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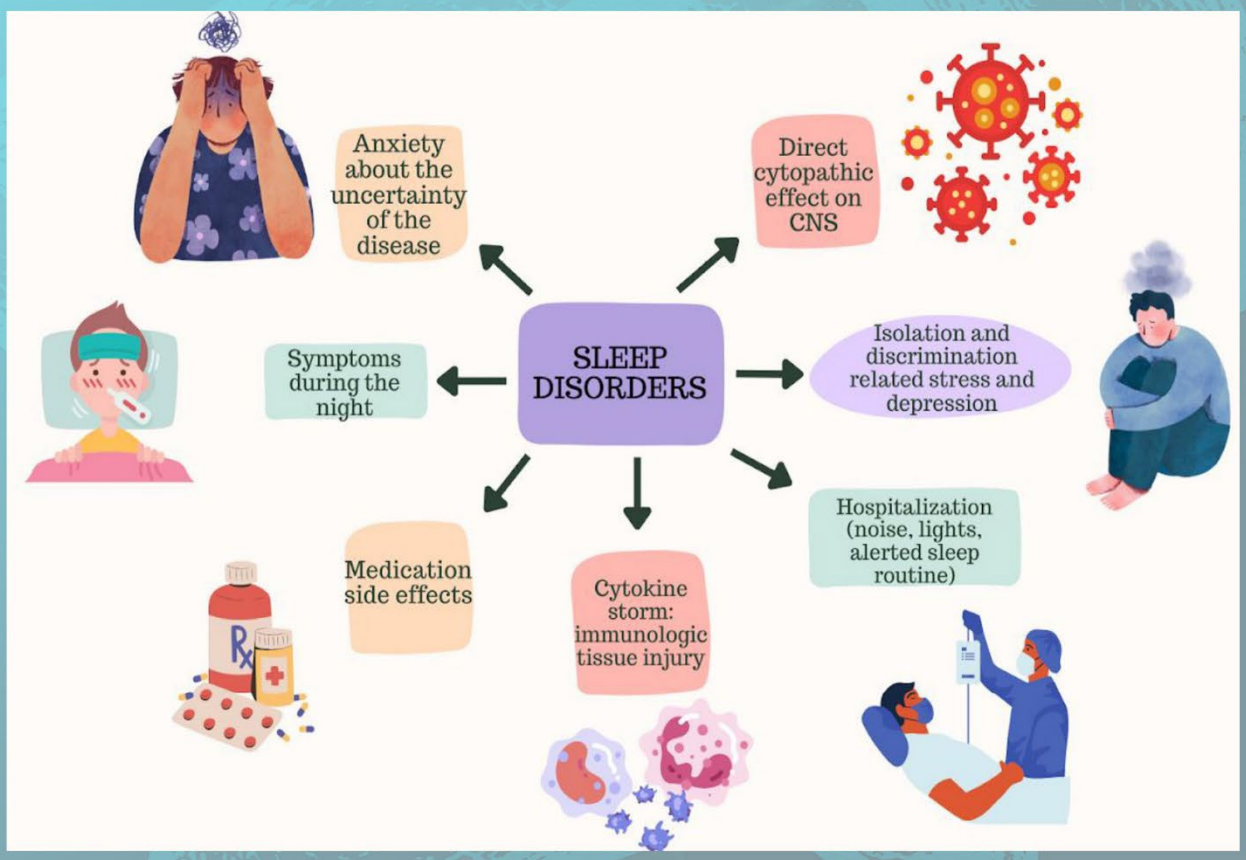

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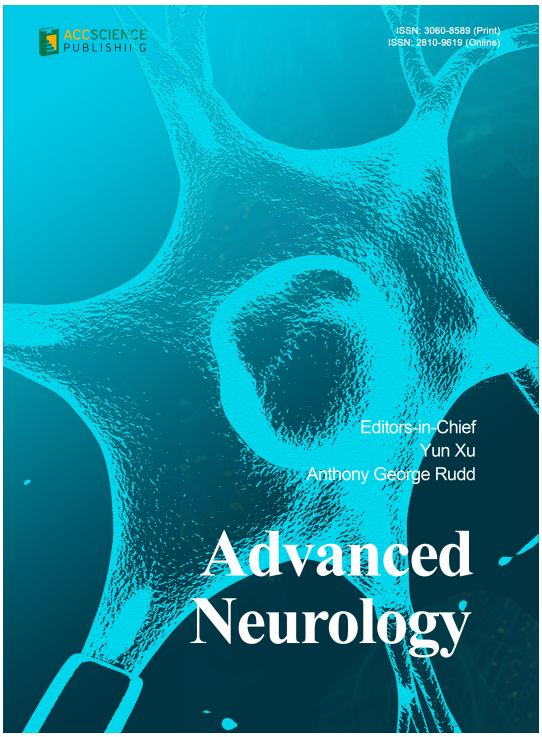

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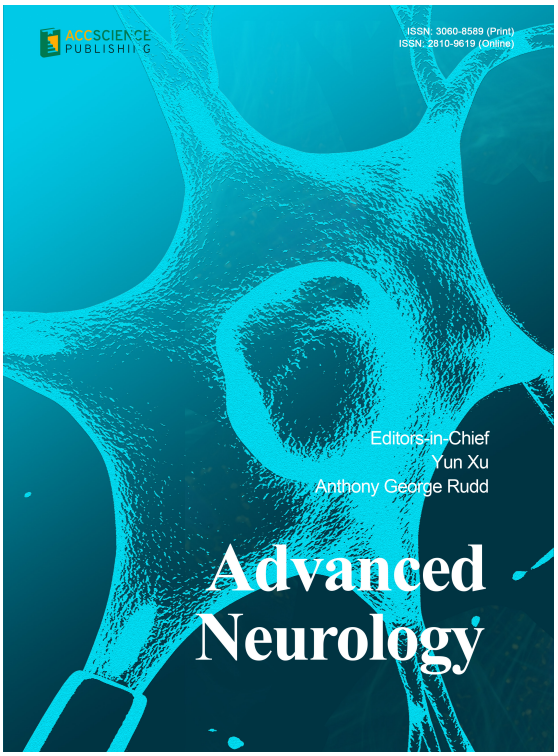

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REVIEW ARTICLE

A holistic strategy for improving cognitive development in children with Down syndrome

Jean Adolphe Rondal*

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Abstract

Down syndrome (DS) is the most frequent autosomal aneuploidy. It is caused by the triplication of human chromosome 21. Disruption of the phenotype is the result of complex gene dosage imbalances regarding chromosome 21 and possibly other chromosomes. The typical DS phenotype is characterized by neurodevelopmental anomalies among which cognitive impairment is prevalent. In recent years, experimental attempts have been made to silence one supernumerary chromosome 21 and correct gene over expressions. They are promising but not practical in human beings. Cognitive pharmacotherapy targeting neurogenesis and synaptic connectivity is in its early stages. In spite of these advances, a complete cure of the condition is not reachable at the present stage and may never be possible given the short time available between syngamy and totipotent or multipotent stem cells in embryonic development. This means that one cannot dispense with behavioral interventions to normalize cognitive functioning in people with DS. The paper reviews current advances in the biomedical treatment of DS and specifies the course and contents of behavioral interventions in memory and language.

Keywords: Down syndrome; Chromosome correction; Epigenetic regulation; Cognitive pharmacotherapy; Memory intervention; Language intervention

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1. Introduction

Down syndrome (DS) is estimated to have a natural incidence of approximately one case in 800 living births.[1] It is caused by the triplication of Hsa21 determining numerous gene dosage imbalances. This impairs the development of the brain and various body organs.[2] Intellectual disability is the common hallmark ranging from mild to severe retardation.

DS exists in several forms: (1) Standard (complete) trisomy 21 (95% of the cases; karyotype 47, XN, +21); (2) mosaic trisomy 21 (1 – 2% of the cases) where only a portion of the cells carries one extra Hsa21; (3) Robertsonian (centric fusion; nonreciprocal) translocations involving C21 are: C21 with C21, C13, C14, C15, and C22: formulae are respectively 46, XN, t (21;21)(q10;q10), + 21, 46, XN, t (13;21)(q10;q10), +21, 46, XN t (14;21)(q10;q10), + 21, 46, XN, t (15;21)(q10;q10), + 21, and 46, XN, t (21;22)(q10;q10), + 21, accounting for 3% of the cases; (4) partial T21 (<1% of the cases) results in only a segment of Hsa21 being triplicated.

DS maps to a region on the long arm of Hsa21 corresponding to band 21q22 possibly containing a so-called DS-critical region the existence of which is contested. Hsa21

contains 225 protein-coding genes and some 400 non-coding genes regulating gene expression.[3]

Screening for trisomy 21 is possible from the 11th week of pregnancy with 95% accuracy in analyzing a sample of maternal blood to isolate fragments of DNA from the fetus. About 99% accuracy is reached in combining the analysis of maternal blood with measures of cardiac rhythm and nuchal translucency of the fetus.[4] However, the screening test is not diagnostical. It cannot substitute more invasive techniques such as amniocentesis and chorionic villus sampling.[5]

Present-day legislation in many countries allows medical termination of pregnancy if the health of the mother is at stake or if the fetus presents a strong likelihood to bear an incurable pathology leading to a major handicap.

According to de Graaf *et al.*,[6] the European mean of medical termination of pregnancy in cases of trisomy 21 is 54% with huge variations between countries (from 0% in Malta to 83% in Spain). In spite of these negative indications, the prevalence of persons with DS in Western countries is important. De Graaf *et al.*[7] report estimations of 419,000 people living with DS in the European countries in 2015 and around 250,000 in the United States in 2014. Decrease in natal prevalence of trisomy 21 due to early diagnosis and medical interruption of pregnancy is compensated by improved medical care along the lifespan.

Life expectancy in persons with DS is close to 65 years and increases regularly. It is reduced by too high a mortality in the first years due to life-threatening comorbidities like congenital cardiopathies and by the early deaths of persons with DS developing a form of Alzheimer's disease, a casualty several times more prevalent in DS than in the rest of the population.[8]

Current work in biomedical sciences and pharmacotherapy aims at improving the phenotype of the persons with DS. The question is whether it has the power of normalizing the cognitive phenotype of these children. As will be argued, the answer to this question is likely to be negative. This should not be taken to mean that experimental and clinical works in these domains are useless. Even if not able to cure the condition, they may end up setting a more robust neurophysiological apparatus in DS children better suited for potentiating the effects of associated behavioral interventions.[9]

The paper is divided in four sections: (1) Chromosome correction; (2) epigenetic reduction of gene overexpression; (3) brain pharmacotherapy; and (4) cognitive behavioral interventions.

