DSM-4
Gawrilow et al. (2016)	Germany	23	24	47/0	10.47	10.47	Trampoline	5 min, 1 weekly, 1 week	AAE	Sedentary task	Consistent with EG	CI	A4;A5	ICD-10
Choi et al. (2015)	Korea	13	17	30/0	15.80	16.00	Sports therapy	90 min, 3 weekly, 6 weeks	CE	Watch video	Consistent with EG	CI	A4;A5	DSM-4
Ziereis and Jansen (2015)	Germany	13	16	21/8	9.2	9.5	Sports therapy	60 min, 1 weekly, 12 weeks	CE	NI	NI	NI	A1;A4;A6	ICD-10
Bustamante et al. (2016)	America	18	16	24/10	9.40	8.70	Physically active games	90 min, 5 weekly, 10 weeks	CE	Sedentary task	Consistent with EG	CI	A2;A5;A6	DSM-4
Memarmoghaddam et al. (2016)	Iran	19	17	NR	8.31	8.29	Selected exercise program	90 min, 3 weekly, 8 weeks	CE	NI	NI	NI	A4;A5	SNAP-4
Pan et al. (2016)	China	16	16	32/0	8.93	8.87	Table tennis exercise	70 min, 2 weekly, 12 weeks	CMT	NI	NI	NI	A2;A3;A4	DSM-4
Lee et al. (2017)	Korea	6	6	12/0	10.46	10.50	Combined exercise program	60 min, 3 weekly, 12 weeks	CE	NI	NI	NI	A4	DSM-4
Benzing et al. (2018)	Switzerland	24	22	38/8	10.46	10.50	Exergame (sports games)	15 min, 1 weekly, 1 week	AAE	Watch video	Consistent with EG	CI	A4;A5;A6	ICD-10
Benzing and Schmidt (2019)	Switzerland	28	23	43/8	10.46	10.39	Exergame for physical and cognitive challenges	30 min, 3 weekly, 8 weeks	CMT	Watch video	Consistent with EG	CI	A5;A6	ICD-10

(Continued)

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TABLE 1 (Continued)

Study	Country	Sample size		Gender (M/F)	Mean age (year)		Intervention						Outcome	ADHD diagnostics
		EG	CG		EG	CG	EG			CG				
							Intervention content	Intervention time, frequency, period	Tape	Intervention content	Interven-tion time, frequency, period	Tape		
Kadri et al. (2019)	Tunisia	20	20	36/4	14.5	14.20	Taekwondo	30 min, 2 weekly, 1.5 years	CMT	Traditional physical education classes	Consistent with EG	TAE	A2;A4;A5;A6	DSM-4
Gelade et al. (2017)	Netherlands	39	CG1:36 CG2:37	85/27	9.96	CG1:9.11 CG2:9.80	Neurofeedback (theta/beta training)	20 min, 3 weekly, 10–12 weeks	NFB	CG1: methylphenidate CG2: aerobic exercise	CG1:NR CG2:20 min 3 weekly, 10–12 weeks	CG1: PCT CG2: TAE	A2;A5;A6	DSM-4
Faramarzi et al. (2016)	Iran	10	10	20/0	Elementary students	Elementary students	Sensory integration training	45 min, 2 weekly, 6 weeks	SIT	NI	NI	NI	A5	Conner's rating scale
Ludyga et al. (2017)	Switzerland	5	8	NR	12.80	13.50	Aerobic exercise	20 min, 1 weekly, 1 week	AAE	Watch video	Consistent with EG	CI	A5	DSM-4
Ahmed and Mohamed (2011)	Egypt	42	42	54/30	13.90	13.80	Aerobic exercises program	40–50 min, 3 weekly, 10 weeks	TAE	NI	NI	NI	A1;A2	DSM-4
García-Gómez et al. (2016)	Spain	9	5	10/4	10.65	10.20	Equestrian therapy	45 min, 2 weekly, 12 weeks	HMS	NI	NI	NI	A5;A6	DSM-4
Hattabi et al. (2019)	Tunisia	20	20	5/35	9.95	9.75	Perceptual motor water exercise	90 min, 3 weekly, 12 weeks	PMT	NI	NI	NI	A1;A5;A6	Conner's rating scale
Hoza et al. (2015)	America	104	98	108/94	6.83	6.83	Aerobic physical activity	31 min, 5 weekly, 12 weeks	TAE	Sedentary task	Consistent with EG	CI	A2	DSM-4
Jensen and Kenny (2004)	Australia	11	8	19/0	10.63	9.35	Yoga	60 min, 1 weekly, 20 weeks	MBE	Cooperative games	Consistent with EG	CI	A2;A4	DSM-4

ADHD, attention-deficit/hyperactivity disorder; EG, experimental group; CG, control group; NR, no report; NI, no intervention; HMS, horsemanship; CBE, combination exercise; PMT, perceptual-motor training; CMT, cognitive-motor training; AE, aquatic exercise; MBE, mind-body exercise; AAE, acute aerobic exercise; TAE, traditional aerobic exercise; SIT, sensory integration training; NFB, neurofeedback; CI, cognitive intervention; PCT, pharmacotherapy; NIMH DISC-IV, national institute of mental health diagnostic interview schedule for children version IV; DSM-4 and DSM-5, diagnostic and statistical manual of mental disorders, fourth edition and fifth edition; ICD-10, international classification of diseases, tenth revision; SNAP-4, Swanson, Nolan and Pelham rating scale, fourth edition; A1, motor ability; A2, attention problems; A3, social problems; A4, cognitive flexibility; A5, inhibition switching; A6, working memory.

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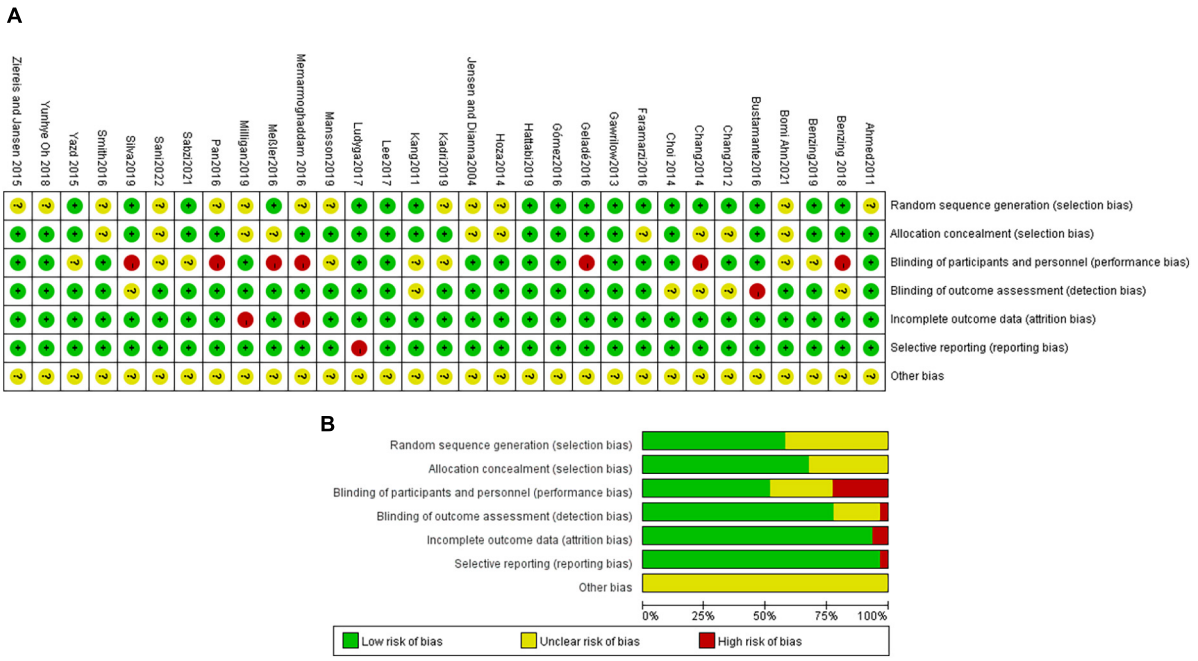

FIGURE 2
(A) Methodological quality of included studies. **(B)** The distribution of the methodological quality of included studies.

participants and personnel; 4. Blinding of outcome assessment; 5. Incomplete outcome data; 6. Selective reporting; and 7. Other bias.

2.7. Statistical analysis

We computed the standardized mean difference (SMD) and 95% CIs for continuous outcomes. The *P*-value of the chi-square test and the I2 index from the heterogeneity test were used to express the level of statistical heterogeneity. Different effect models were selected according to the level of heterogeneity of the test data. When the level of heterogeneity was low, a fixed-effects model ($P \geq 0.1$ and I2 $\leq$ 50%) was used to analyze the data. Otherwise, a random-effects model ($P < 0.1$ or I2 values >50%) was used (Higgins et al., 2003).

According to the PRISMA NMA recommendations, we aggregated and analyzed NMA data using Markov chain Monte Carlo simulation chains in a Bayesian-based framework and Stata software (version 15.1) (Moher et al., 2015; Vats et al., 2019). We will employ the nodal method to quantify and demonstrate the congruence between indirect and direct comparisons, as obtained by Stata software instructions. If the *p*-value is greater than 0.05, the agreement test is passed.

Network meta-analysis was performed by employing a Bayesian model. The data were preprocessed using network group commands, and a mesh evidence map was drawn. The dots in the mesh evidence plot represent one intervention type, and the larger the area of its dots represents, the more significant the number of patients included in the study for the intervention. The line connecting the two dots is a direct comparison of the two interventions, and the thickness of the line represents the number of included studies. The larger the number of included studies, the thicker the line (Chaimani et al., 2013). The effects of the different movement methods were ranked. The effects of the different exercise modalities were ranked, the surface under the cumulative ranking curve (SUCRA) was obtained, and the probability ranking was plotted in a table. SUCRA is expressed as a percentage. The larger the percentage, the more effective the intervention. Additionally, to check for publication bias and minor sample study effects, we generated funnel plots for outcome indicators with study numbers >10 and used symmetry criteria to check (Khera et al., 2016). Stata15.1 was used to perform all statistical analyzes.

3. Results

3.1. Trial selection

A total of 3,052 citations are yielded in the initial search of electronic databases, and an additional seven documents were manually searched. After removing duplicate studies (n = 1,129), 1,930 relevant papers remained. Subsequently, through screening, 1,809 papers were removed, and 121 papers suitable for full-text review remained, of which 90 were further eliminated. Finally, 31 studies were adopted for quantitative synthesis (**Figure 1**).

3.2. Trial characteristics

Characteristics of studies adopted are shown in **Table 1**, all of which were published between 2004 and 2022. The country with the highest number of included studies was Iran, with a total number of five papers. The sample size ranged from 5 to

TABLE 2 League table on motor ability.

PMT	TAE	AE	CBE	CMT	HMS	NI	PCT	NFB
PMT	−0.53 (−6.56, 5.50)	−1.60 (−6.65, 3.46)	−2.38 (−7.72, 2.96)	−2.57 (−10.02, 4.89)	−3.77 (−12.74, 5.20)	−5.26 (−9.38, 1.14)	−9.76 (−14.60, −4.92)	−28.68 (−39.15, −18.20)
0.53 (−5.50, 6.56)	TAE	−1.07 (−6.34, 4.20)	−1.85 (−7.43, 3.72)	−2.04 (−6.45, 2.37)	−3.24 (−13.86, 7.38)	−4.74 (−9.16, −0.31)	−9.23 (−16.70, −1.76)	−28.15 (−40.23, −16.07)
1.60 (−3.46, 6.65)	1.07 (−4.20, 6.34)	AE	−0.78 (−5.27, 3.71)	−0.97 (−7.81, 5.87)	−2.17 (−12.45, 8.11)	−3.66 (−6.60, −0.73)	−8.16 (−15.15, −1.18)	−27.08 (−38.70, −15.46)
2.38 (−2.96, 7.72)	1.85 (−3.72, 7.43)	0.78 (−3.71, 5.27)	CBE	−0.18 (−7.29, 6.92)	−1.39 (−11.82, 9.05)	−2.88 (−6.28, 0.52)	−7.38 (−14.59, −0.17)	−26.30 (−38.05, −14.54)
2.57 (−4.89, 10.02)	2.04 (−2.37, 6.45)	0.97 (−5.87, 7.81)	0.18 (−6.92, 7.29)	CMT	−1.20 (−12.51, 10.10)	−2.70 (−8.93, 3.54)	−7.19 (−15.61, 1.22)	−26.11 (−38.96, −13.27)
3.77 (−5.20, 12.74)	3.24 (−7.38, 13.86)	2.17 (−8.11, 12.45)	1.39 (−9.05, 11.82)	1.20 (−10.10, 12.51)	HMS	−1.50 (−11.36, 8.37)	−5.99 (−13.55, 1.56)	−24.91 (−38.70, −11.12)
5.26 (1.14, 9.38)	**4.74 (0.31, 9.16)**	**3.66 (0.73, 6.60)**	2.88 (−0.52, 6.28)	2.70 (−3.54, 8.93)	1.50 (−8.37, 11.36)	NI	−4.50 (−10.85, 1.86)	−23.42 (−34.67, −12.16)
9.76 (4.92, 14.60)	**9.23 (1.76, 16.70)**	**8.16 (1.18, 15.15)**	**7.38 (0.17, 14.59)**	7.19 (−1.22, 15.61)	5.99 (−1.56, 13.55)	4.50 (−1.86, 10.85)	PCT	−18.92 (−30.45, −7.38)
28.68 (18.20, 39.15)	**28.15 (16.07, 40.23)**	**27.08 (15.46, 38.70)**	**26.30 (14.54, 38.05)**	**26.11 (13.27, 38.96)**	**24.91 (11.12, 38.70)**	**23.42 (12.16, 34.67)**	**18.92 (7.38, 30.45)**	NFB

The bold values represent the signify statistical significance.

104 for the experimental group and 5–98 for the control group, with relatively more men in the included studies. The included experimental and control groups' mean age was less than or equal to 16 years. Interventions included cognitive-motor training (6 studies) (Hoza et al., 2015; Pan et al., 2016; Meßler et al., 2018; Benzing and Schmidt, 2019; Kadri et al., 2019; Mansson et al., 2019), combination exercise (6 studies) (Kang et al., 2011; Choi et al., 2015; Ziereis and Jansen, 2015; Bustamante et al., 2016; Memarmoghaddam et al., 2016; Lee et al., 2017), traditional aerobic exercise (5 studies) (Ahmed and Mohamed, 2011; Hoza et al., 2015; Gelade et al., 2017; Meßler et al., 2018; Kadri et al., 2019), acute aerobic exercise (4 studies) (Chang et al., 2012; Gawrilow et al., 2016; Ludyga et al., 2017; Benzing et al., 2018), aquatic exercise (3 studies) (Chang et al., 2014; Da Silva et al., 2020; Sabzi et al., 2021), horsemanship (3 studies) (García-Gómez et al., 2016; Oh et al., 2018; Ahn et al., 2021), perceptual-motor training (3 studies) (Yazd et al., 2015; Hattabi et al., 2019; Sani et al., 2022), mind-body exercise (2 studies) (Jensen and Kenny, 2004; Milligan et al., 2019), as well as sensory integration training (1 study) (Faramarzi et al., 2016). The outcome indicators for ADHD consisted of motor ability, social problems, attention problems, cognitive flexibility, inhibition switching, as well as the working memory.

3.3. Risk of bias

Eighteen studies (58.1%) had a low risk of bias with respect to random sequence generation. Twenty-one studies (67.7%) had a low risk of bias with respect to allocation concealment. Sixteen studies (51.6%) had a low risk of bias with respect to the blinding of participants and personnel. Twenty-four studies (77.4%) had a low risk of bias with respect to the blinding of outcome assessments. Twenty-nine studies (93.5%) had a low risk of bias with respect to incomplete outcome data. Thirty studies (96.8%) had a low risk of bias with respect to selective reporting. Other biases are not known. Details of the evaluation of bias results for the included literature are shown in **Figures 2A, B**.

3.4. Network meta-analysis

The complete NMA figure will be presented in **Supplementary Appendixes B1–6**.

3.4.1. Motor ability

Seven studies (Chang et al., 2014; Yazd et al., 2015; Pan et al., 2016; Meßler et al., 2018; Oh et al., 2018; Da Silva et al., 2020; Sani et al., 2022) reported on the motor ability of children with ADHD, and a total of nine interventions are involved. As shown in **Table 2**, the statistically significant results of the network meta-analysis were as follows: Perceptual-motor training [*MD* = 5.26, 95% *CI* = (1.14, 9.38)], traditional aerobic exercise [*MD* = 4.74, 95% *CI* = (0.31, 9.16)], and aquatic exercise [*MD* = 3.66, 95% *CI* = (0.73, 6.60)], which were more effective than that with no intervention. Compared with pharmacotherapy, perceptual-motor training [*MD* = 9.76, 95% *CI* = (4.92, 14.60)], traditional aerobic exercise [*MD* = 9.23, 95% *CI* = (1.76, 16.70)], aquatic exercise [*MD* = 8.16, 95% *CI* = (1.18, 15.15)], combination exercise

TABLE 3 League table on attention problems.

AE	PCT	CMT	HMS	MBE	NI	CBE	NFB	CI	TAE
AE	4.71 (−35.80, 45.21)	8.63 (−24.77, 42.03)	9.97 (−23.99, 43.92)	19.34 (−22.39, 61.07)	18.81 (−8.53, 46.16)	21.55 (−19.94, 63.04)	24.65 (−31.56, 80.87)	25.21 (−11.07, 61.48)	26.82 (−5.04, 58.68)
−4.71 (−45.21, 35.80)	PCT	3.92 (−31.05, 38.89)	5.26 (−21.49, 32.01)	14.63 (−27.98, 57.24)	14.11 (−16.82, 45.03)	16.84 (−26.25, 59.94)	19.95 (−27.85, 67.74)	20.50 (−17.47, 58.47)	22.11 (−10.69, 54.92)
−8.63 (−42.03, 24.77)	−3.92 (−38.89, 31.05)	CMT	1.34 (−27.84, 30.51)	10.71 (−23.14, 44.56)	10.18 (−11.66, 32.03)	12.92 (−21.17, 47.01)	16.02 (−35.98, 68.03)	16.58 (−10.65, 43.81)	18.19 (−0.52, 36.90)
−9.97 (−43.92, 23.99)	−5.26 (−32.01, 21.49)	−1.34 (−30.51, 27.84)	HMS	9.37 (−29.70, 48.45)	8.85 (−12.40, 30.10)	11.58 (−27.74, 50.91)	14.68 (−35.98, 65.35)	15.24 (−18.38, 48.86)	16.85 (−10.79, 44.49)
−19.34 (−61.07, 22.39)	−14.63 (−57.24, 27.98)	−10.71 (−44.56, 23.14)	−9.37 (−48.45, 29.70)	MBE	−0.53 (−35.16, 34.11)	2.21 (−26.80, 31.22)	5.31 (−51.68, 62.30)	5.87 (−14.46, 26.19)	7.48 (−21.28, 36.24)
−18.81 (−46.16, 8.53)	−14.11 (−45.03, 16.82)	−10.18 (−32.03, 11.66)	−8.85 (−30.10, 12.40)	0.53 (−34.11, 35.16)	NI	2.74 (−32.04, 37.51)	5.84 (−44.80, 56.48)	6.39 (−21.77, 34.55)	8.00 (−12.63, 28.64)
−21.55 (−63.04, 19.94)	−16.84 (−59.94, 26.25)	−12.92 (−47.01, 21.17)	−11.58 (−50.91, 27.74)	−2.21 (−31.22, 26.80)	−2.74 (−37.51, 32.04)	CBE	3.10 (−54.17, 60.38)	3.66 (−17.09, 24.41)	5.27 (−23.63, 34.17)
−24.65 (−80.87, 31.56)	−19.95 (−67.74, 27.85)	−16.02 (−68.03, 35.98)	−14.68 (−65.35, 35.98)	−5.31 (−62.30, 51.68)	−5.84 (−56.48, 44.80)	−3.10 (−60.38, 54.17)	NFB	0.56 (−52.95, 54.06)	2.17 (−47.69, 52.02)
−25.21 (−61.48, 11.07)	−20.50 (−58.47, 17.47)	−16.58 (−43.81, 10.65)	−15.24 (−48.86, 18.38)	−5.87 (−26.19, 14.46)	−6.39 (−34.55, 21.77)	−3.66 (−24.41, 17.09)	−0.56 (−54.06, 52.95)	CI	1.61 (−18.67, 21.89)
−26.82 (−58.68, 5.04)	−22.11 (−54.92, 10.69)	−18.19 (−36.90, 0.52)	−16.85 (−44.49, 10.79)	−7.48 (−36.24, 21.28)	−8.00 (−28.64, 12.63)	−5.27 (−34.17, 23.63)	−2.17 (−52.02, 47.69)	−1.61 (−21.89, 18.67)	TAE

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TABLE 4 League table on social problems.

HMS	PCT	AE	TAE	CMT	NI
HMS	1.83 (−3.46, 7.12)	6.41 (−12.08, 24.91)	8.10 (−10.78, 26.98)	8.48 (−10.39, 27.35)	10.11 (−8.34, 28.57)
−1.83 (−7.12, 3.46)	PCT	4.58 (−14.65, 23.82)	6.27 (−13.33, 25.88)	6.65 (−12.95, 26.25)	8.28 (−10.91, 27.48)
−6.41 (−24.91, 12.08)	−4.58 (−23.82, 14.65)	AE	1.69 (−2.53, 5.90)	2.07 (−2.11, 6.25)	3.70 (2.37, 5.03)
−8.10 (−26.98, 10.78)	−6.27 (−25.88, 13.33)	−1.69 (−5.90, 2.53)	TAE	0.38 (−0.13, 0.89)	2.01 (−1.99, 6.01)
−8.48 (−27.35, 10.39)	−6.65 (−26.25, 12.95)	−2.07 (−6.25, 2.11)	−0.38 (−0.89, 0.13)	CMT	1.63 (−2.33, 5.60)
−10.11 (−28.57, 8.34)	−8.28 (−27.48, 10.91)	**−3.70 (−5.03,−2.37)**	−2.01 (−6.01, 1.99)	−1.63 (−5.60, 2.33)	NI

The bold values represent the signify statistical significance.

[*MD* = 7.38, 95% *CI* = (0.17, 14.59)] were more effective. Compared with neurofeedback, perceptual-motor training [*MD* = 28.68, 95% *CI* = (18.20, 39.15)], traditional aerobic exercise [*MD* = 28.15, 95% *CI* = (16.07, 40.23)], aquatic exercise [*MD* = 27.08, 95% *CI* = (15.46, 38.70)], combination exercise [*MD* = 26.30, 95% *CI* = (14.54, 38.05)], cognitive-motor training [*MD* = 26.11, 95% *CI* = (13.27, 38.96)], and horsemanship [*MD* = 24.91, 95% *CI* = (11.12, 38.70)] were more effective. In SUCRA, perceptual-motor training ranked first in terms of the probability of the effect of different interventions on motor performance (SUCRA: 82.7%, as shown in **Supplementary Appendix C1**).

3.4.2. Attention problems

Fourteen studies (Jensen and Kenny, 2004; Ahmed and Mohamed, 2011; Kang et al., 2011; Hoza et al., 2015; Bustamante et al., 2016; García-Gómez et al., 2016; Janssen et al., 2016; Pan et al., 2016; Meßler et al., 2018; Oh et al., 2018; Kadri et al., 2019; Milligan et al., 2019; Da Silva et al., 2020; Ahn et al., 2021) reported on the attention problems of children with ADHD, and a total of ten interventions are involved. As shown in **Table 3**, there is no statistical significance for each intervention in the network meta-analysis results. In SUCRA, aquatic exercise ranked first in terms of the probability of the effect of different interventions on the reduction of attention problems (SUCRA: 80.9%, as shown in **Supplementary Appendix C2**).

3.4.3. Social problems

Five studies (Pan et al., 2016; Meßler et al., 2018; Oh et al., 2018; Ahn et al., 2021; Sabzi et al., 2021) reported on the social problems of children with ADHD, and a total of six interventions are involved. As shown in **Table 4**, the statistically significant results of the network meta-analysis were as follows: Aquatic exercise [*MD* = −3.70, 95% *CI* = (−5.03, −2.37)] was more effective than that with no intervention. In the SUCRA, aquatic exercise ranked first in terms of the probability of the effect of different interventions on the reduction of social problems (SUCRA: 79.4%, as shown in **Supplementary Appendix C3**).

3.4.4. Cognitive flexibility

Fourteen studies (Jensen and Kenny, 2004; Kang et al., 2011; Chang et al., 2012, 2014; Hoza et al., 2015; Ziereis and Jansen, 2015; Gawrilow et al., 2016; Memarmoghaddam et al., 2016; Pan et al., 2016; Lee et al., 2017; Benzing et al., 2018; Kadri et al., 2019; Milligan et al., 2019; Da Silva et al., 2020) reported on the cognitive flexibility of children with ADHD, and a total of nine interventions are involved. As shown in **Table 5**, the statistically significant results of the network meta-analysis were as follows: Aquatic exercise [*MD* = 19.65, 95% *CI* = (3.91, 35.40)] was more effective than combination exercise. Aquatic exercise [*MD* = 22.16, 95% *CI* = (8.36, 35.97)] was more effective than that with no intervention. Compared to traditional aerobic exercise, aquatic exercise [*MD* = 43.47, 95% *CI* = (17.05, 69.89)], acute aerobic exercise [*MD* = 32.10, 95% *CI* = (1.38, 62.82)], and cognitive-motor training [*MD* = 30.06, 95% *CI* = (16.23, 43.89)] were more effective. In SUCRA, aquatic exercise ranked first in terms of the probability of the effect of different interventions on cognitive flexibility (SUCRA: 86.6%, as shown in **Supplementary Appendix C4**).

3.4.5. Inhibition switching

Eighteen studies (Kang et al., 2011; Chang et al., 2012, 2014; Choi et al., 2015; Hoza et al., 2015; Bustamante et al., 2016; Faramarzi et al., 2016; García-Gómez et al., 2016; Gawrilow et al., 2016; Janssen et al., 2016; Memarmoghaddam et al., 2016; Ludyga et al., 2017; Benzing et al., 2018; Benzing and Schmidt, 2019; Hattabi et al., 2019; Kadri et al., 2019; Mansson et al., 2019; Milligan et al., 2019; Sani et al., 2022) reported the inhibition switching of children with ADHD, and a total of 13 interventions are involved. As shown in **Table 6**, the statistically significant results of the network meta-analysis were as follows: Cognitive-motor training [*MD* = −67.14, 95% *CI* = (−130.91, −3.37)] was more effective than cognitive intervention. Compared with aquatic exercise, cognitive-motor training [*MD* = −146.75, 95% *CI* = (−257.37, 36.13)], perceptual-motor training [*MD* = −143.08, 95% *CI* = (−262.96, 23.19)], combination exercise [*MD* = −129.35, 95% *CI* = (−232.92, 25.77)], and acute aerobic exercise [*MD* = −112.14, 95% *CI* = (−214.20, 10.09)] were more effective. In SUCRA, cognitive-motor training ranked first in the probability of the effect of different interventions on inhibition switching (SUCRA: 83.5%, as shown in **Supplementary Appendix C5**).

3.4.6. Working memory

Ten studies (Kang et al., 2011; Hoza et al., 2015; Ziereis and Jansen, 2015; Bustamante et al., 2016; Janssen et al., 2016; Benzing et al., 2018; Benzing and Schmidt, 2019; Hattabi et al., 2019; Kadri et al., 2019; Milligan et al., 2019) reported on the working memory of children with ADHD, and a total of nine interventions are involved. As shown in **Table 7**, the statistically significant results of the network meta-analysis were as follows: cognitive-motor training [*MD* = 9.45, 95% *CI* = (2.39, 16.51)] was more effective than traditional aerobic exercise. In SUCRA, perceptual-motor training ranked first in terms of the probability of effectiveness of different interventions on working memory (SUCRA: 73.3%, as shown in **Supplementary Appendix C6**).

TABLE 5 League table on cognitive flexibility.

AE	MBE	CI	AAE	CMT	PCT	CBE	NI	TAE
AE	−8.89 (−43.34, 25.57)	−10.17 (−42.15, 21.80)	−11.37 (−36.93, 14.19)	−13.41 (−35.97, 9.15)	−14.24 (−43.18, 14.70)	−19.65 (−35.40, −3.91)	−22.16 (−35.97, −8.36)	−43.47 (−69.89, −17.05)
8.89 (−25.57, 43.34)	MBE	−1.28 (−14.23, 11.66)	−2.48 (−25.53, 20.56)	−4.52 (−40.47, 31.42)	−5.36 (−45.57, 34.85)	−10.77 (−43.23, 21.70)	−13.27 (−44.90, 18.35)	−34.58 (−72.95, 3.78)
10.17 (−21.80, 42.15)	1.28 (−11.66, 14.23)	CU	−1.20 (−20.34, 17.94)	−3.24 (−36.81, 30.33)	−4.07 (−42.18, 34.04)	−9.48 (−39.30, 20.34)	−11.99 (−40.89, 16.91)	−33.30 (−69.45, 2.86)
11.37 (−14.19, 36.93)	2.48 (−20.56, 25.53)	1.20 (−17.94, 20.34)	AAE	−2.04 (−29.60, 25.51)	−2.87 (−35.80, 30.05)	−8.28 (−31.11, 14.54)	−10.79 (−32.42, 10.84)	−32.10 (−62.82, −1.38)
13.41 (−9.15, 35.97)	4.52 (−31.42, 40.47)	3.24 (−30.33, 36.81)	2.04 (−25.51, 29.60)	CMT	−0.83 (−19.03, 17.37)	−6.24 (−25.63, 13.15)	−8.75 (−26.60, 9.10)	−30.06 (−43.89, −16.23)
14.24 (−14.70, 43.18)	5.36 (−34.85, 45.57)	4.07 (−34.04, 42.18)	2.87 (−30.05, 35.80)	0.83 (−17.37, 19.03)	PCT	−5.41 (−31.95, 21.13)	−7.92 (−33.36, 17.52)	−29.23 (−52.08, −6.37)
19.65 (3.91, 35.40)	10.77 (−21.70, 43.23)	9.48 (−20.34, 39.30)	8.28 (−14.54, 31.11)	6.24 (−13.15, 25.63)	5.41 (−21.13, 31.95)	CBE	−2.51 (−10.09, 5.07)	−23.82 (−47.58, −0.05)
22.16 (8.36, 35.97)	13.27 (−18.35, 44.90)	11.99 (−16.91, 40.89)	10.79 (−10.84, 32.42)	8.75 (−9.10, 26.60)	7.92 (−17.52, 33.36)	2.51 (−5.07, 10.09)	NI	−21.31 (−43.83, 1.21)
43.47 (17.05, 69.89)	34.58 (−3.78, 72.95)	33.30 (−2.86, 69.45)	**32.10 (1.38, 62.82)**	**30.06 (16.23, 43.89)**	**29.23 (6.37, 52.08)**	**23.82 (0.05, 47.58)**	21.31 (−1.21,43.83)	TAE

The bold values represent the signify statistical significance.

3.5. Publication bias

As is vividly shown in **Supplementary Appendix D**, funnel plots were employed to detect publication bias, while no significant publication bias was revealed by the visual inspection of funnel plots for all indicators.

4. Discussion

In this study, motor ability, attention problems, social problems, cognitive flexibility, inhibition switching, and working memory are adopted as outcome indicators to compare the effects of different interventions on each outcome indicator. As shown in **Table 8**, it has been shown in our current study that perceptual-motor training, traditional aerobic exercise, as well as aquatic exercise were the top three interventions for benign development in motor ability. When it comes to attention problems, aquatic exercise, pharmacotherapy, and cognitive-motor training were the top three interventions to reduce attention problems. As for the indicator of the social problem, horsemanship, pharmacotherapy, and aquatic exercise were the top three interventions in reducing social problems. In terms of cognitive flexibility, aquatic exercise, mind-body exercise, and cognitive intervention were the top three interventions to increase cognitive flexibility. For inhibition switching, cognitive-motor training, perceptual-motor training, and combination exercise were the top three interventions to reduce inhibition switching time. Finally, in terms of working memory indicators, perceptual-motor training, pharmacotherapy, and horsemanship were the top three interventions for enhancing working memory. It has been shown in our findings that there is no single intervention most effective across all outcome indicators, and different interventions may be more effective for different outcomes.

Perceptual-motor training is the best physical activity intervention for children with ADHD regarding motor ability and working memory. This type of training combines physical activities such as coordination, balance, and strength with perceptual tasks (Hattabi et al., 2019). Previous research has demonstrated a strong correlation between motor behavior and underlying perceptual processes (Chu and Reynolds, 2007). In particular, when physical activity is designed to improve attention, it will contribute to developing executive functions (Piek et al., 2004; Hung et al., 2013). By combining training activities with perceptual tasks, there is potential for an overall improvement in motor ability and working memory in children with ADHD (Mandich et al., 2001).

The aquatic exercise was the intervention with the highest frequency (4 sessions) in the top three rankings for all outcome indicators and the best physical activity intervention in terms of both attention problems and cognitive flexibility. Aquatic exercise is a form of physical activity in which the training process is completed in an aquatic environment. Due to the fluid nature of water, physical activity in an aquatic environment requires participants to constantly pay attention to the environment's fluctuations (Vivas et al., 2011). At the same time, the buoyancy effect of water provides an auxiliary force, resistance, or support, which makes physical activity in the water environment safer, and children's activity can be more active (Broach and Dattilo, 1996).

TABLE 6 League table on inhibition switching.

CMT	PMT	CBE	NFB	AAE	TAE	MBE	PCT	SIT	HMS	NI	CI	AE
CMT	3.67 (−86.92, 94.26)	17.40 (−56.88, 91.68)	31.40 (−57.04, 119.83)	34.61 (−27.75, 96.96)	44.27 (−29.38, 117.91)	46.46 (−66.93, 159.84)	46.34 (−33.15, 125.82)	54.59 (−54.83, 164.00)	55.91 (−53.49, 165.31)	60.86 (−3.10, 124.82)	67.14 (3.37, 130.91)	146.75 (36.13, 257.37)
−3.67 (−94.26, 86.92)	PMT	13.73 (−76.62, 104.09)	27.73 (−56.77, 112.23)	30.94 (−53.19, 115.06)	40.60 (−61.56, 142.76)	42.79 (−86.99, 172.56)	42.67 (−62.63, 147.96)	50.92 (−67.83, 169.66)	52.24 (−66.49, 170.97)	57.19 (−21.00, 135.38)	63.47 (−26.71, 153.65)	143.08 (23.19, 262.96)
−17.40 (−91.68, 56.88)	−13.73 (−104.09, 76.62)	CBE	13.99 (−83.90, 111.89)	17.20 (−42.57, 76.98)	26.86 (−69.39, 123.12)	29.05 (−83.68, 141.79)	28.93 (−71.39, 129.26)	37.18 (−65.44, 139.81)	38.51 (−64.12, 141.13)	43.46 (−7.17, 94.08)	49.74 (−13.24, 112.71)	129.35 (25.77, 232.92)
−31.40 (−119.83, 57.04)	−27.73 (−112.23, 56.77)	−13.99 (−111.89, 83.90)	NFB	3.21 (−85.40, 91.82)	12.87 (−76.97, 102.71)	15.06 (−117.29, 147.41)	14.94 (−77.44, 107.32)	23.19 (−102.51, 148.89)	24.51 (−101.17, 150.20)	29.46 (−59.43, 118.36)	35.74 (−58.02, 129.50)	115.35 (−11.46, 242.17)
−34.61 (−96.96, 27.75)	−30.94 (−115.06, 53.19)	−17.20 (−76.98, 42.57)	−3.21 (−91.82, 85.40)	AAE	9.66 (−76.47, 95.79)	11.85 (−91.48, 115.18)	11.73 (−78.85, 102.31)	19.98 (−80.77, 120.72)	21.30 (−79.43, 122.03)	26.25 (−23.46, 75.96)	32.53 (−12.79, 77.85)	112.14 (10.09, 214.20)
−44.27 (−117.91, 29.38)	−40.60 (−142.76, 61.56)	−26.86 (−123.12, 69.39)	−12.87 (−102.71, 76.97)	−9.66 (−95.79, 76.47)	TAE	2.19 (−127.24, 131.62)	2.07 (−84.58, 88.71)	10.32 (−114.78, 135.42)	11.64 (−113.44, 136.73)	16.59 (−71.61, 104.79)	22.87 (−66.54, 112.28)	102.48 (−23.74, 228.71)
−46.46 (−159.84, 66.93)	−42.79 (−172.56, 86.99)	−29.05 (−141.79, 83.68)	−15.06 (−147.41, 117.29)	−11.85 (−115.18, 91.48)	−2.19 (−131.62, 127.24)	MBE	−0.12 (−132.62, 132.38)	8.13 (−133.02, 149.27)	9.45 (−131.68, 150.59)	14.40 (−95.82, 124.62)	20.68 (−74.26, 115.62)	100.29 (−41.87, ‘242.45)
−46.34 (−125.82, 33.15)	−42.67 (−147.96, 62.63)	−28.93 (−129.26, 71.39)	−14.94 (−107.32, 77.44)	−11.73 (−102.31,7 8.85)	−2.07 (−88.71, 84.58)	0.12 (−132.38, 132.62)	PCT	8.25 (−119.96, 136.45)	9.57 (−118.62, 137.76)	14.52 (−78.01, 107.05)	20.80 (−72.98, 114.59)	100.41 (−28.93, 229.76)
−54.59 (−164.00, 54.83)	−50.92 (−169.66, 67.83)	−37.18 (−139.81, 65.44)	−23.19 (−148.89, 102.51)	−19.98 (−120.72, 80.77)	−10.32 (−135.42, 114.78)	−8.13 (−149.27, 133.02)	−8.25 (−136.45, 119.96)	SIT	1.32 (−125.96, 128.60)	6.27 (−83.86,96.41)	12.55 (−93.37, 118.48)	92.16 (−36.14, 220.47)
−55.91 (−165.31, 53.49)	−52.24 (−170.97, 66.49)	−38.51 (−141.13, 64.12)	−24.51 (−150.20, 101.17)	−21.30 (−122.03, 79.43)	−11.64 (−136.73, 113.44)	−9.45 (−150.59, 131.68)	−9.57 (−137.76, 118.62)	−1.32 (−128.60, 125.96)	HMS	4.95 (−85.17, 95.07)	11.23 (−94.67, 117.13)	90.84 (−37.47, 219.15)
−60.86 (−124.82, 3.10)	−57.19 (−135.38, 21.00)	−43.46 (−94.08, 7.17)	−29.46 (−118.36, 59.43)	−26.25 (−75.96, 23.46)	−16.59 (−104.79, 71.61)	−14.40 (−124.62, 95.82)	−14.52 (−107.05, 78.01)	−6.27 (−96.41, 83.86)	−4.95 (−95.07, 85.17)	NI	6.28 (−52.26, 64.82)	85.89 (−5.83, 177.62)
−67.14 (−130.91, −3.37)	−63.47 (−153.65, 26.71)	−49.74 (−112.71, 13.24)	−35.74 (−129.50, 58.02)	−32.53 (−77.85, 12.79)	−22.87 (−112.28, 66.54)	−20.68 (−115.62, 74.26)	−20.80 (−114.59, 72.98)	−12.55 (−118.48, 93.37)	−11.23 (−117.13, 94.67)	−6.28 (−64.82, 52.26)	CI	79.61 (−27.84, 187.06)
−146.75 (−257.37, −36.13)	**−143.08 (−262.96, −23.19)**	**−129.35 (−232.92, −25.77)**	−115.35 (−242.17, 11.46)	**−112.14 (−214.20, −10.09)**	−102.48 (−228.71, 23.74)	−100.29 (−242.45, 41.87)	−100.41 (−229.76, 28.93)	−92.16 (−220.47, 36.14)	−90.84 (−219.15, 37.47)	−85.89 (−177.62, 5.83)	−79.61 (−187.06, 27.84)	AE

The bold values represent the signify statistical significance.

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TABLE 7 League table on working memory.

PMT	MBE	HMS	CMT	CBE	AAE	CI	NI	PCT	NFB	TAE
PMT	−1.18 (−21.98, 19.62)	−1.57 (−14.75, 11.60)	−3.71 (−21.19, 13.78)	−3.24 (−16.43, 9.95)	−4.83 (−22.95, 13.30)	−4.82 (−19.54, 9.90)	−4.85 (−14.15, 4.45)	−8.59 (−26.80, 9.61)	−10.24 (−29.38, 8.90)	−13.16 (−31.95, 5.64)
1.18 (−19.62, 21.98)	MBE	−0.39 (−21.20, 20.42)	−2.53 (−19.99, 14.94)	−2.06 (−18.16, 14.04)	−3.64 (−21.82, 14.53)	−3.64 (−18.33, 11.05)	−3.67 (−22.28, 14.94)	−7.41 (−25.62, 10.79)	−9.06 (−28.22, 10.09)	−11.98 (−30.80, 6.85)
1.57 (−11.60, 14.75)	0.39 (−20.42, 21.20)	HMS	−2.13 (−19.63, 15.36)	−1.67 (−14.88, 11.54)	−3.25 (−21.39, 14.89)	−3.25 (−17.98, 11.49)	−3.28 (−12.61, 6.05)	−7.02 (−25.24, 11.20)	−8.67 (−27.82, 10.48)	−11.58 (−30.39, 7.22)
3.71 (−13.78, 21.19)	2.53 (−14.94, 19.99)	2.13 (−15.36, 19.63)	CMT	0.47 (−11.03, 11.96)	−1.12 (−15.27, 13.03)	−1.11 (−10.55, 8.32)	−1.14 (−15.95, 13.67)	−4.89 (−10.07, 0.30)	−6.54 (−14.44, 1.37)	−9.45 (−16.51, −2.39)
3.24 (−9.95, 16.43)	2.06 (−14.04, 18.16)	1.67 (−11.54, 14.88)	−0.47 (−11.96, 11.03)	CBE	−1.58 (−14.10, 10.93)	−1.58 (−8.15, 4.99)	−1.61 (−10.97, 7.75)	−5.35 (−17.92, 7.21)	−7.00 (−20.89, 6.89)	−9.92 (−23.32, 3.49)
4.83 (−13.30, 22.95)	3.64 (−14.53, 21.82)	3.25 (−14.89, 21.39)	1.12 (−13.03, 15.27)	1.58 (−10.93, 14.10)	AAE	0.00 (−10.70, 10.71)	−0.02 (−15.60, 15.55)	−3.77 (−18.80, 11.27)	−5.42 (−21.58, 10.75)	−8.33 (−24.10, 7.44)
4.82 (−9.90, 19.54)	3.64 (−11.05, 18.33)	3.25 (−11.49, 17.98)	1.11 (−8.32, 10.55)	1.58 (−4.99, 8.15)	−0.00 (−10.71, 10.70)	CI	−0.03 (−11.45, 11.39)	−3.77 (−14.52, 6.97)	−5.42 (−17.71, 6.86)	−8.34 (−20.10, 3.42)
4.85 (−4.45, 14.15)	3.67 (−14.94, 22.28)	3.28 (−6.05, 12.61)	1.14 (−13.67, 15.95)	1.61 (−7.75, 10.97)	0.02 (−15.55, 15.60)	0.03 (−11.39, 11.45)	NI	−3.74 (−19.40, 11.91)	−5.39 (−22.13, 11.34)	−8.31 (−24.64, 8.03)
8.59 (−9.61, 26.80)	7.41 (−10.79, 25.62)	7.02 (−11.20, 25.24)	4.89 (−0.30, 10.07)	5.35 (−7.21, 17.92)	3.77 (−11.27, 18.80)	3.77 (−6.97, 14.52)	3.74 (−11.91, 19.40)	PCT	1.65 (−8.12, 4.82)	−4.56 (−10.51, 1.38)
10.24 (−8.90, 29.38)	9.06 (−10.09, 28.22)	8.67 (−10.48, 27.82)	6.54 (−1.37, 14.44)	7.00 (−6.89, 20.89)	5.42 (−10.75, 21.58)	5.42 (−6.86, 17.71)	5.39 (−11.34, 22.13)	1.65 (−4.82, 8.12)	NFB	−2.91 (−9.39, 3.56)
13.16 (−5.64, 31.95)	11.98 (−6.85, 30.80)	11.58 (−7.22, 30.39)	**9.45 (2.39, 16.51)**	9.92 (−3.49, 23.32)	8.33 (−7.44, 24.10)	8.34 (−3.42, 20.10)	8.31 (−8.03, 24.64)	4.56 (−1.38, 10.51)	2.91 (−3.56, 9.39)	TAE

The bold values represent the signify statistical significance.

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TABLE 8 Ranking of SUCRA probabilities for each outcome indicator.

Intervention	Motor ability		Attention problems		Social problems	
	Sucra	Rank	Sucra	Rank	Sucra	Rank
Horsemanship	51.8	6	63.1	4	79.4	1
Combination exercise	60.9	4	40	7	/	/
Perceptual-motor training	82.7	1	/	/	/	/
Cognitive-motor training	58.1	5	68.5	3	29.3	5
Aquatic exercise	69	3	80.9	1	63.7	3
Mind-body exercise	/	/	45.1	5	/	/
Acute aerobic exercise	/	/	/	/	/	/
Traditional aerobic exercise	79.6	2	23.1	10	48.7	4
Sensory integration training	/	/	/	/	/	/
Neurofeedback	0	9	36.3	8	/	/
Cognitive intervention	/	/	28.9	9	/	/
Pharmacotherapy	15.5	8	72.4	2	64.6	2
No intervention	32.4	7	41.7	6	14.4	6
Intervention	**Cognitive flexibility**		**Inhibition switching**		**Working memory**	
	Sucra	Rank	Sucra	Rank	Sucra	Rank
Horsemanship	/	/	43	10	66.3	3
Combination exercise	39.1	7	71.5	3	61.2	5
Perceptual-motor training	/	/	78	2	73.3	1
Cognitive-motor training	55.7	5	83.5	1	62.3	4
Aquatic exercise	86.6	1	4.7	12	/	/
Mind-body exercise	65.7	2	48.6	7	66.9	2
Acute aerobic exercise	60	4	58	5	51.1	6
Traditional aerobic exercise	2	9	48.9	6	10.3	11
Sensory integration training	/	/	43.1	9	/	/
Neurofeedback	/	/	58.2	4	25.6	10
Cognitive intervention	60.9	3	30.1	12	51.1	7
Pharmacotherapy	53	6	47.9	8	33.4	9
No intervention	28	8	34.6	11	48.3	8

For example, swimming in water sports is a highly coordinated and lateralized sport requiring control of the upper and lower limbs in an aquatic environment (Colgate and Lynch, 2004). This feature may allow for further activation of brain regions in the prefrontal cortex and amygdala, thus contributing to improved attentional problems and cognitive flexibility (Faw, 2003).

Horsemanship is the best physical activity intervention in terms of indicators of social problems. Horsemanship is a physical activity modality through learning activities with horses as a vehicle (Kern et al., 2011). It has been shown that because equestrian learning requires participants to establish trust and frequent interaction with the horse, it contributes to developing participants' social competence (Hauge et al., 2014) and self-efficacy (Bizub et al., 2003). With this mutual relationship with the horse, children experience the horse's feelings, which are then internalized in their behavior, enabling further development of empathy. This change will likely transfer to human interactions (Granados and Agis, 2011). At the same time, the horse's rhythmic activity also improves the participants' physiological responses to stress and impulsivity (Tyler, 1994; Jang et al., 2015).

Cognitive-motor training is the best physical activity intervention for inhibiting conversion indicators. Cognitive-motor training is an intervention that integrates cognitive and motor tasks to promote an individual's physical and mental health (Amini et al., 2022). It has been shown that performing two or more cognitive-motor tasks simultaneously, such as computation in postural training and movement under computer games, will contribute more to improvements in cognitive domains compared to single-task training (van der Niet et al., 2016; Luder et al., 2018; Schmidt et al., 2020) while reducing reaction time (Wollesen et al., 2020). Cognitive-motor training requires participants to use both skill and cognitive effort to cope with unpredictable stimuli from the external environment (Chuang et al., 2015). Therefore, some researchers have suggested that this may improve participants' executive functioning, including improvements in inhibitory switching (Kunstler et al., 2018; Gao et al., 2019).

In conclusion, physical activity interventions have varying levels of effect on different indicators related to the symptoms of children with ADHD. This impact is dependent on the components, characteristics, and settings of the intervention. Nevertheless, physical activity interventions have been found to have numerous advantages across multiple indicators.

5. Strengths and limitations

One advantage of our current study is that we are the first network meta-analysis of the effects of physical activity on symptoms related to children with ADHD, which provides some scientific reference for selecting appropriate physical activity therapy for children with ADHD. The second advantage is that this study explored the effects of different physical activities on different symptom indicators in children with ADHD, which can provide some scientific reference for targeted treatment. The third advantage is that the current study only included studies from randomized controlled trials and excluded observational and cross-sectional studies, which helped to enhance the reliability of the findings. However, our reticulated meta-analysis also has some limitations that may affect the interpretation of the results. First, the relatively small number of available studies and the limited number and sample size of studies included in the analysis makes it difficult to give a particularly robust conclusion. Second, the outcome indicators that could be included are still limited. In the future, more outcome indicators of symptoms related to children with ADHD should be included based on an adequate number of studies. Finally, findings should be interpreted with caution because of the small number of studies and the limited evidence for direct comparisons of some interventions. Relevant studies should be further expanded to provide evidence with higher confidence.

6. Conclusion

Our current study showed that the overall performance of aquatic exercise and perceptual-motor training was better. However, different physical activity interventions have different validity and individual differences regarding their effects on different indicators in children with ADHD. Therefore, to ensure that the most suitable physical activity intervention is chosen, it is essential to accurately assess each child's specific ADHD symptoms before implementation.

Author Contributions

CL and DL conceived and designed the study. DL and DW collected the data. DL, DW, and WC analyzed and interpreted the data. DL drafted the manuscript. JY and WZ revised the manuscript. All authors have read and agreed to the published version of the manuscript, and contributed to the study conception and design.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Advances toward precision therapeutics for developmental and epileptic encephalopathies

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Developmental and epileptic encephalopathies are childhood syndromes of severe epilepsy associated with cognitive and behavioral disorders. Of note, epileptic seizures represent only a part, although substantial, of the clinical spectrum. Whether the epileptiform activity *per se* accounts for developmental and intellectual disabilities is still unclear. In a few cases, seizures can be alleviated by antiseizure medication (ASM). However, the major comorbid features associated remain unsolved, including psychiatric disorders such as autism-like and attention deficit hyperactivity disorder-like behavior. Not surprisingly, the number of genes known to be involved is continuously growing, and genetically engineered rodent models are valuable tools for investigating the impact of gene mutations on local and distributed brain circuits. Despite the inconsistencies and problems arising in the generation and validation of the different preclinical models, those are unique and precious tools to identify new molecular targets, and essential to provide prospects for effective therapeutics.

KEYWORDS

epilepsy, rodent models, therapeutics, adeno-associated virus, rare diseases

1. Introduction

A large battery of genes, epigenetic changes, and environmental factors such as pollutants, diet, or brain injuries are key factors that modify brain circuits to generate epileptic disorders that involve multiple interconnected brain regions for epilepsy onset and development (Figure 1A). Brain modifications may result in the alteration of the fine balance between excitation and inhibition (E/I balance; Figure 1C), and a seizure can arise in different brain areas and further spreads to other synaptically-connected brain regions, increasing its severity (Figure 1D).

Worldwide, the prevalence of epileptic diseases is classified into common and rare epilepsies, representing ~95 and ~5%, respectively. Developmental and epileptic encephalopathies (DEEs) are rare diseases (Figure 1B) and comprise a large, heterogeneous group of devastating epileptic disorders occurring in pediatric age. Those are mainly characterized by pharmaco-resistant polymorphous epilepsy, severe electroencephalogram (EEG) abnormalities, and developmental regression (Lin et al., 2022). Patients experience a broad spectrum of neuropsychiatric symptoms ranging from less to more severe, including neurological impairments, intellectual disability, sensory and communication deficits, and other significant psychiatric, motor, and behavioral alterations. DEEs are caused by various factors, among which 30–50% is genetic in origin,

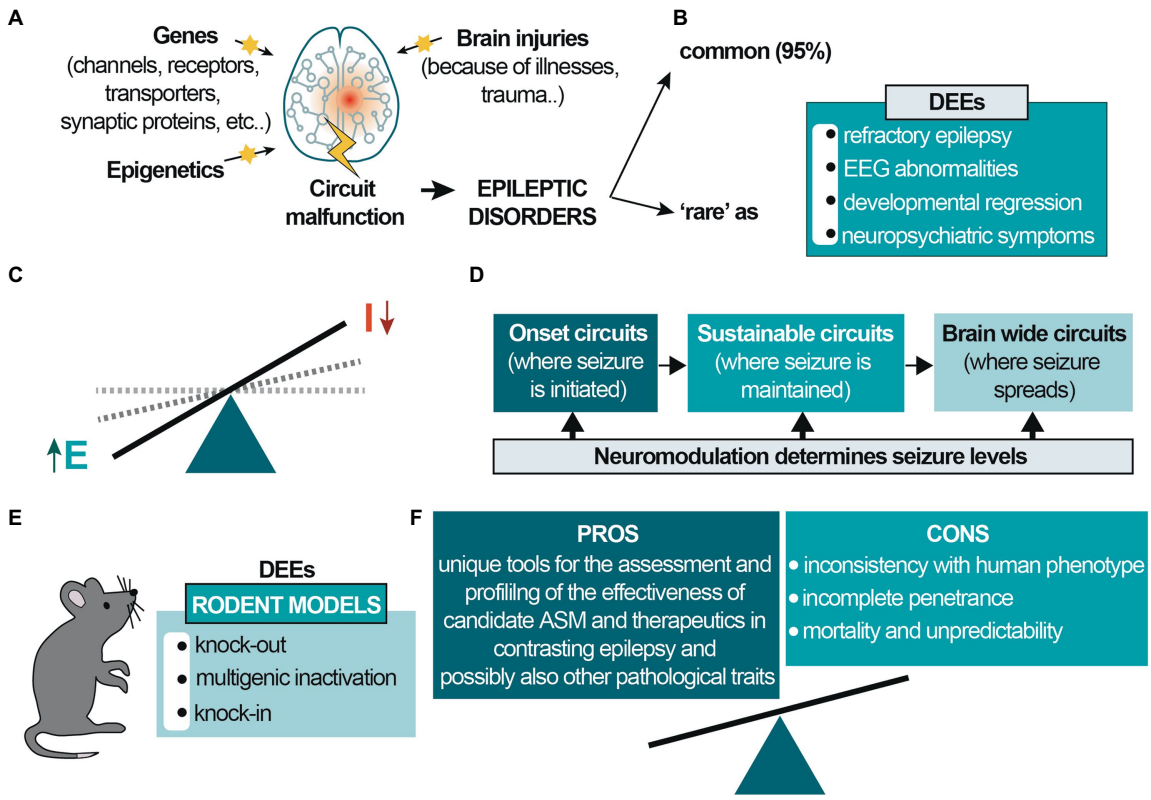

FIGURE 1
Etiology and circuit-based malfunctions in epilepsy. **(A)** Different genes, environmental/epigenetic factors and brain injuries may be the cause of circuit malfunctions that give rise to epilepsy, a network disease involving different interacting brain regions; **(B)** the prevalence of epileptic diseases is classified into common and rare epilepsies (~95 and ~5%, respectively). Developmental and epileptic encephalopathies (DEEs) are rare diseases mainly occurring in pediatric age. **(C)** In epileptic disorders the excitatory drive increases, generating hyperexcitability and E/I dysbalance; **(D)** epilepsy is initiated in different brain regions and activates synaptic brain circuits for seizure maintenance. It can further spread to other brain regions, generating comorbidities and increasing seizure severity. **(E)** Genetic rodent models for DEE mainly consist of knockout, multigenic deletions, and knock-in. These latter may be based on precise human point mutations, satisfying the criteria for 'construct validity completely'; **(F)** Different pros and cons characterize these animal models. The most significant caveats concern the frequent inconsistencies with the human phenotype, particularly the possible absence of the epileptic phenotype and the incomplete penetrance of the mutation. The few models showing spontaneous epileptic seizures are often not viable or affected by a high mortality rate, and the seizures' unpredictability makes them difficult to study. Despite this, genetic animal models are unique and valuable tools for assessing new candidate ASM and other therapeutic strategies and are necessary for subsequent clinical studies, thus benefits prevail.

primarily due to sporadic *de novo* mutations affecting genes involved in the control of neuronal excitability, mainly genes encoding synaptic proteins and ion channels (Allen et al., 2016; von Deimling et al., 2017; Oyrer et al., 2018).

Recent technological advances, among which next-generation whole exome sequencing, have identified many variants. However, due to continuous new diagnoses and mapping of genetic profiles, the number of causative genes for DEEs is continuously growing (McTague et al., 2016; Bayat et al., 2021; Platzer et al., 2022). Despite these progresses, there are no effective therapies approved explicitly for DEEs. Treatment is only symptomatic, aimed at controlling epileptic seizures and restoring abnormal electrical brain activity to reduce the short and long-term sequelae of DEEs. Still, even with polytherapy, partial or complete recovery is rare.

The diagnosis, treatment, and search for a cure for the different DEEs are further complicated by several factors, considering that few cases are currently described and that the patients present variable clinical pictures with multiple comorbidities. Hence, defining an optimal therapy for the various forms of DEEs is still a huge challenge. At the same time, the burden for patients and families remains significant, considering the potential risk of premature mortality, including sudden death (Radaelli et al., 2018). Moreover, one age-related epilepsy syndrome may sometimes evolve into another over time, resulting in many patients with phenotypes not fitting into specific syndromes. Indeed, it is worth considering that mutations in individual genes may give rise to a broad range of phenotypes, and different genes may cause the same syndrome. Many factors contribute to phenotypic heterogeneity, including type and temporal expression of the mutation, mosaicism, convergence of mechanistic pathways, and genome regulators such as epigenetic factors and modifier genes, leading to difficulties in providing genotype/phenotype correlations (Pisani et al., 2018; Guerrini et al., 2023). The complexity of such a scenario makes it clear that a great deal of knowledge is still to be investigated.

Given the phenotypic pleiotropy of gene mutations and the individual heterogeneity of drug responses, a precision medicine targeting the underlying pathology can potentially treat the overall symptomatology and may be considered the most beneficial for DEE

patients (Pisani et al., 2018; Raga et al., 2021). Nowadays, novel advanced precision medicine approaches, such as antisense oligonucleotides (ASOs) and other gene therapy strategies (viral vector systems and CRISPR/Cas9 technology), have raised excitement within the research and medical communities and patients toward effective treatments of rare genetic diseases (Tang et al., 2021). Identifying several causative genes for DEEs has undoubtedly been a tremendous advantage for generating specific genetic animal models. Developing and validating novel preclinical models is fundamental to better understand the general molecular mechanisms that can be common to the different syndromes. Furthermore, the results obtained by testing novel therapies on animal models may help to understand which symptoms' domains are expected to be affected by the new treatment when translated into the clinic.

This minireview briefly focuses on the challenges and opportunities of preclinical genetic models for DEEs, considering a few of them as an example.

Moreover, we will mention some crucial targets for possible circuit-based innovative tools for therapy.

2. Tools and targets

2.1. Rodent models for DEEs: Merits and caveats

There are over a hundred validated DEE-associated genes, the more significant portion including genes that code ion channels or ionotropic receptor subunits (for an exhaustive review, also about animal models, see Guerrini et al., 2023). Despite this, the number is increasing, considering the syndromes' complexity and problematic categorization. Genetically modified rodent models for DEEs consist of classic and conditional knockout, multigenic deletions, and knock-in rodents (Figure 1E). These latter can be based on precise human point mutations, reproducing faithfully the genetic cause responsible for human monogenic disorders. However, the phenotypic spectrum caused by variants in monogenic epilepsy genes is broad and of varying severity, and the mechanisms leading to pleiotropy is not well understood. On one side, the innovative CRISPR-Cas9 technology provides enormous possibilities to quickly generate murine models carrying "patient-specific" mutations for preclinical investigation (Tang et al., 2021). On the other, despite satisfying the criteria for "construct validity," these models still present many caveats, mainly concerning "face validity" (Turner et al., 2021).

First, some of the genetic rodent models generated so far do not show epileptic activity, e.g., Cdkl5 gene knockout or mutated mice (Fallah and Eubanks, 2020) or most of the viable mouse models carrying mutation/deletion in one of the genes coding for the different NMDA receptor subunits: *Grin1*, *Grin2a*, *Grin2b*, or *Grin2d* (Oyrer et al., 2018). These last rodent models are generated to mimic *GRIN*-related disorders, also called "grinpathies" (Santos-Gómez et al., 2021). Compared to rodent models, the alterations of *GRIN* genes in humans have a much more important impact, considering that they are often dominant (XiangWei et al., 2018). Only recently, two mutant mouse models with gain-of-function variants on the GluN2A subunit, which is frequently associated with epilepsy in humans (XiangWei et al., 2018), showed an epileptic phenotype and other symptoms similar to the ones described in DEE patients carrying analogous mutations, becoming, therefore, valuable mouse models for GRIN-related DEE (Amador et al., 2020; Bertocchi et al., 2021). Second, while a mutation affecting one allele in humans is sufficient to lead to DEEs, in animal models the phenotype is largely visible in homozygosity. Attempts have been made to generate preclinical models of mutations present in heterozygosity in humans, but mostly without success, such as for models carrying a loss of function mutations in K^+ channel genes (Oyrer et al., 2018). Moreover, the presence, as well intensity, and frequency of seizures and other symptoms are highly influenced by the genetic background. Finally, even if less investigated, environmental conditions are known to influence disease expression, so that mutants often show traits inconsistent with the human phenotype (Singh et al., 2008).

Besides incomplete penetrance, genetically modified mouse or rats displaying spontaneous epilepsy are rare, considering that they are often either not viable or die in the first days of life. The few viable models that show spontaneous seizures are associated with a high mortality rate and, given that seizure onset and frequency is highly unpredictable, they are difficult to study. Despite this, rodents with spontaneous seizures can still prove to be excellent preclinical tools such as, for example, mutant mice in which the Tsc1 gene was deleted in Emx1-expressing embryonic telencephalic neural stem cells (NSCs), which recapitulate Tuberous Sclerosis Complex (TSC) neuropathological lesions and present spontaneous generalized cortical seizures in 100% of the mutants by postnatal day (PND) 13 (Magri et al., 2011). Notably, the delivery of chronic rapamycin treatment since PND 8 every other day resulted in increased body weight, no seizures and mortality, and reduced anxiety- and depression-like phenotype (Cambiaghi et al., 2013b).

Most preclinical rodent models exhibit reduced thresholds to electrically or pharmacologically induced seizures compared to wild-type littermates, while a minority show reflex epilepsy, meaning that the recurring crisis is provoked by clearly identifiable triggers or stimuli, mimicking the "two-hit" hypothesis (Love, 2005; Faingold, 2012; Spagnoli et al., 2015). A significant portion of this last group shows, in particular, generalized seizures when exposed to a specific acoustic stimulus, namely audiogenic seizures (AGS). AGS is generally induced by an aversive sound with a specific frequency and intensity, to which rodents manifest a stereotyped response that includes, in order: wild running, loss of stability with tonic–clonic crisis, and tonic seizure with limbs extension towards the tail, eventually followed by death from respiratory arrest (AGS-RA; Faingold, 2012). AGS susceptibility usually diminishes with age, as in DBA/1 and DBA/2 mice. These classic inbred strains are commonly used to study sudden unexpected death in epilepsy (SUDEP), given their susceptibility to AGS-RA (Pansani et al., 2016). However, this type of reflex epilepsy is also found in adult rodents, even in models of human syndromes with spontaneous seizures. In the synapsin triple-knockout (Syn-TKO) mice, for example, the seizure susceptibility follows a temporal evolution in which the seizure-provoking maneuver has a peak at about 2 months of age and then rapidly declines (Cambiaghi et al., 2013a). Despite the inconsistency with the human condition, these animals allow for rapid and reliable analysis of the anticonvulsant properties of new antiseizure medications (ASM) or therapeutics. A similar example is the GluN2A(N615S)-expressing mice, carrying a point mutation analogous to those found in children affected by DEE. GluN2A(N615S) mice show high penetrance to AGS-RA (Bertocchi et al., 2021) and no reduction in susceptibility in adult ages,

thus allowing the preclinical assessment of specific antiepileptic treatments at different developmental stages and the possibility to study the mechanisms involved in SUDEP. This feature is difficult to observe in other animal models and opens experimental possibilities impossible to achieve with clinical studies.

In conclusion, preclinical rodent models may not faithfully reproduce the human symptom spectrum, even when carrying the same mutation found in patients. Importantly, however, they offer the unique possibility to study not only epileptic activity but also pathways and disease mechanisms associated with it and related neuropsychiatric impairments that cannot be reliably investigated in other models (Figure 1F).

2.2. E/I balance and activity-dependent plasticity: Two important therapeutic targets

Epilepsy is a network disease involving the development of complex hyperexcitable networks and brain-wide circuits to generate seizures (Englot et al., 2015; Rao and Lowenstein, 2015; Dong et al., 2016; Geier and Lehnertz, 2017; Pfisterer et al., 2020). Early deficits in neuronal activity and connectivity contribute to a developmental cascade affecting brain organization (Lewis et al., 2017) and different interacting brain regions are likely involved in generating E/I imbalance (Rao and Lowenstein, 2015; Shao et al., 2019) for the initiation and propagation of epilepsy (Figure 1). Converging evidence also demonstrates that an imbalance in the E/I ratio in various brain regions is crucially involved in functional deficits at the neural network level, leading to abnormal behavior in different neurodevelopmental disorders (NDDs), such as autism and attention deficit hyperactivity disorder (ADHD), and representing, therefore, a key therapeutic target (Tang et al., 2021). Accordingly, anomalies in E/I balance underlie cerebral response deficits, hyperresponsiveness, and amplified cortical responses to sensory stimuli (Sohal and Rubenstein, 2019). EEG abnormalities and defects in the synchronization and communication of cortical networks are frequently linked with decreased cognitive function and sociability, hyperactivity, impulsivity, and repetitive, stereotyped behaviors (Razak et al., 2021).

Synaptic and circuit E/I balances are established and fine-tuned during brain development (Lee et al., 2017). A substantial portion of the primary pathological changes in this equilibrium occurs during embryonic or early postnatal periods and exerts long-lasting effects. However, the cellular and network-specific changes that characterize the E/I imbalance are still poorly understood, highlighting the importance of pursuing detailed and integrative analyses of E/I imbalance in animal models, particularly during perinatal and postnatal critical periods.

Preclinical models are essential for studying E/I imbalance and age-related changes in brain circuits due to activity-dependent plasticity that likely develops over time. For example, during juvenile development, the experience-dependent formation of a specialized sheath made of extracellular matrix components around a portion of neurons, named "perineuronal net" (PNN), marks the closure of critical periods of neuronal plasticity (Carulli et al., 2010). Interestingly, the formation of PNNs happens in concomitance with the well-known developmental switch in the expression of NMDAR GluN2B/A subunits, increasing the system "maturity" particularly in higher brain structures (Acutain et al., 2021). Moreover, it has been suggested that extracellular sulfated proteoglycans, enriched in PNN, play an exquisite role in regulating intracellular chloride ion concentrations in neurons and, consequently, the direction of inhibitory/excitatory GABA responses (Glykys et al., 2014). Not surprisingly, therefore, with GABAergic signaling, PNNs are frequently altered in animal models for different NDDs, leading to E/I imbalance and hyperexcitability of local cortical circuits. For example, the hyperexcitability of auditory cortex pyramidal neurons in mouse models of fragile X syndrome (Fmr1KO mice) is well described to lead to hyper-responsivity to sound (Rotschafer and Razak, 2013). Here, pharmacologically or environmentally induced E/I balance and PNN readjustment have rescued hyperresponsivity and susceptibility to AGS (Lovelace et al., 2016, 2020; Pirbhoy et al., 2020).

Fragile X Mental Retardation Protein (FMRP1) loss, as the effects of other gene mutations, may alter neuronal excitability and plasticity in opposite directions in different brain areas, generating heterogeneous regional and temporal effects on synaptic E/I balance to modulate neuronal firing (Lee et al., 2017). The allostatic establishment of excessive activation of glutamatergic neurons may impair plasticity, brain functions, physiology, and behavior. It would be, therefore, crucial to selectively target and dampen the activity of hyper-excitable neurons involved in these abnormalities. Understanding the dynamics of E/I balance-related alterations in brain circuitry that subserves cognitive and behavioral functions and the developmental refinements of the altered circuitry during critical periods of plasticity could provide novel insights into the nature of different NDDs.

2.3. Importance of video-EEG recording in seizure monitoring

Preclinical models represent a necessary step in developing new treatments for epilepsy and comorbidities. In addition to tolerability, the identification of ASM effectiveness is mainly based on its effects on seizure threshold or their spreading and, more generally, on behavior (Swinyard and Kupferberg, 1985). When studying the (chronic) effects of possible ASM, the combination of time-locked video and EEG recording (usually called video-EEG monitoring) and different behavioral tests can be considered the best option. Video-EEG allows a precise correlation between a given event and brain activity (superficial or deep). Off-line analysis can be helpful to confirm the association between a specific behavior and an epileptic seizure or a different episode (such as scratching, movement disorder, artifacts, or other false positives, for example, grooming; Cambiaghi et al., 2013b). Moreover, video-EEG monitoring is essential to classify seizures and determine their temporal dynamics. Lastly, besides qualitative observation, quantitative EEG data analysis can be associated with a particular behavior or task, such as sleep or a cognitive test (for an exhaustive review, see Cambiaghi et al., 2015).

2.4. Circuit-based innovative rAAV tools for therapy

Animal models can be rapidly generated, and they are valuable tools to investigate neurobiological processes across brain regions *in vivo* and in acute brain slices and would help provide prospects for

precision medicine in humans to treat epilepsies (Figure 2A). With the advent of recombinant adeno-associated viruses (rAAVs) delivery for local targeting of brain circuits (Dogbevia et al., 2016) and intravascular delivery for brain-wide-targeting (Goertsen et al., 2022), it is possible to treat or perhaps even cure these disorders. Specific brain circuits can be targeted and manipulated by inducible gene expression systems using rAAVs to control plasticity (Hasan et al., 2013) and E/I balance (Reus-García et al., 2021). Introducing normal gene(s) to brain cells to overcome the negative effects of the mutated gene(s) is becoming a feasible approach (Murphy et al., 2013; Dogbevia et al., 2016; Figures 2B,C). It is also possible to selectively target hyperactive neurons using the c-fos-equipped genetic switches (Hasan et al., 2019; Keller et al., 2022; Figures 2B,D). These inducible switches can be controlled by different levels of the chemical inducer provided to the animals (and potentially humans) to control graded levels of gene expression for proper titration of E/I balance (Figure 2E) and effective recovery of mutant gene dysfunctional activities.

3. Discussion

DEEs encompass a wide range of highly multifaceted syndromes, predominantly of genetic origin, with the genes involved mainly coding for ion channels or proteins controlling neuronal excitability. Patients with DEEs display severe symptoms and high heterogeneity, even if carrying the same mutation. Additionally, the genetic background, plasticity, and epigenetic mechanisms significantly impact mutation expressivity (Guerrini et al., 2023). This variability adds complexity to uncovering the correlation between genotype and phenotype and the development of effective therapies. Preclinical models constitute an invaluable tool for better understanding the multifaceted pathophysiology of various DEEs and for studying the effect of candidate drugs. Knock-in mice reproduce more faithfully the unique human disorders; however, many DEE syndromes still lack comprehensive genetic models.

A key aspect of studying candidate ASM effects in animal models is linked to the possibility of seizure characterization and the analysis of background EEG abnormalities and their correlation with behavior.

EEG abnormalities *per se* can have a detrimental effect on the developing brain, and the results that a candidate ASM may have on EEG in animal models can provide valuable insight into how much clinical improvement can be obtained by treatment-induced EEG modulation. We highlight the importance of adopting time-locked video-EEG recordings to detect the changes and synchronization of different brain areas' activity and their correlation with specific/abnormal behaviors.