2. Chromosome correction

Several techniques have been tested for reducing supernumerary chromosomes. Amano *et al.*[10] claim to have normalized up to 40% of iPSCs generated from fibroblasts obtained from persons carrying a standard trisomy 21. They suggest that introducing a ZSCANA-mRNA into aneuploid cells allows to detect unpaired chromosomes during cell division and eliminate them.

Li *et al.*[11] introduced a TKNEO fusion transgene at the locus 21q21.3 of the gene *APP* in iPSCs generated from fibroblasts of persons with DS. This resulted in the loss of an entire copy of Hsa21 in a large majority of the treated clones.

The most promising correction technique may be the one experimented by Jiang *et al.*[12] involving the use of a *XIST* gene. In humans, the male Y chromosome contains a few dozen genes compared to about 3000 for the X one. As females have two X chromosomes, a dosage reduction is needed to maintain a balance. Natural X dosage reduction is driven by a non-coding RNA, named *XIST*, produced from the inactive X chromosome. Jiang *et al.* reprogrammed fibroblasts from male persons with DS into iPSCs through genetic engineering. They inserted a transgene *XIST* at locus 21q22 of the gene *DYRK1A* in one of the three Hsas21. This silenced this chromosome in 85% of the treated clones.

Most importantly, Czerminsky and Lawrence[13] report that epigenetic plasticity in DS iPSCs is retained at least 35 days beyond the pluripotent stage. At that time, it is still strong enough to initiate chromosome-wide repression in NSC differentiating into neurons. Correcting a deficiency in the process of differentiation of neural stem cells into neurons could be possible by inducing *XIST* at different stages in neurogenesis. However, it is unclear how such corrected cells would behave *in vivo*.

Full chromosome correction requires intervening in the very first days of life. A plausible scenario involves blastomere biopsy and genetic analysis to ascertain trisomy 21 before inserting the biologic agent able to normalize the aneuploidy and reimplanting the treated cells into the embryo expecting cell proliferation to proceed normally.[14]

To cure DS all of the eight cells at day 3 post-insemination would have to undergo chromosome correction. At this stage, embryonic cells are multipotent. They no longer have the power to generate a complete organism but can differentiate into any organic tissue. An intervention on fewer stem cells at that stage would induce a mosaic of cells, some with the normal number of chromosomes and others with three copies of Hsa21. Chromosome correction at day 2 after syngamy would not need to be

performed on the four blastomeres as these earlier stem cells are totipotent, which means that each one is capable of generating a complete organism while eliminating the other three blastomeres. However, whether performed at day 2 or 3 embryonic life, there would not be enough time available between syngamy and chromosome editing for rendering the therapeutic intervention practical.

3. Epigenetic reduction of gene overexpression

Identifying genes on Hsa21 with a dosage imbalance contributing to alterations in brain, behavior, and health in persons with DS is a foremost task. Ait Yahya-Graison *et al.*[15] counted 120 genes expressed in lymphoblastic cells derived from individuals with DS. About 20% of these genes were overexpressed in correspondence with the gene dosage effect (i.e., 1.5) or even amplified beyond that level, including genes *DYRK1A*, *APP*, and *EURL* that play important roles in cell functions and neurogenesis. *DYRK1A* transgenic mice exhibit neurogenesis alterations, brain, and behavioral abnormalities comparable to those of persons with DS.[16] In humans, gene expression studies of amniotic fluid, placenta, and cardiac tissues during fetal development suggest that in DS at least 40 genes are involved in craniofacial changes, nervous system development, intellectual disability, cardiac anomalies, and myeloproliferative disorders.[17]

Epigenetics offers hope for improving the biological development of individuals with DS by regulating gene expression.

A natural product, epigallocatechin-3-gallate (EGCG), has generated much interest in recent years. It is a polyphenol of green tea with antioxidant properties and the capacity to inhibit the expression of the kinase encoded by gene *DYRK1A*. Experiments with mouse models of DS show that when administered early in development, EGCG rescues neurogenesis.[18,19] Controlling product dosage and treatment duration is essential. Chronic administration of doses of 100 mg EGCG/kg/daily from embryonic time to early postnatal days in Ts64Dn mice has detrimental effects on craniofacial development whereas doses of 30 mg/kg/day improve the facial skeleton.[20]

De la Torre *et al.*[21] tested the effect of a one-year treatment with EGCG green tea extracts, 9mg per kilo of weight daily, administered orally and coupled with cognitive training, in a sample of 54 adolescents and adults with DS, women, and men, aged between 16 and 34 years. Participants treated with green tea extracts containing EGCG and simultaneously enrolled in a cognitive training demonstrated a significant superiority in memory, visual recognition, and daily routines abilities over the subjects having been exposed to a drug placebo and the same training program. A retest 16 months later showed partial persistence of the effects. This experiment is inconclusive, however. Its design does not allow to quantify the respective influences of drug and training in the results. The design should have included three groups of subjects matched for age and sex, that is, 1: EGCG alone; 2: Cognitive training alone; and 3: EGCG + cognitive training.

More genes located on Hsa21, but *DYRK1A* is overexpressed in trisomy 21. They also need to be downregulated. Alternatively, natural variation in gene expression may also modulate the outcome of gene dosage imbalances. Prandini *et al.*[22] have suggested that overexpression of some gene sequences is compensated back post-transcriptionally toward typical dosage levels. However, Hunter *et al.*[23] insist that gene dosage compensation is not a common mechanism in DS. It depends on the aneuploid chromosome, the tissue analyzed, and the stage of development.

A huge task awaits biomedical research in testing safe molecular products with the capacity to regulate the overexpressed genes in trisomy 21.

Complicating the matter is the outcome of an investigation by Donovan *et al.*[24] revealing an important molecular heterogeneity in trisomy 21. These researchers analyzed blood samples from 356 participants with DS and 146 euploid controls matched for age and sex. They identified several subtypes of trisomy based on differential overexpression patterns of genes on chromosome 21, both among different Hsa21 genes and across DS individuals. Comparative analyses among these subtypes revealed a strong heterogeneity in the dysregulation of key pathophysiological processes across the molecular subtypes of trisomy 21. Future research linking these molecular differences with DS symptoms may force clinical strategies into a more personalized approach to DS.

4. Brain pharmacotherapy

Neurogenesis impairment during the fetal stage in DS has three major causes: (1) abnormalities in the neural differentiation of iPSCs into NSCs and cell cycle alterations reducing proliferation of NSCs leading to brain hypotrophy (between 10 and 30% reduction in weight, size, and volume); (2) augmented differentiation of NSCs into glial elements (oligodendrocytes and astrocytes) at the expense of neural cells; and (3) abnormal neuron maturation with reduced dendritic areas, spine density, and reduced neuronal connectivity.[25-27]

Stagni and Bartesaghi[28] have identified a series of genes responsible for neurogenesis impairment in DS, among

which *DYRK1A* and *APP* are highly expressed in early development. A few genes involved in neuron maturation anomalies have also been identified. They include *DSCAM* that plays a role in dendritic and synaptic development. The same authors have provided a meta-analysis of 40 prenatal and neonatal pharmacological attempts to improve neurogenesis and neural connectivity in DS mice using either natural or non-natural substances.

The resulting picture varies with the product and the developmental aspect considered. Study that targeted neural progenitor cells with natural substances showed a short-term effect, except melatonin that had no effect. Among non-natural substances, chlorhydrate of fluoxetine (a selective inhibitor of the recapture of neurotransmitter serotonine) was the only one to have an effect. Regarding dendritic development, the effects of all substances tended to fade over time.[28]

Natural substances, except oleic acid, administered during the first two postnatal weeks (corresponding to the third trimester of gestation in humans) have only a short-term effect on hippocampal proliferation and dendritic development. In contrast, study with non-natural substances demonstrated longer-term efficiency.[28]

The neurobiology of DS also results in a reduction of synaptic density and plasticity. Much attention has been devoted to the neurotransmitters. Gotti *et al.*[29] have reviewed a series of studies on the alterations of brain circuits that can be identified in murine models of DS. It shows that different neurotransmission systems are downgraded in several cerebral regions including the hippocampus, the locus coeruleus, and the frontal cortex. Drugs are in the pipeline for reducing the neurotransmission deficits caused by DS. A current strategy is to inhibit the enzymatic cleavage of the neurotransmitter in the synaptic space.

Bartesaghi *et al.*[30] have analyzed the results of a series of experimental and clinical attempts to upgrade the cholinergic, the glutamatergic, and the GABAergic systems in DS. They found no solid support for the treatments aiming at increasing acetylcholine recapture in children, adolescents, and young adults with DS. In contrast, treatment with the NMDA (N-methyl-D-aspartic acid) receptor antagonist memantine (a molecule that mimics the action of neurotransmitter glutamate) improves cognitive measures in TS65Dn mice and young adults with DS. Individual variability is important. Many participants in the human studies show little to no gain but a subset of individuals respond positively to the drugs.[30]

Brain pharmacotherapy is still in its beginning stages. The equation is complex. It involves several interacting variables: the precise calendar of development as to the organic aspect targeted, drug safety, applicability, short- and longer-term effects, and people's intrinsic variability.

5. Cognitive behavioral interventions

This type of intervention started being developed several decades ago in response to the wishes of the associations and the parents of children with DS and it has been refined and extended since through clinical and experimental research. This type of intervention should not be confounded with psychotherapy that relates to attempts to help people cope with their psychological and psychiatric problems.

Memory and language are the most important areas in cognitive functioning. They are interdependent functions. Language implies good short- and longer-term storage abilities. Memory is largely based on linguistic coding. In what follows, one specifies the course of current behavioral interventions in memory and language with children and adolescents with DS.

5.1. Memory intervention

What we call memory is an interconnected set of treatment and storage systems and sub-systems. A key distinction is between short-term (also called working) memory (STM) maintaining the information active for a few seconds before eliminating or transferring it to the long-term store (LTM). Evidence suggests that the auditory-vocal store (AV-STM) and the visual-spatial one (VS-STM) are separate entities. LTM is also divided along several lines: explicit (conscious) and implicit (non-conscious) components. The former is related to declarative memory (recording facts and events), the latter to procedural memory (recording sequential procedures for doing things).[31]

A long-standing finding in the DS literature is that AV-STM is more impaired relative to VS-STM.[32] In most individuals with DS, the AV-STM span is limited to a few items.[33] They tend not to repeat verbal sequences, which, as a result, rapidly decay. The major sub-component in Baddeley's working memory scheme is an articulatory control process based on sub-vocal rehearsal and operating on a phonological loop. The limitations in AV-STM in individuals with DS are linked to dysfunctional aspects of their speech. Their articulatory development is delayed and often incomplete.[34]

Neuropsychological studies show that the memory limitations in DS correspond to the underdevelopment of the hippocampal and prefrontal systems, and possibly the cerebellar structures.[35]

Remediation efforts target the articulatory control process of AV-STM for increasing its span. Results show

that it is possible to augment the span capacity by several units, especially in young children, by training overt cumulative rehearsal and phonological processing.

Regarding LTM, the evidence suggests that explicit memory abilities are more impaired in individuals with DS[36] than implicit ones, which appear to be more independent from cognitive level.[37] The correct functioning of explicit memory depends on the integrity of the medial temporal lobe, in particular the hippocampal and peri hippocampal structures, in relationship with the prefrontal cortex, the caudate nucleus, and the anterior cingulate cortex.[38,39] Volumetric and magnetic resonance imaging studies of individual with DS reveal a disproportionately smaller volume of the frontal, temporal, and cerebellar regions.[40]

Implicit memory involves the frontal, parietal, and superior temporal cortices of the left brain, the left-basal ganglia (most importantly the striatum with the caudate nucleus and the putamen), and the right-neocerebellar structures. Research in brain hemodynamics, electrophysiology, and magnetoencephalography has documented differences between the two kinds of memories at the neuronal and molecular level.[41]

A key aspect in episodic memory is the degree of organization of the material to be recorded. At the coding stage, the relevant characteristics of the information need to be related to each other in relevant ways, which helps retrieving them from LTM. The hippocampal cortex codifies entering information for computing an index of the contents of the LTM store.

Memory intervention at this level is directed toward increasing the subjects consciousness regarding organizational characteristics of the material to be remembered.[42]

5.2. Language intervention

Twenty years of functional imagery have yielded a precise picture of the relationship between particular brain areas and language functions.[43] The brain of persons with DS is characterized by an underdevelopment of the frontal and temporal lobes hosting two of the major language areas, that is, the Broca area (Brodmann areas 44, 45, and 47) on the production side of language functioning and the Wernicke area (Brodmann areas 22, 41, and 42) on the reception one.[44,45]

Language intervention in DS centers on the major components of the language system, that is, speech, lexicon, and morphosyntax.

As said, speech development is retarded in individuals with DS and is often incomplete.

Specific craniofacial dysmorphia is responsible for a good part of these difficulties. They involve: cranial doming, shortening of the skull along the anteroposterior axis, midfacial hypoplasia with maxillary deficiency, flattened nose bridge, insufficient lip seal, high palate with reduction in length, and narrowed oropharynx. These anomalies determine masticating, swallowing, and often breathing difficulties with sleep apnea. Sleep disturbance may interfere with slow brain waves during NREM sleep and fragilize synaptic consolidation in LTM, further hindering cognitive development.[46] Dental malocclusion with maxillary transverse discrepancy and posterior crossbite is frequently observed.[47]

At least four dosage-sensitive genes determine the craniofacial phenotype in Dp1Tyb mice that recapitulates key aspects of the human DS craniofacial dysmorphology.[48] One of them is *DYRK1A* whose overexpression results in a decreased proliferation of neural crest cells and a decrease in the size of frontal bone primordia. This causes defective skull bones with aberrant mineralization of the growth plates between cranial base bones. EGCG molecules attach to the adenosine triphosphate binding site of a *DYRK1A* protein altering its function. As indicated, doses of 30mg/kg/day of EGCG administered to Ts65Dn mice improve the facial skeleton of the animals. Complicating the dosage matter is the fact that a similar dose chronically administered from embryonic time in these mice reduces mineral density in longer bones.[49]

Analyses of the phonological processes highlight close similarities between children with DS and those with typical development. This means that the strategies developed for helping typical children with articulatory delays can be used with DS children.[50]

Specific programs for boosting lexical acquisitions in children with DS have been tested and found efficient.[51] Early vocabulary training is all the more important as the first words are usually quite delayed in children with DS, which contributes to slowing down the whole language development. Joining manual signed expressions (for example, borrowing from the lexicon of the American sign language of the deaf) with oral productions can help the child to progress more rapidly in the early stages of lexical development. Fast mapping of novel objects names and the acquisition of internal state vocabulary (words referring to sensory perception, positive and negative effects, affective behaviors, moral judgments, and volition) can then proceed toward building extended lexical networks. Here also, the typical patterns of development in non-retarded children are found in children with DS.[51]

Morphosyntactic training concentrates on promoting the understanding and expression of the major semantic

relations underlying sentence structures, increasing children's consciousness of the respective positions of the words and mutual relationships in phrases and sentences, and drawing attention to nominal, pronominal, and verbal inflections.[52]

Artificial intelligence can be of help. Many children with DS are able to learn to read at least to some extent.[53] A personal computer equipped with adequate programs can assist morphosyntactic remediation even in DS children with limited reading ability. Animated computer screens with pointing mechanisms can be used for visually demonstrating meaning relations between sequences of words. In the same way, computer programs displaying arrows, colors, or other pedagogical devices can assist children to identify syntactic patterns in sentences, including between non-adjacent units and discontinuous constituents, as well as to illustrate the grammatically authorized displacements of constituents in sentences.[54] The relationships between the words in phrases and sentences that need to be identified to comply with the requirements of grammatical morphology, can also be illustrated on the screen. Dual afferentation with combined visual and auditory information is useful for maintaining children's attention and foster learning.

Meta-analyses show that intervention programs are efficient in improving speech, lexical, and morphosyntactic abilities in children with DS.[55,56] Table A1 supplies a summary of the prospects for cognitive improvement in Down syndrome as discussed in the paper.

6. Conclusion

Promising results in chromosome correction and gene regulation have been obtained *in vitro* and with mouse models of DS. The question of their applicability to human individuals is open. To be complete, the repression of a supernumerary Hsa21 must be implemented early in embryonic life. This is not authorized in humans nor is it practical at the present point. New research findings point toward the possibility of epigenetic corrections beyond the first stages of embryonic development but it remains to be seen how the corrected cells would behave in a full organism.

No biological therapy initiated after the first embryonic days could completely cure the trisomic condition. Moreover, there is only little time available for a confirmed diagnosis between syngamy and the end of the totipotent and multipotent stem cells. However, future genetic and epigenetic interventions later in prenatal development and efficient brain pharmacotherapy in late prenatal and early postnatal times may help constructing a more robust neurophysiological apparatus in children with DS. On this basis, cognitive behavioral programs will be able to operate efficiently, helping to transform the negative image of persons with DS for the better, favoring a fuller social acceptance, and contributing to reduce the abortion rates of these innocent and lovely babies.

The main point on which one has insisted in this paper is that a holistic approach associating biomedical and behavioral strategies from prenatal stages onto childhood and adolescence offers the best chance for bringing the cognitive phenotype of the individuals with DS as close as possible to normality.

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Appendix

Table A1. Summary of the prospects for cognitive improvement in Down syndrome

Strategy	Objective	Effect
1. Chromosome correction	Silencing supernumerary Hsa21	Positive
– *ZSCAN4* fusion transgene *in vitro*		
– *TKNEO* fusion *transgene in vitro*		
– *XIST RNA transgene in vitro*		
2. Cellular therapy	Correcting deficiency in the differentiation of NSCs into neurons	In experimentation
– *XIST chromosome*-wide repression in NSCs		
3. Epigenetic correction	Regulating gene overexpression	Positive
– EGCG inhibitor of kinase *DYRK1A* in mice and humans		
– EGCG improvement of *craniofacial* dysmorphias in mice and infants		
4. Brain pharmacotherapy	Improving neurogenesis in mice	Positive
	Improving cognition in mice and humans	Positive fluoxetine
		Positive memantine
5. Behavioral intervention	Improving memory	Positive
	STM	
	LTM	
6. Behavioral intervention	Improving language	Positive
	Speech	
	Lexicon	
	Morphosyntax	

Abbreviations: EGCG: Epigallocatechin-3-gallate; LTM: Short-term memory; NSCs: Neural stem cells; STM: Long-term memory.

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REVIEW ARTICLE

The hidden impact of sleep disorders in the post-pandemic world

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Abstract

Studying sleep disorders is crucial due to their substantial influence on behavior and health in all age groups. The complex interplay of genetic, environmental, and lifestyle factors, including hormonal fluctuations, stress, and ozone exposure, is the cause of these disorders, which include insomnia, parasomnia, central hypersomnolence disorder, sleep-disordered breathing, and circadian rhythm sleep–wake disorders. They impact behavior, deteriorate social connections, and impair cognitive abilities, physical well-being, and emotional stability. The complex relationship between sleep and mental health frequently eludes attention, with approximately 17% of adults experiencing mental disorders during their lifetime. Frequently, insomnia is associated with anxiety and depression, indicating a bidirectional relationship. Conversely, individuals with mental health issues are susceptible to sleep disorders such as narcolepsy, restless legs syndrome, and sleep apnea. Treatment modalities such as relaxation therapy, stimulus control, and cognitive-behavioral therapy provide prospects for improvement, along with preventive measures such as maintaining a regular sleep schedule and exercising daily. The pandemic's effect on sleep health has led to the introduction of terms such as "coronasomnia" and "COVID-somnia," urging further studies on post-COVID-19 conditions. This review examines various sleep disorders, their effects, treatments, and the pandemic's impact on sleep patterns, aiming to guide readers through the evolving landscape of sleep health during this global upheaval.

Keywords: Coronasomnia; COVID-somnia; Anxiety; Depression; Post-COVID-19

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1. Introduction

Sleep disorders pose a multifaceted challenge to the health and well-being of individuals, disrupting the body's natural sleep–wake cycle and substantially impacting cognitive function, emotional stability, and social interactions. The characteristics, triggers, and consequences of these conditions, which range from insomnia to narcolepsy, necessitate a nuanced understanding of effective diagnosis and treatment. The global landscape of sleep disturbances has evolved as the aftermath of the COVID-19 pandemic, resulting in

the phenomenon known as "coronasomnia" or "COVID-somnia," further underscoring the complex relationship between sleep and mental health. As societies navigate the complexities of the post-pandemic era, it is becoming increasingly crucial to investigate the emergence of unique sleep disorders and their link to mental well-being. This comprehensive overview aims to delve into the defining characteristics, etiology, diagnostic methods, treatment modalities, and preventive measures for common sleep disorders, shedding light on their widespread impact across diverse demographic groups and highlighting the importance of proactive management in enhancing the overall quality of life.