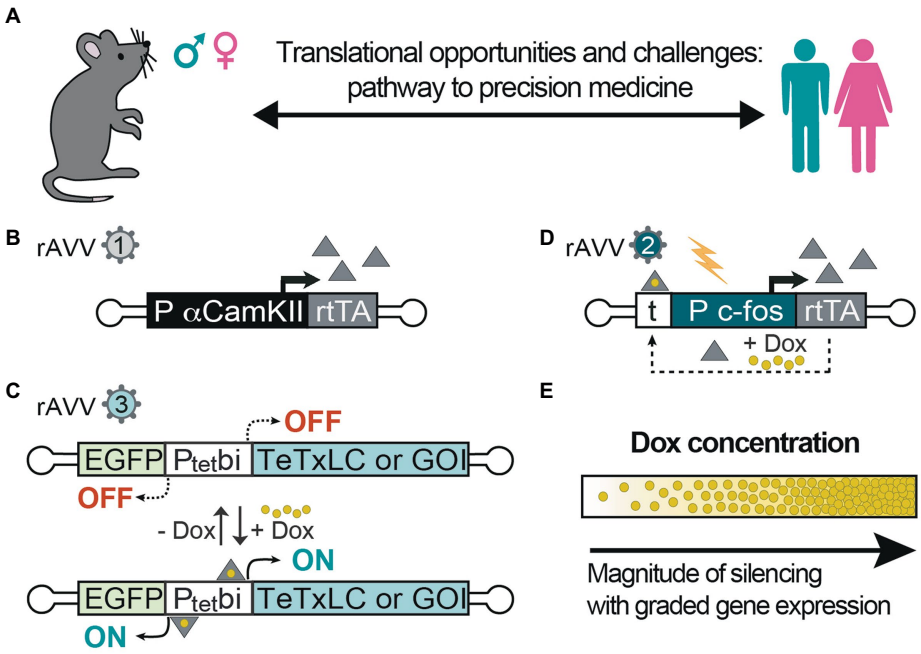

FIGURE 2
Inducible control of excitation-inhibition balance and gene activity. **(A)** Animal models are useful for testing specific hypotheses-driven experiments, also regarding gender differences in various pathologies and in response to therapy. Thus far, they represent a powerful approach to move preclinical studies into clinical trials; **(B)** Recombinant adeno-associated virus (rAAVs) can be equipped with α-calmodulin kinase-2 promoter (αCaMK2) to express reverse tetracycline transactivator (rtTA; rAAV1), which upon **(C)** binding to the Ptetbi promoter (rAAV3) in the presence of doxycycline (Dox) activate the expression of tetanus toxin light chain (TeTxLC) to block evoked synaptic transmission in excitatory neurons. In the absence of Dox, the expression is switched-off and synaptic transmission can resume; **(D)** With another system based on c-fos promoter with upstream tetracycline operators (t), hyperactive cells are specifically targeted in the presence of Dox to express rtTA, which establishes an autoregulated loop for rtTA-dependent rtTA expression, thereby E/I can be controlled in the tagged cells using TeTxLC or any gene of interest (GOI) as in **(C)**. Indeed, these technologies shown here **(B/C)** or **(D/C)** can also be used to express any gene of interest to override the mutations responsible for epilepsy. **(E)** Moreover, the level of expression and synaptic silencing can be controlled at different concentrations of Dox. Controlled levels of gene expression can allow for proper titration of E/I balance.

Preclinical and clinical studies suggest that the high-fat, low-carbohydrate ketogenic diet (KD) effectively controls drug-resistant seizures (Nei et al., 2014; Ruan et al., 2022). Evidence also demonstrated some efficacy in improving cognitive abilities (Rho and Boison, 2022; Zhu et al., 2022). However, such dietary therapy is difficult to adhere to, needs continuous monitoring, is unsuitable for all patients, and has several side effects (Rho and Boison, 2022; Zhu et al., 2022; Operto et al., 2023). A better understanding of the molecular mechanisms through which KD and novel ASM can alleviate seizures and cognitive deficits is fundamental for developing new drugs with an overall efficacy while avoiding side effects as much as possible. Animal models are well suited for this purpose; they offer the advantage of studying pathways relevant to the disease pathology, as often the principal target (i.e., a defective gene and encoded protein) is not druggable.

The search for effective treatment for various forms of DEE is a priority for science and society. In recent years, a drive for precision medicine using new candidate ASM and targeted gene therapy has emerged as a viable treatment strategy for different diseases and holds much promise for drug-refractory epilepsies. Further studies, both preclinical and in children suffering from resistant seizures, are needed. Considering the variability and phenotypic pleiotropy typical of DEEs, genetic therapeutic approaches able to precisely target common pathological mechanisms, such as the E/I imbalance, might open prospects for these devastating syndromes and more common types of epilepsy. With large-scale changes in brain networks during early disease onset, developing effective therapies at neonatal or even prenatal stages would be imperative to treat these debilitating disorders.

In conclusion, there is a great need for novel preclinical models for the different forms of DEEs, considering their potential to assess and profile the effectiveness of therapeutic strategies aimed at contrasting not only the epileptic phenotype, but also the wide range of comorbidities experienced by the patients, from behavioral to cognitive deficits, despite the difficulties in transferring the results from preclinical to clinical practice.

Author contributions

IB conceived and wrote the first draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Contribution of the dihydropyrimidinase-like proteins family in synaptic physiology and in neurodevelopmental disorders

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The dihydropyrimidinase-like (DPYSL) proteins, also designated as the collapsin response mediators (CRMP) proteins, constitute a family of five cytosolic phosphoproteins abundantly expressed in the developing nervous system but down-regulated in the adult mouse brain. The DPYSL proteins were initially identified as effectors of semaphorin 3A (Sema3A) signaling and consequently involved in regulation of growth cone collapse in young developing neurons. To date, it has been established that DPYSL proteins mediate signals for numerous intracellular/extracellular pathways and play major roles in variety of cellular process including cell migration, neurite extension, axonal guidance, dendritic spine development and synaptic plasticity through their phosphorylation status. The roles of DPYSL proteins at early stages of brain development have been described in the past years, particularly for DPYSL2 and DPYSL5 proteins. The recent characterization of pathogenic genetic variants in *DPYSL2* and in *DPYSL5* human genes associated with intellectual disability and brain malformations, such as agenesis of the corpus callosum and cerebellar dysplasia, highlighted the pivotal role of these actors in the fundamental processes of brain formation and organization. In this review, we sought to establish a detailed update on the knowledge regarding the functions of *DPYSL* genes and proteins in brain and to highlight their involvement in synaptic processing in later stages of neurodevelopment, as well as their particular contribution in human neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASD) and intellectual disability (ID).

KEYWORDS

dihydropyrimidinase-like proteins, collapsin response mediator proteins, neurodevelopmental disorders (NDDs), human genetics research, neuronal development, synaptic physiopathology, missense variants, animal model

1. Introduction

The Dihydropyrimidinase-like (DPYSL) proteins, also designated as the Collapsin response mediators (CRMP) proteins, constitute a family of five cytosolic phosphoproteins (Quinn et al., 1999), abundantly expressed in the developing nervous system but down-regulated in the adult mouse brain (Minturn et al., 1995; Byk et al., 1996; Wang and Strittmatter, 1996; Fukada et al., 2000; Yuasa-Kawada et al., 2003). The DPYSL proteins were initially identified as effectors of

semaphorin 3A (Sema3A) signaling and consequently involved in regulation of growth cone collapse (Goshima et al., 1995).

To date, it has been established that DPYSL proteins mediate signals for numerous intracellular/extracellular pathways and play major roles in variety of cellular process including cell migration (Yamashita et al., 2006), neurite extension (Brot et al., 2010), axonal guidance (Goshima et al., 1995; Arimura et al., 2005; Uchida and Goshima, 2005; Yoshimura et al., 2005), dendritic spine development (Yamashita et al., 2007) and synaptic plasticity (Yamashita et al., 2011) through their phosphorylation status.

The roles of DPYSL at early stages of brain development have been described in the past years, particularly for DPYSL2 and DPYSL5. In this review, we sought to establish a detailed synthesis on the functions of DPYSL genes and proteins in brain and to highlight their involvement in synaptic processing in later stages of neurodevelopment, as well as their contribution in human neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASD) and intellectual disability (ID). This synthesis will likely provide a new perspective regarding the specific function of *DPYSL* genes and proteins in developing and functioning brain, as well as their respective role in the fine regulation of brain developmental stages.

2. The DPYSL proteins family

In mammals, five DPYSL (or CRMP) proteins have been identified and are encoded by their respective coding-genes (*CRMP1 or DPYSL1*, *DPYSL2, DPYSL3, DPYSL4, and DPYSL5*) (Table 1). The predicted secondary structure of human DPYSL proteins family is well described and allowed to determine that DPYSL1-4 display around 75% sequence homology with each other, whereas DPYSL5 is more distant phylogenetically with only 50% of sequence homology (Schmidt and Strittmatter, 2007; Tang et al., 2015). Alignment and comparison of amino acid sequence of mouse DPYSL5 with other DPYSL proteins show that the conservation level is lower at the C-terminal region (Fukada et al., 2000; Tang et al., 2015). The DPYSL associate generally in homo-tetramer or hetero-tetramer complexes with single or multiple isoforms.

3. Physiological pathways involving DPYSL proteins

The DPYSL proteins participate in several major physiological pathways, from cellular migration, neurite growth and guidance to synapse maturation, particularly through their C-terminal domain, which includes the last 50 amino-acids, and which is the target of numerous post-translational modifications sites that regulate the interaction between DPYSL and various types of proteins, including receptors, ion channels, cytoskeletal and motor proteins.

As an example, numerous kinases such as glycogen kinase 3β (GSK3β), cyclin-dependent kinase 5 (Cdk5), dual specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) and Rho-associated kinase 2 (ROCK2) target the C-terminal regions of DPYSL proteins (Uchida and Goshima, 2005; Yoshimura et al., 2005; Cole et al., 2006; Arimura and Kaibuchi, 2007; Uchida et al., 2009; Yamashita and Goshima, 2012; Nakamura et al., 2014). Despite differences in the C-terminal part of DPYSL5 compared to other DPYSL proteins, it is noted that many consensus sequences for DPYSL phosphorylation sites are retrieved for DPYSL5 (Fukada et al., 2000).

The C-terminal domain is extensively conserved among DPYSL isoforms and across species, and is sufficient to associate with assembled microtubules *in vivo* (Soutar et al., 2009). Precisely, phosphorylation/dephosphorylation status of DPYSL proteins is essential to control their spatiotemporal functions, by modulating their binding to cytoskeleton and signaling proteins (Yamashita and Goshima, 2012).

Thus, DPYSL proteins can coordinate cytoskeleton dynamic regulating filopodia formation, axonal guidance, neurite outgrowth and establishment of neuronal polarity by interacting with tubulin and actin in brain (Fukata et al., 2002; Arimura et al., 2005; Hotta et al., 2005; Rosslenbroich et al., 2005; Brot et al., 2010; Higurashi et al., 2012; Ji et al., 2014; Khazaei et al., 2014; Tan et al., 2015; Gong et al., 2016; Yu-Kemp and Brieher, 2016). Non-phosphorylated DPYSL2 promotes axonal elongation and branching by binding to tubulin heterodimer (Schmidt and Strittmatter, 2007) whereas its phosphorylation by GSK3β, ROCK2 and Cdk5 lowers binding affinity of DPYSL2 to tubulin leading to growth cone collapse and arrest of axonal outgrowth (Fukata et al., 2002; Schmidt and Strittmatter, 2007; Khanna et al., 2012). The binding of DPYSL1-3 to tubulin allows polymerization and stabilization of microtubules (Fukata et al., 2002; Lin et al., 2011; Khazaei et al., 2014) while the tubulin-DPYSL4 or tubulin-DPYSL5 complex interaction causes inhibition of microtubule polymerization (Aylsworth et al., 2009; Brot et al., 2010).

In addition to phosphorylation, the functions of DPYSL proteins are also regulated by other post-translational modifications including acylation, SUMOylation and O-GlcNAcylation (Leney et al., 2017; Myllykoski et al., 2017; Chew and Khanna, 2018). For instance, DPYSL2 phosphorylation at Serine 522 by Cdk5 promotes

TABLE 1 List of known human *DPYSL/CRMP* genes and corresponding proteins.

Human gene (HGNC)	Locus	Transcript RefSeq	Human protein	Alias for protein name	Protein RefSeq
CRMP1	4p16.2	NM_001014809.3	DPYSL1	CRMP1	NP_001014809.1
DPYSL2	8p21.2	NM_001197293.3	DPYSL2	CRMP2	NP_001184222.1
DPYSL3	5q32	NM_001197294.2	DPYSL3	CRMP4	NP_001184223.1
DPYSL4	10q26.3	NM_006426.3	DPYSL4	CRMP3	NP_006417.2
DPYSL5	2p23.3	NM_001253723.2	DPYSL5	CRMP5	NP_001240652.1

The official name of the human genes is indicated according to the Hugo Gene Nomenclature Committee (HGNC) nomenclature. The chromosomal locus of each gene corresponds to the Genome Reference Consortium Human Build 38 patch release 14 (GRCh38.p14). The reference sequence (RefSeq) for genes and protein was extracted from the National Center for Biotechnology Information (NCBI) database.

association between DPYSL2 and cytoplasmic loops of CaV2.2 (Brittain et al., 2012; Chew and Khanna, 2018), leading to an increase of Ca^{2+} influx through the Cav2.2 channel and the release of neurotransmitters (Brittain et al., 2009, 2012). Similarly, SUMOylation of DPYSL2 alters calcium influx (Ju et al., 2013) and increases cell surface expression of NaV1.7 channel (Dustrude et al., 2016). Dephosphorylation of DPYSL2 at Thr514 and deSUMOylation at Lys374 sites promote the formation and maturation of dendritic spines, however, no interference is found between these two post-translational modifications in the regulation of dendritic spine morphology (Zhang et al., 2018).

4. Neuronal expression of DPYSL genes and proteins

Based on *in situ* hybridization (Wang and Strittmatter, 1996) and immunostaining analyses (Bretin et al., 2005), the DPYSL proteins are detected at a higher level in post-mitotic neural cells during the embryonic stage than in adult mouse brain stage (Wang and Strittmatter, 1997; Ricard et al., 2001). The mRNA expression level of *Dpysl* genes is intense during the neonatal period (Embryonic day 18 – Postnatal day 5) in the central nervous system of mice (Charrier et al., 2003). At Postnatal day 1, all DPYSL except DPYSL4 are strongly expressed in cortex and hippocampus (Wang and Strittmatter, 1996; Ricard et al., 2001) essential for social communication and cognitive functions. In addition, a peak of the expression level of DPYSL proteins is observed during the first postnatal week corresponding to a period of neuronal maturation and synaptogenesis (Byk et al., 1996; Wang and Strittmatter, 1996; Bretin et al., 2005; Schmidt and Strittmatter, 2007).

The BrainSpan transcriptome of the developing human brain shows a similar kinetics of *DPYSL* genes expression level, indicating their preponderant role in prenatal and perinatal periods when neurogenesis, dendritic development and synaptogenesis stages occur (Figures 1A–E) (Sunkin et al., 2013). Interestingly, DPYSL2 displays similar expression levels from prenatal to adult stages, suggesting that it may also have a role in later stages of development such as in myelination or synaptic pruning (Figures 1A–E). Indeed, DPYSL2 mediates Semaphorin 3F dependent synapse pruning (Ziak et al., 2020).

A transcriptomic study of human fetal brain development (BBI Allen single cell atlases) using single-cell RNA sequencing indicated that around 25–30% of cells display *DPYSL expression* in cerebrum, except *DPYSL4* which is very weakly expressed (only 2% of cells) (Cao et al., 2020). *DPYSL* mRNAs are mainly found in neurons (excitatory/inhibitory) but also in glial cells (Figure 1F), which suggest a contribution in neuronal degeneration/regeneration, as well as in inflammatory pathways in the context of neurological diseases inflammation and neurodegeneration pathways (Nagai et al., 2017).

5. Antagonistic/synergistic roles of DPYSL proteins

Each DPYSL protein displays a distinct subcellular neuronal localization both in time and space demonstrating their divergent functions during development (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996; Wang and Strittmatter, 1996; Byk et al., 1998; Kamata et al., 1998; Bretin et al., 2005).

In primary hippocampal mouse neurons, during the axonogenesis, DPYSL2 is specifically enriched in neurite which is the future axon while DPYSL5 is strongly retrieved in dendrites maintaining dendrites at a quiescent state (Brot et al., 2010, 2014). At Days *in vitro* (DIV) 4–5, a switch is observed. DPYSL5 is detected at a very low level in dendrites and DPYSL2 level remains constant in same area allowing dendritic outgrowth (Brot et al., 2010). The transient DPYSL5 expression in different neuronal compartments regulates the establishment of neuronal polarity (Bretin et al., 2005; Brot et al., 2010, 2014). DPYSL5 forms a ternary complex with tubulin and microtubule associated protein 2 (MAP2) and inhibits the neurites outgrowth by reducing DPYSL2-tubulin interaction complex (Brot et al., 2010; Brot, 2014). It is not yet excluded that the inhibition of DPYSL2 activity by DPYSL5 may occur through their hetero-oligomerization as DPYSL2 and DPYSL5 have a very similar structure and form hetero-tetramer *in vivo* (Fukada et al., 2000; Brot et al., 2010; Petratos and Lee, 2013; Ponnusamy and Lohkamp, 2013).

Despite that DPYSL5 does not inhibit axonal growth (Brot et al., 2010), its deficiency results in an increase on DPYSL2-induced axon elongation and on multiple axon formation (Inagaki et al., 2001). These results suggest that *in vivo* DPYSL5 also modulates DPYSL2 activity on axonal growth and formation.

Although the biological functions associated with each homo- or hetero-tetramer of DPYSL proteins are still poorly known, it has been demonstrated that DPYSL2 and DPYSL3 are complexing and work together to regulate growth cone development and axonal elongation *in vivo* (Tan et al., 2015). For instance, overexpression of DPYSL2 and DPYSL3 in hippocampus stimulate axonal growth and this effect is abolished when DPYSL2 is co-transfected with the truncated construct DPYSL3ΔC471 (unable to bind actin) or when DPYSL3 is co-transfected with DPYSL2ΔC322 (unable to bind tubulin). These findings suggest that DPYSL2/DPYSL3 hetero-tetramer complex creates a link between microtubules and actin, aiming to coordinate cytoskeleton dynamics, and axonal development regulation in hippocampal neurons (Tan et al., 2015). These findings illustrate the various actions of DPYSL proteins and highlight their ability to form homo- or hetero-tetramer complex, in order to modulate and regulate the function of other DPYSL proteins during neural network formation.

DPYSL proteins appear to play antagonistic but also complementary roles during neurodevelopment (Byk et al., 1998; Yuasa-Kawada et al., 2003; Brot, 2014; Makihara et al., 2016). *In vivo* studies demonstrate that DPYSL1 and DPYSL2 have synergistic but distinct roles in mediating Sema3A signaling in order to regulate dendritic development and spine maturation (Hamajima et al., 1996; Sasaki et al., 2002; Morita et al., 2006; Yamashita et al., 2007, 2012).

In fact, abnormalities were observed in dendritic patterning (branching and length dendritic) of cortical (layer V) neurons from distinctly *Dpysl1*$^{-/-}$ and *Sema3A*$^{-/-}$ mouse model, compared to their littermate neurons. These defects in dendritic morphology are not retrieved in KO *Dpysl2*$^{-/-}$ and double-heterozygous KO *Dpysl1*$^{+/-}$ *Dpysl2*$^{+/-}$ mouse models. Moreover, the level of DPYSL1 increases in *Dpysl2*$^{-/-}$ compared to wild-type cortical brain lysates,

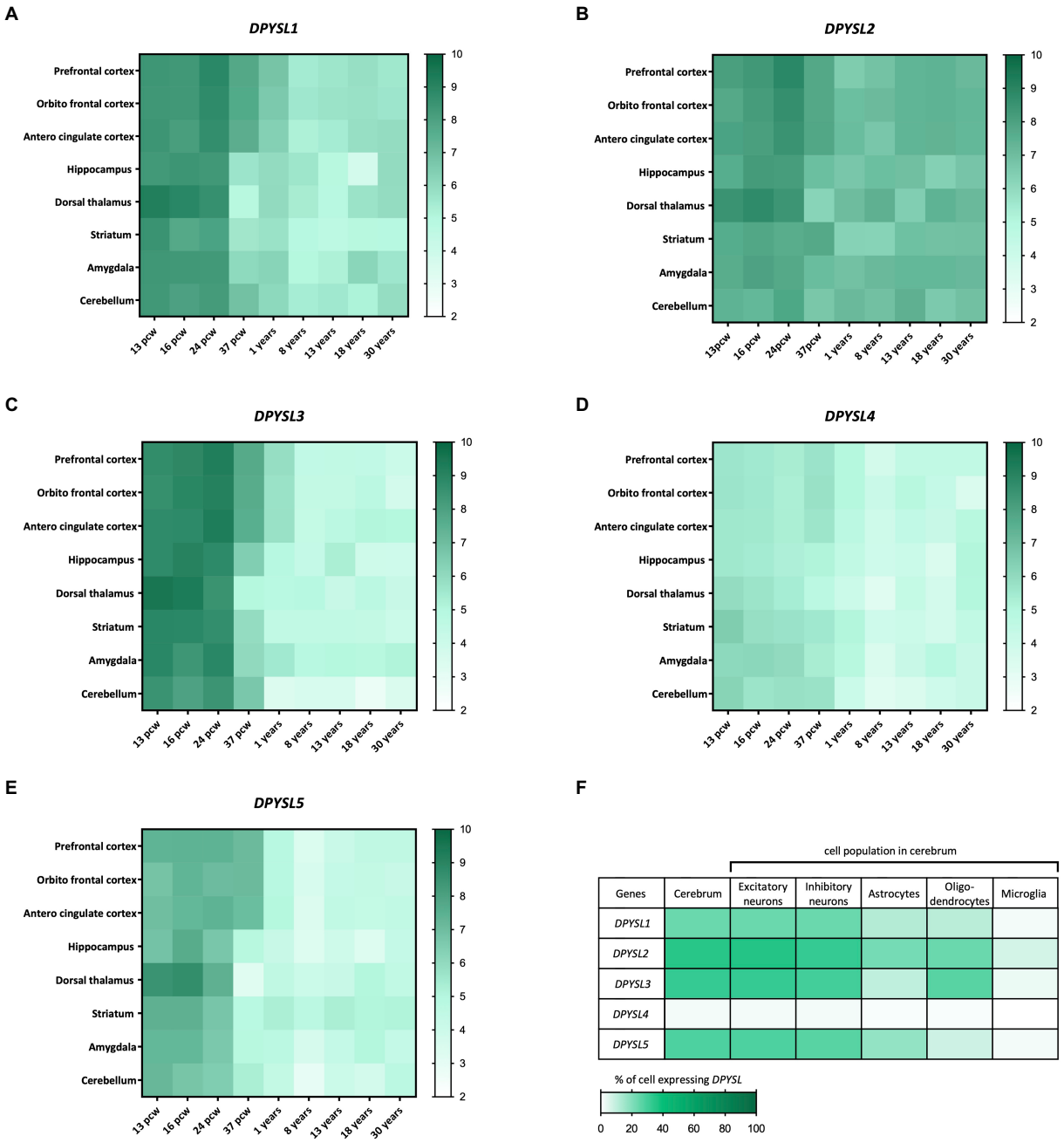

FIGURE 1
Dynamic expression profile of *DPYSL* genes in the human brain. **(A–E)** Spatio-temporal profile of expression of *DPYSL* genes. The database used to perform the gene expression heatmap is BrainSpan. The level expression is expressed in RPKM (reads per kilobase of transcript per million reads mapped) and these data are obtained from RNA sequencing and exon microarray. Pcw, post-conceptual week. **(F)** *DPYSL* expression in different cell populations of the humain cerebrum. Data are from BBI allen single cell atlases. The single-cell atlases is realized from human fetal samples (72–129days post-conceptual age).

highlighting a DPYSL1 compensatory mechanism for DPYSL2 deficiency (Diss et al., 2014; Makihara et al., 2016). A proteomic analysis in cortex of *Dpysl2*$^{ki/ki}$ mice (where serine 522 is mutated to alanine preventing its potential phosphorylation) demonstrated an increase of DPYSL3, DPYSL4 and DPYSL5 (Nakamura et al., 2018) as well as in *Dpysl2*$^{-/-}$ (Nakamura et al., 2016), thereby suggesting that the phosphorylation or loss of functions of DPYSL2 have an impact on other DPYSL proteins.

A study from Yamashita and colleagues showed that both DPYSL1 and DPYSL2 are required for regulating dendritic branch trajectory in cerebral cortical neurons reinforcing their synergistic role in dendritic organization (Yamashita et al., 2012). In addition, DPYSL1-4 may have a redundant role in dendritic growth and maturation in neurons (Quach et al., 2008; Khazaei et al., 2014; Cha et al., 2016; Makihara et al., 2016; Takaya et al., 2017; Kawashima et al., 2021).

6. Synaptic functions of DPYSL proteins

6.1. Role in the formation and maturation of dendritic spines

When maturation of neurons and synaptic connections is strongly active (around first postnatal week in rodents), DPYSL expression is the highest (Charrier et al., 2003). All five DPYSL proteins are expressed in synaptosomes from rat brain at neonatal postnatal day 1 (P1) (Charrier et al., 2006; Brittain et al., 2009; Yamashita et al., 2012) and are postsynaptic density (PSD) proteins (Collins et al., 2006; Laumonnier et al., 2007), suggesting a role in synaptogenesis and neurotransmission. Dendrites are the first site of synapse formation (Purves and Hume, 1981) and synaptogenesis represents an essential process for the establishment of cognitive and communication function as well as for learning and memory (Elston, 2000).

Studies on genetic deletion of *Dpysl* members in mice establish a direct link between loss of DPYSL and impairment of dendritic patterning and spine development (Table 2; Charrier et al., 2006; Quach et al., 2008; Yamashita et al., 2012). The synaptic density is reduced in *Dpysl1*$^{-/-}$, *Dpysl2*$^{-/-}$ mutant mice (Yamashita et al., 2007; Makihara et al., 2016).

The SEMA3A protein is essential for induction of mature spines formation through the Fyn-Cdk5 cascade in cultured cortical neurons (Sasaki et al., 2002; Li et al., 2004; Cole et al., 2006; Morita et al., 2006; Figure 2). Nevertheless, Sema3A is not able to induce an increase in functional synapses density in cortical neurons from *Dpysl1*$^{-/-}$ and *Cdk5*$^{-/-}$ mice (Yamashita et al., 2007). Several studies revealed the importance of CDK5 phosphorylation of DPYSL1 at Thr509 and Ser522 sites and of DPYSL2 at Ser522 site for SEMA3A-induced spine development and maturation (Yamashita et al., 2007; Jin et al., 2016; Makihara et al., 2016). Conversely, DPYSL2 dephosphorylated forms increase the number of dendritic spines and the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Zhang et al., 2018). This suggest that dephosphorylated forms of DPYSL2 promotes polymerization of tubulin (Fukata et al., 2002; Uchida and Goshima, 2005) and thus, spinogenesis. A recent study demonstrated that DPYSL2 is not only a mediator of Sema3A-signaling regulating spine development but also plays a key role in synaptic refinement through Semaphorin 3F (Ziak et al., 2020). Loss of *Dpysl2* causes axonal pruning defects and inadequate elimination of dendritic spines in multiples areas of the brain and in cultures of hippocampal neurons (Table 2). This defect is accompanied by social behavior abnormalities (see section "*DPYSL* genes and neurodevelopmental disorders").

DPYSL3 is also critical for spine formation and maturation in cultured hippocampal neurons *via* the interaction with actin cytoskeleton by its C-terminal region (Rosslenbroich et al., 2005; Cha et al., 2016). Overexpression of DPYSL3 wild-type or DPYSL3 with actin-binding domain constructs increase frequency of mEPSCs in comparison with control GFP or with form of *DPYSL3ΔC471* (lacking the domain of interaction with actin) transfected neurons. These results indicate that DPYSL3-actin interaction increases number of functional synapses and thus, influences synaptic transmission (Cha et al., 2016). Similarly, DPYSL5 deficiency in cerebellum induces an aberrant Purkinje cell morphology. In *Dpysl5*$^{+/-}$ mice, Brain-derived neurotrophic factor (*BDNF*) increased the number of primary dendrites per neurons in the hippocampus while this effect is lost in neurons from complete KO *Dpysl5*$^{-/-}$ brains (Table 2). Consequently, they demonstrate that DPYSL5 phosphorylation by TrkB is involved in BDNF–TrkB signaling to regulate dendritic morphology and synaptic plasticity in Purkinje cells (Yamashita et al., 2011).

The phosphorylated/dephosphorylated state of DPYSL proteins seems to be crucial for the regulation of their interaction with cytoskeleton proteins and for the control of dendritic architecture (Arimura et al., 2005; Yamashita and Goshima, 2012; Makihara et al., 2016; Zhang et al., 2018). Several post-translational modifications of DPYSL2 allow modulation of membrane addressing of the CaV2.2 and NaV1.7 ion channels, as well as the formation and maturation of dendricic spines (see section Physiological pathways involving DPYSL proteins). These data highlight the importance of future research on their post-translational modifications and associated signaling pathways to clarify their function in synapse formation and in neurotransmission.

6.2. Role in physiology and synaptic plasticity

In addition to synapse formation process, DPYSL proteins interact with presynaptic and postsynaptic machinery and may also have a role in synaptic plasticity. For instance, loss of *Dpysl1-4* in murine models cause dysregulation of genes expression related to excitatory and/or inhibitory synaptic transmission explaining synaptic plasticity dysfunction (Yamashita et al., 2011; Tsutiya et al., 2015; Zhang et al., 2016; Tsutiya et al., 2017). In fact, abnormal NMDA receptor composition, including GluN2B and GluN1, is observed in hippocampus of *Dpysl2* knock-out (KO) mice resulting in a reduction of long-term potentiation (LTP) induction and in defects in learning function (Zhang et al., 2016). *Dpysl2*$^{-/-}$ mice also showed altered expression of proteins involved in GABAergic synapse (NSF, PRKACB, GNAI1), glutamatergic synapse (GRIA2, PRKACB, GNAI1, SHANK3, SHANK2, GRIA1) and neurotrophin signaling pathways (Table 2). These alterations of both inhibitory and excitatory synapse related proteins may contribute to the behavioral phenoytpe of these mice (Nakamura et al., 2016).

An altered LTP is found in CA1 hippocampi neurons of *Dpysl1*$^{-/-}$ and *Dpysl4*$^{-/-}$ mice models (Table 2; Su et al., 2007; Quach et al., 2008). Deletion of *Dpysl1* leads to a decrease in the expression of GAP43 and PSD95 proteins (Su et al., 2007) and inactivation of *Dpysl3* also disturbs the mRNA expression levels of genes encoding GluR1, GluR2, VgluT1, VgluT2, GABAα1, GABAAγ2, GABAB receptor 1 and vGAT, in a region-dependent manner (Tsutiya et al., 2017). To date, no reports have shown that DPYSL5 is required for LTP formation, but in cerebellum of *Dpysl5*$^{-/-}$ mice the induction of long-term depression (LTD) is deficient between parallel fibers and Purkinje cells (Table 2; Yamashita et al., 2011). Consequently, involvement of DPYSL in LTP and in LTD is a critical mechanism for memory and learning processes (Malenka and Bear, 2004; Stacho and Manahan-Vaughan, 2022).

In parallel, three studies showed the involvement of DPYSL proteins in the dynamic trafficking of AMPA receptors (AMPARs) (Khazaei et al., 2014; Lin et al., 2019) and NMDA receptors (NMDARs) (Bretin et al., 2006). It is well-known that the trafficking of glutamatergic receptor, which enables the endocytosis, recycling and exocytosis of receptors is crucial for synaptic strength and plasticity. Moreover, the interaction between dephosphorylated

TABLE 2 Neuronal and behavioral phenotypes observed in mouse models invalidated for the *Dpysl/Crmp* genes.

Mouse genotype	Neuroanatomical and cellular phenotype	Molecular defect	Electrophysiology	Behavioral phenotype	References
Dpysl1$^{-/-}$ *or* *Crmp1*$^{-/-}$	- Abnormal dendritic development of CA1 pyramidal neurons - Reduction of synapse density in CA1 hippocampus - Reduced number of mature dendritic spines in cortical neurons	- Decreased PDS95 and GAP-43 protein levels in CA1 hippocampus	- Decreased LTP in CA1 hippocampus	- Hyperactivity, impaired emotional behavior - Decreased pre-pulse inhibition - Impaired learning and memory	Su et al. (2007) and Yamashita et al. (2007, 2013)
Dpysl2$^{-/-}$ *or* *Crmp2*$^{-/-}$	- Altered dendritic morphology (number spine and dendritic branching) of CA1 pyramidal neurons and cortical neurons (layer V) - Altered dendritic spine pruning in dentate gyrus - Abnormal axon pruning arising from hippocampus and visual cortex - Dysgenesis of corpus callosum	- Abnormal NMDA receptor composition - Decreased level of synaptic proteins NSF, PRKACB, GNAI1, GRIA2, SNAP25 - Increased level of synaptic proteins SHANK3, SHANK2, GRIA1	- Reduced LTP induction in hippocampus	- Decreased anxiety - Hyperactivity - Impaired social behavior, learning and memory - Defects in locomotor activity	Nakamura et al. (2016), Zhang et al. (2018), and Ziak et al. (2020)
Dpysl3$^{-/-}$ *or* *Crmp4*$^{-/-}$	- Increased dendritic total length and branching in primary hippocampal neurons - Defective infrapyramidal bundle of mossy fibers of the dentate gyrus (DG) pruning in the hippocampus	- Altered mRNA expression levels of genes related to neurotransmission and cell adhesion in hippocampus, cortex and olfactory bulb		- Decreased social interaction - Alterations of sensory responses (temperature and olfactory)	Niisato et al. (2012), Tsutiya et al. (2016), Takaya et al., 2017, and Tsutiya et al. (2017)
Dpysl4$^{-/-}$ *or* *Crmp3*$^{-/-}$	- Abnormal dendrite and spine morphogenesis in hippocampus - Defect in infrapyramidal bundle of mossy fibers pruning in hippocampus		- Impairment of LTP induction in CA1 hippocampus		Quach et al. (2018)
Dpysl5$^{-/-}$ *or* *Crmp5*$^{-/-}$	- Aberrant Purkinje cell morphology in cerebellum		- Impaired LTD induction between parallel fibers and Purkinje cells	- Abnormal limb-clasping reflexes	Yamashita et al. (2011)

DPYSL2 and endophilin2 promotes insertion of the GluA1 subunit of AMPARs to the post-synaptic membrane and increases amplitude and frequency of mEPSCs in cultured hippocampal neurons (Figure 3; Zhang et al., 2018). In contrast, DPYSL2 downregulates the amount of the NR2B subunit of the NMDARs on the surface of cortical neurons (Bretin et al., 2006). DPYSL5 protein also regulate the endocytosis of GluA1 subunit of the AMPARs *via* phosphorylation of GluA2 at Serine 880, illustrating a specific function of DPYSL5 at glutamatergic synapses (Lin et al., 2019; Figure 3). On the other hand, it is also shown that DPYSL5 could modulate GluA2 endocytosis *via* GluA2 phosphorylation site on Serine 880 (S880), triggering social deficit (Lin et al., 2019).

Complementary to their role at the postsynaptic level, DPYSL are also expressed at presynaptic terminal. DPYSL2 and DPYSL4 have been identified as main regulators of ion currents voltage dependent (Brittain et al., 2009; Quach et al., 2011, 2013). Alike, DPYSL4 facilitates the depolarization-evoked Ca^{2+} response of L- and N-type Ca^{2+} channels to promote dendrite morphogenesis of hippocampal neurons (Quach et al., 2013; Figure 3). DPYSL2 binds and regulates the trafficking to membrane of both presynaptic voltage-gated Na^{2+}channels (NaV1.7) (Dustrude et al., 2013, 2016, 2017) and N-type voltage-gated Ca^{2+} channel (CaV2.2) (Brittain et al., 2009, 2011; Moutal et al., 2016).