2. Methods

A systematic literature review approach was employed to determine the prevalence of sleep disorders and their effect on behavior before and after the COVID-19 pandemic. We conducted a comprehensive search across multiple databases, including PubMed, Scopus, ScienceDirect, and Google Scholar, to identify pertinent studies published between 2018 and 2024. Our search strategy included keywords such as "Coronasomnia," "COVID-somnia," "Anxiety," "Depression," and "Post-COVID." The search process involved multiple stages. Initially, we conducted broad searches using the predefined keywords across the selected databases. We reviewed the titles and abstracts of potential studies to determine their relevance after identifying them. This initial search yielded 173 articles. After eliminating duplicates, 86 distinct studies remained. Subsequently, we reviewed the titles and abstracts to screen for relevance based on our predefined inclusion and exclusion criteria. This screening resulted in the exclusion of 87 studies that did not fulfill the criteria. We contrasted the prevalence and patterns of sleep disorders before and after the pandemic, highlighting any significant trends or changes. In addition, we investigated the emerging unique sleep disorders during and after the pandemic, commonly referred to as "Coronasomnia" or "COVID-somnia." The inclusion and exclusion criteria are delineated in Table 1 below.

Table 1. Inclusion and exclusion criteria for studies on post-pandemic sleep disorders

Inclusion criteria	Exclusion criteria
Individuals of all ages and sexes affected by or experiencing sleep pattern alterations or sleep disorders during or after the COVID-19 pandemic.	Studies focusing exclusively on non-human participants or populations that are not impacted by COVID-19.
Studies focusing on the impact of COVID-19 on sleep quality, sleep disorders (including insomnia, sleep apnea, and restless legs syndrome), and related mental health issues (anxiety, depression, and PTSD).	Studies not directly linked to sleep patterns, sleep disorders, or mental health impact during or after the COVID-19 pandemic.
Studies conducted during or following the onset of the COVID-19 pandemic.	Studies lack defined methodologies, data, or outcomes related to sleep or mental health.
Both qualitative and quantitative studies, including surveys, observational studies, cohort studies, and clinical trials, evaluate sleep patterns and associated factors.	Studies conducted exclusively before the onset of the COVID-19 pandemic.
Studies reporting on changes in sleep duration, sleep quality, prevalence of sleep disorders, mental health outcomes associated with sleep disturbances, and the relationship between COVID-19 and sleep.	Studies that are not published in English, unless translations are provided for review.

Abbreviation: PTSD: Post-traumatic stress disorder.

3. Results

The COVID-19 pandemic has significantly disrupted sleep patterns and exacerbated mental health challenges worldwide. Before the pandemic, sleep habits typically followed more consistent patterns, with fewer interruptions and higher overall sleep quality. However, COVID-19 onset resulted in significant alterations: individuals began staying awake later into the night, slept for extended hours, and experienced more frequent awakenings. Health-care personnel and individuals who were directly impacted by the virus were particularly affected by this disruption. During this period, the prevalence of sleep disorders, notably insomnia, saw a marked increase, which was primarily attributed to increased stress and anxiety that resulted from the pandemic's uncertainties.

The impact on mental health was equally profound, as evidenced by the increased prevalence of post-traumatic stress disorder (PTSD), depression, and anxiety. The pandemic's disruption of daily life and social isolation measures contributed to a decrease in overall psychological resilience. Terms such as "coronasomnia" were coined to encompass the spectrum of sleep-related issues intensified by the pandemic's stressors. Beyond the immediate effects, a substantial number of COVID-19 survivors continue to experience insomnia and other sleep disturbances, emphasizing the long-term consequences on sleep health. The research highlights the intricate relationship between sleep quality and mental well-being, where poor sleep worsens existing mental health conditions and vice versa. Comprehensive strategies that prioritize both mental health support and sleep management are necessary to address these challenges. Individualized interventions backed by ongoing research are essential in mitigating

the long-term impact of the pandemic on sleep and mental health outcomes. As societies traverse the recovery phases, understanding and resolving these interconnected issues remain vital for facilitating overall well-being after COVID-19.

4. Discussion

Many behavioral alterations can result from sleep disorders, including insomnia, sleep apnea, and parasomnia. Difficulties in focusing, mood swings, irritability, and daily exhaustion can all be induced by insufficient or poor-quality sleep. Anxiety, depression, and other mental health issues have been associated with long-term sleep deprivation. Certain sleep disorders may also trigger unusual behaviors during sleep. For instance, people with rapid eye movement sleep behavior disorder may experience vivid dreams, which could cause them to move or behave in potentially harmful ways while asleep. Children with sleep-disordered breathing, such as obstructive sleep apnea, have been shown to manifest more severe behavioral issues, including hyperactivity, inattention, and aggression.[1] Furthermore, sleep disturbances can exacerbate existing clinical disorders and impede social cognition, behavior, and social interaction in individuals with autism spectrum disorder.[2]

Comprehending the association between behavioral changes and sleep disorders is crucial. It can facilitate the early identification of potential problems, the implementation of appropriate therapies, and the enhancement of the overall quality of life. Research has demonstrated a link between sleep disorders and emotional and behavioral issues such as anxiety, depression, inattention, and aggression in children and adolescents.[3,4] The utilization of behavioral therapy to address sleep disorders can enhance behavior, mood, and cognitive performance throughout the day.[4]

4.1. Sleep disorders and their characteristics

Sleep disorders such as insomnia, circadian rhythm disruptions, sleep apnea, narcolepsy, parasomnias, and restless legs syndrome have a substantial influence on both physical and mental health. Their reasons range from genetic predispositions to lifestyle variables, and treatments include drugs and lifestyle changes, with an emphasis on tailored methods for optimal management. Table 2 describes the overview of sleep disorders and their characteristics.

4.2. Impact during COVID

The coronavirus disease 2019 or COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a pandemic in March 2020. Many affected patients also suffer from chronic disorders such as obstructive sleep apnea (OSA) and central sleep apnea (CSA). Studies have increasingly identified OSA in hospitalized COVID-19 patients, indicating a higher mortality risk.[8]

Insomnia symptoms were prevalent during the pandemic, primarily because of anxiety regarding the virus and disruptions in daily life. The quality of sleep has been reported to be worse among younger individuals, which has been further exacerbated by concerns regarding limited supplies and lifestyle changes. Health-care workers encounter unique challenges, including inadequate education, isolated work environments, and job insecurity, which contribute to anxiety, stress, PTSD, and depression on a global scale.

Insomnia rates have also increased significantly among older adults, who are particularly concerned about their increased susceptibility to COVID-19 symptoms. Research suggests that the prevalence of insomnia symptoms is not directly correlated with age or sex.[9,10]

Figure 1 is a mind map that effectively summarizes the cumulative impact of various factors, such as stress, lifestyle adjustments, and health anxieties, on sleep patterns across different demographics during the pandemic.

4.3. Sleep trends before and after COVID-19

Significant research has been conducted on the effects of the COVID-19 pandemic on mental health and sleep. In this overview from Tables 3-5, we examine the pre- and post-COVID-19 impact on sleep trends and behavioral changes by analyzing a collection of studies and research findings.

4.4. Unique sleep disorders are emerging during and after COVID-19

The terms "coronasomnia" or "COVID-somnia" refer to various sleep issues, including insomnia, disruptions in sleep patterns, alterations in the sleep–wake cycle, and diminished sleep quality. These problems frequently result from stress associated with the fear of the virus, its psychosocial impacts on daily life, or the physical and mental aftermath of contracting COVID-19.[1]

One study identified various forms of sleep disturbances and their prevalence rates: insomnia at 60%, frequent nocturnal awakenings at 41%, breathing difficulties at 36%, restless legs syndrome at 18%, sleep apnea at 10%, vivid dreams at 33%, nightmares at 26%, and lucid dreams at 15%.[20,21]

Figure 2 provides a detailed insight into the physiological, neurological, and psychological factors

Table 2. An overview of sleep disorders and their characteristics

Condition			References
Insomnia	Diagnostic criteria	Sleep disturbances: 1. Difficulty initiating sleep 2. Difficulty maintaining sleep 3. Waking up earlier than desired 4. Reluctance to adhere to an appropriate bedtime schedule Associated daytime symptoms: 1. Fatigue/malaise 2.Attention, concentration, or memory impairment 3.Mood disturbance/irritability 4.Behavioral problems 5.Susceptibility to errors/accidents 6.Concerns about or dissatisfaction with sleep (>3 times/week and >3 months)	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]
	Etiology	• Idiopathic • Inadequate sleep hygiene • Improper sleep training • Secondary or concurrent psychiatric or medical condition • Drug or substance use	
	Treatment	• Hypnotics • Antidepressants • Melatonin agonists • Orexin antagonists	
Circadian rhythm sleep–wake disorders	Diagnostic criteria	1. Delayed sleep–wake phase disorder: The patient's sleep pattern is consistently delayed beyond what is considered necessary 2. Advanced sleep–wake phase disorders: The patient's sleep consistently commences earlier than deemed necessary. 3. Symptoms present for at least 3 months 4. Sleep log and actigraphy monitoring for a minimum of 7 days, demonstrating a shift in sleep schedule, either shifting later or earlier than usual.	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]
	Etiology	• Polymorphism in the hPer3 gene • Increased or decreased exposure to light • Behavioral, social, and occupational activities • Psychiatric disorders	
	Treatment	• Melatonin–combined with morning blue light	
Sleep-disordered breathing: obstructive and central sleep apnea	Diagnostic criteria	1. The patient complains of sleepiness, non-restorative sleep, fatigue, or insomnia symptoms 2. The patient wakes up with breath-holding, gasping, or choking 3. Habitual snoring, breathing interruptions, or both during the patient's sleep 4. The patient was diagnosed with hypertension, a mood disorder, cognitive dysfunction, coronary artery disease, stroke, congestive heart failure, atrial fibrillation, or type 2 diabetes mellitus.	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]
	Etiology	• Obesity • Upper airway obstruction • Structural abnormalities of the head and neck • Endocrine disorders • Alcohol consumption • Use of sedatives • Genetic predisposition	
	Treatment	• Continuous or bilevel positive airway pressure • Dental or oral appliances • Surgery for specific indications • Weight loss • Avoiding triggers	
Narcolepsy	Diagnostic criteria	Type 1: 1. The patient experiences daily episodes of an uncontrollable urge to sleep or daytime sleep lapses, lasting for a minimum of 3 months 2. Cataplexy and a mean sleep latency of ≤8 min and two or more SOREMPs* 3. CSF hypocretin-1 concentration ≤110 pg/mL Type 2: 1. Narcolepsy without cataplexy 2. Hypersomnolence is not explained by other causes such as insufficient sleep, obstructive sleep apnea, and medication or substance use	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]

(Cont'd...)

Table 2. (*Continued*)

Condition			References
	Etiology	• Hypocretin deficiency • Head trauma • Sustained sleep deprivation • Unspecified viral illness • Sudden changes in sleep–wake patterns • HLA subtypes DR2/DRB1*1501 and DQB1*0602	
	Treatment	Sleepiness: 1. Modafinil 2. Armodafinil 3. Methylphenidate 4. Amphetamine salts 5. Sodium oxybate Cataplexy: 1. Sodium oxybate 2. SSRI	
Parasomnia	Diagnostic criteria	REM-related parasomnia: 1. Repeated episodes of sleep-related vocalization and/or complex motor behaviors Non-REM-related parasomnia: 1. Recurrent episodes of incomplete awakening from sleep 2. Inappropriate or absent responsiveness to the efforts of others to intervene or redirect the person during the episode 3. Limited or no associated cognition or dream imagery 4. Partial or complete amnesia for the episode	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]
	Etiology	• Sleep deprivation • Situational stress • Febrile states • Use of psychotropic medications • Comorbid conditions such as migraine and encephalitis • Alcohol consumption	
	Treatment	• Clonazepam • Other benzodiazepines • Melatonin • Treatment of associated disorders and other precipitating factors • Antidepressants	
Restless legs syndrome and periodic limb movements of sleep	Diagnostic criteria	An urge to move the legs is usually accompanied by or thought to be caused by uncomfortable and unpleasant sensations in the legs. These symptoms must 1. Begin or worsen during periods of rest or inactivity. 2. Be partially or relieved by movement. 3. Occur exclusively or predominantly in the evening or night rather than during the day.	Schreck *et al.*, Pavlova *et al.*, Holder *et al.*, Sateia[2,5-7]
	Etiology	• Family history • Iron deficiency (ferritin level<50 μg/L) • Certain medications • Female sex • Pregnancy • Chronic renal failure • Prolonged immobility	
	Treatment	• Dopamine agonists • Pramipexole • Ropinirole • Gabapentin Enacarbil • Iron supplementation • Opiates • Benzodiazepines	

Abbreviations: REM: Rapid eye movement; SOREMPs: Sleep-onset rapid eye movement periods; SSRI: Selective serotonin reuptake inhibitor.

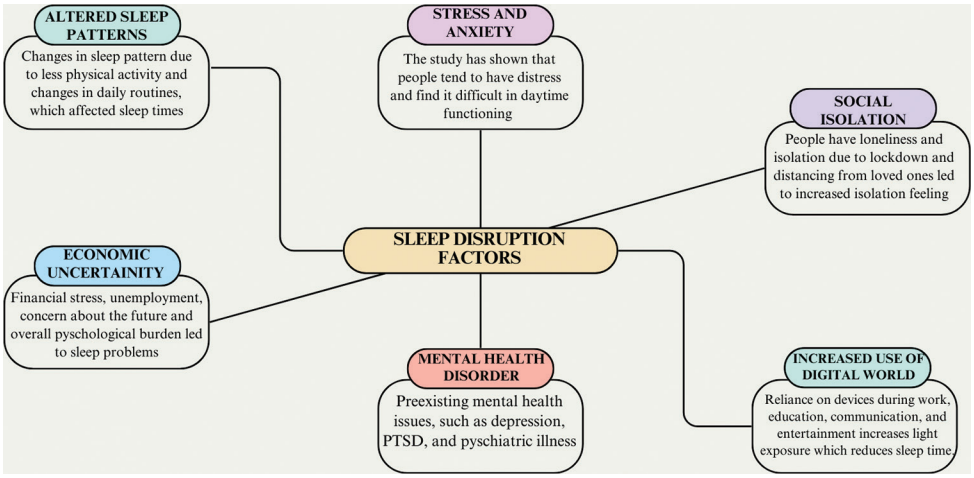

Figure 1. Factors that can disrupt sleep

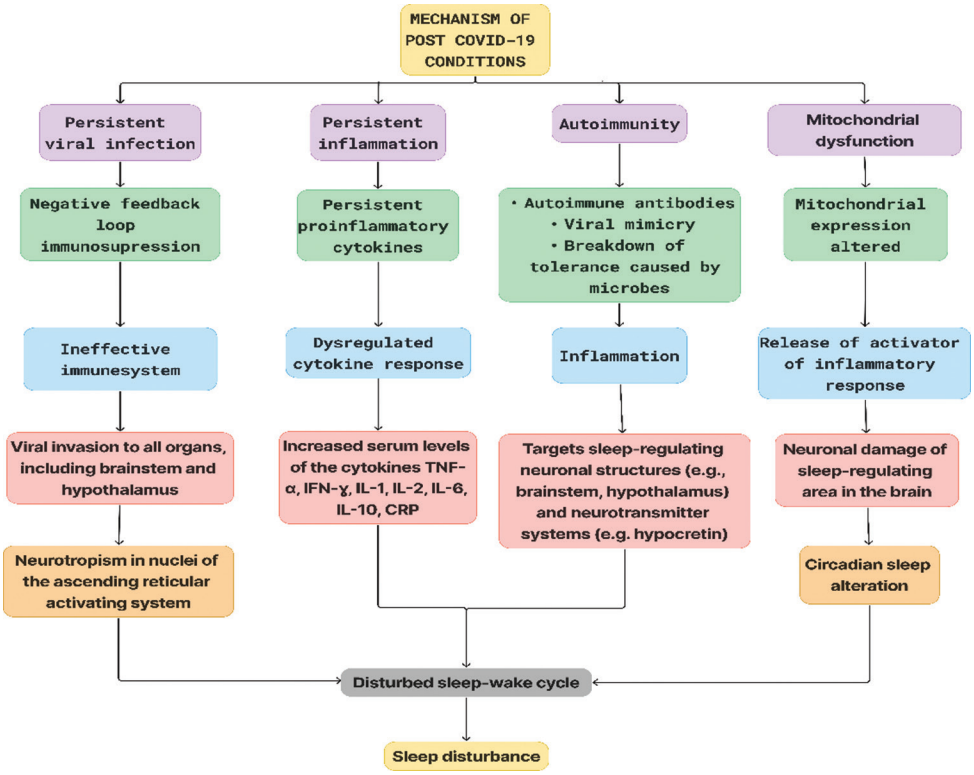

Figure 2. Mechanism of post-COVID-19 conditions leading to sleep disturbances

that contribute to insomnia and other sleep-related issues following the recovery from COVID-19. It elucidates the mechanisms underlying the post-COVID-19 conditions that precipitate sleep disturbances, providing a thorough understanding of their development.

The SARS-CoV-2 virus has been observed to target specific regions of the brain, including the prefrontal cortex, basal ganglia, and hypothalamus, which are essential for the regulation of sleep.[22] It directly disrupts sleep by infiltrating the central nervous system through the nasal mucosa and olfactory pathway or indirectly by entering the bloodstream through the blood–brain barrier. Infection of endothelial cells and leukocytes, in conjunction with the cytokine storm phenomenon, can increase blood–brain barrier permeability, resulting in immune-mediated tissue damage, neuropsychiatric symptoms, and sleep disorders.[23]

Table 3. Analysis of sleep patterns pre- and post-COVID-19

Pre-COVID-19	Post-COVID-19	References
Self-reported sleep times generally exhibited consistent patterns with minimal variation.	Self-reported sleep times shifted later during the COVID-19 outbreak, with more significant changes observed early in the pandemic than later in the year.	Yuan *et al.*[11]
Before the COVID-19 pandemic, individuals typically slept on time, woke up early, and experienced fewer awakenings.	After the COVID-19 pandemic, individuals began sleeping and waking up later, with an increased number of awakenings.	Gao and Scullin[12]
Although 17.1% of the individuals slept less than usual, a considerable proportion slept more. Two meta-analyses also reported high rates of insomnia – 23.8% and 37% – with a more significant impact on healthcare workers compared to the general population (47.3% vs. 18.2%).	Significant alterations in sleep patterns were observed among the US adult population during the COVID-19 pandemic, with the strongest correlation with symptoms of mental health impairment. Approximately 53.9% of the cohort reported increased sleep duration, most likely due to increased stress impacting sleep.	Batool-Anwar *et al.*[13]
Approximately 51.7% of individuals reported no difference in sleep quality during the stay-at-home order compared to before the pandemic.	Recent results indicate that during COVID-19 home confinement, sleep profiles changed, with delayed sleep phases, prolonged sleep duration, and diminished sleep quality reported.	Dai *et al.*[14]
-	People reported worse overall sleep health during the COVID-19 pandemic, with lower ratings across all six dimensions of sleep health – regularity, satisfaction, alertness, timing, efficiency, and duration – compared to before the infection. Poor sleep health was more prevalent among those with a history of COVID-19, particularly those with severe symptoms, and was associated with a lower quality of life.	Alzueta *et al.*[15]
There is no information on the clinical conditions before the lockdown, which could influence the perceptions of sleep quality and pre-sleep arousal during the pandemic.	During the lockdown, Italian mothers and their pre-school children experienced diminished sleep quality, heightened emotional symptoms, and self-regulation issues. These problems also impacted school-age children. Maternal psychological distress and employment status significantly affected both maternal and child sleep and mental health, particularly for parents of children under 9 years of age.	Gorgoni *et al.*[16]
In accordance with this, 39.3% of individuals reported experiencing more difficulty falling asleep, whereas 35.6% reported waking up more frequently than usual compared to the pre-pandemic period. Over one-third of the participants experienced increased trouble falling asleep or more frequent night awakenings, indicating substantial sleep disruptions.	During the COVID-19 pandemic, the initial regional studies identified a rise in sleep disturbances and alterations in sleep patterns among the general public and frontline healthcare workers.	Yuksel *et al.*[17]
No other significant differences in subjective sleep parameters were noted before the outbreak, except for delayed waking up times. Furthermore, approximately 36.0% of individuals reported experiencing sleep difficulties before the outbreak.	Sleep patterns were affected, with the "delayed sleep" subgroup reflecting a U.S. survey that reported that Gen Z and millennials experienced the most delayed bedtimes during confinement. This subgroup was more likely to work from home and had fewer dependents, which enabled more flexibility in adjusting their sleep schedule.	Robillard *et al.*[18]
This pattern was most pronounced among women with relatively normal sleep patterns before the pandemic, as they experienced a substantial deterioration in sleep and increased distress.	Overall, sleep patterns significantly deteriorated in comparison to those that existed before the pandemic.	Petrov *et al.*[19]

A retrospective cohort study by Zhang *et al.* suggested a potential correlation between poor sleep and increased susceptibility to COVID-19 infection. This correlation may be potentially linked to the lower absolute lymphocyte counts and an elevated neutrophil–to–lymphocyte ratio observed in Chinese patients.[1] Figure 3 illustrates various stressors, lifestyle changes, and health impacts associated with the pandemic that contribute to disrupted sleep patterns and insomnia.