Several post-translational modifications of DPYSL2 allow modulation of membrane addressing. For instance, DPYSL2 phosphorylation at Serine 522 by Cdk5 promotes association between DPYSL2 and cytoplasmic loops of CaV2.2 (Brittain et al., 2012; Chew and Khanna, 2018), leading to an increase of Ca^{2+} influx through the Cav2.2 channel and the release of neurotransmitters (Brittain et al., 2009, 2012). Similarly, SUMOylation of DPYSL2 alters calcium influx (Ju et al., 2013) and increases cell surface expression of NaV1.7 channel (Dustrude et al., 2016). These findings suggest that DPYSL2 can regulate synaptic activity and plasticity by modifying the membrane localization of ion channels and thus controlling associated currents (Figure 3). Moutal et al. identified syntaxin1 as a novel DPYSL2 protein partner (Moutal et al., 2017), and this protein is

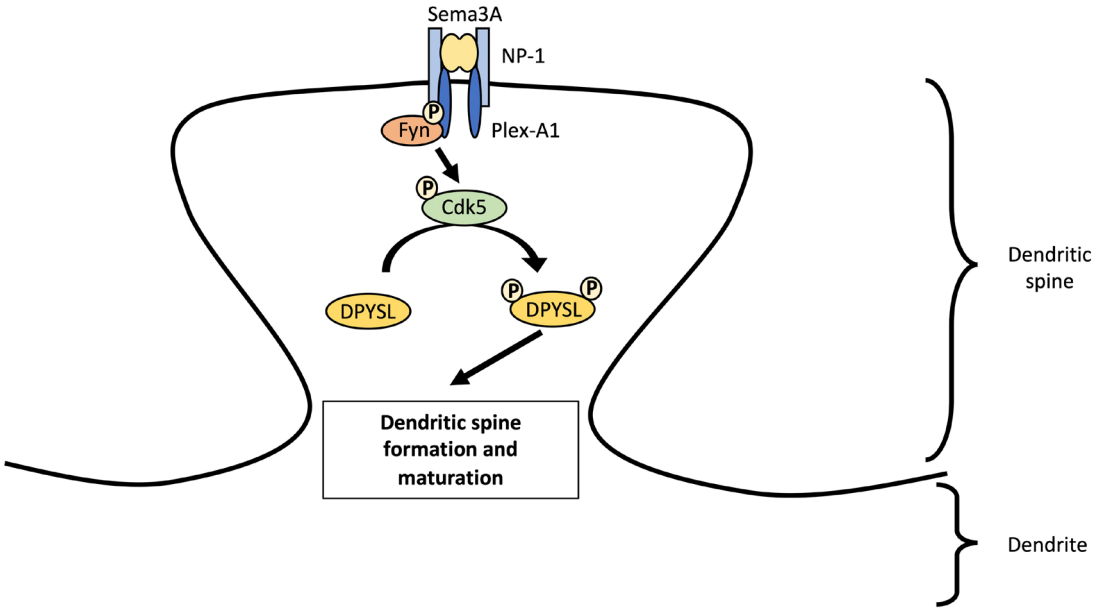

FIGURE 2
Regulation of dendritic spine maturation in cortical neurons by DPYSL proteins through Sema3A-cdk5 signaling pathway. We propose a model involving the Sema3A-DPYSL pathway in the formation and maturation of dendritic spines. Fyn phosphorylates semaphorin receptor (Plexin A2) and facilitates PlexinA2 interaction to Sema3A. Fyn also promotes the phosphorylation of kinases like cyclin dependent kinase-5 (Cdk5) which in turn phosphorylates DPYSL proteins. We suggest that DPYSL proteins will thus play a role in the genesis and maturation of dendritic spines. AS an example, DPYSL1 can regulate spine development through Sema3A–Cdk5 signaling. Sema3A binds a receptor complex of the transmembrane proteins Neuropilin-1 (NP-1) and Plexin A1 (Plex-A1). After interacting with plexin A1, Fyn stimulates kinase activity of Cdk5 via Tyr15 phosphorylation of Tyr15. Cdk5 Tyr15 phosphorylation will then phosphorylates DPYSL1 tetramer at Thr509 and Ser522 locations. Sema3A through phosphorylation of DPYSL1 by Cdk5 will increase the density of PSD-95 and synapsin I clusters at dendrites and thus, promote formation of mature spines in cultured cortical primary neurons.

involved in synaptic vesicle endocytosis neurotransmitter release (Rizo, 2022).

Interestingly, DPYSL3 interacts with proteins involved in synaptic vesicle recycling (Quinn et al., 2003) and electrophysiological experiments demonstrated that DPYSL3 enhances Ca^{2+} current density in hippocampal neurons (Wang et al., 2010).

Together, the DPYSL proteins act as neuromodulators of Ca^{2+} channel function and seem to play a major role in synaptic vesicle exocytosis and transmitter releasing in synaptic cleft (Figure 3).

Thus, the combination of these findings converged on the fact that DPYSL proteins might be key regulators of synapses architecture and activity *via* an interaction with cytoskeletal proteins but also with synaptic scaffolding proteins. Future protein interaction studies shall further clarify DPYSL protein interactome at the synapses.

7. *DPYSL* genes and neurodevelopmental disorders

Consistently with the major role of DPYSL proteins in dendritic organization and in formation and maturation of synapse, various studies suggested that they would contribute in the pathophysiology of psychiatric diseases such as schizophrenia and NDDs (Table 3; Edgar et al., 2000; Charrier et al., 2003; Hong et al., 2005; Beasley et al., 2006; Bader et al., 2012; Braunschweig et al., 2013; Yamashita et al., 2013; Lee et al., 2015; Quach et al., 2015; Tsutiya et al., 2017; Quach et al., 2021; Murtaza et al., 2022) (database SFARI, denovo-db). Interestingly, dendritic and spine dysfunctions are described in NDDs including schizophrenia, Down's syndrome, Fragile X syndrome, Rett syndrome and ASD (Huttenlocher, 1991; Kaufmann and Moser, 2000; Martínez-Cerdeño, 2017; Nelson and Bender, 2021; Quach et al., 2021). *Dpysl* KO mouse models displayed morphological abnormalities in neurons as well as behavioral defects similar to those found in schizophrenia (hyperactivity, learning and memory deficits...) or in ASD (Yamashita et al., 2013; Nakamura et al., 2016; Tsutiya et al., 2017; Ohtani-Kaneko, 2019).

Fragile X mental retardation protein (FRMP) encoded by the *FMR1* gene, is an RNA binding protein involved in fragile X syndrome, and regulates the function of many neuronal mRNAs crucial for neuronal development, synaptic plasticity and dendritic spine architecture (Banerjee et al., 2018). Interestingly, a proteomic analysis on extracts of nucleus laminaris from chicken identified CRMP1 and DPYSL2 as candidate substrates for FMRP (Sakano et al., 2017). Post-transcriptional modifications of DPYSL proteins, such as SUMOylation, impact their function in synapse, which is of particular interest for Fragile X syndrome since the activation of mGluR5 receptors promotes the SUMOylation of FRMP, leads to the dissociation of FRMP from mRNA granules to regulate spine elimination and maturation (Khayachi et al., 2018). Moreover, DPYSL2 protein expression can be controlled by the mTOR signaling pathway that is dysregulated in fragile X syndrome (Sharma et al., 2010). Both DPYSL2 and mTOR are associated with common physiological functions such as neuronal polarity, axonal outgrowth and synaptic strength as well as brain disorders including schizophrenia (Pham et al., 2016; Na et al., 2017; Izumi et al., 2022). Taken together, these data suggest that deregulation of the mTor-DPYSL2 molecular pathway may be involved in NDDs such as schizophrenia or ID.

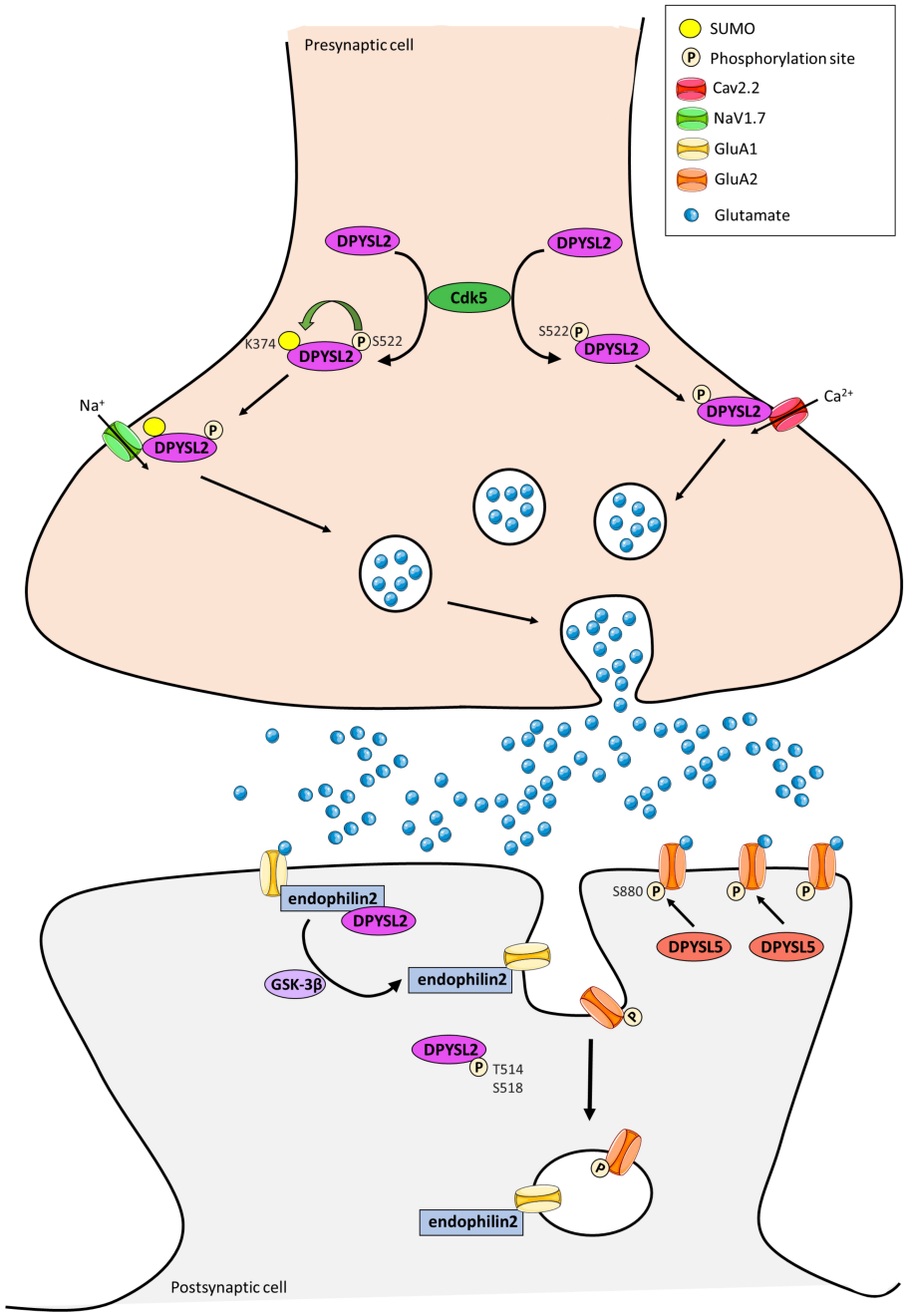

FIGURE 3
Representation of the contribution of DPYSL2 and DPYSL5 proteins in the control of synaptic plasticity. DPYSL2 binds and regulates the trafficking of both voltage gated Na^{2+} (NaV1.7) and Ca^{2+} (CaV2.2) channels at presynaptic terminal. DPYSL2 phosphorylation at Ser 522 by Cdk5 promotes its binding to Cav2.2. This interaction causes an increased number of CaV2.2 at cell surface leading to an increase in Ca^{2+} influx and glutamate release. SUMOylation is enhanced by phosphorylation of DPYSL2 through CDK5 action. This SUMOylation induces an increase of NaV1.7 channel at surface and in neuronal excitability. At postsynaptic level, DPYSL2 phosphorylation by GSK3β inhibits interaction with endophilin2 and reduces the number of GluA1 subunits of AMPARs at membrane. Similarly, DPYSL5 *via* GluA2 S880 phosphorylation can modulate traffic at the surface of the GluA2 subunit of AMPA receptors. (Adapted from Lin et al., 2019; Stratton et al., 2020; Henley et al., 2021).

For instance, genetic variants of *DPYSL1* or *DPYSL2* genes and alteration of DPYSL1 and DPYSL2 proteins levels have been reported in post-mortem brains of schizophrenic patients (Beasley et al., 2006; Martin-de-souza et al., 2010; Nomoto et al., 2021). Additionally, a link between DPYSL1 and DPYSL2 and the maternal antibody-related ASD subtype (MAR ASD) has been established (Braunschweig et al., 2013; Ramirez-Celis et al., 2021). Maternal antibodies in the placenta target fetal proteins and would cause alterations in neurodevelopment leading to behaviors associated with autism. A recent study highlighted that maternal IgG reactivity during pregnancy to both DPYSL1 + DPYSL2 increased at 16-fold the odds of an ASD diagnosis compared to the control group and over 6-fold relative to the ID group (Ramirez-Celis et al., 2022). This pattern DPYSL1 + DPYSL2 of MAR ASD is associated with ASD + ID diagnosis and ASD no-ID (Ramirez-Celis et al., 2022).

TABLE 3 Summary table of *de novo* heterozygous missense variants in *DPYSL* genes and their contribution in neurodevelopmental diseases.

Genes	Allele change	Residue change	Behavioral phenotype	References
DPYSL1	c.1052T > C	p.Phe351Ser	ID, behavioral problems	Ravindran et al. (2022)
	c.1280C > T	p.Thr427Met	ASD, no ID, delayed motor development	Ravindran et al. (2022)
	c.1766C > T	p.Pro589Leu	ID, ASD, delayed motor development	Ravindran et al. (2022)
DPYSL2	c.42C > A	p.Ser14Arg	ID	Suzuki et al. (2022)
	c.1028G > A	p.Arg343His	ASD	Satterstrom et al. (2020); Database: SFARI
	c.1312C > A	p.His438Asn	ASD	De Rubeis et al. (2014); Database: SFARI, *de novo*-db
	c.1693C > T	p.Arg565Cys	ID	Suzuki et al. (2022)
	c.1801C > T	p.Arg601Cys	ASD	Iossifov et al. (2014); Database: SFARI, *de novo*-db
DPYSL3	c.415G > A	p.Val139Ile	ASD	Iossifov et al. (2014); Database: SFARI, *de novo*-db
	c.1622C > A	p.Ser541Tyr	ASD	Tsutiya et al. (2017)
DPYSL5	c.121G > A	p.Glu41Lys	Severe ID Behavioral problems	Jeanne et al. (2021); Database: *de novo*-db
	c.139G > A	p.Gly47Arg	ID Ritscher-Schinzel syndrome	Jeanne et al. (2021)
	c.241G > A	p.Asp81Asn	Developmental disorder	Database: *de novo*-db
	c.1090G > A	p.Val364Ile	ASD	Database: *de novo*-db

In addition to DPYSL1-2, DPYSL3, and DPYSL5 are also involved in psychiatric disorders with the description of *de novo* missense mutations in *DPYSL2, 3,* and *5* in individuals with NDDs (Table 3 and Figure 4).

7.1. Genetic variants in *DPYSL1*

A recent study reported heterozygous *de novo* variants in the *DPYSL1* gene in three unrelated individuals with muscular hypotonia, ID and/or ASD (Table 3, Figure 4) (Ravindran et al., 2022). Whole exome sequencing identified two variants associated with ID (p.Pro589Leu for the long isoform of *DPYSL1* or p.Pro475Leu for the short isoform; p.Phe351Ser for the long isoform or p.Phe237Ser for the short isoform), and one variant in an individual with ASD (p.Thr427Met for the long isoform or p.Thr313Met for the short isoform). These variants are predicted to affect the ternary structure of *DPYSL1* and to impact the oligomerization of DPYSL1 proteins. When using the short isoform of *DPYLS1* protein, the p.Thr313Met and p.Pro475Leu variants are positioned next to the dimer/tetramer interface of CRMP1B, and they impair the homo-oligomerization of *DPYSL1*. The overexpression of variants p.Thr313Met and p.Pro475Leu in mouse cortical neurons caused a decrease in neuritic outgrowth (Ravindran et al., 2022), which is a morphological phenotype associated with many neurodevelopmental disorders (Quach et al., 2015; Prem et al., 2020). Interestingly, *Dpysl1*$^{-/-}$ mice have defects in dendritic spines (Yamashita et al., 2007; Makihara et al., 2016) and an inability to induce LTP (Su et al., 2007). In addition, these mice show schizophrenia like behaviors (Yamashita et al., 2013).

7.2. Genetic variants in *DPYSL2*

Three *de novo* missense variants in *DPYSL2*, predicted deleterious *in silico*, were described in individuals with ASD from the Autism Sequencing consortium (variant p.His438Asn) (Veron et al., 2018) and in two individuals of the Simons Simplex Collection (p.Arg343His, p.Arg601Cys) (Iossifov et al., 2014; Satterstrom et al., 2020). Of interest, a recent study described two unrelated patients with ID and hypoplasia of the corpus callosum associated *with a de novo* missense variant (p.Ser14Arg or p.Arg565Cys) of *DPYSL2* (Table 3 and Figure 4; Suzuki et al., 2022).

Functional assays in zebrafish model showed that p.Ser15Arg and p.Arg566Cys variants (corresponding to codons Ser14 and Arg 565 of human *DPYSL2*) led to the loss of function of DPYSL2 protein. Cell transfection experiments of DPYSL2 protein variants demonstrated that both mutations caused a decrease in DPYSL2 protein levels, probably due to increased degradation by the proteasome. Moreover, both variants impaired DPYSL2 interaction with tubulin. These results collectively support the pathogenity impact of p.Ser14Arg and p.Arg565Cys variants causing intellectual disability in humans (Suzuki et al., 2022). It is interesting to note that the patients described by Suzuki et al., have dysplasia of the corpus callosum which has also been found in *Dpysl2*$^{-/-}$ mouse model that display a dysgenesis of corpus callosum and defects in callosal axon guidance (Ziak et al., 2020).

In mice, *Dpysl2* deficiency induces a reduction of spine density and dendritic branching in CA1 hippocampal neurons and in layer V of cortical neurons of mice (Makihara et al., 2016; Zhang et al., 2016). Moreover, brain-specific *Dpysl2*-KO mice display hyperactivity and social, cognitive and affective behavioral impairments, reminiscent of deficits associated with schizophrenia (Zhang et al., 2016). On the other hand, total deletion of *Dpysl2* in mice leads to histological and behavioral alterations similarly to "ASD-related phenotype" such as axonal pruning defects and inadequate elimination of dendritic spines in dentate gyrus of hippocampi (Ziak et al., 2020). Very interestingly, a defect in synaptic pruning in layer V pyramidal neurons has been reported in temporal lobe of postmortem ASD patients (Tang et al., 2014). Furthermore, *Dpysl2*$^{-/-}$ mice exhibit ASD-related social behavior changes such as ultrasonic vocalization deficits in the early postnatal period (P8, P12) and social behavioral deficits in adult (Ziak et al., 2020).

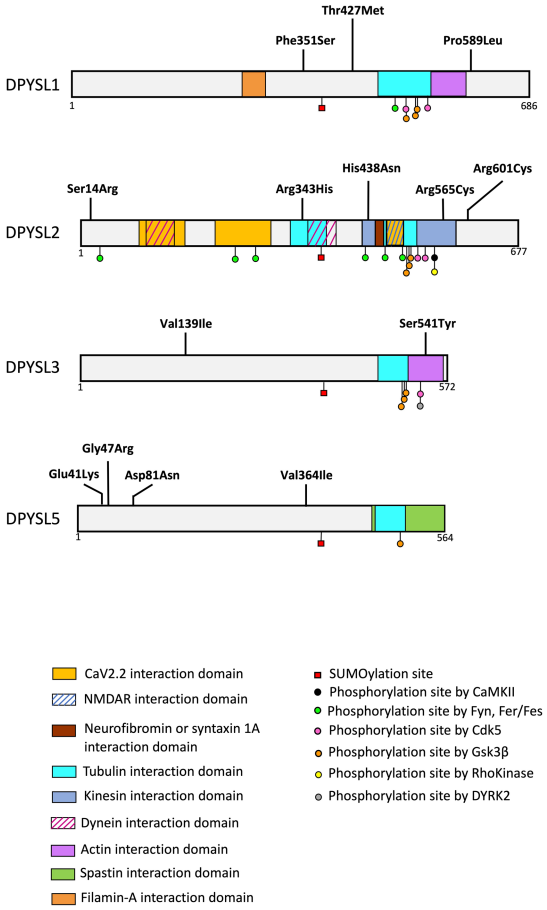

FIGURE 4
Schematic representation of protein interaction domains, post-translational. The interaction between DPYSL proteins and the represented proteins was identified in animal models (mouse, rat). Alignment of the amino acid sequences corresponding to these domains demonstrates sequence conservation in humans. The list of binding sites and post translational modifications provided in the figure is non-exhaustive. (Adapted from Nakamura et al., 2020).

7.3. Genetic variants in *DPYSL3*

The genetic analysis of the Simon Simplex Collection reported two *de novo* missense variants (p.Ser541Tyr and p.Val139Ile) of the *DPYSL3* gene associated with ASD (Iossifov et al., 2014; Tsutiya et al., 2017; Table 3 and Figure 4).

The study of *Dpysl3*-KO cultured hippocampal neurons showed that *DPYSL3* deficiency was associated with longer dendrites with more branching (Tsutiya et al., 2017). The *Dpysl3*-KO neurons transfected with pEGFP-DPYSL3^{S540Y} exhibited an increasing in dendritic branching compared to control *Dpysl3*-KO neurons transfected with pEGFP-DPYSL3^{WT} (the human DPYSL3 Serine 541 corresponds to mouse DPYSL3 codon Serine 540). To conclude, the p.Ser541Tyr mutation alters dendritic morphology and impairs the function of DPYSL3 (Tsutiya et al., 2017).

In addition, *Dpysl3*-KO mice exhibit several ASD-like phenotypes, including deficits in social interaction (determined by the three-chambers test) and alterations of sensory response measured by the emission of ultrasonic vocalization of mouse pups after different sensory stimuli. Interestingly, the serine 541, which is mutated into Tyrosine in an ASD patient (Tsutiya et al., 2017), is a phosphorylation site of DPYSL3 (Figure 4; Mertins et al., 2016) (database PhosphositePlus). As phosphorylation is essential for DPYSL cellular functions, it is plausible that DPYSL3 p.Ser541Tyr mutation may cause a loss-of-function of DPYSL3 leading to defects in dendritic arborization associated with behavioral deficits. Furthermore, Tsutiya and colleagues highlighted that *Dpysl3* deficiency altered mRNA expression of *Gria1* and *Gria2* (encoding GLUR1 and GLUR2 subunits of the AMPA receptor), essential for dendritic development and maturation (Chen, 2009). In addition, it remains essential to highlight that previous studies also revealed contribution of these two AMPA receptors subunits in mice with social deficits and in patients with ASD or other NDDs (Purcell et al., 2001; Ramanathan et al., 2004; Essa et al., 2013; Erickson et al., 2014; Uzunova et al., 2014; Kim et al., 2019).

7.4. Genetic variants in *DPYSL5*

The *DPYSL5* gene (and its respective protein DPYSL5) is the latest discovered member of DPYSL family (Fukada et al., 2000; Inatome et al., 2000; Ricard et al., 2001), and has been recently described as a novel candidate gene for NDDs (Table 3 and Figure 4). An international collaboration allowed to identify nine families including patients with ID associated with cerebral malformations, and carriers of *de novo* heterozygous missense variants in *DPYSL5*. A recurrent *de novo* variant p.Glu41Lys has been identified in eight unrelated subjects with ID, corpus callosum agenesis and posterior fossa abnormalities. Furthermore, a p.Gly47Arg variant was found in two sisters with Ritscher-Schinzel syndrome (Jeanne et al., 2021). It is critical to note that all individuals with p.Glu41Lys and p.Gly47Arg mutations in *DPYSL5* display an agenesis of corpus callosum which is a neuroanatomical malformation already associated with ASD and ID (Halgren et al., 2012; Wegiel et al., 2018; Li et al., 2019; Mimura et al., 2019; Nabais Sá et al., 2020; Qi et al., 2022).

Very interestingly, a dysgenesis of the corpus callosum has also been described for the two patients with ID and carrying the mutations p.Ser14Arg and p.Arg565Cys in *DPYSL2* gene. Another similarity worth mentioning is a hypoplasia of the cerebellum in patients carrying both variants of *DPYSL5* which is also found in the patient with mutation p.Ser14Arg of *DPYSL2* gene (Jeanne et al., 2021; Suzuki et al., 2022). DPYSL5 protein may form a homo- or hetero-tetramer with DPYSL2-4 (Wang and Strittmatter, 1997; Stenmark et al., 2007). As previously described in Jeanne et al. publication, *DPYSL5* p.Glu41Lys and p.Gly47Arg variants do not affect oligomeric assembly but by adding a positive charge to the electrostatic surface of the protein, which may alter the interaction between DPYSL5 and its partners (Jeanne et al., 2021). It is well-characterized that primary neuronal cultures, overexpressing DPYSL5 inhibits tubulin polymerization and neurite growth (Brot et al., 2014). However, overexpression of missense variants of DPYSL5 results in the loss of the inhibitory regulation of DPYSL5 on dendritic growth. Both mutations altered the function of DPYSL5 by preventing the formation of the complex with MAP2 and βIII-Tubulin. In addition, p.Gly47Arg substitution increased the binding of DPYSL5 to DPYSL2 (Jeanne et al., 2021). Thus, it has been hypothesized that p.Gly47Arg modulates the neuronal function of DPYSL2 by increasing the formation of DPYSL2/DPYSL5 complex. This study highlighted the importance of

DPYSL5 in neuronal development and put forward that defect in these regulatory mechanisms is responsible for a syndromic form of NDD with brain anomalies.

No studies have reported behavioral defects related to NDDs in *Dpysl5* mutant animal models but Lin et al., demonstrated that the hippocampal overexpression of DPYSL5 triggers social interaction deficits in both control mice and in 3xTg-Ad mice, a classical mouse model of Alzheimer's disease (Lin et al., 2019) suggesting that DPYSL5 closely controls social behavior. Overall results reveal that impairments in DPYSL2, DPYSL3 and DPYSL5 functions can lead to ID, ASD or schizophrenia. Although genetic causes of ASD and ID include mutations in genes coding for proteins involved in various pathways, such as chromatin remodeling, transcriptional regulation or the dynamics and reorganization of the cytoskeleton, a majority of genes/proteins mutated in NDDs contribute to the architecture and activity of the synapses (Guilmatre et al., 2009; Pavlowsky et al., 2012). Thus, in this review we have provided compelling evidence that dysregulation of DPYSL expression may also impair synaptic function and consequently lead to early-onset cognitive disorders, demonstrating that DPYS*L* genes and proteins defects may also contribute to "synaptopathies."

8. Discussion

The DPYSL proteins family appear to be involved in various biological events during the development including differentiation, axon guidance, neurites extension, dendritic branching and axonal regeneration (Ip et al., 2014). Here, we gathered various evidence from an extensive review of the literature that DPYSL genes and proteins are necessary for regulating the formation and the maturation of synapses, the neurotransmission and synaptic plasticity, mainly due to their synaptic localization at both pre and post synaptic terminals (Collins et al., 2006; Laumonnier et al., 2007; Brittain et al., 2009, 2011).

Genetic deletion of *Dpysl* in mice leads to synaptic impairment as well as cognitive and behavioral disorders, which are common defects associated with NDDs. As summarized in Table 3, genetic studies uncovered the contribution of *de novo* missense mutations in the *DPYSL* genes in NDDs (Iossifov et al., 2014; Veron et al., 2018; Satterstrom et al., 2020; Jeanne et al., 2021; Suzuki et al., 2022) (database: SFARI; *denovo*-db), suggesting their central role in the brain formation and functioning and the pathogenesis of NDDs. This review also highlights that DPYSL may have antagonistic or complementary activity and that their predisposition to homo- and hetero-oligomerization may have a direct impact on their physiological role. It is likely that the localization of variants in specific interaction and/or functional domains necessary for oligomerization of DPYSL proteins may have a consequence on their synaptic functions and thus lead to NDD.

Further understanding of signaling pathways located upstream and downstream of DPYSL for each homo or hetero-tetramer assembly will likely help to elucidate the physiological contribution of DPYSL proteins during brain formation and maturation and the pathogenic mechanisms leading to neurodevelopmental disorders.

Online database

BBI-Allen single cell atlases, https://descartes.brotmanbaty.org/ (accessed January 19, 2023).

National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/ (accessed January 19, 2023).

SFARI gene, https://gene.sfari.org/ (accessed January 19, 2023).

Denovo-DB, https://denovo-db.gs.washington.edu/denovo-db/ (accessed January 19, 2023).

PhosphoSitePlus, https://www.phosphosite.org/ (accessed January 19, 2023).

Author contributions

FD and FL wrote the first draft of the manuscript. DU, PV, and MJ contributed in the revision of the initial version. All authors revised and approved the final version of the manuscript submitted for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Fragile X Syndrome as an interneuronopathy: a lesson for future studies and treatments

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Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability (ID) and a primary genetic cause of autism spectrum disorder (ASD). FXS arises from the silencing of the *FMR1* gene causing the lack of translation of its encoded protein, the Fragile X Messenger RibonucleoProtein (FMRP), an RNA-binding protein involved in translational control and in RNA transport along dendrites. Although a large effort during the last 20 years has been made to investigate the cellular roles of FMRP, no effective and specific therapeutic intervention is available to treat FXS. Many studies revealed a role for FMRP in shaping sensory circuits during developmental critical periods to affect proper neurodevelopment. Dendritic spine stability, branching and density abnormalities are part of the developmental delay observed in various FXS brain areas. In particular, cortical neuronal networks in FXS are hyper-responsive and hyperexcitable, making these circuits highly synchronous. Overall, these data suggest that the excitatory/inhibitory (E/I) balance in FXS neuronal circuitry is altered. However, not much is known about how interneuron populations contribute to the unbalanced E/I ratio in FXS even if their abnormal functioning has an impact on the behavioral deficits of patients and animal models affected by neurodevelopmental disorders. We revise here the key literature concerning the role of interneurons in FXS not only with the purpose to better understand the pathophysiology of this disorder, but also to explore new possible therapeutic applications to treat FXS and other forms of ASD or ID. Indeed, for instance, the re-introduction of functional interneurons in the diseased brains has been proposed as a promising therapeutic approach for neurological and psychiatric disorders.

KEYWORDS

interneurons, Fragile X Syndrome, interneuronopathy, FMRP, ASD, excitation/inhibition balance

Introduction

Fragile X Syndrome (FXS) is the most prevalent genetic form of intellectual disability, following an X-linked inheritance, associated with deficits in cognition, language, Autism Spectrum Disorder (ASD), anxiety, epilepsy and Attention Deficit Hyperactivity Disorder (ADHD) (Hagerman et al., 2017). In FXS, the *FMR1* gene is silenced and, consequently, its product, the Fragile X Messenger Ribonucleoprotein Protein (FMRP), is entirely absent. FMRP is an RNA-binding protein involved in different steps of mRNA metabolism, such as translational control both in soma and at the synaptic level, RNA transport along dendrites and from nucleus

to cytoplasm (Maurin et al., 2014; Richter and Zhao, 2021; Kieffer et al., 2022). FMRP regulates the shaping of sensory circuits during the critical period, which is a time during early postnatal life when the development and maturation of functional properties of the brain is strongly dependent on experience or environmental influences. Indeed, early sensory activity is pivotal for the maturation of visual (Burbridge et al., 2014) and somatosensory networks (Tuncdemir et al., 2016). FMRP loss causes alterations in maturation and pruning of dendritic spines and dysregulates the expression of a large number of synaptic proteins, which are essential for the correct function of cerebral circuits (Richter and Zhao, 2021). The information flow between brain regions occurs due to a fine balance between excitatory and inhibitory neurons that control the output signal. Excitatory (E) and Inhibitory (I) synapses have different architectures. Pyramidal cells comprise the majority of the neuronal population and are primarily responsible for long-range glutamatergic transmission in the mammalian forebrain. GABAergic interneurons (INs) are the major inhibitory neurons in the central nervous system (Zhang et al., 2021), where they control and synchronize the synaptic activity of excitatory neurons. They represent 10–25% of the total number of cortical neurons and are classified based on their morphology, molecular markers, postsynaptic targets, origin area, electrophysiological properties and functions, according to the Petilla terminology (Ascoli et al., 2008). Cognition, behavior and sensory information processing depend on this efficient balance. The control of neuronal excitability and ability of synapses to strengthen or weaken in response to an enhancement or decrease in their activity provide an efficient mechanism to tune up the E/I responses (Sears and Hewett, 2021). Synapses are extremely plastic structures, modifying their activity based on changes in neuronal activity or sensory experiences. Nevertheless, it is mandatory that these changes are synchronized with other synapses to maintain E/I inputs. Due to the fine regulation of the ratio between E/I synapses, its disruptions induce a broad range of neurological and psychiatric disorders, such as FXS. This pathology can be classified as an interneuronopathy, where an alteration in inhibitory activity occurs rendering some neuronal circuits hyper-responsive and hyper-excitable (Sohal and Rubenstein, 2019).

The GABAergic inhibitory system is impaired in FXS

Most of the altered excitatory mechanisms in FXS are described in the framework of the mGluR theory, according to which the absence of FMRP exaggerates mGluR-dependent protein synthesis, leading to altered synaptic plasticity (Bear et al., 2004). However, FMRP is also expressed in GABAergic neurons at post-natal day 21 (PND 21) (Olmos-Serrano et al., 2010) and regulates the expression of different components of GABAergic transmission (Paluszkiewicz et al., 2011a). Indeed, $GABA_A$ receptor δ subunits in neocortex are downregulated in adult *Fmr1* KO mice at age of 8–12 weeks (d'Hulst et al., 2006; Figure 1). In human patients, a reduction of the $GABA_A$-mediated intracortical inhibition associated to an increase of intracortical circuit excitability was reported (Morin-Parent et al., 2019). Moreover, a decreased GABA concentration in the frontal cortex and thalamus of neonatal PND 5 *Fmr1* KO mice was found (Reyes et al., 2020). In line with the reduced excitability showed by INs, also the availability of GABA is decreased at PND 21 in the *Fmr1* KO amygdala, due to a decline in the number of inhibitory synapses and a reduced expression of GAD65/67, a rate-limiting enzyme for GABA synthesis (Olmos-Serrano et al., 2010; Figure 1). All these alterations lead to a hyper-activity of neuronal circuits that can explain the typical behavioral disturbances of FXS such as exaggerated fear, anxiety and hyperactivity (Figure 1).

In the somatosensory cortex of 1 year-old *Fmr1* KO mice, a reduction of parvalbumin (PV)-positive density, but not calbindin (CB) and calretinin (CR)-positive INs was described (Selby et al., 2007). In addition, PV INs present a bigger soma and an impaired distribution in the lamina. Interestingly, PV INs reduction mainly occurs in somatosensory cortical layers II/III/IV of 8-week-old *Fmr1* KO mice, but not in deeper layers V and VI where PV INs number is increased (Selby et al., 2007; Lee et al., 2019). The density of somatostatin (SOM)-positive INs in layer II/III does not change between WT and *Fmr1* KO mice at PND 19–31, as well as the proportion of layer II/III SOM/CR-positive INs (Paluszkiewicz et al., 2011b). Moreover, *Fmr1* KO fast-spiking (FS) INs display an immature dendritic morphology during the critical period at PND 5–6 (Nomura et al., 2017), while at PND 9–10 there are no differences compared to normal (Crair and Malenka, 1995). These interneuronal in impairments could result into an alteration in the physiological onset of critical period, cell migration, differentiation of neurons and refinement of neuronal connectivity (Hensch and Fagiolini, 2005; Luhmann et al., 2015; Begum and Sng, 2017).