Patients with obstructive sleep apnea often present with various comorbidities that are strongly linked to severe

Table 4. Analysis of sleep disorders pre- and post-COVID-19

Pre-COVID-19	Post-COVID-19	References
Insomnia was a prevalent sleep disorder both before and during COVID-19.	Studies have revealed that although some individuals with pre-existing sleep disorders or poor sleep quality experienced improvements during the pandemic, others experienced disruptions or worsened sleep, particularly during periods of increased stress or anxiety.	Yuan *et al.*[11]
-	Some individuals may have experienced a decline in their sleep quality due to the pandemic, potentially due to disorders such as insomnia, sleep apnea, or sleep-related anxiety. Certain studies suggest that the psychological impact of the pandemic contributed to these sleep disturbances.	Gao and Scullin[12]
-	These alterations are consistent with sleep pattern disturbances, which may indicate various sleep disorders, including insomnia, hypersomnia, or circadian rhythm disorders.	Batool-Anwar *et al.*[13]
Although the text does not identify a specific sleep disorder, the symptoms described align with issues such as insomnia (difficulty falling or staying asleep), circadian rhythm disorders (delayed sleep–wake patterns), and sleep disturbances caused by increased stress and anxiety.	The alterations include delayed sleep–wake schedules, increased sleep duration, and prolonged sleep latency. Although the text does not specify a particular sleep disorder diagnosed post-COVID-19, these symptoms are indicative of various potential sleep issues.	Dai *et al.*[14]
-	Commonly reported sleep disturbances that have persisted for over 12 months post-COVID-19 include newly diagnosed insomnia, sleep apnea, and restless leg syndrome.	Alzueta *et al.*[15]
The female sex is linked to somatic pre-sleep arousal, indicating potential sex-specific vulnerabilities in mental health. Women generally report higher rates of insomnia and greater stress and emotional reactivity. In addition, the absence or interruption of work before COVID-19 was associated with elevated somatic pre-sleep arousal, indicating that employment status can affect mental health and sleep quality.	Comorbid sleep pathologies render individuals more susceptible to sleep disturbances associated with specific pandemic-related factors.	Gorgoni *et al.*[16]
In a large U.S. sample studied before the pandemic, although women exhibited more insomnia symptoms and lower sleep self-efficacy than men, overall sleep health did not differ by sex.	-	Yuksel *et al.*[17]
Sleep-related difficulties included problems with sleep initiation, maintenance, and early morning awakenings; however, specific sleep disorders or diagnoses were not identified in the provided excerpt.	After the COVID-19 outbreak, the study noted an increase in clinically meaningful sleep-related difficulties, such as an increased prevalence of sleep initiation problems, sleep maintenance issues, and early morning awakenings.	Robillard *et al.*[18]
The excerpt refers to specific sleep disorders, such as insomnia and depressive symptoms, but it lacks comprehensive information regarding the occurrence of these disorders before the onset of COVID-19.	The excerpt describes four distinct profiles of sleep pattern alterations in response to the COVID-19 pandemic: delayed sleep, dysregulated and distressed, sleep opportunist, and sleep lost and fragmented.	Petrov *et al.*[19]

clinical outcomes if they develop COVID-19. Beyond these factors, OSA itself induces sleep deprivation, which can dysregulate the immune system, exacerbate hypoxia induced by COVID-19, and disrupt the renin–angiotensin system, thereby increasing susceptibility to severe infection.[23]

In addition, the pandemic resulted in significant alterations in dream activity, including an increase in nightmares, heightened dream recall, and parasomnia. Liu *et al.* highlighted that patients with COVID-19 exhibited a higher prevalence of dream enactment behaviors, which correlated with the severity of their illness. This indicates that there is a need for further research to explore the potential neurodegenerative effects of COVID-19.[23]

Table 5. Impact on mental health pre- and post-COVID-19

Pre-COVID-19	Post-COVID-19	References
-	Factors such as the severity of the pandemic and the quality of sleep before the pandemic were associated with changes in sleep duration and quality. The impact on mental health varied by individual and region, with conditions such as anxiety, stress, depression, PTSD, and an overall decline in mental health observed.	Yuan *et al.*[11]
Individuals who reported higher susceptibility of their sleeping patterns to stress at baseline were more likely to experience worsening sleep quality during the pandemic. This suggests a link between stress and mental health, as stress can lead to mental health disorders and affect sleep quality.	Mental health was adversely impacted, with deteriorating sleep quality often linked to conditions such as anxiety, depression, or stress. Post-pandemic, individuals with declining sleep quality may continue to face these mental health challenges unless they are addressed. Those with high post-pandemic stress levels are at risk of persistent mental health challenges.	Gao and Scullin[12]
Before the pandemic, regular sleep schedules probably did not impact mental health.	Surveys on loneliness associated with social isolation resulting from COVID-19 mitigation strategies during the pandemic highlighted a substantial impact on sleep duration. Interventions aimed at enhancing mental health could potentially lead to improvements in sleep health.	Batool-Anwar *et al.*[13]
Individuals were already experiencing acute psychological stress before the pandemic, suggesting that stress and lifestyle changes were adversely impacting mental health.	Results demonstrate that the SARS pandemic, including COVID-19, similarly affected people's mental health, leading to negative emotions such as fear, loneliness, and annoyance.	Dai *et al.*[14]
-	Sleep health is linked to physical and mental health outcomes and quality of life in community populations both before and during the COVID-19 pandemic. However, currently, there is a lack of data on the long-term consequences of COVID-19 on sleep health. Consequently, poor sleep health following COVID-19 may have severe long-term consequences for both mental and physical health.	Alzueta *et al.*[15]
Poor sleep quality and increased arousal levels have been commonly linked to mental health issues such as anxiety and depression. This is consistent with previous research linking stress, depression, and sleep problems to mental health disorders.	Younger adults reported greater concerns and severity of insomnia during the pandemic, with a higher risk of psychological distress, anxiety, and depression, as well as more frequent and intense dreams and nightmares.	Gorgoni *et al.*[16]
-	Preliminary evidence indicates the prevalence of high depression and anxiety levels during COVID-19, with poorer sleep quality likely associated with these conditions. Current research suggests that sleep disturbances during the pandemic increase anxiety, depression, and suicidal behavior, thereby increasing the risk of long-term health effects. This highlights the need for public health interventions.	Yuksel *et al.*[17]
-	Across the behavioral change subgroups, the proportion of individuals who experienced a clinically significant increase in stress and depression during the pre-outbreak period was significantly different from that during the outbreak period.	Robillard *et al.*[18]
-	Research on chronic stress and new infectious diseases suggests that sleep and mental health consequences can have long-term effects that extend beyond the immediate stressors, potentially leading to subclinical or poor health outcomes. The findings, in conjunction with findings from other studies, indicate that the sleep and psychological impact of this pandemic could be significant, underscoring the necessity for rapid, coordinated policy and program initiatives.	Petrov *et al.*[19]

Abbreviation: PTSD: Post-traumatic stress disorder.

4.5. Post-COVID-19 impact

As the world moves beyond the COVID-19 pandemic, evolving sleep patterns continue to significantly impact overall well-being globally, given their crucial role in recovery.[23] Post-COVID-19, long-term COVID arises as a persistent condition with symptoms lasting 1 – 3 months following infection.[24]

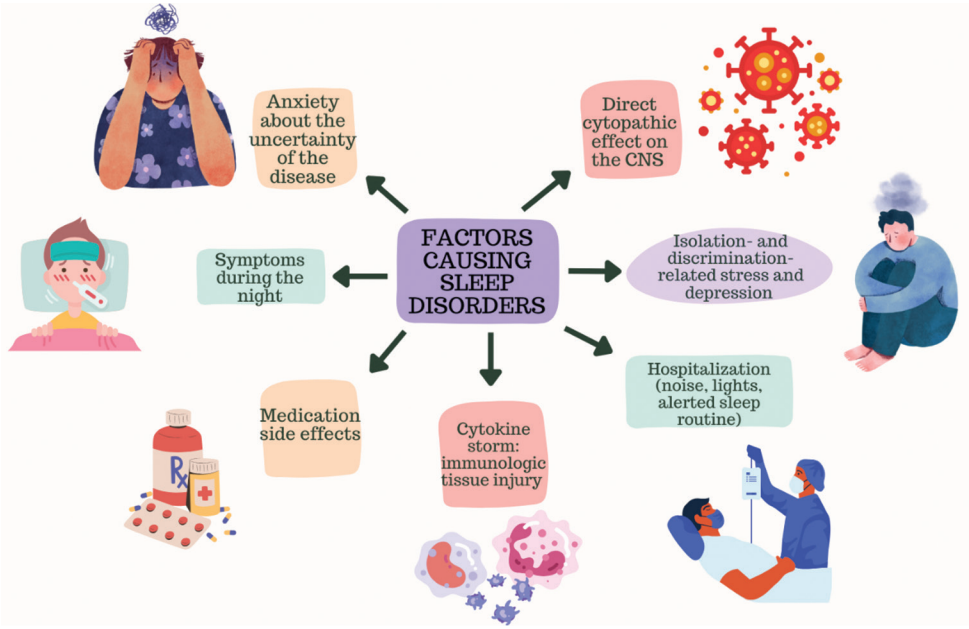

Figure 3. COVID-19-related factors triggering sleep disorders

In February 2024, a study published in Frontiers in Public Health investigated anxiety, depression, and sleep disturbances among 1,000 adult COVID-19 survivors in Vietnam. The study, which was conducted from June to September 2022, concentrated on individuals who were not hospitalized and had no prior history of insomnia. Results revealed that 76% of the study participants experienced post-COVID-19 insomnia, with a quarter reporting severe symptoms. In addition, one-third of the participants reported diminished sleep quality, shorter duration, and increased difficulty falling asleep than they did before their infection.[23,24]

In conclusion, a significant portion of the population continues to be affected by long-term COVID-19, characterized by persistent symptoms, especially sleep disturbances. The study underscored the severity and frequency of post-COVID-19 insomnia, highlighting the virus's long-term impact on sleep patterns.[24] Although many aspects of long-term COVID remain unknown, ongoing research is actively investigating its causes and potential interventions. A majority (77%) of those affected attribute pandemic-related factors to their post-outbreak insomnia symptoms.[25]

4.5.1. Connection between sleep and mental health

An individual's mental, social, and emotional health is critical, influencing all aspects of life, including work productivity, emotional resilience, and decision-making. Maintaining strong mental health enables individuals to make positive life choices, manage stress effectively, and preserve overall well-being. Conversely, poor mental health can impair judgment and lower quality of life. Therefore, improving sleep quality and developing healthy sleep routines are essential for promoting mental health.

The prevalence of mental health issues among adults has increased significantly in recent years. Recognizing the complex relationship between sleep patterns and mental well-being is vital. Figure 4, a mind map, illustrates the complicated relationships between sleep patterns and mental health, offering valuable insights into their reciprocal relationship.[24,26-34]

4.6. Long-term consequences of sleep disorders if left untreated

Untreated sleep disorders can significantly impact a person's overall quality of life, influencing both physical and mental health beyond occasional restless nights. Obstructive sleep apnea, for instance, is significantly linked to an elevated risk of stroke. Individuals with conditions such as Down syndrome or Prader–Willi syndrome (PWS) frequently experience untreated OSA, leading to excessive daytime sleepiness and risks such as impaired cognitive function and cardiovascular complications. Moreover, untreated OSA complicates the diagnosis of other sleep disorders, especially in patients with attention deficit hyperactivity disorder (ADHD).[35]

Untreated OSA increases the chance of death by about 6% every 5 – 8 years owing to cardiovascular events.

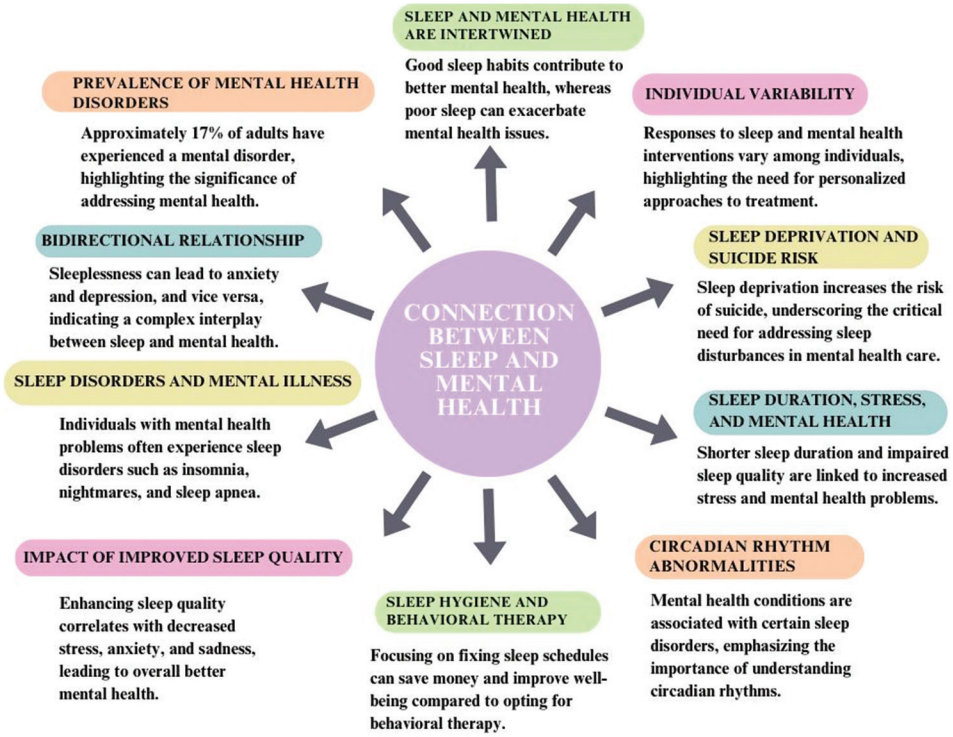

Figure 4. Exploring the relationship between sleep and mental health

Managing sleep disorders is crucial in preventing strokes, given their close association with cardiovascular conditions and increased stroke risk.[36] Neglecting sleep disorders after a stroke also increases the risk of stroke recurrence and premature death,[37] in addition to hindering rehabilitation efforts. ADHD symptoms can worsen with untreated sleep disorders such as sleep-disordered breathing, restless legs syndrome, and narcolepsy, further compromising cognitive function and overall well-being.[38]

Untreated sleep disorders raise the risk of developing and progressing cancer and are recognized as risk factors for various tumors.[39] In older adults, untreated insomnia correlates with increased healthcare utilization and costs, even after accounting for other health conditions. Sleep-disordered breathing affects roughly 53% of women during their midlife, particularly post-menopausal women, and can have a negative impact on both mental and physical health.[40]

Untreated insomnia is linked to a greater incidence of major depression and anxiety, particularly in high-stress professions such as professional truck driving. Studies demonstrate a significant prevalence of sleep disorders among truck drivers, including insomnia (27.5%) and OSA (25.8 – 51%), with insomniacs having 2 – 8 times higher risk of motor vehicle accidents.[8]

4.7. Limitations

Studies that use data from specific populations, such as hospitalized patients, health-care professionals, or survey participants, may not generalize findings to broader demographics. This limitation can compromise the precision and reliability of data collection, making it difficult to establish causation or understand the long-term effects of COVID-19 on sleep and mental health. In addition, the positive impacts of COVID-19 on sleep and mental health may not be fully captured by current studies, as these effects are still evolving. Long-term research that monitors individuals' sleep patterns and mental health before, during, and after the pandemic could offer a more comprehensive understanding of these impacts. Such studies would help clarify how these factors interact over time and contribute to ongoing endeavors to enhance public health interventions and support systems.

5. Conclusion

The diverse array of sleep disorders presents a complex challenge affecting millions of individuals worldwide, affecting their physical health, mental well-being, and overall quality of life. Understanding the multifaceted nature of these conditions, including their genetic, environmental, and societal influences, underscores the

need for thorough assessment and personalized treatment strategies.

As we navigate the post-pandemic landscape, it is crucial to acknowledge the long-term effects of COVID-19 on sleep patterns and mental health, emphasizing the interdependence of these domains. By prioritizing research, education, and support services, health-care systems can promote resilience and enable patients to regain restful nights and strengthen their psychological well-being.

Implementing evidence-based strategies and promoting healthy sleep hygiene are crucial steps toward enhancing the overall well-being of individuals affected by sleep disturbances at every stage of life. This comprehensive approach strives to address the complexities of sleep disorders and aid individuals in achieving optimal health and vitality.

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Conflict of interest

The authors declare they have no competing interests.

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Writing – original draft: All authors
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Consent for publication

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Availability of data

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ACCSCIENCE PUBLISHING

Advanced Neurology

REVIEW ARTICLE

Ferroptosis in neonatal hypoxic-ischemic brain injury and implications for therapeutic development

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Abstract

The morbidity and mortality associated with neonatal hypoxic-ischemic brain injury (HIBI) and the related clinical syndrome, hypoxic-ischemic encephalopathy (HIE), remain substantial. Consequently, treatment alternatives to therapeutic hypothermia, the current standard of care, are urgently needed. Therefore, unique aspects of the complex mechanistic underpinnings of neonatal HIBI and HIE must be studied. This review focuses on ferroptosis, a unique form of cell death, which was first described in 2012 and is characterized by iron-dependent lipid peroxidation, leading to lethal reactive oxygen species buildup. The role of ferroptosis in neonatal HIBI has been indirectly supported by decades of research and directly demonstrated using *in vivo* and *in vitro* models in recent years. Molecular targets, including nuclear factor erythroid 2-related factor 2, cystine/glutamate antiporter (system x_c^-), and glutathione peroxidase-4, have been identified as key mediators of ferroptosis in neonatal HIBI. In preliminary experiments, agents modulating the activity of these and other targets effectively suppress ferroptosis and attenuate the structural and functional consequences of neonatal HIBI. While considerable work is still required to establish the underlying mechanisms and therapeutic importance of these effects, the foundational studies show that antiferroptotic agents may reduce the lasting burden of neonatal HIBI and HIE.

Keywords: Hypoxic-ischemic brain injury; Hypoxic-ischemic encephalopathy; Neonatal brain injury; Ferroptosis; System x_c^-; Glutathione peroxidase-4; Nuclear factor erythroid 2-related factor 2

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1. Introduction

Neonatal hypoxic-ischemic brain injury (HIBI) involves inadequate blood and oxygen supply to the brain during birth, often resulting in hypoxic-ischemic encephalopathy (HIE). HIE affects 1 – 8 in every 1,000 individuals born in high-income countries.[1] Affected neonates present with impaired reflexes, abnormal muscle tone, seizures, respiratory difficulties, and low heart rate.[2,3] HIE carries a substantial risk of mortality

and neurodevelopmental disability, with many survivors experiencing developmental delays, sensorimotor and memory impairments, learning disabilities, epilepsy, and cerebral palsy.[1-3] HIE's burden is also massive in terms of healthcare and social costs, accumulated through acute treatment and lifelong supportive care.[4-6] Therapeutic hypothermia (TH) has been widely implemented as the standard of care for neonatal HIBI and HIE.[7] It involves reducing the brain's temperature through localized or systemic cooling to combat excessive membrane excitability and induce molecular changes that prevent further neuronal injury and promote recovery.[7,8] TH has remarkably reduced overall HIBI-associated mortality and disability; however, the number of neonates affected by both remains striking.[7-9] According to a meta-analysis by the Canadian Pediatric Society, TH protects against mortality and moderate-to-severe disability at the age of 18 – 24 months, with an overall relative risk (RR) of 0.75 and an RR of 0.68 and 0.82 in moderate and severe HIBI groups, respectively.[7] The insufficient success of TH is partially explained by limited eligibility for this treatment because it requires initiation within 6 h of HIBI onset and fulfillment of specific gestational age, weight, and umbilical pH criteria. Furthermore, incomplete efficacy is a contributing factor, considering that TH potentially fails to target all key pathophysiological processes.[1,7-9] Consequently, there is a major need for alternative therapeutic strategies to prevent neonatal HIBI and HIE. Significant research has aimed at addressing the gaps in understanding of their mechanistic underpinnings and identifying unique strategies to exploit them.[10-14] However, there is still much work to be done in this regard.