Indeed, alterations in sensory experience processing, like in FXS, induce a disruptive development not only in synaptic plasticity of excitatory neurons, but also in cortical INs-afferent connectivity. This hypothesis is supported by the description of an alteration of cortical INs-afferent connectivity of the PVs and SOM cortical INs in PND 30 *Fmr1* KO mice (Pouchelon et al., 2021). The number of synapses and neurons is strongly regulated by experience influence during development. Layers I-IV of the auditory cortex present a developmental enhancement of PV cell density in both WT and *Fmr1* KO mice at PND 21, but *Fmr1* KO auditory cortex has less PV cell density than WT (Wen et al., 2018b). Like PV INs, perineuronal nets (PNNs), which are proteins in the extracellular matrix often associated with PV cells, show a developmental increase. However, *Fmr1* KO mice show a reduction of PNNs selectively at PND 21 in layer II-IV of the auditory cortex. This loss of PNNs around PV cells is associated with abnormal critical period plasticity and reduced excitability of PV cells (Figure 1).

The endopeptidase Matrix Metalloproteinase-9 (MMP-9) cleaves the extracellular matrix components of PNN and is over-expressed in *Fmr1* KO mice, leading to an altered PNN formation (Sidhu et al., 2014; Figure 1). The PNN pattern can be rescued by MMP-9 genetic deletion (Wen et al., 2018a) or by its pharmacological inhibition at PND 22 (Pirbhoy et al., 2020).

Electrophysiological and Ca^{2+} alterations in FXS interneurons

The ElectroEncephaloGram (EEG) power represents the amount of neurons that fire synchronously in a certain frequency band (Willerman et al., 1991), while coherence is used to highlight if two or more brain regions have comparable oscillatory activity (Bowyer, 2016). In FXS patients, the resting-state EEG recordings showed an

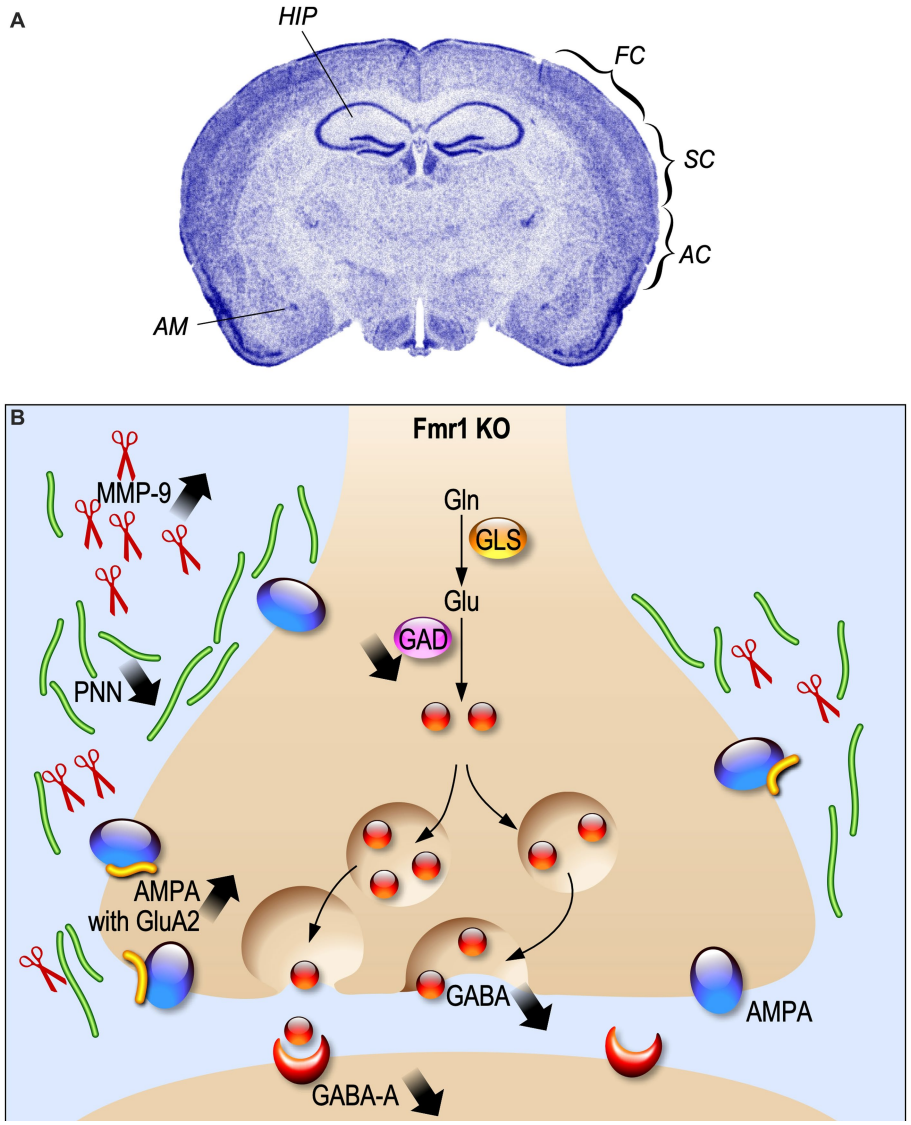

FIGURE 1
Electrophysiological alterations in Fragile X Syndrome. **(A)** Fmr1 KO brain regions where the inhibitory system is impaired are indicated. HIP, hippocampus; FC, frontal cortex; SC, somatosensory cortex; AC, auditory cortex; AM, amygdala. **(B)** Schematic representation of synaptic alteration in GABAergic synapses.

increased relative theta power (4–8 Hz), a reduced relative upper-alpha (10–12 Hz) and beta (12–30 Hz) power (Van der Molen and Van der Molen, 2013; van der Molen et al., 2014), and a heightened gamma frequency (30–80 Hz) band power (Wang et al., 2017). These alterations in EEG power are a readout of elevated excitatory cortical activity and a decrease of the inhibition process (Contractor et al., 2015; Chen et al., 2017; Ethridge et al., 2017; Donoghue et al., 2020; Guyon et al., 2021). Analogous EEGs are recorded in murine models of FXS. Indeed, adult *Fmr1* KO mice show an increased delta and gamma resting EEG power between 1.5 and 3 months of age (Lovelace et al., 2018; Wen et al., 2019). Consistent with these results, it was shown that *Fmr1* deletion in forebrain excitatory neurons affects neuronal oscillations, enhancing the resting EEG gamma power in the auditory cortex of mice at PND 60–70 (Lovelace et al., 2020). Higher theta oscillations and coherence in the slow gamma band were recorded in the hippocampus of *Fmr1* KO mice at 8 weeks of age (Arbab et al., 2018). In addition, adult *Fmr1* KO mice display a cortical reduction of sound-evoked gamma synchrony (Kulinich et al., 2020; Lovelace et al., 2020). Consistent with the human and mice EEG recordings, *Fmr1* KO rats display a reduction in alpha power and enhanced baseline of gamma power at 5 weeks of age (Kozono et al., 2020). This alteration in gamma band power is correlated to impairment in social and sensory processing and it is influenced by the abnormal activation and development of PV positive – fast-spiking (FS) interneurons. These types of neurons undergo developmental maturation during the early postnatal days, displaying modifications in membrane capacitance (C_m), input resistance (R_{in}) and neuronal activity (Itami et al., 2007). PV – FS interneurons in the FXS somatosensory cortex show a delay in the development of their intrinsic membrane properties during the critical period (Nomura et al., 2017). Indeed, in *Fmr1* KO INs, C_m is significantly lower during the critical period, whereas R_{in} is higher compared to WT INs.

Moreover, *Fmr1* KO FS interneurons show a delay in the maturation of their firing properties, displaying an adaptation on the spiking activity, while FS mature INs are characterized by a non-adaptive spiking pattern. During the neurodevelopmental period, the local excitation of PV-FS inhibitory neurons is also altered in *Fmr1* KO mice, showing a decrease in the neocortex (Gibson et al., 2008; Patel et al., 2013; Nomura et al., 2017). These neuronal and synaptic delays in neonatal *Fmr1* KO mice can be rescued by chronic administration of a TrkB receptor agonist between PND 1 and PND17 (Nomura et al., 2017; Figure 2). Moreover, the GABA switch from depolarizing to hyperpolarizing currents is delayed in cortical neurons of *Fmr1* KO mice (He et al., 2014; Figure 2).

In addition, SOM- low threshold spiking (LTS) INs of *Fmr1* KO mice at PND 19–31 are less activated by the group 1 metabotropic glutamate receptor (mGluR), generating inhibitory synaptic events with a reduced frequency (Paluszkiewicz et al., 2011b; Figure 2). LTS INs also present unsynchronized activity with pyramidal neurons, leading to the conclusions that those disruptions in neuronal synchrony could be the effect of disrupted LTS IN activity.

Alterations in the primary visual cortex of *Fmr1* KO mice are also present at 6–8 weeks of age: PV INs display a reduced visually evoked activity with lower frequency of the calcium peak induced by a visual stimulus compared to WT cells (Goel et al., 2018). Hypersensitivity was also displayed in neurons of the auditory cortex of *Fmr1* KO mice (Rotschafer and Razak, 2013), showing an increased response to a stimulus than WT mice (Wen et al., 2018a). Consistent with the general hyper-activation of the auditory cortex, there is an expanded frequency tuning in *Fmr1* KO neurons, where sound responses become abnormally high between PND 14 and PND 21, suggesting that a higher number of neurons in the auditory cortex are activated by a stimulus at the same time. This enhancement in responses could be caused by an alteration of the interneuronal activity (Patel et al., 2013; Wen et al., 2018a). Indeed, a decreased number of PV INs and impairments in the perineuronal and extracellular matrix components were described in the auditory cortex (Wen et al., 2018a). The genetic reduction of MMP-9 restores the magnitude of auditory cortex response in *Fmr1* KO neurons at PND 19–23 to WT levels (Wen et al., 2018a). These findings demonstrate the pivotal role of extracellular matrix to control the development and the functions of GABAergic neurons.

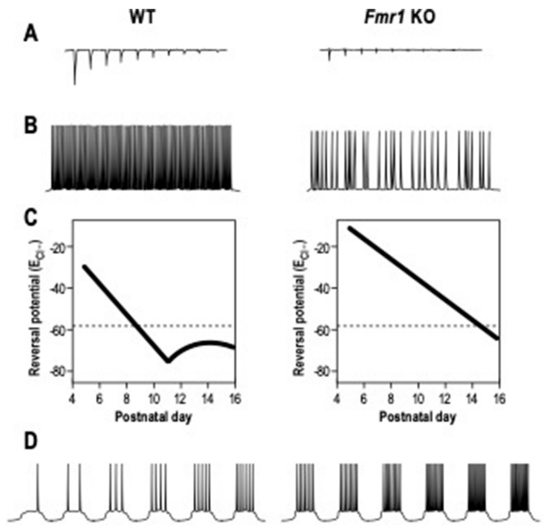

FIGURE 2
Electrophysiological alterations in Fragile X Syndrome. **(A)** The local excitation of interneurons, induced by excitatory neurons and measured as amplitude of excitatory post-synaptic currents, is reduced in *Fmr1* KO mice compared to WT mice. **(B)** The action potentials firing of interneurons induced by DHPG, agonist of group 1 metabotropic glutamate receptor (mGluR), is decreased in *Fmr1* KO mice. **(C)** The chloride reversal potential (E_{Cl^-}) remains depolarized in excitatory neurons of *Fmr1* KO mice during neuronal development. **(D)** The firing action potential rates in excitatory neurons is increased in *Fmr1* KO mice than in WT.

Due to the connections between the cortex and the amygdala, a disrupted cortical spike synchronization could then affect amygdala neuronal activity, leading to hyper-responsivity (Olmos-Serrano et al., 2010; Prager et al., 2016). Indeed, a significant neuronal hyperexcitability in pyramidal neurons of the amygdala was shown in *Fmr1* KO mice at PND 20–30 (Olmos-Serrano et al., 2010; Figure 2). Those neurons had a higher action potential (AP) frequency in response to a series of depolarizing current steps and also showed a decreased threshold for AP generation compared to WT. The synaptic response can be rescued by bath application of the GABA agonist gaboxadol (THIP), indicating a deficit in inhibitory transmission. Moreover, adult *Fmr1* KO pyramidal neurons in the amygdala display a reduced amplitude and frequency of inhibitor post-synaptic currents (sIPSC) (Olmos-Serrano et al., 2010). In young mice, at PND 10, amygdala neurons in *Fmr1* KO show reduced sIPSC amplitude and frequency, increasing at PND 14 (Vislay et al., 2013). In contrast, at PND 16, sIPSC amplitude returned to WT level, but the frequency remained high. At PND 21, sIPSC amplitude and frequency returned to control levels. These results show alterations at specific developmental points of inhibitory neurotransmission in the *Fmr1* KO amygdala.

Conversely, the cerebellar absence of FMRP reduces the spontaneous firing rate of Purkinje neurons at PND 26–32, due to an increased GABA release from IN basket cells (Yang et al., 2020). This interneuronal hyperactivity is induced by an altered activity of Kv1.2, a potassium channel highly expressed in fast-spiking GABAergic neurons. The deletion of *Fmr1* induces higher Ca^{2+} transients because of a lower interneuronal expression of Kv1.2, leading to an over-inhibition of Purkinje neurons.

Inhibitory INs display a form of synaptic plasticity which is independent from the activation of the NMDA receptor for glutamate, due to Ca^{2+} influx through AMPA receptors (Kullmann and Lamsa, 2007). The Ca^{2+} permeability of AMPA relies on the absence of the GluA2 subunit in the structure of the receptors (Akgül and McBain, 2016). In *Fmr1* KO mice at 2–3 weeks of age, CA1 inhibitory INs present an increased expression of the GluA2 subunit in AMPA receptor, which induces a decreased inwardly rectification of AMPAR-mediated excitatory synaptic current and a higher rectification index at glutamatergic synapses onto inhibitory INs (Hwang et al., 2022; Figure 1).

Recently, an altered AMPA response of the *Fmr1* KO cell fraction, enriched in INs, was highlighted thanks to the use of Agonist-induced functional analysis and cell sorting (ai-FACS) (Castagnola et al., 2020). This innovative tool allows to sort living cells on the base of their response to Ca^{2+} concentration changes in real time, using a fluorescent indicator after the application of a pharmacological agent. These analyses resulted in the identification of altered interneuronal

populations during the early post-natal development of *Fmr1* KO brain (PND 18). In particular a reduced number of *Fmr1* KO INs express *Meis2*, a transcription factor involved in ASD, at PND18 and this alteration was restored at PND19 (Castagnola et al., 2020). These results confirmed at the molecular level the presence of a transient altered interneuronal phenotype during early post-natal brain development in the absence of FMRP.

Involvement of interneurons in the behavioral phenotype of FXS

Fmr1 KO mice exhibit a cognitive deficit, autistic features and hyperactivity. Many studies investigated extensively the sensory phenotypes in both patients and animal models of FXS (Dölen et al., 2007; Knoth et al., 2014). In particular, these mice display increased sensory responses and impaired sound selectivity (Rotschafer and Razak, 2013). Altered expression of PV and PNN in amygdala, hippocampus and auditory cortex of *Fmr1* KO mice were showed to be linked to impaired tone-associated memory formation in adult mice following fear conditioning (Reinhard et al., 2019). Indeed, lower levels of PNN in amygdala and auditory cortex could be the cause of impaired tone-associated fear memory in *Fmr1* KO mice as well as a reduced PNN density in hippocampal CA2. In addition, auditory cortex PV cell density is decreased after fear conditioning in both WT and *Fmr1* KO mice, while it is increased during learning in hippocampal CA3 only in WT mice, indicating a link between tone-associated memory and PV cells. Impaired visual discrimination in FXS mice at 6–8 weeks of age was also shown to be correlated to decreased activity of PV INs and to an orientation tuning deficit of pyramidal neurons (Goel et al., 2018). Goel et al. used an excitatory DREADD strategy, targeting PV cells in *Fmr1* KO mice that restored their visually evoked response and learning capacity in a visual discrimination task. More recently, the selective deletion of the *Fmr1* gene in PV- and SOM- expressing cells in mice induced an aberrant behavioral phenotype in adult mice at 6–8 weeks of age (Kalinowska et al., 2022). Mice with PV *Fmr1*-lacking INs showed anxiety-like behavior, altered social behavior and dysregulated *de novo* protein synthesis. Conversely, *Fmr1* loss in SOM-expressing neurons did not result in behavioral abnormalities and did not significantly impact *de novo* protein synthesis. This suggests that PV cells alteration contribute more in the *Fmr1* KO impaired behavior.

Remarkably, increased PV levels and enhanced PNN formation in the auditory cortex of *Fmr1* KO mice following MMP-9 inhibition is correlated with decreased anxiety and hyperactivity during adolescence (PND 27–28) (Pirbhoy et al., 2020). Consistent with these findings, MMP-9 deletion in *Fmr1*/*Mmp-9* double KO mice at the age of 2 months ameliorates anxiety, tested in an open field task, and social interaction (Sidhu et al., 2014). Consistent with these results, the reduced level of MMP-9 in *Mmp9* $^{+/-}$ /*Fmr1* KO mice rescue abnormal sensory gating tested with pre-pulse inhibition (PPI) of acoustic startle response (Kokash et al., 2019). Interestingly, in 3-month old *Fmr1* KO mice, the altered PPI can be rescued by $GABA_A$ activation by the $GABA_A$ receptor agonist THIP, supporting the aberrant GABAergic transmission theory in FXS (Olmos-Serrano et al., 2011). Another evidence of an altered inhibition of GABA signaling in FXS is represented by audiogenic seizures in *Fmr1* KO mice, which consist in an extreme manifestation of auditory hypersensitivity after loud sound stimuli (Chen and Toth, 2001). This behavioral phenotype can be reversed by intraperitoneal administration of $GABA_A$, agonists to *Fmr1* KO mice at PND 21–25 (Heulens et al., 2012). Moreover, *Fmr1* KO mice exposed to passive sound postnatally (PND 9–21) have a significantly increased number of PV cells (Kulinich et al., 2020), showing again the correlation between INs and auditory cortex development.

Conclusion and therapeutic perspectives

Overall, the studies we summarized here strongly suggest that FXS is a form of interneuropathy. However, to advance the research in the field several aspects could be taken into account to design future studies:

I. To date, most of the studies have characterized FXS INs in adult mice (Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011b; Arbab et al., 2018; Goel et al., 2018; Kokash et al., 2019; Lee et al., 2019; Reinhard et al., 2019; Lovelace et al., 2020; Yang et al., 2020; Pouchelon et al., 2021; Kalinowska et al., 2022), while only a few studies have taken in consideration interneuronal impairment during the critical window of postnatal development (Nomura et al., 2017; Castagnola et al., 2020; Reyes et al., 2020; Rais et al., 2022). It would be interesting to study and compare various ages in *Fmr1* KO mice, which are associated to an altered function of INs through the different steps of neurodevelopment.
II. The different brain areas have been studied differently: more attention has been paid to cortex (Selby et al., 2007; Gibson et al., 2008; Paluszkiewicz et al., 2011b; Patel et al., 2013; He et al., 2014; Nomura et al., 2017; Goel et al., 2018; Wen et al., 2018a,b; Lee et al., 2019; Pirbhoy et al., 2020; Reyes et al., 2020; Pouchelon et al., 2021) compared to other brain regions, such as the hippocampus (Arbab et al., 2018; Reinhard et al., 2019; Hwang et al., 2022), leading to missing molecular and behavioral information to understand the physiopathology of FXS.
III. Another aspect that should be better considered in the future is the interneuronopathy in both sexes. Recently, it was shown that an altered activation of PV INs in mice during the critical period, especially in the limbic structures of the brain, has an impact on anxio-depressive behavior in adulthood (Banerjee et al., 2022). Indeed, adult male and female animals in which PV-positive INs have been activated during the critical period were less anxious and showed a reduction in despair-like behavior in adulthood. However, this reduction was dependent on the task and on the sex, leading to the conclusion that also the female phenotype should be taken into consideration in the behavioral test. FXS is a X-linked disorder, for this reason female *Fmr1* $^{-/-}$ mice are poorly studied since not representative of patients affected by this syndrome, however behavioral differences have been described in *Fmr1* KO females compared to males (Nolan et al., 2017) as well as sex differences in molecular pathways have been highlighted (Jiang et al., 2021). These results suggest that the analysis of this underrepresented population could help in the full understanding of brain function.

IV. Even if multiple pre-clinical studies have been carried out, the impact of various drugs was only episodically tested on interneuronal-associated phenotypes, as in the case of the modulation of TrkB or MMP-9 in infant *Fmr1* KO brain (Nomura et al., 2017; Pirbhoy et al., 2020).

The use of compounds directly linked to the GABAergic system (e.g., Baclofen, R-Baclofen and Ganaxolone that are $GABA_B$ agonist) has been shown to rescue some of the molecular and behavioral phenotypes which characterize FXS in patients and in murine models (Heulens et al., 2012; Schaefer et al., 2015; Veenstra-VanderWeele et al., 2017; Jonak et al., 2022), suggesting that the rectification of the E/I imbalance through an enhancement of the GABAergic system could be a potential treatment for FXS. Although positive results were obtained in preclinical studies and in a Phase II clinical trial, these therapeutic approaches did not result into a broad treatment for FXS patients (Castagnola et al., 2017).

Due to the absence of significant results from the clinical studies, it remains a challenge to increase GABAergic system activity in those interneuropathies characterized by an excessive reduction in the GABA response. Among the drugs currently available we can mention metformin, an anti-hyperglycemic drug prescribed against diabetes mellitus type 2. The off-label use of metformin in FXS children improves language development and behavior (Biag et al., 2019). Furthermore, chronic treatment with metformin for 10 days in adult *Fmr1* KO mice rescues different behavioral deficits, such as social deficits and repetitive behavior and normalizes the over-expression of MMP-9 (Gantois et al., 2017). We hypothesize that metformin could have an effect also on IN development and maturation due to its effect on MMP-9 expression. In addition, cannabidiol has a positive allosteric modulation on $GABA_A$ receptors (Bakas et al., 2017), enhancing GABAergic transmission, and improves the balance in inhibitory and excitatory transmission, restoring neuronal function and synaptic plasticity in patients with FXS (Palumbo et al., 2023).

Furthermore, a useful tool used to increase synaptic inhibition could be neuronal transplantation, which has the effect to improve the behavioral phenotype in several nervous system pathologies. In Alzheimer's disease-related mouse models, transplanted embryonic IN progenitors restore normal cognitive functions (Tong et al., 2014). Moreover, the replacement of INs improves memory precision after traumatic brain injury, showing to be a powerful therapeutic strategy for correcting post-traumatic memory and seizure disorders (Zhu et al., 2019). In the same path, preclinical studies performed on an epilepsy animal model highlighted a reduction of seizures after transplantation of GABAergic INs or their progenitors (Cunningham et al., 2014; Hammad et al., 2015). In this context, it is interesting to underline that human induced pluripotent stem cell (iPSC)-derived cortical neurons were transplanted into the adult mouse cortex with human synaptic networks substantially restructured over 4 months, suggesting the potential usefulness of this technology (Real et al., 2018). Thus, the precise definition of affected INs subtypes during development in FXS, as well as in other forms of brain developmental disorders, could provide a new therapeutic approach for the most severe forms of developmental brain disorders. To reach this goal, single-cell sequencing and spatial omics technologies will be very useful in combination with functional analyses.

Author contributions

AT: writing—original draft. AT, AB, BB, and SD: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Advanced genetic therapies for the treatment of Rett syndrome: state of the art and future perspectives

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Loss and gain of functions mutations in the X-linked *MECP2* (methyl-CpG-binding protein 2) gene are responsible for a set of generally severe neurological disorders that can affect both genders. In particular, *Mecp2* deficiency is mainly associated with Rett syndrome (RTT) in girls, while duplication of the *MECP2* gene leads, mainly in boys, to the *MECP2* duplication syndrome (MDS). No cure is currently available for *MECP2* related disorders. However, several studies have reported that by re-expressing the wild-type gene is possible to restore defective phenotypes of *Mecp2* null animals. This proof of principle endorsed many laboratories to search for novel therapeutic strategies to cure RTT. Besides pharmacological approaches aimed at modulating MeCP2-downstream pathways, genetic targeting of *MECP2* or its transcript have been largely proposed. Remarkably, two studies focused on augmentative gene therapy were recently approved for clinical trials. Both use molecular strategies to well-control gene dosage. Notably, the recent development of genome editing technologies has opened an alternative way to specifically target *MECP2* without altering its physiological levels. Other attractive approaches exclusively applicable for nonsense mutations are the translational read-through (TR) and t-RNA suppressor therapy. Reactivation of the *MECP2* locus on the silent X chromosome represents another valid choice for the disease. In this article, we intend to review the most recent genetic interventions for the treatment of RTT, describing the current state of the art, and the related advantages and concerns. We will also discuss the possible application of other advanced therapies, based on molecular delivery through nanoparticles, already proposed for other neurological disorders but still not tested in RTT.

KEYWORDS

Rett syndrome, gene therapy, genome-RNA editing, read-through therapy, nanoparticle

Introduction

Rett syndrome (RTT; OMIM 312750) is an X-linked neurodevelopmental disorder that almost exclusively affects girls (Amir et al., 1999). With an incidence of 1 in 10,000 it represents the most common genetic cause of severe intellectual disabilities in females worldwide (Neul et al., 2010). Apparently normal at birth, typical (or classic) RTT patients seem to grow up fine through the first 6–18 months of life, then their neurological development arrests and a regression phase occurs leading to manifestation of distinct symptoms, including loss of speech, impaired motor skills, stereotypical hand movements, gait abnormalities and seizures, that together

constitute the hallmarks of the disease. Autistic features and other severe clinical traits such as apnea, hyperventilation, scoliosis, weight loss and cardiac abnormalities are often seen in affected girls (Chahrour and Zoghbi, 2007). In addition, atypical RTT patients manifesting either milder or more severe clinical features also exist. Notably, in contrast to what was initially reported, *MECP2* mutant males also have been described and they generally display greater clinical harshness compared to females; they can experience mild mental retardation or die from severe neonatal encephalopathy (Neul et al., 2018).

Originally characterized in 1966 by Andreas Rett, an Austrian pediatrician who observed two girls having same unusual behavior, it was only in 1999 that the laboratory of Huda Zoghbi discovered that variations in the X-linked methyl-CpG-binding protein-2 (*MECP2*) gene are causative of RTT. Indeed, 90–95% of individuals with typical RTT and 70% of atypical cases are mutated in *MECP2*. Additionally, genetic changes in the X-linked Cyclin-Dependent Kinase-Like 5 (*CDKL5*; OMIM #300203) (Evans et al., 2005) or the Forkhead box G1 (*FOXG1*; OMIM #164874) (Philippe et al., 2010) genes have been related with atypical and rarely classic forms of RTT (Neul et al., 2010). Most of the mutations in *MECP2* are hypomorphic, thus leading to partial or complete "loss of function" of the protein; however hypermorphic mutations, associated with duplication of portions of Xq28 spanning the *MECP2* locus, have been related to a neurodevelopmental disorder called MeCP2 duplication syndrome (MDS) (van Esch et al., 2005). MDS predominantly affects males, who manifest severe intellectual disability, delayed psychomotor development, seizures, respiratory infection, feeding difficulties and progressive spasticity. They often die before 25 years of age because of frequent infections and neurological decline. Remarkably, while RTT is a sporadic disease, *MECP2* duplications are inherited with >90% penetrance from mothers, who carry the mutated copy of the gene on the silenced X chromosome (Ramocki et al., 2010). Eventually, *MECP2* mutations have been associated with neurodevelopmental disorders such as Angelman-like syndrome and Attention-Deficit Hyperactivity Disorders (ADHD), and occasionally with autism (Ramocki et al., 2009). Collectively, these results of molecular genetics prove that *MECP2* can cause a broad spectrum of neuropsychiatric disorders and intellectual disabilities that can be gathered as MeCP2-related disorders.

To date there is no cure for RTT and ongoing treatments are meant to alleviate disease symptoms. For example, medications are provided to mitigate breath irregularities and sleep problems while antiepileptic drugs are administered to relief patients affected by seizures (about 60%) (Vignoli et al., 2017). Other treatment options that overall improve the quality of life of RTT girls include occupational and physical therapy, scoliosis equipment and nutritional programs. About 70% of individuals with typical RTT may survive longer than 45 years old with appropriate medical and care management (Tarquinio et al., 2015).

Importantly, a breakthrough study by Adrian Bird's teams proved that restoration of endogenous *Mecp2* expression in symptomatic hemizygous male and heterozygous female adult mice reversed many of the Rett-like phenotypes even at the late stages of syndrome progression (Guy et al., 2007). The reversal of disease condition has led the scientific community to consider gene replacement therapy as the most amenable strategy to cure RTT and MeCP2-related disorders (Gadalla et al., 2013, 2017; Garg et al., 2013; Sinnett et al., 2017). However, pre-clinical studies of gene therapy in RTT demonstrated that overexpression of MeCP2 resulted in severe neurological defects and liver damage in injected animals (Collins et al., 2004; Gadalla et al., 2013; Matagne et al., 2021; Li et al., 2023). For these reasons, in the last decades, novel strategies were explored to identify the most appropriate delivery vector that could finely transduce *MECP2* mainly in brain cells without triggering deleterious side-effects.

By describing gene therapy strategies and engineered vectors able to control MeCP2 expression, we will review and comment on the most recent progresses of genetic interventions; further, we will discuss the latest development of DNA/RNA editing approaches and reactivation of the *Mecp2* allele placed on the silenced X-chromosome and open to future approaches for molecular delivery through nanoparticles that are still not tested in RTT.

Genetics of RTT: the *MECP2* gene and its pathogenic mutations

The *MECP2* gene is located on the long arm of X-chromosome (Xq28) where it spans almost 76 kilobases (kb). Its 3′-UTR (untranslated region) is one of the longest known in the human genome as well as its second intron is atypically long (60,000 nucleotide) (Reichwald et al., 2000). *MECP2* is present in all vertebrates, and in both human and mouse consists of four exons from which two different protein isoforms are generated: the MeCP2 E1 and MeCP2 E2 (Figure 1A). In human, the MeCP2 E1 is encoded by exons 1, 3 and 4 while exon 2 is excluded via alternative splicing. The resulting product is a longer protein of 498 amino acid containing 21 unique N-terminal residues. The MeCP2 E2 is translated from exons 2, 3, and 4 and has 9 unique residues (Mnatzakanian et al., 2004) (Figure 1B). While MeCP2 E1 is conserved across vertebrates and predominantly expressed in adult brain, MeCP2 E1 is only present in mammals and highly expressed in peripheral tissues (Tillotson and Bird, 2019). Remarkably, recent evidence suggests that mutations in MeCP2 E1 might be associated with RTT (Yasui et al., 2014). The human MeCP2 E2 protein is structurally composed by 486 amino acid residues and functionally characterized by five main domains: N-terminal domain (NTD, 1–78 amino acid); methyl-CpG binding domain (MBD; 78–162) spanning 85-amino acid; intervening domain (ID); transcriptional repression domain (TRD) of 104- amino acid and carboxyterminal domain (CTD; 310–486 amino acid). MeCP2 binds to methylated cytosine via the MBD, while the CTD facilitates its interaction to naked or nucleosomal DNA thus mediating chromatin compaction (Figure 1B). Two other regions are mostly relevant, and both located in the TRD domain: the NCoR1/2 co-repressor complex interaction domain (NID, amino acid 285–313), a short region of 29-amino acid, and a nuclear localization signal (NLS; 255–271) of 16 residues. The estimated molecular weight of MeCP2 is approximately 53 kDa; however, in western blot the protein is detected at 72 kDa.

Over 500 different *MECP2* mutations have been identified as causative of RTT and documented in the web database (RettBase: http://mecp2.chw.edu.au). Among those, there are eight major point mutations (p.Arg106Trp (R106W), p.Arg133Cys (R133C), pThr158Met (T158M), p.Arg168* (R168X), p.Arg255* (R255X), p.Arg270* (R270X), p.Arg294* (R294X) and p.Arg306Cys (R306C)) that account for almost 65% of all variations found in typical RTT individuals (Neul et al., 2008). In addition, small deletions,

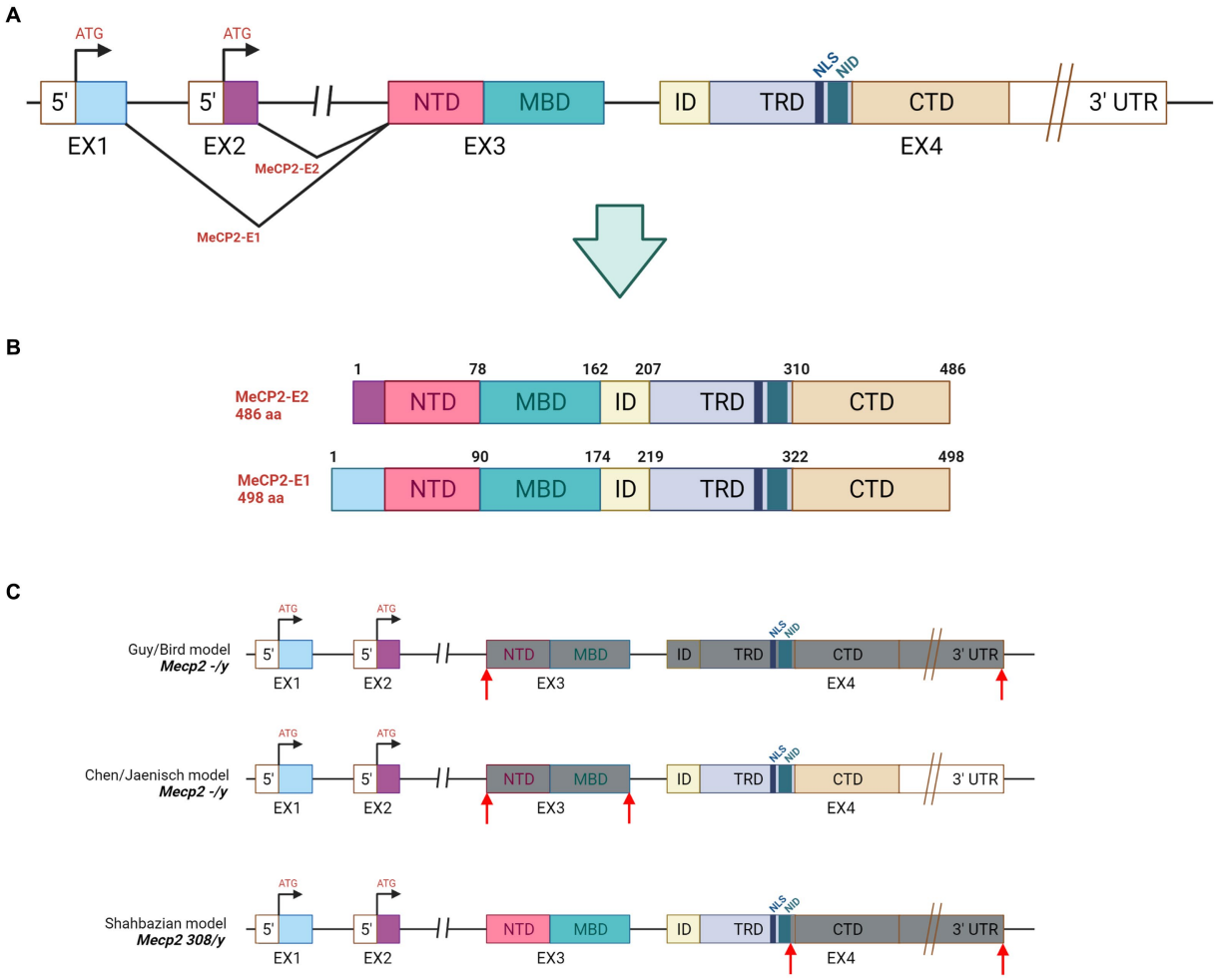

FIGURE 1
Gene and protein structure of MeCP2. **(A)** Gene structure: NTD=N-Terminal domain, MBD=Methyl-binding domain, ID=Intervening domain, TRD=Transciption repression domain, CTD=C-terminal domain, NID=NCoR intreraction, NLS=Nuclear localizatoin signal. **(B)** Illustration of MeCP2-E1 and MeCP2-E2 isoforms derived by alternative splicing in *MECP2*. **(C)** Genetic features of the mostly used mouse models of RTT. *Mecp2*$^{-/y}$ mice were obtained by deleting the exon 3 and 4 (Guy et al., 2001) or by removing the exon 3 (Chen et al., 2001) from the *Mecp2* gene. The *Mecp2*$^{308/y}$ animal model was produced by inserting the T308X nonsense mutation in exon 4. The resulting truncated protein lacks the C-terminal domain while maintaining the MBD and the TRD portions (Shahbazian et al., 2002). Grey boxes depict the exons that are missing in the MeCP2 protein, while the red arrows indicate the deleted portion.

predominantly falling in the C-terminal domain of MeCP2 are featured in 5–10% of classical RTT patients. Several studies supported the relationship between the clinical severity of RTT and the type of mutations in *MECP2* (Frullanti et al., 2019). Indeed, partial loss-of-function variations such as the R133C and late truncating mutations often lead to a milder phenotype, while missense mutations (e.g., R306C or T158M) and early truncating variations such as R294X are associated to phenotypes of medium severity. Complete loss-of-function mutations (e.g., R255X or other early truncations) and large deletions often yield to a very severe clinical course of RTT. Interestingly, mutations occurring within the NID region (e.g., R294X) destroy the interaction with transcriptional co-repressor complex NCoR/SMRT and disrupt the repressive activity of MeCP2. Similarly, variations falling in the MBD (e.g., R133C) partially abolish the binding to methylated DNA, thus confirming the biological relevance of these two domains (Cuddapah et al., 2014; Frullanti et al., 2019). However, other factors such as X-chromosome inactivation (XCI) participate to the phenotypic variability of RTT individuals. XCI defines the pattern of silencing of one X-chromosome that occurs in all cells during early female embryogenesis. Consequently, a female patient generally features half cells expressing the wild type *MECP2* allele and the other half the mutated one (random XCI). However, if this process is skewed and it favors the inactivation of mutant *MECP2* in most cells, no (as seen in silent carriers) or very mild clinical manifestations are overt; alternatively, the preferential inactivation of the wild-type allele causes an aggravation of the phenotype. Therefore, the degree of XCI skewness determines the greater clinical variability seen in RTT patients (Takahashi et al., 2008). Finally, genetic modifiers of *MECP2*, which mainly remain undisclosed, may also affect the variability of the disease.

In conclusion, the variability and clinical severity of RTT patients is the result of the complex combination among the type of *MECP2* mutation, the presence of genetic modifier(s), X-chromosome inactivation status, and environment.

Neuron and glia: partners in RTT?

MeCP2 is ubiquitously expressed but its highest levels are reached in brain, lung and spleen. Its amounts are modest in heart and kidney and are almost undetectable in stomach and liver. In particular, in brain MeCP2 protein levels correlate with neuronal maturation, rising when neurons project dendritic arbors and axons, and when connectivity is established (Kishi and Macklis, 2004; Neul and Zoghbi, 2004). In addition, its expression remains high throughout adulthood thus supporting its main role of sustaining the activity of mature neurons. However, increasing evidence underlined its fundamental role in early stages of neuronal development (Ronnett et al., 2003; Sun et al., 2019). Indeed, MeCP2 expression was detected in embryonic and postnatal neocortical cells, including neuronal precursors (Kishi and Macklis, 2004; Bedogni et al., 2016). Further, transcriptional analyses of *Mecp2* null embryonic cortices revealed an enrichment of genes expressed by progenitors and early postmitotic neurons, and a decrease of transcripts involved in neuronal differentiation and responsiveness to external stimuli, thus providing another relevant role of MeCP2 in neuronal fate refinement and activity (Cobolli Gigli et al., 2018). However, the most conspicuous consequence of MeCP2 deficiency in the CNS is the reduced size and weight of the RTT brain (reduction of 12–34%). Other more subtle alterations include decreased dendritic complexity, defects in spine density and morphology, and increased neuronal packing (Armstrong et al., 1995; Bauman et al., 1995). *In vivo* studies performed on different mouse models showed that *Mecp2* deficiency also disrupts, with a pattern that varies among different brain regions, the balance of synaptic excitation and inhibition (Dani et al., 2005; Shepherd and Katz, 2011). Finally, long-term synaptic plasticity (i.e., Long Term Potentiation (LTP) and Long Term Depression (LTD)) which underlies the processes for cognitive functions and long-term memory formation is also generally affected (Li et al., 2016).

Even though MeCP2 was detected in many non-neuronal cell types, the neuropathology observed in RTT was exclusively ascribed to its loss in neurons. However, recent studies started to investigate whether glial cells might contribute to the RTT pathogenesis. Indeed, increased levels of genes encoding glia-specific proteins (e.g., α B-crystallin, glial fibrillary acidic protein (GFAP), excitatory amino acid transporter 1 (EAAT1) and S100 A13) were documented in post-mortem brain of RTT girls (Colantuoni et al., 2001).

Consistently, transcriptional and proteomic analyses of *Mecp2* null brain cortices revealed a perturbation of pathways involved in astrocytic maturation and morphology (Yasui et al., 2013; Delépine, 2015). In a more detailed study, Mandel's team showed the inability of wild-type neurons to grow fine in the presence of *Mecp2* null astrocytes and proposed that, in RTT females, the activity of healthy neurons might be affected by *MECP2*-mutant astrocytes in a non-cell autonomous manner, probably through the impaired release of morphogenic factor(s) or/and secretion of toxic molecule(s) (Ballas et al., 2009). Few years later, by using inducible Cre mouse lines, they selectively removed or induced *Mecp2* in astrocytes and proved that while neurons are mainly responsible for the neurological phenotypes in RTT, astrocytes largely contribute to the progression of the disease (Lioy et al., 2011).

Emerging evidence has also reported a possible involvement of microglia and oligodendrocytes in RTT pathogenesis (Maezawa and Jin, 2010; Nguyen et al., 2013; Kahanovitch et al., 2019).

Mouse models of *MECP2*-related disorders

Given their large-scale utility, several mouse models of RTT and *MECP2*-related disorders have been generated to investigate MeCP2 functions and the mechanisms underlying disease pathology (Ricceri et al., 2008; Lombardi et al., 2015). The first two *Mecp2*-null mouse models, generated with the Cre-Lox technology in the early 2000s, were developed in the laboratories of Rudolf Jaenisch and Adrian Bird (Chen et al., 2001; Guy et al., 2001). Both models carried deletion of exon 3 (Jaenisch) or exons 3–4 (Bird) of *MECP2* gene and well recapitulate many RTT features, thus further providing the genetic cause of the disease (Figure 1C). In particular, *Mecp2* null males ($Mecp2^{-/y}$) have no apparent phenotype until 4 to 5 weeks of age, when they become underweight and exhibit hindlimb clasping, abnormal gait, tremors, breathing irregularities, and often seizures. Symptoms worsen with aging and the animals die approximately in 10–12 weeks. Compared to wild-type animals, null mice have smaller brains, shrinked cortices, more densely and packed neurons with immature synapses. Heterozygous female mice (HET, $Mecp2^{-/+}$) display similar RTT-like phenotypes, including hypoactivity, ataxic gait, hindlimb clasping, breathing irregularities starting much later, at 3–4 months of age. In contrast to null males, they are fertile, become overweight and survive longer than 10 months. Although HET females should be the appropriate genetic mouse model of RTT, the long time required for symptoms to become overt and the associated phenotypic variability, led researchers to set their experiments on the *Mecp2* null male model, which manifests earlier and highly consistent phenotypes. However, the majority of RTT patients harbors missense or truncating mutations leading to a hypofunctional MeCP2 rather than to its complete loss. Accordingly, missense variations such as R106W, T158M, p.Thr158Ala (T158A) and p.Tyr120Asp (Y120D) express reduced protein levels compared to the wild-type product (Goffin et al., 2012; Johnson et al., 2017; Lamonica et al., 2017; Gandaglia et al., 2019). Interestingly, among nonsense mutations, the R294X produced stable truncated proteins whereas the R168X, R255X, and R270X did not yield to a detectable product (Collins and Neul, 2022). It is subject of debate whether global MeCP2 deficiency correctly recapitulates the molecular features of the disease. For this reason, other models harboring common *MECP2* mutations have been generated offering a long list of disease modeling animals for the comprehension of molecular consequences, pathophysiology and genotype–phenotype correlations of specific genetic lesions (Katz et al., 2012). The first RTT mouse model ($Mecp2^{308/y}$) expressing a hypomorphic truncated form of Mecp2 and lacking of the C-terminal domain, was developed in 2002 (Shahbazian et al., 2002) (Figure 1C). The overall phenotype of the animal was milder with respect to the full null line; heterozygous females confirmed to be less sick and to manifest more variable phenotypes. Mice carrying truncating mutations (i.e., Mecp2 R168X or R255X), or mimicking the most common missense variations (i.e., Mecp2 p.Ala140Val (A140V), R133C, R306C, T158A/M, R106W) (Jentarra et al., 2010; Goffin et al., 2012; Lyst et al., 2013; Johnson et al., 2017; Lamonica et al., 2017) or the rare one Y120D (Gandaglia et al., 2019) have been generated, although the mostly used RTT mouse models still remain the $Mecp2^{-/y}$ and $Mecp2^{308/y}$. To better investigate the circuits involved in RTT pathogenesis and understand the etiology of the disease, conditional knockout mice have been developed and characterized. For instance, to achieve loss of *Mecp2* in cell types such

as neurons and glia, a Nestin-driven Cre recombinase mouse line was used. The resulting animals showed reduced Mecp2 expression from embryonic day 12 (E12) and phenotypes similar to null mice, thus suggesting that the absence of Mecp2 in CNS is the leading cause of RTT symptoms (Chen et al., 2001). Subsequent studies addressed the consequences of *Mecp2* inactivation in specific neuronal subtypes or brain areas. For example, loss of Mecp2 in dopaminergic neurons caused impairment of motor coordination, while its absence in serotoninergic neurons induced augmented aggression (Samaco et al., 2009). Similarly, deletion of *Mecp2* in the basolateral amygdala resulted in anxiety behavior and learning deficits, while its loss in hypothalamic Sim1-expressing neurons revealed a role of Mecp2 in the regulation of social and feeding behavior and response to stress (Fyffe et al., 2008). Overall, each mouse line recapitulated some of the typical RTT features suggesting that MeCP2 function is important across brain regions, that all together contribute to the RTT features seen in patients. Conditional mice have been instrumental also to assess the neuropathological consequences of postnatal inactivation of *Mecp2*. Indeed, depletion of *Mecp2* at different ages (3 weeks-old and 10 weeks-old in males and 20 weeks-old in females) always caused the appearance of RTT-like phenotypes, brain shrinking and premature death (McGraw et al., 2011; Cheval et al., 2012; Nguyen et al., 2012). Although *Mecp2*-inactivating mutations have strong consequences on brain functioning, they do not lead to neuronal loss (Akbarian, 2003). Accordingly, a breakthrough study in 2007 demonstrated that reactivation of *Mecp2* in adult male and female mutant mice rescued neurological defects. These experiments proved that RTT and *MECP2*-related disorders are not an irreversible condition and that therapies focused on MeCP2 restoring could be beneficial in patients also after symptoms onset (Guy et al., 2007). Finally, mice overexpressing *Mecp2* (Mecp2-TG1) have been generated to investigate the gain of function consequences of the protein. Similarly to human pathology, severity of MDS-like phenotypes positively correlates with the levels of Mecp2 protein. Animals that overexpress modest level of Mecp2 showed enhanced motor learning, forepaw clasping, and increased contextual fear conditioning (Collins et al., 2004). In contrast, mice expressing from two to four-fold levels of Mecp2 displayed enhanced anxiety-like behavior and motor dysfunction. To conclude, mouse models represent the species mainly used to understand specific aspects of RTT pathology and to address preclinical studies of the disease; however other animal models such as rats, zebrafish and non-human primates have been developed and are available for RTT community to corroborate scientific findings (Veeraragavan et al., 2016; Chen et al., 2017).

MeCP2: a multifunctional protein inducing a plethora of possible pathogenic mechanisms

By the time that *MECP2* was identified as a causative gene of RTT, Adrian Bird and his collaborators had already partially characterized its functions (Lewis et al., 1992). Indeed, their studies had shown that MeCP2 selectively binds to methylated DNA independently from the specific sequences, thereby repressing transcription *in vitro*. On the same line, it was proved that the TRD domain promotes gene silencing by binding to corepressor complexes (Sin3A and NCoR) that contain histone deacetylase activities (Jones et al., 1998; Nan et al., 1998) (Figure 2). Subsequently, Skene and colleagues proposed that in mature neurons, Mecp2 might serve as an alternative linker histone and organize a specialized chromatin structure, thus dampening overall transcriptional noise (Skene et al., 2010). In addition, MeCP2 function was linked to mRNA splicing for its interaction with Y-box binding protein 1 (YB-1), a member of the family of DNA- and RNA-binding proteins, implicated in many RNA/DNA dependent processes including the regulation of alternative splicing (Eliseeva et al., 2011). Since then, many other splicing factors have been associated with MeCP2, especially through its CTD or TRD domains (Good et al., 2021), leading Cheng and colleagues to propose that the majority of MeCP2-bound proteins are involved in RNA splicing and processing (Cheng et al., 2017). However, few years later Chhatbar and co-workers demonstrated that regulation of splicing is not a primary function of MeCP2 (Chhatbar et al., 2020). To increase the repertoire of roles attributed to MeCP2, other reports have proposed for MeCP2 a regulatory function in microRNA (miRNA) post-transcriptional processing (Cheng et al., 2014). Moreover, contrary to expectation, *ex-vivo* studies on purified hypothalami and cerebella from RTT mice suggested that MeCP2 may also positively regulate gene expression by interacting with the transcriptional activator cAMP response element-binding (CREB) (Chahrour et al., 2008). In 2011, the protein synthesis was found significantly impaired in both *Mecp2* null mice and heterozygous females as a consequence of reduced AKT/mTOR signaling pathway (Ricciardi et al., 2011), thus suggesting yet another function of MeCP2. However, this study did not address how the translational deficits contribute to RTT pathogenesis, nor indicated whether they are a direct or indirect effect of Mecp2 loss. Finally, we recently published that MeCP2 localizes at the centrosome and in primary cilium thus providing another role for the protein outside the nucleus (Bergo et al., 2015; Frasca et al., 2020). Interestingly, we showed that *Mecp2* loss affects cilium formation and signaling transduction of the Sonic Hedgehog pathway, a key regulator of processes involved in brain development and growth (Frasca et al., 2020) (Figure 2). All together, these findings emphasize the multifunctional role of MeCP2 in brain, even though its main role appears to be its capacity to bridge the NCoR1/2 corepressor complex to methylated DNA (Tillotson and Bird, 2019). Although all these studies suggest a primary role for MeCP2 in regulating gene expression, it is important to mention that Johnson et al. (2017) have recently proven that most of the transcriptional changes observed in *Mecp2* deficient neurons are cell type-specific, therefore reflecting the high cellular heterogeneity featured by brain. Further, by analyzing, in *Mecp2* heterozygous female mice, gene expression profiles of neurons expressing either the wild type or mutant *Mecp2* allele, they revealed that both cells feature differentially expressed genes (DEGs). Interestingly, most DEGs occur in neurons expressing the mutant allele, thereby indicating cell autonomous changes; however, non-cell autonomous DEGs were also reported. Notably, cell and non-cell autonomous DEGs represent different biological processes (Johnson et al., 2017).

Genetic approaches targeting the *MECP2* gene or its transcript

Since the ground-breaking idea of using viruses as vector for gene therapy, much progress has been made to develop an efficient

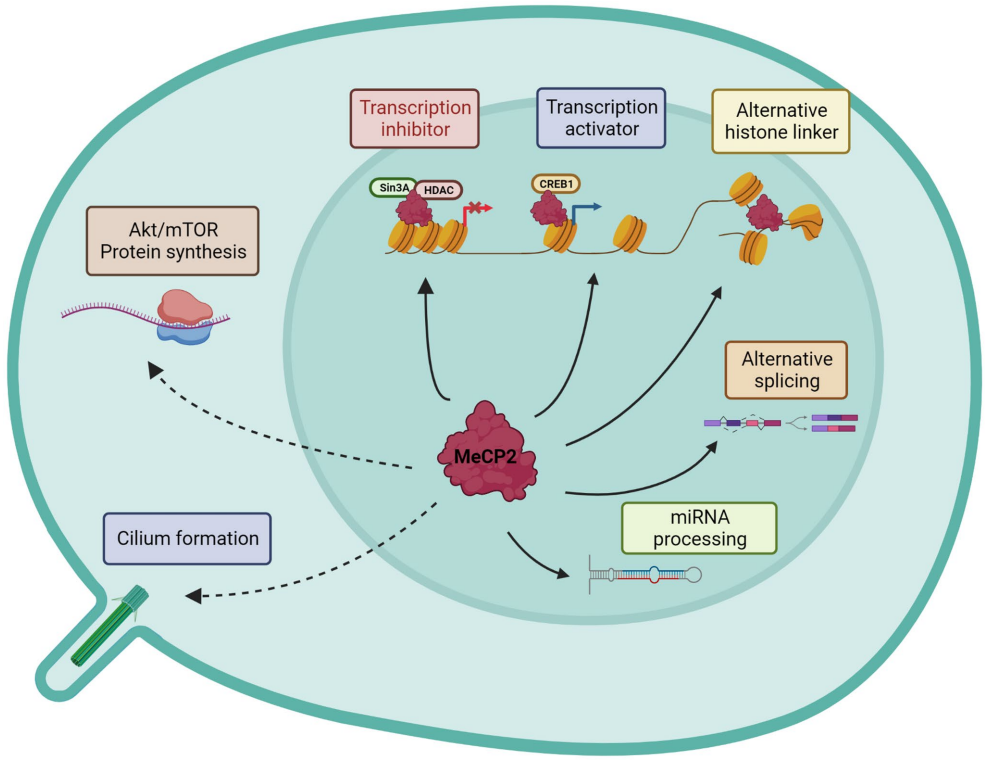

FIGURE 2
Graphical representation of known MeCP2 functions. Nuclear and cytoplasmatic activities are distinguished. Continuous lines describe direct MeCP2 actions, while dotted lines the indirect effects.

methodology. Retroviruses, lentiviruses, adenoviruses and adenovirus-associated viruses (AAV) have been widely studied to deliver therapeutic genes in diverse applications and in the past decade to treat multiple disorders (Bulcha et al., 2021). In particular, lentiviral and retroviral vectors were the earlier employed to treat disease models (Miller, 1992). However, their big advantage of carrying larger DNA payload was mitigated by the fact that the integration of fragment of their genetic material into the genome of the host cell increased the probability of insertional mutagenesis and carcinogenesis (McCormack and Rabbitts, 2004; Hacein-Bey-Abina et al., 2008). In addition, these viruses do not cross the blood brain barrier (BBB), thus resulting less appropriate for clinical trials of neurological disorders. In contrast, adenoviruses and AAVs can efficiently bypass the BBB, infect post-mitotic cells such as neurons, and provide stable transgene expression without integrating within the host genome (Foust and Kaspar, 2009). Unfortunately, adenoviruses induced an elevated immune response from the target cells which limited their use in clinics. Remarkably, AAVs triggered very low levels of immune-response and for this reason were the first to be used in clinical trial in 2012 to treat lipoprotein lipase deficiency, a rare autosomal recessive disorder of lipid metabolism (Carpentier et al., 2012). Since then, several clinical trial for CNS disorders (Sanfilippo type A and B, ClinicalTrials.gov: NCT02053064 and NCT03300453, respectively; Batten disease, ClinicalTrials.gov: NCT01414985; metachromatic leukodystrophy, ClinicalTrials.gov: NCT01801709; and spinal muscular atrophy, ClinicalTrials.gov: NCT02122952) have utilized AAVs as gene delivery tool (Gray et al., 2011b; Saraiva et al., 2016). However, one limitation of these vectors is their small packaging size (~5.0 kb) which restricts the product that can be packed to less than 4.7 kb for single stranded (ss) and approximately to 2.3 kb in the more efficacious self-complementary (sc) packaging approach (McCarty et al., 2001; Lykken et al., 2018). Currently, two methods have been employed to deliver these vectors into the brain: systemic (e.g., intravenous injection) and direct CNS route (e.g., intrathecal, intra-cisterna magna or direct injection into the neuropil). Systemic delivery is more desirable from a translational point of view because it is less invasive than the direct routes, although the number of transduced cells in the CNS is limited and the choice of AAVs vector able to cross the BBB is restricted to serotype 9 (AAV9) (Gray et al., 2011b; Saraiva et al., 2016) as demonstrated in mice and large animal models (Foust et al., 2009; Bevan et al., 2011; Mendell et al., 2017). The translational feasibility of systemic administration of AAV has a big limitation though: the high doses required to efficiently transduce the CNS and promote diffuse expression of the transgene often lead to off-target-based toxicity, especially in the liver (Ronzitti et al., 2020; Verdera et al., 2020). To improve tissue tropism and avoid systemic immune responses, AAV viral genome has been widely engineered with the inclusion of cell-type specific promoters (Gray et al., 2011a; de Leeuw et al., 2014), enhancers elements (Vormstein-Schneider et al., 2020; Graybuck et al., 2021), microRNA target sites (Hordeaux et al., 2020) and viral capsid variants (Matsuzaki et al., 2018; Ravindra Kumar et al., 2020). In the next paragraph, we will describe the evolution of AAVs constructs (Figure 3) and their pros and cons in pre-clinical studies of RTT (Supplementary Table S1).

Gene replacement therapy in RTT

Given that RTT is a monogenic disorder caused by loss of function mutations in *MECP2* and that its protein has crucial roles in several physiological pathways, gene delivery of a wild copy of *MECP2* to mutant brain cells have been widely contemplated for the treatment of RTT. A seminal study by Rastegar and colleagues demonstrated the capacity of retroviral vectors comprised of self-inactivating (SIN) long term repeat (LTR) regions and of the *Mecp2* promoter (MeP) to efficiently transduce *MECP2-e1* in neuronal stem cells (NSCs). Differentiated neurons derived by infected NSCs showed restoration of protein levels and rescue of dendritic growth and branching. Consequently, the authors employed SIN lentiviral vectors to directly transduce mature cortical neurons. However, lentiviral vectors only reached a restricted brain area near to the administration site, indicating that they are probably unsuitable for the treatment of RTT (Rastegar et al., 2009).

1st generation of expression cassettes

The first and encouraging report of gene therapy in mouse model of RTT was obtained by administrating recombinant adeno associated viruses, that still appears as the preferred vectors. In particular, brain injection of single stranded AAV2/9-*MECP2,* driven by the chicken β-actin promoter (AAV2/9-CBA-*MECP2;* Figure 3A) in postnatal day (P) *Mecp2*-null mice (P0-2), efficiently transduced different areas (almost 40% in thalamus and hypothalamus, 20–25% in motor cortex and hippocampus, 15% in brain stem and only 7% in the striatum) (Gadalla et al., 2013). Transgene expression was found mostly in neurons and at near physiological levels. Infection efficiency was sufficient to ameliorate some RTT-like phenotypes, such as motor functions and life span. In the same study, systemic administration in juvenile mice (4- to 5-weeks old) of a vector (scAAV9-MeP-*MECP2;* Figure 3A) in which *MECP2* expression was guided by a short region of its own promoter (229 bp), although drastically reducing transduction efficiency in brain cells (2–4%), was able to extend life span (Gadalla et al., 2013). Studies of biodistribution revealed higher transduction efficiency in peripheral tissues, mostly in liver. Notably, high levels of alanine aminotransferase, a liver damage marker, were observed in treated animals, suggesting that MeCP2 overexpression leads to deleterious side effects. Parallelly, systemic delivery in RTT male mice of 4- to 5-weeks of a vector containing the *Mecp2*e1 cDNA guided by a longer fragment of its own promoter (~700 bp; Figure 3B) robustly improved RTT features and stabilized phenotypes (i.e., survival and phenotypic score). The good transduction efficiency in CA3, brain stem and cortex (20–25%) could explain the observed stabilization, while the lower efficacy in cerebellum (5%) might indicate the minor importance of reaching this area (Garg et al., 2013). Accordingly, a less prominent role for cerebellum in the progression of RTT mouse phenotype has been recently suggested (Achilly et al., 2021; Carli et al., 2023). Notably, systemic injection in 10- to 12- months old heterozygous (HET) females also improved phenotypic score and motor function while the effects on respiration were inconclusive and liver damage was not observed (Garg et al., 2013). In addition, Roux's team demonstrated that systemic injection in 5 months-old *Mecp2* HET female mice of the sc-AAV9-MCO vector, expressing the codon-optimized *Mecp2e1* (MCO) transgene controlled by a short portion of the *Mecp2* promoter (264 bp; Figure 3C), rescued breathing deficits and improved locomotor functions (Matagne et al., 2021). Administration of the same virus to *Mecp2*-null mice improved motor functions, explorative behaviors and extended the median life span; respiratory patterns were also normalized (Matagne et al., 2017). All together these studies highlighted that an efficacy of brain cells transduction of 25–40% is sufficient to ameliorate mouse phenotypes, but it could not rescue RTT-like features to wild-type levels which probably requires a higher transduction efficiency. Moreover, it raised a concern about the toxic consequences induced in peripheral tissues by systemic delivery of the MeCP2 therapeutic gene. More recently, the endogenous *Mecp2* promoter was replaced with the human synapsin (hSyn) (Figure 3H) or the cytomegalovirus early enhancer/chicken β-actin (CAG) promoter (Figure 3H) (Yang et al., 2023). Similar to previous reports, brain injections into the later ventricle (LV) of P2 *Mecp2*$^{-/y}$ mice resulted in higher transduction efficiency for hypothalamus and lower for hippocampus and cortex. Interestingly, when mice were injected with AAV9-CAG-*MECP2,* MeCP2 levels increased less (38,9%) compared to the AAV-hSyn-*MECP2* vector, but the median survival was extraordinary prolonged, possibly because of the capacity of the AAV9-CAG-*MECP2* vector to restore MeCP2 expression in astrocytes and oligodendrocytes (Yang et al., 2023). None of these vectors raised liver toxicity.

2nd generation of expression cassettes

With the aim to control the levels of MeCP2 and deliver the therapeutic gene more efficiently without toxicity, a second generation of expression cassette was investigated (Gadalla et al., 2017; Sinnett et al., 2017). This new AAV9 vector, in addition to the *MECP2e1* cDNA controlled by its own promoter (MeP426), included a modified 3′ UTR incorporating its highly conserved polyadenylation (pA) signal, and a panel of miRNA-binding sites relevant to control *MECP2* mRNA levels (Figure 3D). Intravenous delivery of the generated virus in juvenile mice (4- to 5-weeks old) did not affect hepatic architecture despite its higher transduction and, similarly to the first generation of AAV9, improved life span and body weight of mutant animals while it did not ameliorate the severity score. In contrast, direct intracerebroventricular (ICV) injection into neonatal *Mecp2*-null mice resulted in higher brain transduction efficiency (comparable to that one described in Gadalla et al., 2013), increased survival and ameliorated RTT-like phenotypes, outlining the importance of endogenous regulatory elements and the need of high transduction efficiency throughout the brain (Gadalla et al., 2017). The same 2nd generation vector was injected into the cisterna magna of juvenile *Mecp2* mutant mice (Sinnett et al., 2017) (Figure 3D). Although treated animals improved lifespan and body weight, behavioral traits were not rescued in contrast with previous reports. To further ameliorate the AAVs strategy, novel capsid variants were engineered. A recent work used the synthetic vector, AAV-PHP.B which features higher permeabilization of the BBB in adult mice and more efficient transduction of neurons and glia (Deverman et al., 2016; Morabito et al., 2017) (Figure 3F). An instable *Mecp2* (i*Mecp2*) transgene cassette was then inserted to limit supra-physiological levels of Mecp2. The intravenous injection of the PHP.eB-iMecp2 vector in 4 to 5 weeks old male mice revealed a sustained behavioral improvement when at

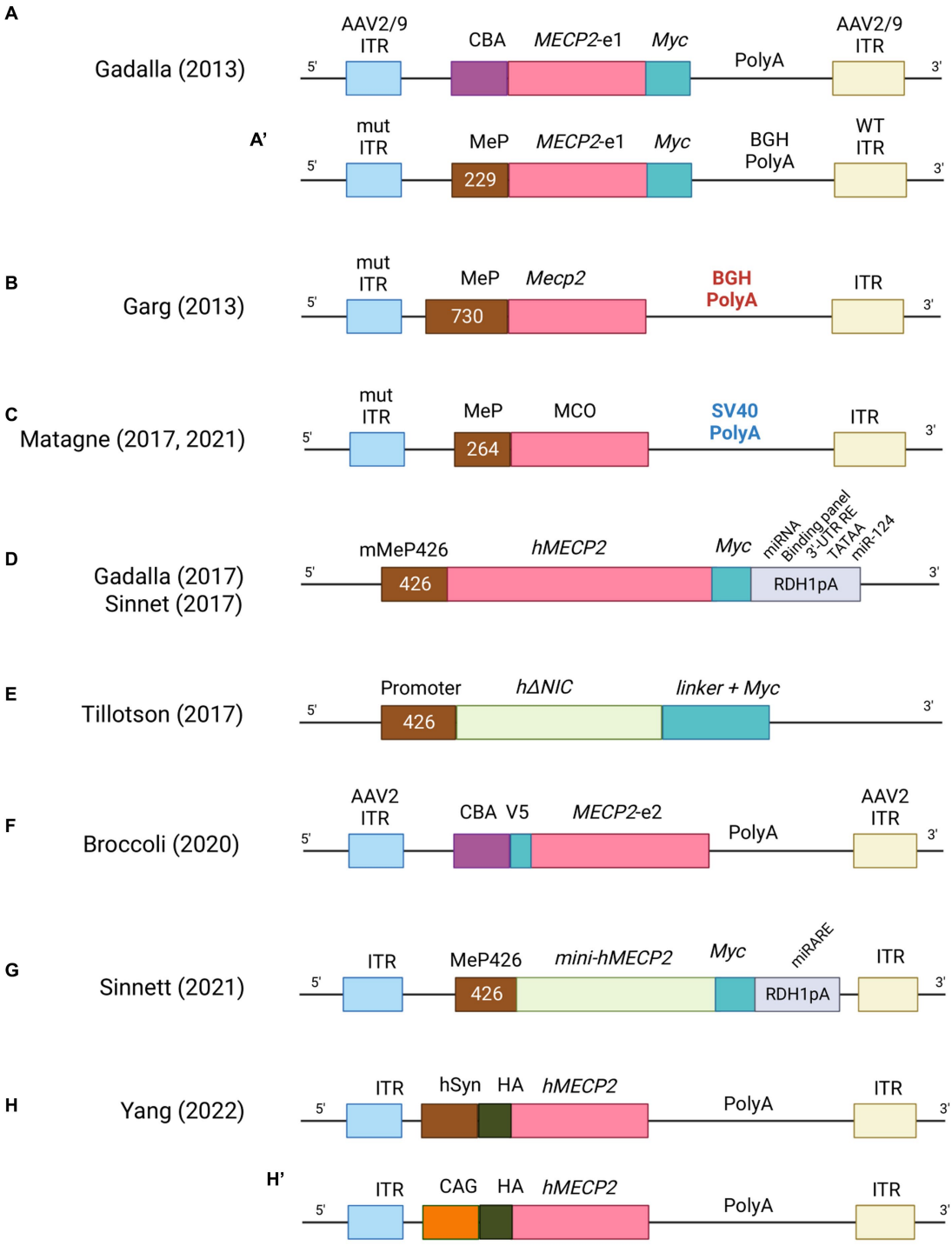

FIGURE 3
List of AAV constructs used in gene therapy for RTT. For each construct the main features are indicated. CBA=Chicken β-actin promoter, MeP=MeCP2 promoter, ITR=Inverted terminal repeats, BGHpolyA=bovine growth hormone polyadenylation signal, SV40polyA=Simian virus 40 hormone polyadenylation signal, hSyn=human synapsin 1 gene promoter, CAG=cytomegalovirus early enhancer/chicken β-actin promoter.

least 70% of the brain cells were infected and physiological levels of the protein were maintained (Luoni et al., 2020). Importantly, an efficiency of 15% of brain cells transduction was not sufficient to ameliorate RTT-like phenotype; further, infected *Mecp2* null male mice displayed a strong immune response to the exogenous protein which severely affected their lifespan. To overcome this issue, chronic immunosuppression was employed leading to strikingly ameliorated general health conditions and prolonged life span. Systemic delivery of the PHP.eB-iMecp2 vector in 5 months old *Mecp2*$^{+/-}$ HET females improved locomotor phenotypes and pathological features; no

hepatotoxicity was observed (Luoni et al., 2020). Although these findings reinforced the idea that gene therapy could be a promising strategy for RTT, it has to be noticed that the brain tropism of this vector is unfortunately restricted to C57Bl/6J mice and the LY6A receptor mediating its efficient transport through the BBB is not expressed in non-human primates (NHPs) (Hordeaux et al., 2018).

The most successful study based on engineered capsid variants was recently described by the Gradinaru's lab. By applying the Multiplex-Cre recombination-based AAV targeted evolution (M-CREATE) method (Ravindra Kumar et al., 2020; Goertsen et al., 2022), the authors identified the variant AAV.CAP-B10 which showed higher tropism for neurons and negligible specificity for all peripheral tissues including liver. Intravenous administration of this vector in mice and adult marmosets resulted in broad and robust transgene expression across cortex and cerebellum as well as spinal column and dorsal root ganglia (DRG) regions (Goertsen et al., 2022). On the contrary, the delivery of AAV-PHP.B failed to increase transgene expression in the brain of marmosets confirming previous data in NHP (Matsuzaki et al., 2018). In general, this study posed an important step forward for the treatment of neurological disorders by gene therapy.

Mini*MECP2* expression cassettes

To increase the efficiency of transduction, several laboratories focused on scAAVs; the reduced packaging capacity (2.2 kb) (McCarty et al., 2001) led the Bird's team to include only essential domains of the transgene. Interestingly, intracranial injection in neonatal mice of vectors encoding a minimal-MeCP2 protein (scAAV-mini *MECP2*) constituted by the methyl-binding domain and the NCOR- interaction domain (NID) improved phenotypes and survival in the absence of toxic effects (Tillotson et al., 2017) (Figure 3E). As logic consequence, Sinnet and colleagues paired the strategy of inserting microRNA targets into the 3' UTR of *MECP2* with scAAV9 vector carrying mini*MECP2*. In particular, they designed a novel miRNA target panel (named miR-responsive autoregulatory element or miRARE) able to "tune" mini*MECP2* expression through a negative feedback mechanism that is responsive to *MECP2* overexpression (Sinnett et al., 2021) (Figure 3G). This work showed that the inclusion of the autoregulatory element improved the safety of AAV9/mini*MECP2* gene therapy without compromising its efficacy (Sinnett et al., 2021). Moreover, biodistribution analysis revealed that while miRARE inhibited the expression of MeCP2 in wild-type mice (~8% of brain cells expressed transduced MeCP2), in mutant animals, 40% of brain cells expressed the therapeutic gene. Importantly, injected null mice showed delayed onset of gait abnormalities and extended life span.

Although gene delivery is becoming a promising strategy for several neurological disease, an important concern for developmental disorders is to define the optimal time-window of intervention. Published data from preclinical studies of RTT gene therapy indicated that delivery of *MECP2* either in newborn pre-symptomatic or symptomatic adult mice can ameliorate survival and recover phenotypes, thus suggesting that therapeutic interventions can be potential effective across different ages. However, a systematic study exploring different time windows of MeCP2 delivery has not been performed yet. Regardless of the time of intervention, the long-lasting expression of transgene at appropriate therapeutic dosage is of primary importance to avoid repetitive treatments that are not always accessible especially in case of neurological disorders. Indeed, to the best of our knowledge, no study has reported how long the *MECP2* transgene remains expressed or when it turns off. Further, we find it relevant to disclose which is the right number of brain cells that have to be transduced for significant clinical improvement and if specific brain areas have to be primarily transduced; further, although intuitive, the importance of expressing the *MECP2* transgene also in glial cells remain to be proven. On this line, considering that: i) gene replacement occurs both in cells expressing the WT or the mutant allele and with uneven doses and ii) *MECP2* dosage appears to affect gene expression with cell and non-cell autonomous effects (Johnson et al., 2017), in the future, it might be highly informative to assess at the cellular level the transcriptional consequences of gene therapy. In light of that, AAV9 vectors carrying different promoters may be chosen to preferentially target specific cell types of the brain.

MECP2 gene and RNA editing

A valid alternative strategy to maintain correct MeCP2 levels and avoid overexpression side effects is represented by genome editing which directly repairs the mutated gene. The most popular method of gene editing is CRISPR-Cas9 composed by the Cas9 enzyme, a nuclease capable of cutting the genome at predefined location indicated by a guide RNA (gRNA) and a repair template, which contains a wild-type sequence. These two components form a ribonucleic complex that recognizes and cleaves the target sequence (Figure 4A). Depending on cell type and its growth phase, cleaved DNA can be repaired either by non-homologous end joining (NHEJ) or homology-based repair (HDR) (Ran et al., 2013; Jiang and Doudna, 2017). HDR requires long homology arms to precisely introduce the exogenous DNA template (ssDNA or dsDNA) and finely edit the break site (Wu et al., 2018). Its action is favored during S, G2 and M phases, but it is strongly repressed in G1. NHEJ repairs the double strand breaks (DSBs) during all cell cycle phases, but its highest activity has been found in G1. For these reasons NHEJ-based methods have been mostly employed for genome editing in neurons, although they usually generate small insertions/deletions (indels) around the break site. Notably, HDR has been recently demonstrated to be effective in terminally differentiated neurons, although at lower levels (Nishiyama et al., 2017). However, one of the main caveats that limits the use of the CRISPR-Cas9 system in gene therapy is the necessity of co-transducing in the same cell two different AAVs, respectively containing the Cas9/gRNA and the "template." Another concern is their long-term presence which may lead to off-target cleavage mediated by the Cas9 enzyme or to alteration of relevant and "dangerous" genes (Zhang et al., 2015). In spite of that, because of its high efficiency and feasibility, CRISPR-Cas9 approaches are nowadays leading research studies for the treatment of several genetic diseases. Regarding RTT, the first *in vitro* study was conducted into human induced pluripotent stem (iPS) cells (le et al., 2019). By co-transfecting the sg5/Cas9 and the ssODN-R270X template vectors, the MECP2^{R270X} mutation was initially knocked-in leading to two homozygous and five heterozygous clones out of 22, thus reaching an insertion efficiency of 9 and 23%, respectively. With the focus to correct the variation, homozygous iPSC clones (*MECP2*$^{R270X/R270X}$) were then transfected

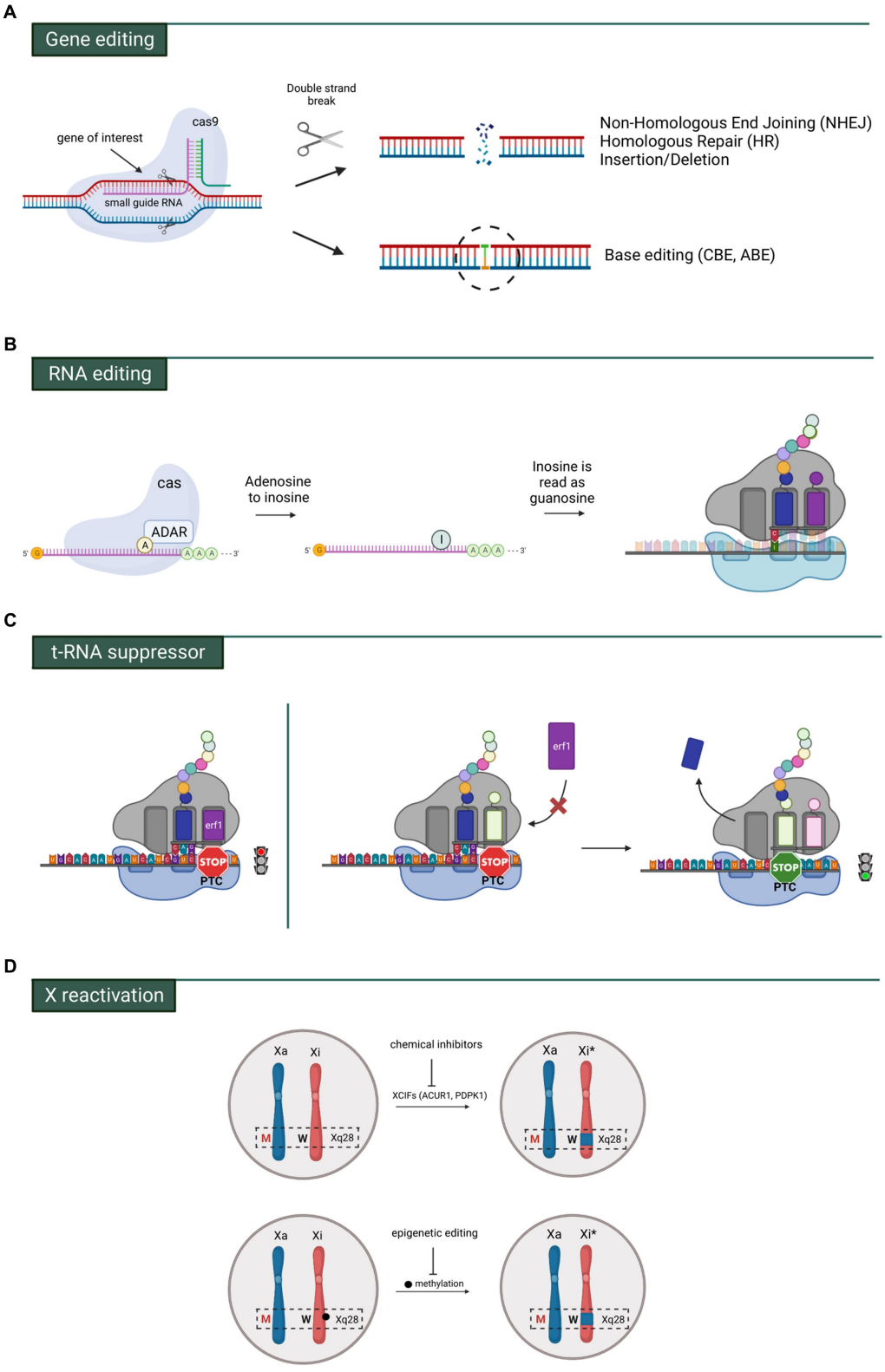

FIGURE 4
Main genetic strategies investigated for RTT. **(A)** Gene editing by means of Crisp-CaS9 allowing broad changes in portions of DNA or precise single base modifications. **(B)** RNA editing comprising the ADAR system which allows to modify an adenosine with inosine through deamination. Inosine is then recognized as a guanosine and paired with a cytosine in the anticodon tRNA. **(C)** On the left, a prematur termination codon (PTC) interrupts the translation of the mRNA. On the right, a specific tRNA suppressor binds the PTC allowing the translation readthrough. Erf1=eukaryotic translation terminator factor 1. **(D)** The mutated *MECP2* allele (red M) is localized in the active X-chromosome (Xa), while the wild type *MECP2* allele (black W) is in the inactive chromosome (Xi). By means of chemical (silencing of inactivating factors) or epigenetic actions (inhibiting DNA methylation), the region containing the wt allele of *MECP2* (Xi*) can be reactivated.

with sg3/Cas9 and donor wild type template vectors. Consequently, sequencing analysis revealed the successful repair of mutant *MECP2* in iPSCs and the recovery of its mRNA levels (le et al., 2019). Another encouraging study of genome editing in RTT was performed in patient fibroblasts and neurons derived from iPSCs carrying the most common *MECP2* variant, c.473C > T p.Thr158Met (Croci et al., 2020). Co-transfection efficiency of the dual vector approach was about 8,6% in RTT fibroblasts. Next generation sequencing of sorted cells harboring both vectors revealed that on average 55% of alleles were correctly edited, leading to a significant increase of MeCP2 levels. Similarly, 14% of mutant alleles were reverted to the WT sequence in iPSC-derived neurons (Croci et al., 2020). The lower editing efficiency in neuronal cells was probably due to the aforementioned HDR deficiency in post-mitotic cells (Chapman et al., 2012; Bonnerjee and Bagh, 2021). Although these *in vitro* data represented a first step towards gene therapy approaches based on the application of CRISPR/Cas9 system, to date no study has ever tested this tool in animal models of RTT.

To circumvent the generation of DSB and reduce indel modifications, a DNA base editing tool has been recently developed that enables a direct and irreversible conversion of one base pair to another at the target site (Gaudelli et al., 2017). This strategy is based on the fusion of Cas9 mutants, that cannot make DSBs, with specific nucleotide-converting enzymes named editors. Two different base switching are nowadays possible: C-G to T-A mediated by cytosine base editors (CBEs) and A-T to G-C catalyzed by adenosine base editors (ABEs) (Gaudelli et al., 2017). However, the only base-editing strategy so far tested in *in vivo* model of RTT is the RNA Editing for Programmable A to I Replacement (REPAIR). This approach takes advantage of a family of naturally occurring enzymes named Adenosine Deaminase Acting on RNA (or ADAR) (Maas et al., 1996; Melcher et al., 1996; O'Connell et al., 1998), which catalyze the hydrolytic deamination of adenosine (A) to inosine (I) on RNA (Bass and Weintraub, 1988). The inosine present on the mRNA codon pairs with a cytosine (C) present on the tRNA anticodon, therefore resulting in codon change (Figure 4B). One member of ADAR family, ADAR2, is highly expressed in brain and mostly engineered for site-direct RNA editing. Its domains have been fused with heterologous RNA binding proteins (i.e., Cas13 or the bacteriophage λN peptide) to improve the capability to specifically target endogenous RNA by recognizing short hairpin RNA (shRNA). Indeed, ADAR2 binds to the shRNA that is complementary to the mRNA of interest and deaminases the target sequence (Cox et al., 2017). Considering that approximately 55% of pathogenic variants causing RTT are G > A changes, repair by targeted RNA editing represents a valid alternative approach (Fyfe et al., 2003). With this purpose, the laboratory of Gail Mandel generated a Knock-in (KI) mouse carrying the c.317G > A, p.Arg106Gln (R106Q) mutation in the *Mecp2* gene (Sinnamon et al., 2017). Infection of cultured hippocampal neurons with AAVs containing a hyperactive ADAR2 enzyme fused to a bacteriophage peptide and a guide shRNA sequence, resulted in *in vitro* RNA repair and the recovery of Mecp2 protein level (about 40%) together with its ability to bind to heterochromatin (Sinnamon et al., 2017). Few years later, the same group tested the efficacy of RNA editing in a mouse model of RTT. AAVs expressing the RNA editing enzyme were injected into the *Mecp2* null hippocampus and, 3 weeks later, 50% of *Mecp2* mRNA was edited (i.e DG, CA1 and CA3) (Sinnamon et al., 2020). Very recently, the same authors provided the first evidence that targeted RNA-editing approach can alleviate behavioral phenotypes in a mouse model carrying a human mutation. To assess a different adenosine context, they generated a novel RTT mouse model carrying the patient mutation *MECP2*^{G311A} (Sinnamon et al., 2022). Systemic injection of viral vectors into retro-orbital region of P28 and P35 adult animals showed the highest efficiency of RNA-editing in the brainstem (18%) and midbrain (13%) after 4 weeks from the delivery. The other brain areas such as cerebellum, cortex, olfactory bulb, thalamus, hypothalamus and striatum only reached 3–5% of efficiency. In particular, MeCP2 protein expression was restored in 20% of brainstem cell populations where its association with heterochromatic foci was almost fully rescued (75% of Mecp2 intensity in heterochromatic foci). The half lifespan of injected mice with *Mecp2*-targeting virus was extended of about 6 weeks compared to control animals. Moreover, since the brainstem is a brain region tightly linked to respiratory function, the authors mainly focused on the evaluation of apneas and breathing patterns, which were improved in treated animals, while motor or cognitive functions were not tested (Sinnamon et al., 2022).

Although further *in vivo* studies are necessary to confirm its therapeutic efficacy, RNA editing could represent, in near future, the approach of choice for patients with suitable mutations.

Readthrough of *MECP2* nonsense codon mutations and t-RNA suppressor therapy

Nonsense mutations are responsible for 10–15% of all genetic lesions and for almost 1,000 deleterious genetic disorders (Mort et al., 2008). Nearly 35% of typical RTT patients harbor *MECP2* nonsense mutations which lead to early protein truncation (Lyst and Bird, 2015; Ip et al., 2018). A very attractive pharmacogenetic strategy that mediates the suppression of these mutations by small molecules is provided by translational readthrough inducing drugs (TRIDs) (Figure 4C). Basically, aminoglycosides, such as gentamicin, can restore the expression of full-length proteins by allowing readthrough of premature termination codons (PTCs). Mechanistically, gentamycin weakly binds to the eukaryotic ribosomal decoding center, leading to misincorporation of near-cognate aminoacyl-tRNAs at the PTC, therefore permitting to continue protein synthesis albeit inducing, in some cases, a missense mutation (a concerning issue considering that MeCP2 is particularly sensitive to residue alterations) (Wilhelm et al., 1978; Palmer et al., 1979). In any case, several studies have showed elevated toxicity for gentamycin at required therapeutic doses (Karijolich and Yu, 2014) and its restricted ability to cross the BBB (Nau et al., 2010). To overcome these limitations, novel-related compounds (including Ataluren (or PTC124), ELX-02, and NB54) were tested in animal models of human diseases, leading to successful clinical trials for Duchenne muscular dystrophy (DMD) and cystic fibrosis (CF) (Hamed, 2006). However, a phase 2 clinical trial of non-aminoglycoside drug, Ataluren, was recently showed to be not effective for the treatment of nonsense mutations in CDKL5 deficiency disorder (CDD) and Dravet syndrome (DS) (Devinsky et al., 2021). Accordingly, *in vitro* studies of Landsberger's team demonstrated the inability of this drug to induce read-through activity on CDKL5 PTCs. Moreover, they also reported that conversely, aminoglycosides efficiently suppressed CDKL5 nonsense mutations and partially recovered the protein activity (Fazzari et al., 2019). Similarly, aminoglycosides administration in cultured cells overexpressing several common nonsense

MECP2 mutations, or cultured fibroblasts derived from RTT patients induced the expression of MeCP2 that correctly localized into the nucleus (Brendel et al., 2011; Vecsler et al., 2011). Interestingly, only one publication demonstrated, in an animal model carrying the *Mecp2*-R294X PTC, the capacity of gentamicin to increase the expression of full-length Mecp2 (Merritt et al., 2020); however, whether this increase was sufficient to ameliorate RTT phenotypes has yet to be tested. In spite of that, readthrough therapy has several disadvantages including low efficiency, high toxicity, not specificity and nucleotide context-dependency, that need to be overcome to make this approach suitable in the next future. A valid alternative strategy could be represented by nonsense suppressor tRNA (sup-tRNA) approach, which makes use of anticodon-engineered tRNAs able to recognize the PTC but charged with correct amino acid to permit translational readthrough. The delivery of sup-tRNAs through AAVs would thus stabilize the RNA and rescue the expression of the full-length protein (Chang et al., 1979; Temple et al., 1982). This concept was introduced decades ago and very recently it was tested in a mouse model of human lysosomal storage disease, the mucopolysaccharidosis type I (MPS I) caused by α-L-iduronidase (IDUA) enzymatic activity deficiency (Wang et al., 2022). Systemic injection of an rAAV9.2 sup-tRNA^{Tyr} restored IDUA activity in liver and heart lysates up to 9.5 and 27% of WT level respectively, however no activity was recorded in brain. In contrast, unilateral intrahippocampal injection of rAAV9.2 sup-tRNA^{Tyr} restored IDUA activity to 10% in the injected hippocampus (Wang et al., 2022), suggesting that sup-tRNA^{Tyr} works in various tissues although its feasibility is still limited by the efficiency of gene delivery. Although this technology is still at early development, and no study is reported in RTT, we envisage that in future it will largely benefit from the already available advanced approaches of gene transduction in brain.

Reactivation of the inactive X chromosome

Due to XCI, RTT girls are mosaics of cells expressing either normal or mutant *MECP2* (Takahashi et al., 2008). Reactivation of the wild type allele on the inactive X chromosome (Xi) represents a potential therapeutic approach for RTT. Because of XCI reversibility, several laboratories attempted to find molecules able to promote its reactivation, by targeting its inactivating factors (Figure 4D). One of the first study conducted by Green and his collaborators reported that pharmacologically targeting of X chromosome inactivation factors (XCIF) such as ACVR1 (Activin A receptor type I) and PDPK1 (Pyruvate Dehydrogenase Kinase 1) reactivated *Mecp2* in the nuclei of differentiated mouse ES cells (Bhatnagar et al., 2014). *In vitro* combined inhibition of ACVR1 and PDPK1 effectors rescued morphological defects in RTT neurons (i.e., soma size and dendritic branches) upon *Mecp2* reactivation. *In vivo* intracerebral injection of the same combined treatment in *XistΔ:Mecp2/Xist:Mecp2-GFP* female mice, harboring the deletion of the *Xist* gene and expression of wild-type *Mecp2* in the active X chromosome, and *Mecp2* fused to GFP on the inactive one, resulted in reactivation of Xi-Mecp2-GFP in 30% of cells (Przanowski et al., 2018). By digging into the mechanisms of Xi reactivation, Lee's lab used a combination of an inhibitor of DNA methylation (5-Aza) with an antisense oligonucleotide (ASO) directed against *Xist* RNA to activate the inactive X chromosome. After five days of treatment with Xist ASO + 5-Aza, mouse embryonic fibroblasts (MEFs) carrying the *Mecp2:luciferase* reporter showed an encouraging strong increment of *Mecp2:luciferase* levels (Carrette et al., 2018). Additionally, luminescence-based high-throughput screens on mouse fibroblasts carrying an inactive *MeCP2-luciferase* reporter identified two inhibitors (AG490 and Jaki) of the JAK/STAT pathway as XCI reactivating agents. This study revealed that reactivation is cell-type dependent. Indeed, while AG490 and 5-Aza reactivated *Mecp2* in mouse fibroblasts, only 5-Aza increased MeCP2 levels in a humanized Xi-containing cell line (THX88) (Lee et al., 2020). To date, the mixed modality approach represents a valid strategy for the treatment of X-linked disorders and encourages further screening for Xi-reactivating drugs. However, one concern could be the tissue/cell-type specificity and the toxicity at high doses, that together with reactivation of other X-linked genes could lead to deleterious effects. Alternatively, a targeted approach, consisting in epigenetic editing, was recently tested in RTT-like hESCs carrying on Xi a wild-type allele of *MECP2* and its methylated promoter, and on the active X a *MECP2* null allele produced by a GFP-polyA stop cassette after exon 3. Transduction of hESC derived neurons with dCas9-Tet1/sgRNA proved that demethylation of the *MECP2* promoter reactivated the wild type allele located on the Xi (82% of protein expression). Moreover, direct epigenome editing of neurons, carrying on Xi MeCP2 exon 3 fused with GFP and its methylated promoter and on Xa MeCP2 exon 3 fused with tdTomato, showed a moderate reactivation of MeCP2-GFP (17,7% of protein expression) without affecting the expression of other genes on both X chromosomes (Qian et al., 2023). Although precise DNA methylation editing of *MECP2* displayed encouraging results, further validation in *in vivo* animal models of RTT is essential for the translational value of this approach. For instance, the big size of the current editor (dCas9-Tet1) that have to be packed in a single AAV for *in vivo* delivery could represents a big challenge for the pre-clinical studies.

Future direction for RTT therapy: nanoparticles' delivery

An attractive alternative method for gene delivery might be represented by nanoparticles; because of their lower immunogenicity, higher genetic payload and moderate costs, it is an emerging field that is being tested for several neurological disorders and brain cancers. There are three categories of nanomaterials: (i) lipid-based, (ii) polymer-based, and (iii) inorganic nanoparticles (Ramamoorth and Narvekar, 2015). Lipid-based nanoparticles (LBNP) represent a valid vehicle from a safety perspective because of their biodegradability and low toxicity. Even though they are not much stable, they have been widely explored and already used in clinic for a range of diseases including cancer (Vaughan et al., 2020) and the recent COVID-19 vaccines (Andresen and Fenton, 2021). Polymer-based nanoparticles (PBNP) are more stable than LBPN and display controlled degradation and elimination of polymer (Moku et al., 2021). The main disadvantages are constituted by poor targeting and quick nanoparticles clearance (Gagliardi et al., 2021) which make insufficient the efficiency of gene transfer for the desired application (Dizaj et al., 2014). However, modifications of the nanoparticle surface, such as PEGylation or addition of zwitterionic molecules, have been used to delay the clearance (Shi et al., 2021). The third class of nanomaterials is represented by inorganic particles, which includes: gold-nanoparticles, carbon-dots (CDs), silica nanoparticles,

iron-oxide magnetic nanoparticles, and spherical nuclei acid nanoparticles (SNA, NPs). While they are less expensive and easier to produce, the big challenges of these nanomaterials are their biodistribution and neurotoxicity (Guo et al., 2021). As for viral delivery, nanoparticles can be directed into the CNS systematically or locally, therefore sharing the same advantages and concerns already discussed for both routes. Of note, systemic delivery of nanoparticles was proven successful in trespassing the BBB when modifications such as PEGylation and conjugation to transferrin were applied (Huang et al., 2007; Gregory et al., 2020). Local delivery, which include intrathecal (IT), intracerebroventricular (ICV), and intranasal injection although more invasive, offers the advantage of bypassing the BBB. The ICV route delivers the particles through the cerebral ventricles with the help of a device consisting of a dome and a catheter that are implanted under the scalp (Duma et al., 2019). The IT injection, instead, takes place into the CSF of the lower spinal cord (Fowler et al., 2020). Finally, in intranasal injection the particles are delivered into the olfactory bulb that has direct access to the CNS (Bryche et al., 2020). Although this route is the less invasive compared to the previous ones, it has also the lowest delivery rate. Clinical trials based on gene delivery of non-viral particles-based (i.e., antisense oligonucleotide or RNA interference) have been employed for the treatment of neurodegenerative disorders such as spinal muscular atrophy (NCT04591678), Parkinson's disease (NCT03976349) and Alzheimer's disease (NCT03186989). However, most of the studies exploring nanoparticle were focused on the treatment of brain cancer, especially glioblastoma, given the lethality of the grade IV of this tumor. Recently, a phase 0 clinical trial (NCT03020017), that utilized gold nanoparticles packed with siRNAs targeting the *Bcl2L12* oncogene (Kumthekar et al., 2021), showed interesting results in patients after systemic delivery. As the field is exponentially growing, advanced approaches aim at ameliorating nanoparticle stability, batch-to-batch consistency, and neurotoxicity: the objective is to render their use feasible for clinical trials of neurological disorders. We believe that nanoparticles delivery holds tremendous promises that could be pursued also for therapeutic treatments of RTT.

Discussion

The ground-breaking idea that RTT is not an irreversible condition boosted studies investigating the beneficial effects of advanced gene and RNA therapies. In the last decade, reversion of disease symptoms upon *MECP2* re-expression was exponentially reported by several research groups. In spite of positive observations, the dosage-sensitivity of *MECP2* gene complicated the feasibility of this approach: reduced levels of MeCP2 lead to RTT-like phenotypes, likewise its overexpression results in neurological defects. The situation could be even more hard to manage in female patients who already express normal levels of WT MeCP2 in approximately 50% of transduced cells; perhaps the expression of the transgene in these cells will result in gain of function phenotypes (Takagi, 2001). Moreover, RTT patients generally carry hypomorphic mutations which determine a partial loss-of-function of MeCP2, therefore increasing the risk for complications due to protein overexpression. Accordingly, whereas most of the gene therapy studies have been conducted in a *Mecp2* null genetic background, two recent studies evaluated *MECP2* gene therapy benefits in mouse models harboring either the truncated mutation R294X (Collins et al., 2022) or the missense mutation R133C (Vermudez et al., 2022). When a wild-type copy of *MECP2* was supplied to male animals, most of RTT features were rescued and, importantly, no "over-expression phenotype" was observed. In contrast, similar experiments performed in heterozygous female mice resulted in behavioral tasks associated with adverse effects of MeCP2 supplementation (Pitcher et al., 2015; Collins et al., 2022), confirming the risk of transducing high levels of *MECP2* in this gender. This study also suggested the importance of evaluating partial loss-of-function alleles in female mouse models, in addition to the vastly used null models, and including in pre-clinical studies the assessment of behavioral abnormalities associated with MeCP2 overexpression.

After years of refinement protocols for *MECP2* gene therapy, remarkable news came from the study named NGN-401 by Stuart Cobb, the chief scientific officer of Neurogene.[1] Cobb's team used the Expression Attenuation via Construct Tuning (EXACT) technology to deliver AAV9:*MECP2* directly into the cerebrospinal fluid of male mice. EXACT technology enables to self-regulate the transgene expression by tuning *MECP2* levels within a "safe range," thus avoiding the toxicity associated with its overexpression. Male mice treated with a high dose of transducing particles (3×10^{11}) extended their lifespan to approximately 37 weeks, while heterozygous females did not show any sign of toxicity. Encouraging results of NGN-401 were obtained also in non-human primates. Thus, very recently (24th January, 2023), Neurogene announced the clearance by FDA to initiate a clinical trial of the investigational gene therapy, NGN-401, in female children with RTT.[2] In similar way, Steven Gray and Sarah Sinnet at UT Southwestern Medical Center used the mi-RARE platform to safely control the level of MeCP2 protein upon its delivery. After promising results in preclinical studies (as described above), a gene therapy program, named TSHA-102, has initiated the first clinical trial – the REVEAL Adult study – in females of 18 years or older living with RTT. This study will evaluate the safety, tolerability and preliminary efficacy of a single intrathecal administration of TSHA-102 into the spinal fluid[3] of affected girls.

An important advance in the field will probably derive from genome editing that, despite some concerns, appears the most promising and definitive approach to restore physiological levels of functional MeCP2. Direct correction of the endogenous mutant *MECP2* would in fact bypass risks of toxicity and inflammation related to MeCP2 overexpression. However, both approaches, conventional gene therapy and genome editing, are limited by the low tropism of most viruses. In this regard, more studies should be addressed to reveal which brain area have a more detrimental role from unbalanced levels of MeCP2 and therefore need an urgent intervention. The objective might be a localized therapeutic intervention to recover more severe disease symptoms. Beside the genetic approaches so far explored and summarized in Figure 5, several research groups attempted to normalize the downstream pathways altered in RTT. Even though many approaches were proved successful in preclinical studies, sadly, only one completed a phase 3

1 https://rettsyndromenews.com/news/non-toxic-gene-therapy-rett-syndrome-extends-lifespan-mice/

2 https://rettsyndromenews.com/news/rett-gene-therapy-ngn-401-cleared-advance-clinical-trials/?cn-reloaded=1link

3 https://clinicaltrials.gov/ct2/show/NCT05606614?cond=rett+syndrome&draw=2&rank=3

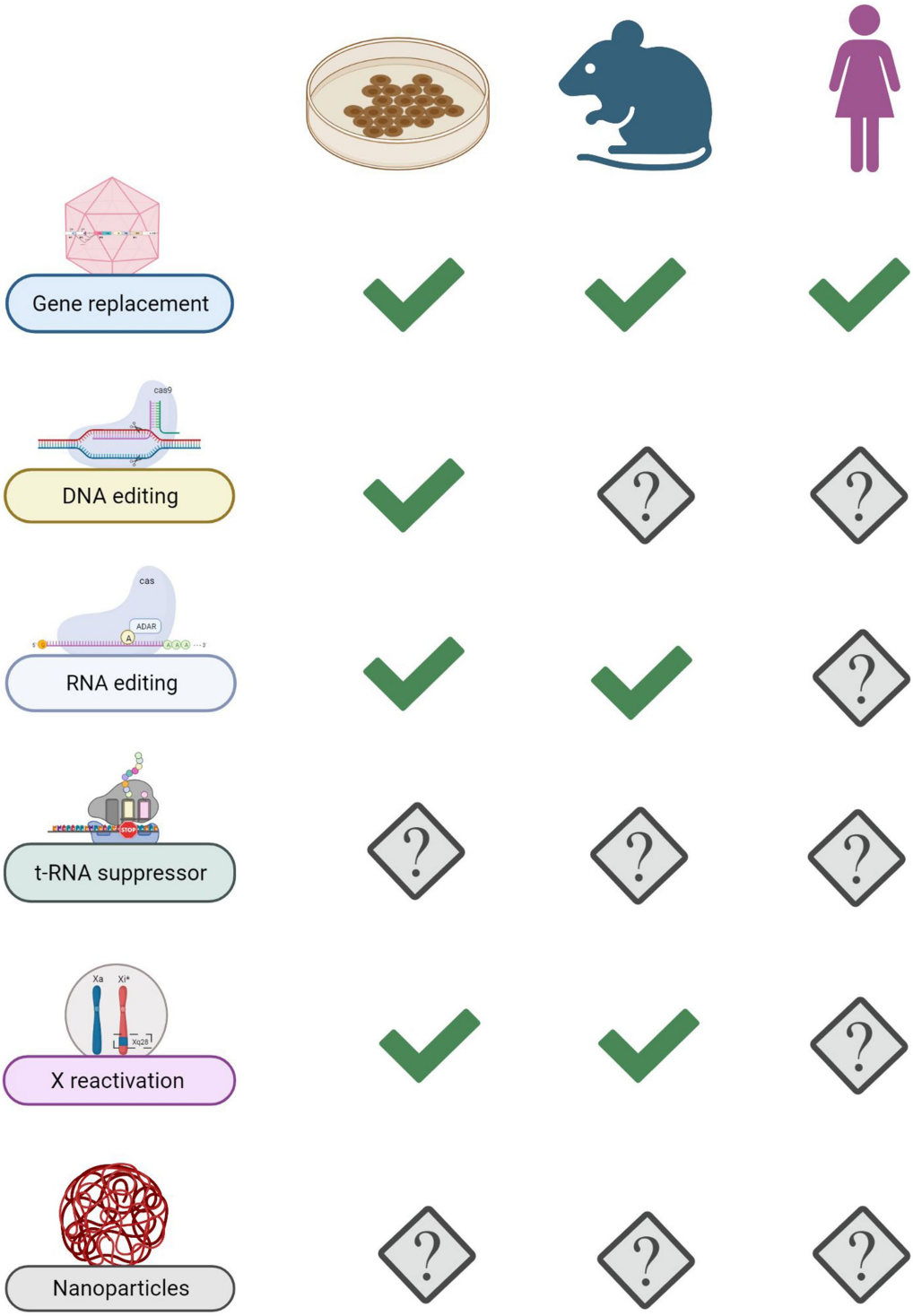

FIGURE 5
State of the art of advanced RTT therapies. Summary of progresses made with various genetic approaches in *in vitro* and *in vivo* models of disease. Gene replacement therapy stands out as the only one so far that reached RTT girls.

clinical trial.[4] Indeed, oral administration of Trofinetide, analog of the amino-terminal tripeptide of insulin growth factor 1 (IGF-1), in children and adolescent with RTT, improved primary and secondary clinical endpoints.[5] The difficulty in finding effective therapies, in spite of numerous promising pre-clinical trials might imply that

4 https://clinicaltrials.gov/ct2/show/NCT04181723

5 https://acadia.com/media/news-releases/acadia-pharmaceuticals-announces-positive-top-line-results-from-the-pivotal-phase-3-lavender-trial-of-trofinetide-in-rett-syndrome/

we must adhere more strictly to optimal rules (Katz et al., 2012) and use ideal cellular models for drug screening such as patients' iPSC-derived neurons and organoids. Indeed, neurons derived from human MeCP2 deficient iPSCs recapitulated deficits previously observed in mouse primary neurons and human RTT brain (Marchetto et al., 2010; Farra et al., 2012; Haase et al., 2021). Since these cells offer an unlimited source and various genetic backgrounds, iPSCs hold enormous promise for drug discovery. Considering animal models, we believe in the necessity to establish whether models recapitulating human pathogenic mutations, or the null line are equally suitable for preclinical trials, which gender should be treated, and in general, which is the best time frame to assess efficacy.

Author contributions

NL conceived the idea of this review. NL and MP wrote and refined together the manuscript. DP produced all the schematic illustrations for the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Glutamatergic synaptic deficits in the prefrontal cortex of the Ts65Dn mouse model for Down syndrome

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Down syndrome (DS), the most prevalent cause of intellectual disability, stems from a chromosomal anomaly resulting in an entire or partial extra copy of chromosome 21. This leads to intellectual disability and a range of associated symptoms. While there has been considerable research focused on the Ts65Dn mouse model of DS, particularly in the context of the hippocampus, the synaptic underpinnings of prefrontal cortex (PFC) dysfunction in DS, including deficits in working memory, remain largely uncharted territory. In a previous study featuring mBACtgDyrk1a mice, which manifest overexpression of the *Dyrk1a* gene, a known candidate gene linked to intellectual disability and microcephaly in DS, we documented adverse effects on spine density, alterations in the molecular composition of synapses, and the presence of synaptic plasticity deficits within the PFC. The current study aimed to enrich our understanding of the roles of different genes in DS by studying Ts65Dn mice, which overexpress several genes including *Dyrk1a*, to compare with our previous work on mBACtgDyrk1a mice. Through *ex-vivo* electrophysiological experiments, including patch-clamp and extracellular field potential recordings, we identified alterations in the intrinsic properties of PFC layer V/VI pyramidal neurons in Ts65Dn male mice. Additionally, we observed changes in the synaptic plasticity range. Notably, long-term depression was absent in Ts65Dn mice, while synaptic or pharmacological long-term potentiation remained fully expressed in these mice. These findings provide valuable insights into the intricate synaptic mechanisms contributing to PFC dysfunction in DS, shedding light on potential therapeutic avenues for addressing the neurocognitive symptoms associated with this condition.

KEYWORDS

intellectual disability, Down syndrome, Ts65Dn, excitatory synapse, long-term synaptic plasticity, prefrontal cortex, pyramidal neuron

1. Introduction

Down syndrome (DS), the most common form of intellectual disability, is a chromosomal disorder caused by having all or part of an extra chromosome 21. This additional copy introduces ~300 extra genes, resulting in a wide range of significant clinical phenotypes. The shared feature among all individuals with DS is intellectual disability, which manifests as

specific deficits in learning and memory (Chapman and Hesketh, 2000). The underlying neural mechanisms of these alterations remain poorly understood, but potential factors could include defects in neural network formation, information processing, and brain plasticity.

Mouse models of neurological disorders are invaluable tools for identifying the cellular mechanisms at play in these conditions. Several animal models mimicking DS alterations have been developed, with the most extensively studied being the Ts65Dn mouse. This mouse model has a segmental trisomy of mouse chromosome 16, bearing three copies of genes equivalent to those on human chromosome 21 (Hsa21), many of which are conserved between mice and humans (Davisson et al., 1990; Gardiner et al., 2003; Sturgeon and Gardiner, 2011; Rueda et al., 2012). This trisomic region includes ~140 genes and largely overlaps with the Hsa21 region believed to be responsible for many DS phenotypes, including intellectual disability (Korenberg et al., 1994). Amongst these triplicated genes, the *Dyrk1a* gene has been proposed as a major contributor to the intellectual disability and microcephaly in DS (Courcet et al., 2012). Ts65Dn mice exhibit DS-relevant behavioral, cellular, and molecular phenotypes, such as working memory alterations, long-term memory deficits, hyperactivity, disrupted neurogenesis, and synaptic plasticity, and a general over-inhibition (Holtzman et al., 1996; Gardiner and Davisson, 2000; Belichenko et al., 2004, 2007; Kleschevnikov et al., 2004; Rueda et al., 2012; Ruiz-Mejias, 2019). Much of the research on synaptic plasticity has focused on the hippocampus, a brain structure vital for learning and memory (Cramer and Galdzicki, 2012).

The prefrontal cortex (PFC) is acknowledged as a key brain region controlling executive cognitive processes like planning, cognitive flexibility, working memory, and emotional behavior (Goldman-Rakic, 1990; Seamans et al., 1995). Dysfunctions in the PFC are common in numerous neuropsychiatric diseases, including DS (Goto et al., 2010). Neuropsychological assessments of individuals with DS have highlighted deficits in executive functions (Lanfranchi et al., 2010). The specific role of the triplication of these ~140 genes in PFC malfunction and the synaptic basis for the working memory impairments seen in DS individuals is not yet fully understood (Pennington et al., 2003; Lanfranchi et al., 2010).

In this study, we analyzed the intrinsic characteristics and glutamatergic synaptic plasticity of deep-layer pyramidal neurons in the PFC of adult male Ts65Dn mice. Our research focus was directed toward the synapses in the deep layers of the prefrontal cortex (PrPFC), specifically from layer II-III to V-VI. We targeted the deep-layer pyramidal neurons situated in layer V-VI, which serve as a central source of output from the prefrontal cortex. Notably, the downward projections from layers II/III to layers V/VI play a pivotal role in information processing, with the pyramidal cells in layer V projecting to subcortical regions.

We focused solely on male Ts65Dn mice as significant over-expression of the DS candidate gene *Dyrk1a* in the cerebral cortex has been detected in males, but not females (Hawley et al., 2022). Our findings revealed alterations in the intrinsic properties of pyramidal neurons and specific changes in synaptic plasticity. These results uncover functional abnormalities in the PFC of male Ts65Dn mice, which may contribute to the cognitive and behavioral impairments observed in individuals with DS.

2. Materials and methods

2.1. Animals and ethics statement

All animal experiments were performed according to the criteria of the European Communities Council Directive (86/609/EEC). The B6EiC3Sn.BLiA-Ts(1716)65Dn/DnJ [known as Ts65Dn] mice were obtained from Dr. Jean Delabar's laboratory. Ts65Dn mice (Davisson et al., 1993) were maintained on a B6/C3H background and genotyped as described previously (Reinholdt et al., 2011). All mice were weaned at 21 days. After weaning, they were caged socially in same-sex groups. Male Ts65Dn mice and wild type littermate controls were used at 4 to 6 months of age.

2.2. Acute prefrontal cortex slice preparation

PFC slices were prepared as described (Lafourcade et al., 2011). Mice were anesthetized with isoflurane and decapitated. The brain was sliced (300 µm) in the coronal plane (Integraslice, Campden Instruments, Leicester, U.K.) and maintained in physiological saline (4°C). Slices were stored for 30 min at 32–35°C in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 2.4 mM MgCl2, 1.2 mM CaCl2, 18 mM NaHCO3, 1.2 mM NaH2PO4 and 11 mM glucose, equilibrated with 95% O2/5% CO2. Slices were stored at 22 ± 2°C until recording.

2.3. Electrophysiology

Whole-cell patch-clamp and field excitatory postsynaptic potential (fEPSP) were recorded from layer V/VI pyramidal cells in coronal slices of mouse prelimbic PFC (Lafourcade et al., 2011; Thomazeau et al., 2014). For recording, slices were superfused (2 ml/min) with ACSF at 32–35°C. Picrotoxin (100 µM) was added to block GABA-A receptors. To evoke synaptic currents, 150–200 µs stimuli were delivered at 0.1 Hz through a glass electrode placed in layer II/III.

For whole-cell patch-clamp experiments, pyramidal neurons were visualized using an infrared microscope (BX-50, Olympus). Experiments were performed with electrodes containing 128 mM potassium gluconate (KGlu), 20 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.3 mM CaCl2, 2 mM Na2 + -ATP, 0.3 mM Na + -GTP, 10 mM glucose buffered with 10 mM HEPES, pH 7.3, osmolarity 290 mOsm. Electrode resistance was 4–6 MOhm. If access resistance (no compensation, <25 MOhm) changed by >20%, the experiment was rejected (Thomazeau et al., 2014). Neuronal intrinsic excitability was assessed during a current clamp protocol. Membrane voltage response, resting membrane potential, input resistance, rheobase and the number of action potentials were determined by applying current steps ranging from −400 to +400 pA in increments of +50 pA, each lasting 500 ms. The membrane voltage response was evaluated based on the steady-state voltage during hyperpolarizing and depolarizing current injections (Figure 1B). Input resistance was computed as the change in membrane voltage (ΔmV) divided by the injected current (pA) (Figure 1C). Resting membrane potential was assessed at the beginning of the whole-cell recording during the current clamp protocol (Figure 1D). Rheobase was defined as the

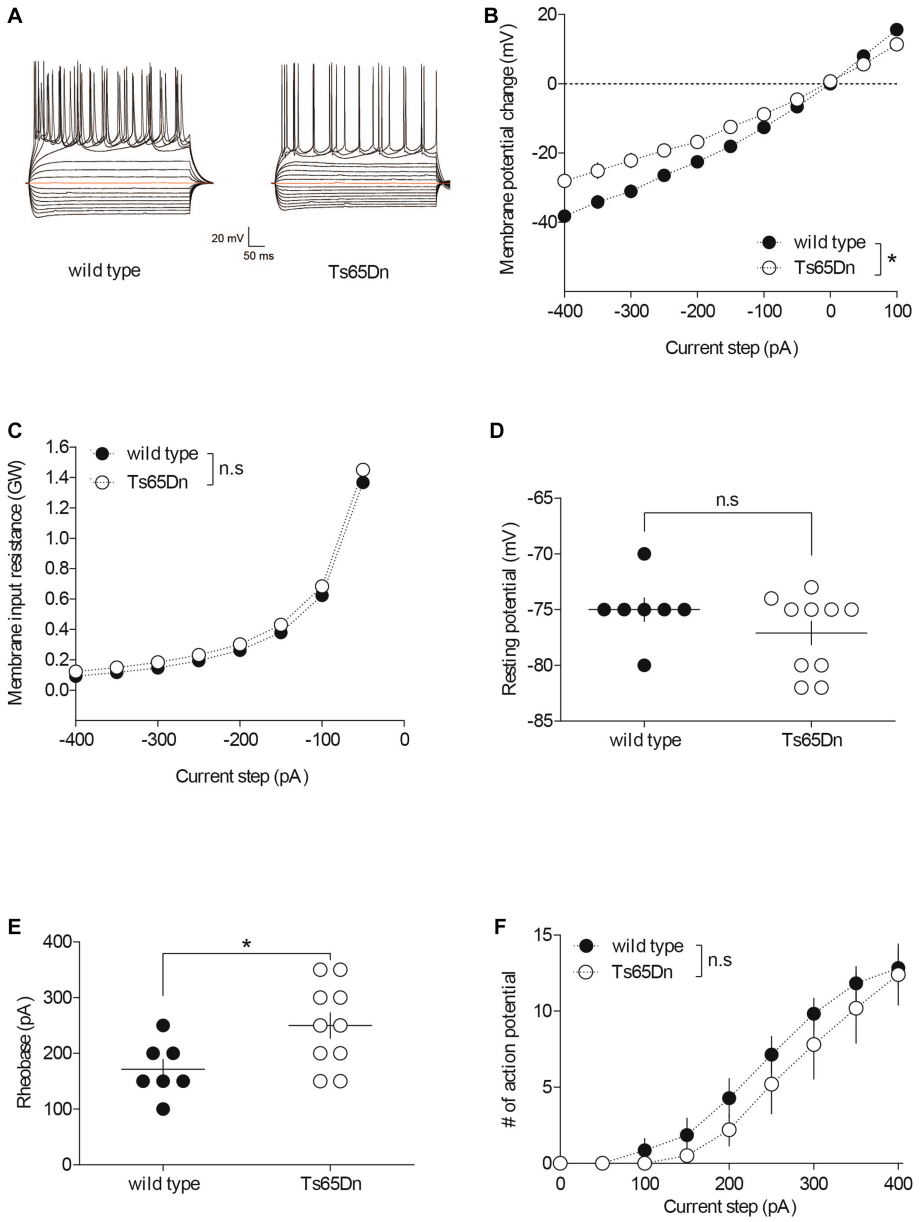

FIGURE 1
The intrinsic properties of pyramidal neurons in layers V/VI of the prefrontal cortex are altered in Ts65Dn mice. PFC pyramidal neurons were patched in current-clamp mode and current was injected. The stimulation protocol consisted in 19 steps of current, 500 ms-long, with increment of 50 pA, starting at −400 pA. **(A)** Typical membrane responses to somatic current steps of PFC pyramidal neurons from wild-type and Ts65Dn mice. **(B)** Summary of current-voltage (I-V) curves recorded in pyramidal neurons of both strains showing an increase in inward rectification in Ts65Dn mice (white symbols; $n = 10$ neurons from 4 mice) compared to wild-type mice [black symbols; $n = 7$ neurons from 5 mice; two-way repeated measures ANOVA, genotype: $F_{(1,15)} = 9.932$, $^{**}p = 0.0066$]. **(C)** The membrane input resistance was similar in wild-type and Ts65Dn mice (two-way repeated measures ANOVA, genotype: $F_{(1,15)} = 0.6498$, n.s. $p = 0.4328$). Resting membrane potential is similar in both genotypes (**D.** −75.00 ± 1.09 mV, $n = 7$ neurons from 5 wild-type mice, black symbols; −77.10 ± 1.10 mV, $n = 10$ neurons from 4 Ts65Dn mice, white symbols; n.s. $p > 0.05$, Mann-Whitney test). In contrast the rheobase, i.e. the minimal current necessary to evoke action potential firing, was higher in Ts65Dn mice (**E.** 171.40 ± 18.44 pA, $n = 7$ neurons from 5 mice wild-type mice, black symbols; 250.00 ± 23.57 pA, $n = 10$ neurons from 4 Ts65Dn mice, white symbols; $^{*}p = 0.0438$, Mann-Whitney test). The horizontal line represents the mean value. (**F.** The summary of current-discharge curves indicates that the number of action potentials elicited in response to current injection steps is similar in pyramidal neurons of Ts65Dn mice ($n = 10$ neurons from 4 mice, white symbols) and wild type mice ($n = 7$ neurons from 5 mice, black symbols; two-way repeated measures ANOVA, genotype: $F_{(1,15)} = 0.697$, n.s. $p = 0.4169$). Error bars represent standard error to the mean.

minimum current necessary to elicit an action potential (Figure 1E). The number of action potentials was determined for each depolarizing current step lasting 500 ms (Figure 1F). For voltage-clamp experiments (Figures 2A,B), evoked EPSCs were recorded at −70 mV and LTD was induced by 10 min stimulation at 10 Hz (Lafourcade et al., 2011).

For fEPSP experiments (Figures 2C–F), input–output profiles were recorded for all fEPSP recordings. In time course experiments,

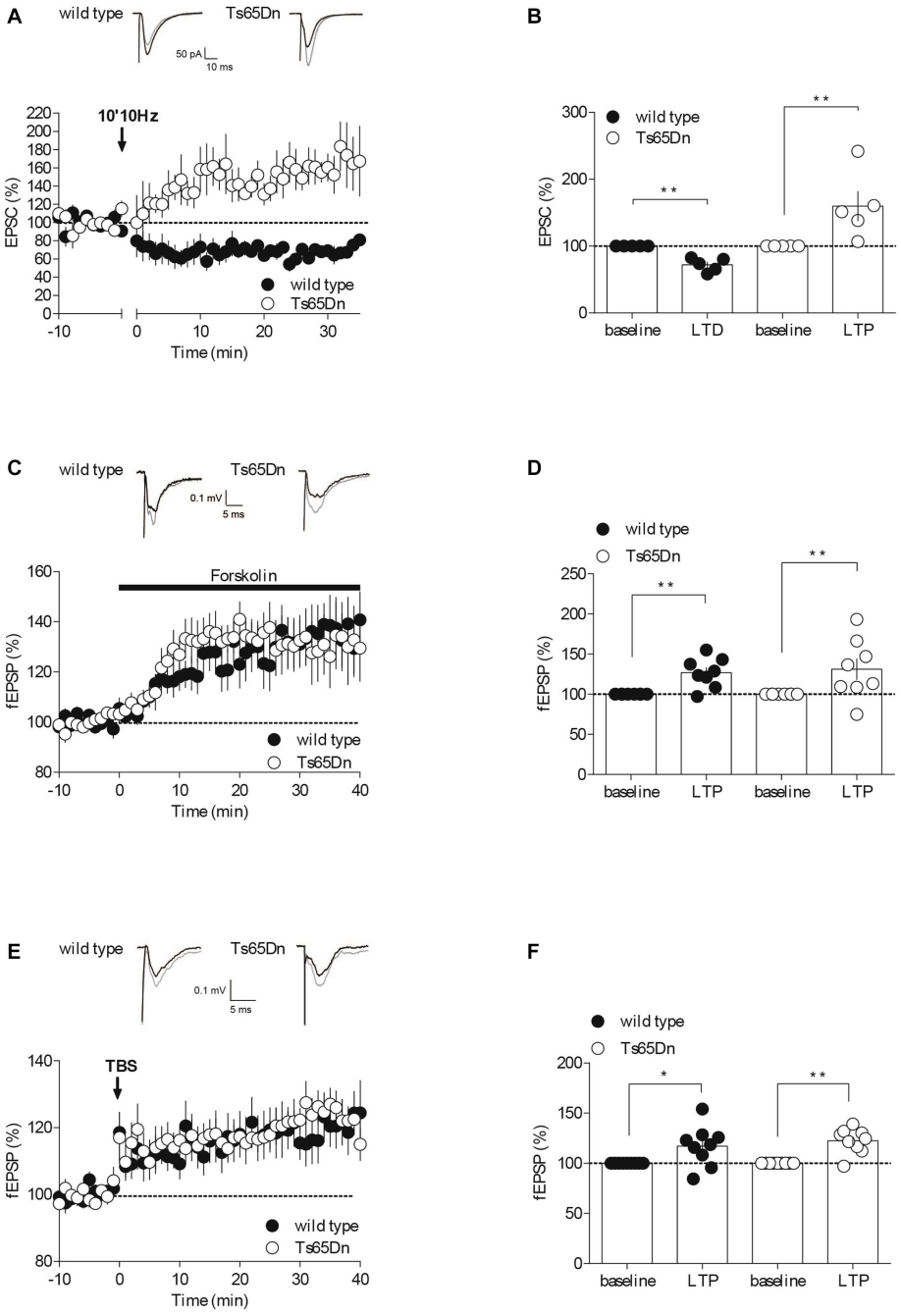

FIGURE 2
Synaptic plasticity is partially affected in Ts65Dn mice. Endocannabinoid-dependent LTD is absent in Ts65Dn mice. **(A)** Low frequency stimulation of layers II/III induces the long-term depression of the amplitude of excitatory post-synaptic currents (EPSCs) recorded in pyramidal neurons from layers V/VI of the PFC in wild-type mice. In Ts65Dn mice, this protocol triggered instead a long-term potentiation in the amplitude of EPSCs recorded in pyramidal neurons from layers V/VI of the PFC. Representative traces averaged from EPSC response before (black) and 30 min after plasticity induction (gray) in mice from either genotype. Average time courses of mean EPSC amplitude were normalized to baseline. Error bars represent standard error to the mean. **(B)** Average EPSC as percentage of the baseline recapitulating the data (wild-type: $n = 5$ neurons from 5 mice, black symbols, 72.14 ± 4.53, 30–35 min versus 100% baseline; $^{**}p = 0.0079$, Mann–Whitney test; Ts65Dn mice: $n = 5$ neurons from 5 mice, white symbols, 159.83 ± 22.42, 30–35 min versus 100% baseline; $^{**}p = 0.0079$, Mann–Whitney test). Error bars represent standard error to the mean. The AC/cAMP-dependent facilitation is intact in Ts65Dn mice. **(C)** Incubation with the adenylate cyclase activator, forskolin, in the perfusion bath leads to an increase in the amplitude of the field excitatory postsynaptic potentials (fEPSPs) recorded in layers V/VI in wild-type mice and in Ts65Dn mice. Representative traces averaged from fEPSP response before (black) and 35 min after plasticity induction (gray) in mice from either genotype. Average time courses of mean fEPSP amplitude were normalized to baseline. Error bars represent standard error to the mean. **(D)** Average fEPSP as percentage of the baseline recapitulating the data (wild-type: $n = 8$ slices from 8 mice, black symbols, $126.82 \pm 6.62\%$, 35–40 min versus 100% baseline; $^{**}p = 0.0104$, Mann–Whitney test; Ts65Dn mice: $n = 8$ slices from 8 mice, white symbols, $131.2 \pm 13.26\%$, 35–40 min versus 100% baseline; $^{**}p = 0.0057$, Mann–Whitney test). Error bars represent standard error to the mean. Long-term potentiation dependent induced by TBS is preserved in the Ts65Dn mouse model. **(E)** A theta burst stimulation of PFC layers II/III induces long-term potentiation of the amplitude of the field excitatory postsynaptic potentials (fEPSPs) recorded in layers V/VI in wild-type mice and in Ts65Dn. Representative traces averaged from fEPSP response before (black) and 35 min after plasticity induction (gray) in mice

(Continued)

FIGURE 2 (Continued)
from either genotype. Average time courses of mean fEPSP amplitude were normalized to baseline. Error bars represent standard error to the mean. **(F)** Average fEPSP as percentage of the baseline recapitulating the data (wild-type: $n = 9$ slices from 9 mice, black symbols, 117.07 ± 6.69, 35–40 min versus 100% baseline; $^{*}p = 0.0361$, Mann–Whitney test; Ts65Dn mice: $n = 9$ slices from 9 mice, 122.43 ± 4.16, 35–40 min versus 100% baseline; $^{**}p = 0.0019$, Mann–Whitney test). Error bars represent standard error to the mean.

the stimulation intensity was that necessary give a response 40–60% of the maximal. fEPSPs were recorded at 0.1 Hz. The presynaptic adenylate cyclase (AC)/cAMP signaling pathway-dependent facilitation was induced by bath application of the AC activator forskolin at 10 µM. NMDAR-LTP was induced using a theta-burst stimulation (TBS) protocol consisting of five trains of burst with four pulses at 100 Hz, at 200 ms interval, repeated four times at intervals of 10 s (Thomazeau et al., 2014). The glutamatergic nature of the fEPSP was confirmed at the end of the experiments using the glutamate receptor antagonist 6,7-dinitroquinoxaline-2, 3-dione (DNQX, 20 µM), that blocked the synaptic component without altering the non-synaptic component (not shown).

2.4. Data acquisition and analysis

Due to the strong phenotypes, the experimenters were not blind to the genotypes, but no data were excluded before statistical analysis (GraphPad Software Inc., La Jolla, CA). Data were recorded on a MultiClamp700B, filtered at 2 kHz, digitized (20 kHz, DigiData 1440A), collected using Clampex 10.2 and analyzed using Clampfit 10.2 (all from Molecular Device, Sunnyvale, USA). Both amplitude and area of fEPSPs and EPSCs were computed, and only amplitude was display, the area giving the same results. The magnitude of LTD and LTP was calculated, respectively, 30–35 min and 35–40 min after induction as percentage of baseline responses. To perform current–voltage (I–V) curves and to test the excitability of pyramidal neurons, a series of 500 ms long hyperpolarizing and depolarizing current steps, from −400 to +400 pA with 50 pA increment, were applied immediately after breaking in the cell. The rheobase has been determined as the minimum current intensity required to induce a first action potential.

2.5. Drugs

Drugs were added at the final concentration to the ACSF. Picrotoxin was from Sigma (St. Quentin Fallavier, France). DNQX was from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Rockville, MD, USA). Forskolin was from Tocris (Bristol, UK).

2.6. Statistical analysis

The value n corresponds to the number of individual cells or slice per animal. All values are given as mean ± standard error to the mean (s.e.m). Statistical analysis was performed with Prism 7.0 (GraphPad Software Inc., La Jolla, CA). Two sample comparisons were made with the Mann–Whitney *t*-test. Multiple comparisons (genotype and current injection) were analyzed using repeated measure two-way ANOVA with *post hoc* Sidak's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Intrinsic properties of PFC pyramidal neurons are changed in Ts65Dn mice

Neuronal pathways are plastic, continually adapting in response to stimuli. These alterations can occur at synaptic locations (areas where neurons connect) as well as non-synaptic locations throughout the neuron. Non-synaptic, or intrinsic, plasticity can be defined as a change in the neuron's inherent excitability that is independent of changes in synaptic transmission. To understand the impact of the segmental trisomy 16 of Ts65Dn mice on neuronal membrane properties, the basic intrinsic properties of prefrontal cortex (PFC) pyramidal neurons were first examined. Pyramidal neurons from layers V/VI of the PFC were recorded in the "current-clamp" mode to assess their membrane profile in response to a range of somatic current steps, from −400 to +400 pA in 50 pA increments (Figure 1A). The average curves illustrating the relationship between the current injected into the neuron and the resulting change in membrane potential (I/V curves) revealed differences between Ts65Dn mice and their wild-type euploid littermates. Notably, the pyramidal neurons of the aneuploid mice exhibited increased inward rectification, evidenced by a differential deviation from linearity in the I/V plots (Figure 1B). However, there was no change detected in the membrane input resistance (Figure 1C) and the membrane potential (Figure 1D). The rheobase, or the minimum current intensity needed to trigger the first action potential, was higher in Ts65Dn mice, indicating a decrease in membrane excitability (Figure 1E). Lastly, the number of action potentials emitted following incremental current injection was found to be similar between the two mouse strains (Figure 1F).

3.2. PFC synaptic plasticity in the Ts65Dn mice

The intrinsic properties of neurons, which dictate their spiking patterns and level of excitability, are critical for their computational capabilities. Changes in these non-synaptic properties, as well as in synaptic properties, can influence synaptic efficacy. Long-term modifications of synaptic efficacy, referred to as synaptic plasticity, are believed to play crucial roles in cognition, learning, and the formation of memory. Following our assessment of neuronal intrinsic properties, we next evaluated synaptic plasticity in the prefrontal cortex of Ts65Dn mice. We compared opposite forms of synaptic plasticity that rely on distinct mechanisms in our mouse groups. These forms included endocannabinoid-mediated long-term depression

(eCB-LTD), presynaptic adenylyl cyclase/cyclic adenosine monophosphate signaling pathway-dependent facilitation (AC/cAMP-facilitation), and N-methyl-D-aspartate receptor-dependent long-term potentiation (NMDAR-LTP) (Figure 2).

3.2.1. Induction of endocannabinoid-dependent long-term depression appears to fail in Ts65Dn mice

The endocannabinoid (eCB) system plays a significant role in synaptic plasticity, and it might contribute to the development of mood disorders associated with prefrontal cortex (PFC) dysfunction (Hill et al., 2009; Scheyer et al., 2017). In response to neuronal activity, eCBs are released by the postsynaptic compartments, and they retrogradely activate presynaptic cannabinoid receptors, resulting in long-term depression (LTD) by reducing neurotransmitter release (Robbe et al., 2002; Puente et al., 2011; Araque et al., 2017). To assess whether eCB-LTD was impaired in the PFC of Ts65Dn mice, we recorded excitatory postsynaptic currents (EPSCs) isolated with Picrotoxin (100 μM) in layer V/VI pyramidal neurons patch-clamped at −70 mV. We then gauged the impact of a low-frequency tetanic stimulus protocol (10 min at 10 Hz). Consistent with our previous studies (Lafourcade et al., 2007, 2011; Thomazeau et al., 2014), low-frequency tetanic stimulation in the prefrontal layers II/III triggered a lasting decrease in the efficacy of excitatory synapses, as measured by the amplitude of EPSCs, onto PFC layer V/VI pyramidal neurons in wild-type mice. In stark contrast, the identical protocol led to an increase in the EPSCs recorded in the prefrontal layer V/VI pyramidal neurons of Ts65Dn mice (Figures 2A,B).

3.2.2. Protein kinase A-mediated long-term potentiation occurs to remain unaltered in Ts65Dn mice

To determine if the release probability was generally affected, we next investigated a presynaptic form of LTP, specifically Adenylyl cyclase (AC)/cAMP-facilitation. Adenylyl cyclase is an enzyme that transforms ATP into cAMP, which subsequently activates protein kinase A, thereby facilitating the release of the transmitter (Chavez-Noriega and Stevens, 1994; Weisskopf et al., 1994). We used the AC activator Forskolin in a bath application and observed its effect on the amplitude of field excitatory postsynaptic potentials (fEPSPs) recorded in PFC layers V-VI following electrical stimulation of layer II-III. The synaptic enhancement induced by the pharmacological activation of AC with Forskolin appeared to be typical in the aneuploid strain (Figures 2C,D), indicating that this form of plasticity seems to be preserved in Ts65Dn animals. The synaptic enhancement resulting from the pharmacological activation of AC using Forskolin seemed to exhibit a typical response in the aneuploid strain (Figures 2C,D). This suggests that this specific form of plasticity is conserved in Ts65Dn animals.

3.2.3. Theta burst stimulation-induced long-term potentiation is unaffected in Ts65Dn mice

NMDAR-dependent long-term potentiation (LTP) is arguably the most widely expressed and comprehensively studied form of synaptic potentiation (Volianskis et al., 2015). We evaluated this postsynaptic form of LTP at the PFC synapses from layers II-III to V-VI in both wild-type and Ts65Dn mice by recording evoked fEPSPs and examining the impact of a theta burst stimulation (TBS) protocol (five burst trains of four pulses at 100 Hz, 200 ms apart, repeated four times at 10 s intervals). The LTP was not affected in Ts65Dn mice (Figures 2E,F). Indeed, TBS of PFC layers II/III induced a comparable increase in the measured amplitude of the evoked field potential response in V/VI layers in both mouse strains.

4. Discussion

In this study, we found altered intrinsic properties and some impaired synaptic plasticity in the PFC of Ts65Dn mice, a model of DS with overexpression of around 130 genes from the mouse analog of Hsa21, Mmu16, including Dyrk1a. Notably, our results contrast with those reported in mBACtgDyrk1a mice.

4.1. Intrinsic neuronal properties

Whole-cell patch clamp recordings allowed investigating PFC pyramidal neurons' cellular intrinsic properties to detect alterations in neuronal excitability. In an earlier study, we showed that the intrinsic properties of PFC layer V/VI pyramidal neurons were not affected in mBACtgDyrk1a mice (Thomazeau et al., 2014). In marked contrast, we report here that Ts65Dn mice exhibit an augmented inward rectification and a higher rheobase (Figure 1), reflecting a decrease in neuronal excitability. This is in line with the decreased neuronal excitability already reported in somatosensory cortex layer IV neurons and in dissociated neuronal cultures from the hippocampi of Ts65Dn mice (Cramer et al., 2015; Stern et al., 2015). This reduced intrinsic excitability has been linked to an increase expression level of the inwardly rectifying potassium channel Kir3.2, encoded by the *Kcnj6* gene, one of the triplicated genes in the Ts65Dn mice (Harashima et al., 2006; Best et al., 2007, 2012). Strategies to pharmacologically reduce this channel function could be explored to normalize the intrinsic properties of PFC pyramidal neurons in Ts65Dn mice.

4.2. Synaptic plasticity range

Changes in intrinsic, i.e., non-synaptic, properties of neurons can impact their ability to integrate synaptic information. To assess whether dynamic changes at the layers II-III to V-VI pyramidal cell synapses level of pyramidal neurons from PFC were affected in Ts65Dn mice, we then examined several forms of synaptic plasticity that rely on distinct pre- and postsynaptic mechanisms. Interestingly, eCB-LTD appears to be affected in both mouse models (Figures 2A,B; Thomazeau et al., 2014). In contrasts, TBS induced-LTP and AC/cAMP-facilitation was solely disrupted in the mBACtgDyr1a mice (Figures 2C–F; Thomazeau et al., 2014, unpublished data). Thus, glutamatergic synaptic plasticity appears to be much less altered in Ts65Dn mice compared to mBACtgDyrk1a mice. Further investigations should be conducted to assess basic synaptic properties in Ts65Dn PFC synapses, in order to understand whether changes in synaptic properties, such as PFC synaptic excitability, would explain the alteration in synaptic plasticity observed in Ts65Dn mice, i.e., by a change in the number of active excitatory synapses recruited during plasticity induction. Basic synaptic properties could be profiled by (i)

establishing as input/output relationships to evaluate synaptic efficacy; (ii) quantifying the progressive inhibition of NMDAR-mediated EPSCs by an open-channel blocker of NMDAR (Chavis and Westbrook, 2001; Thomazeau et al., 2014) or measuring paired-pulse ratio -a form of short-term synaptic plasticity that depends on release probability- to evaluate glutamate release probability; (iii) measuring excitation-spike coupling (Daoudal et al., 2002; Thomazeau et al., 2014) to assess how excitatory synaptic inputs are integrated into action potential; and (iv) by identifying NMDAR subunit composition of PFC pyramidal synapses in Ts65Dn mice. Moreover, TBS induced-LTP appears to be intact, but it would be crucial to pharmacologically verify that its induction is mediated by activation of NMDARs. Out of all the plasticity tested, only eCB-LTD seems to be impaired. Mechanistically, this form of plasticity is more complex than NMDAR- or mGluR-dependent plasticity, as it involves a series of pre- and postsynaptic loci and processes. This could explain why it is more sensitive to genetic modifications. Surprisingly, the eCB-LTD protocol, lead to LTP in Ts65Dn mice.

Additional experiments are required to unravel the mechanisms facilitating the induction of LTP rather than LTD in response to low-frequency tetanic stimulation in Ts65Dn mice. The most straightforward explanation is that the absence of the eCB-LTD mechanism permits LTP to occur. If CB1R concurrently mediate both LTP and LTD, the application of a CB1R antagonist could potentially clarify this situation. Indeed, there have been reports of eCB-dependent LTP in different brain aeras, resulting in an increase neurotransmitter release mediated by either homosynaptic mechanisms, or heterosynaptic processes involving CB1Rs located on astrocytes or GABAergic neurons (Navarrete and Araque, 2010; Piette et al., 2020). Lastly, it is worth noting that while eCBs may potentially enhance the induction of hippocampal LTP by dampening inhibitory transmission (as suggested by Carlson et al., 2002), this scenario can be ruled out in our current experiments that were conducted in the presence of a GABAAR antagonist.

Numerous studies have shown that LTP is affected in the hippocampus of Ts65Dn mice (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Fernandez et al., 2007; Duchon et al., 2020), in the perirhinal cortex (Roncacé et al., 2017) and the striatum (Di Filippo et al., 2010), as well as in the hippocampus of other mouse models of DS; see for review (Bartesaghi, 2023). Most of these studies were done without blockade of inhibitory transmission. The deficit of LTP can be rescued by reducing the magnitude of GABA-mediated signaling through treatment with $GABA_AR$ antagonist (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Fernandez et al., 2007; Duchon et al., 2020), or by reversing $GABA_A$ R signaling, GABA being excitatory rather than inhibitory in Ts65Dn mice (Deidda et al., 2015). In our study, it is possible that the induction of LTP was facilitated by pharmacological inhibition of inhibitory transmission (Thomazeau et al., 2017). It would be compelling to replicate the experiment without the pharmacological blockage of inhibitory transmission to confirm our findings. However, a study conducted on the somatosensory cortex demonstrated that in Ts65Dn mice, the balance between excitatory and inhibitory transmission might remain mostly unaltered, which was indicated by a decrease in the frequency of both excitatory and inhibitory spontaneous synaptic activities (Cramer et al., 2015).

Importantly, the alteration in synaptic function observed in Ts65Dn mice could also be explained by non-neuronal changes, such as defects in astrocyte and/or microglia function. Indeed, while both have been shown to participate actively in synaptic transmission and plasticity mechanisms (Papouin et al., 2017; Marinelli et al., 2019; Stogsdill et al., 2023), new findings are challenging the neurocentric view of DS and providing new insights into the contribution of other brain cell types, such as astrocytes and microglia, to the pathophysiology of DS (Ponroy Bally and Murai, 2021; García and Flores-Aguilar, 2022).

4.3. Polygenic effects

How can we account for the differences in LTP observed between Ts65Dn and mBACtgDyrk1a mice? The partial Ts65Dn model provides a genetic environment more akin to that found in DS, with trisomy of over one hundred genes. However, the regulatory relationships between these genes are currently unknown, and overexpression of genes on chromosome 21 could lead to modified expression of genes on other chromosomes. While comparative analyses of gene expression on other chromosomes have demonstrated that gene deregulations are specific to Hsa21 in DS fetuses (Mao et al., 2003), research on the Ts65Dn model has shown that there is a global alteration in gene expression in these mice (Chrast et al., 2000; Lyle et al., 2004). Furthermore, gene products can interact with one another, creating compensatory or exacerbating mechanisms, which can affect synaptic parameters in Ts65Dn mice differently, reflecting the intricacy of the gene effects associated with this condition. In simpler terms, the deficits observed in Ts65Dn mice that are not present in mBACDyrk1a mice could be attributed to detrimental mechanisms arising from the overexpression of genes other than Dyrk1a. Conversely, the absence of defects in Ts65Dn that are seen in mBACDyrk1a mice could indicate the synergistic and/or compensatory activities of these three-copy genes. According to Dowjat et al. (2007), the amount of DYRK1A protein in the brains of Ts65Dn animals is 1.5 times higher. However, it is important to confirm whether this rate is consistent specifically within the PFC of the Ts65Dn animals. Currently, little is known about the spatiotemporal regulation of DYRK1A expression, or whether the overexpression of Dyrk1a and other trisomal genes in mouse models of DS varies across age, sex, and brain region throughout development (Stringer et al., 2017). Recent research has shown that the expression of DYRK1A protein in P15 mice is sex dependent in Ts65Dn mice, with significant overexpression in males but not in females (Hawley et al., 2022). This suggests that brain development in male and female Ts65Dn mice may have different trajectories, underscoring the significance of conducting future DS mouse model studies that include both males and females.

4.4. Limitations of the Ts65Dn mouse

It is important to note that the Ts65Dn animals have triplication of 50 non-orthologous Mmu17 genes in addition to Mmu16 orthologous genes, which may complicate the genotype/phenotype relationship and contribute to the observed synaptic phenotypes. To address this issue, future experiments could be performed on the refined Ts65Dn model recently developed, the Ts66Yah, which retains the major features of DS, but showed an overall milder phenotype

than Ts65Dn mice (Duchon et al., 2022). However, it is worth noting that the partial Ts65Dn model does not overexpress the Hsa21 orthologous genes carried by Mmu10 and Mmu17, which raises questions about the relevance of this partial mouse model as a standard for DS research (Guedj et al., 2023). Therefore, it is important to conduct comparative studies in different mouse models for DS, each with its limitations in recapitulating the full spectrum of human DS. To fully compare the Ts65Dn and mBACtgDyrk1a mouse models, additional experiments such as examining basal glutamatergic transmission, mGluR3-LTD, dendrites and spines morphology would be necessary (see Thomazeau et al., 2014).

5. Conclusion

Overall, the parallel between this more complete Ts65Dn DS model and the monogenic Dyrk1a overexpression model highlights the complexity of the physiological and pathological mechanisms responsible for the neurocognitive symptoms of DS and underscores the importance of exploring new therapeutic strategies other than targeting DYRK1A activity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animals were treated in compliance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the care and use of laboratory animals. The French Ethical committee authorized this project (APAFIS#3279–2015121715284829 v5). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AT and OM designed the research. AT and OL carried out the experiments. AT analyzed the data. AT and OM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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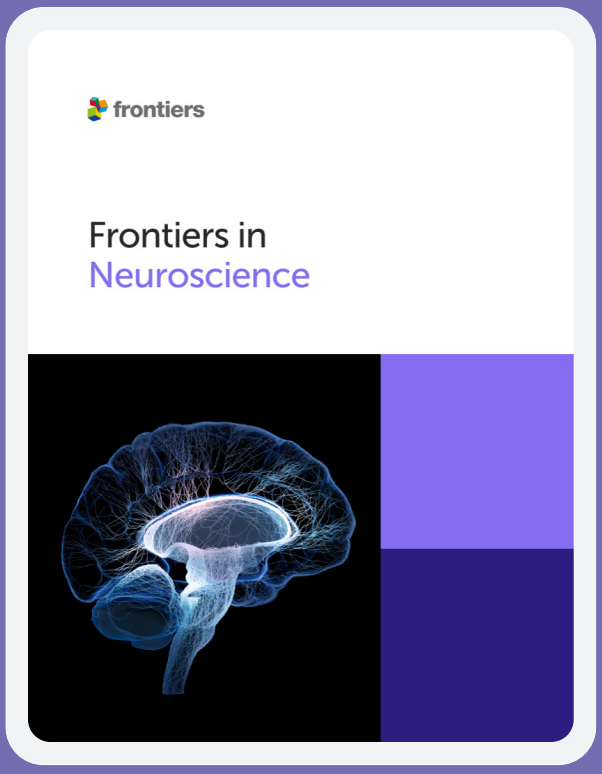

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