A series of complex, interacting mechanisms contribute to neonatal HIBI and consequential HIE (Figure 1). These occur in two phases: primary neuronal injury, which consists of acute cellular consequences of blood and oxygen loss, and secondary neuronal injury, which involves higher-volume cell death, beginning 6 – 24 h later.[8,14] The hallmarks of primary neuronal injury are intracellular ATP depletion, anoxic depolarization, excessive glutamate release, and microglial activation.[15-17] Blood and oxygen deprivation necessitates cellular reliance on anaerobic metabolism, which significantly reduces ATP production and triggers the release of lactic acid and hydrogen ions (H^+). This promotes extracellular, and consequently, intracellular, acidosis, impairing cell signaling.[15] Moreover, this decline in cellular energy leads to anoxic depolarization – the dysregulation of ion gradients across neuronal membranes – resulting in excessive accumulation of calcium (Ca^{2+}), sodium (Na^+), and chloride (Cl^-) intracellularly and potassium (K^+) extracellularly.[15,18,19] The ion buildup stimulates cell swelling and activation of Ca^{2+}-dependent enzymes that promote production of free radicals, including reactive oxygen species (ROS) and nitric oxide (NO). ROS and NO exert cytotoxic effects during the secondary neuronal injury phase. Mitochondrial dysfunction follows, exacerbating ATP loss and free radical production.[15,20] Primary neuronal injury is also associated with excessive glutamate release. Although glutamate is the primary excitatory neurotransmitter in physiological signaling, its aberrant release overactivates *N*-methyl-D-aspartate (NMDA) receptors, promoting further Ca^{2+} influx and excitotoxicity.[16,20] This is exacerbated by anoxic depolarization because it enables passive NMDA receptor activation.[16] The neonatal brain is more susceptible to the effects of excitotoxicity than the adult one due to higher NMDA receptor expression and activity.[21,22] In addition, the byproducts of primary neuronal injury, such as ROS and NO, activate microglia, leading them to adopt phagocytic capabilities and release proinflammatory cytokines and chemokines. This disrupts blood-brain barrier integrity, allowing for migration of peripheral immune cells into the brain, which enhances delayed inflammatory signaling for secondary injury.[17,23] In addition to directly killing cells, these primary neuronal injury mechanisms supply effector molecules, which mediate large-scale cell death in the secondary neuronal injury phase of neonatal HIBI.[24]

Three forms of secondary cell death occur due to primary neuronal injury-induced oxidative stress, excitotoxicity, and inflammation: apoptosis, necrosis, and autophagy.[15,17] Apoptosis involves chromatin condensation, DNA fragmentation, and cytoskeletal structure loss, followed by the collection of disassembled cellular contents in vesicles known as apoptotic bodies. Apoptosis is mediated by the proteolytic activity of caspases; therefore, caspase activation is another characteristic feature.[15,25-27] Necrosis, often observed in severe HIBI, involves nuclear degradation and swelling of organelles and whole cells, leading to membrane damage and consequential lysis.[15,27-29] Autophagy involves the formation of vesicles – autophagosomes – in which disassembled cellular contents are collected before they are broken down via fusion with large vacuoles. These vacuoles are the primary observable feature of autophagy.[27,29,30] Essential involvement of an additional form of cell death – ferroptosis – in neonatal HIBI has been suggested, which is the focus of our review.

2. Ferroptosis

2.1. Overview

Ferroptosis is a unique form of cell death, first described by Dr. Brent Stockwell's group[31] in 2012, which involves iron-dependent lipid peroxidation, leading to a fatal buildup of ROS. Ferroptosis is induced by Ras-selective lethal (RSL)

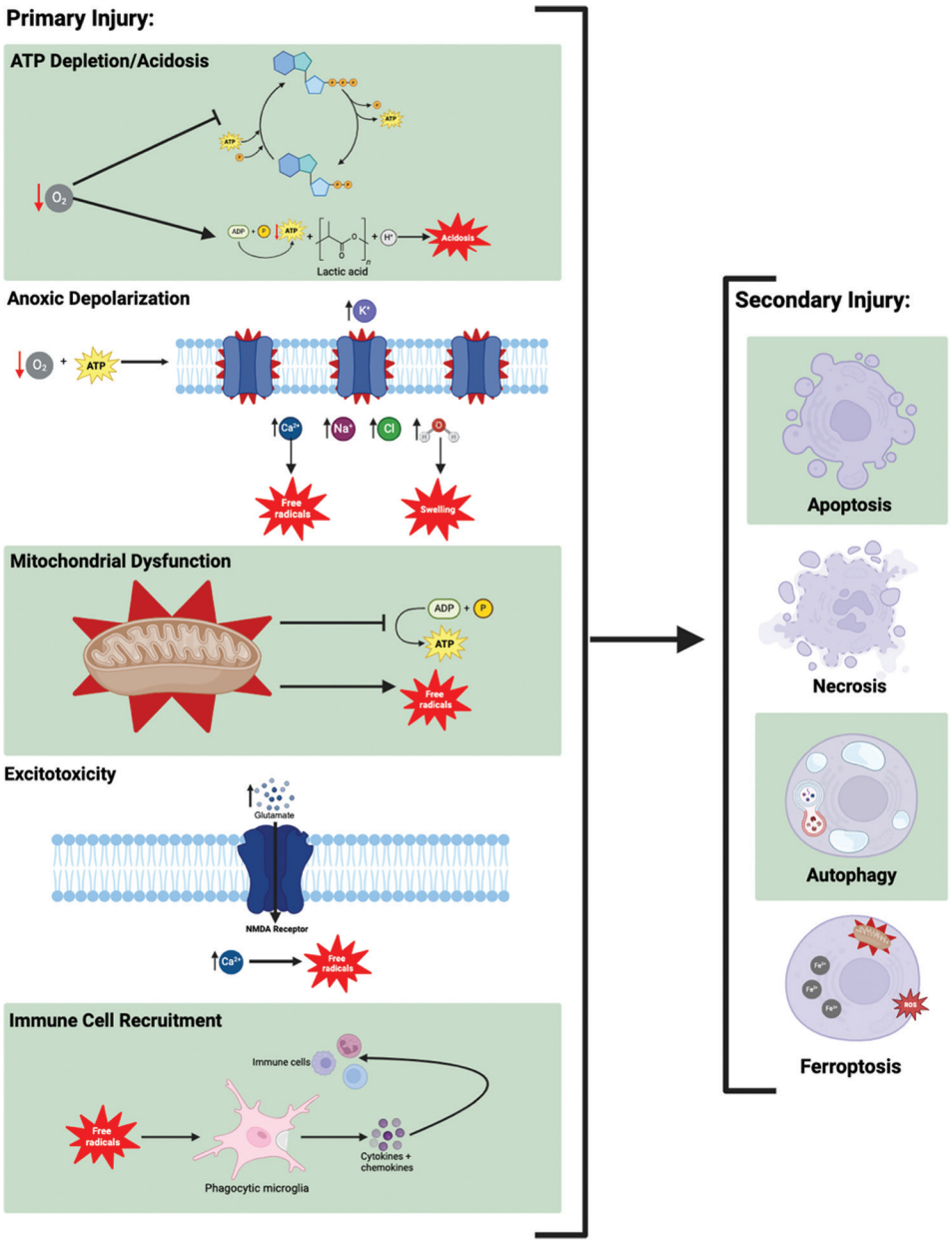

Figure 1. Mechanisms of primary and secondary neuronal injury in neonatal HIBI. Under hypoxic conditions, neurons rely on anaerobic metabolism, leading to ATP depletion and acidosis in the primary neuronal injury phase. Another primary neuronal injury mechanism is anoxic depolarization. This results in dysregulated ion and water accumulation, promoting free radical production and cell swelling. Mitochondrial dysfunction also contributes by reducing ATP production and stimulating free radical release. Excessive glutamate accumulation leads to NMDA receptor hyperactivation, promoting calcium-dependent free radical production. Microglia adopt phagocytic properties and release proinflammatory cytokines and chemokines, facilitating the recruitment of peripheral immune cells into the brain. Effector molecules from these primary neuronal injury mechanisms promote larger-scale secondary cell death through apoptosis, necrosis, autophagy, and ferroptosis, each with its distinct mechanisms and cellular features. Figure was created using BioRender.com.
Abbreviations: HIBI: Hypoxic-ischemic brain injury; NMDA: *N*-methyl-D-aspartate.

compounds, which are selectively activated by the Ras family members, a group of >150 guanosine triphosphatases with essential signaling functions in diverse cellular processes.[31,32] Key to the discovery of ferroptosis were the Stockwell group's former observations that cell death induced by erastin – an RSL – does not exhibit the defining features of apoptosis and is unaffected by a pan-caspase inhibitor. They therefore concluded that it occurs through

a non-apoptotic mechanism.[33] The group subsequently found that erastin-treated cells failed to exhibit other signs of apoptosis but showed unique morphological changes in their mitochondria indicative of mitochondrial dysfunction. These changes were not seen in cells treated with agents that induce apoptosis, necrosis, or autophagy.[34] In addition, ROS accumulation was noted in erastin-treated cells, and antioxidants protected against erastin-mediated death, implicating an oxidative mechanism.[34] In a final foundational study,[35] they found that RSL-mediated cell death was iron-dependent, as iron chelators notably decreased cell death observed in response to erastin, RSL3, or RSL5. In summary, before the 2012 study, the Stockwell group had concluded that RSL-mediated cell death is non-apoptotic, involves ROS accumulation, and requires iron. The study integrated these findings to characterize a novel mechanism.[31]

In cultured HT-1080 fibrosarcoma cells, the Stockwell group detected an increase in cytosolic and lipid ROS starting 2 h after erastin treatment, with cell detachment and death 4 h later. ROS buildup and cell death were inhibited by cotreatment with an iron chelator but enhanced by exposure to exogenous iron. They concluded that RSL compounds mediate cell death through iron-dependent ROS buildup and termed this process *ferroptosis*.[31] Through microscopy and bioenergetic analysis, the group further supported the fact that ferroptosis is a unique phenomenon that does not resemble apoptosis, necrosis, or autophagy. They also identified a small molecule – ferrostatin-1 – as a potent, specific inhibitor of ferroptosis, which prevents erastin-mediated ROS buildup, possibly by acting as a scavenger. In addition, they presented evidence for the involvement of ferroptosis in cancer cell death and excitotoxic neuronal damage.[31] Ferroptosis has since been studied by numerous groups and identified to participate in physiological functions, such as embryonic development, aging, inflammation, and tumor suppression.[36-38] Its important role in various diseases, including myocardial infarction,[39] neurodegenerative diseases,[40] acute renal failure,[41] intestinal ischemia-reperfusion injury,[42] and, most relevant to this review, neonatal HIBI,[43] has also been described.

2.2. Signaling mechanisms

Since the discovery of ferroptosis, research has focused on delineating its underlying signaling mechanisms. In the instrumental study by the Stockwell group,[31] the cystine/glutamate antiporter (system x_c^-) was implicated as a key mediator of erastin-induced ferroptosis, a finding that has since been well-supported.[44-47] System x_c^- promotes cystine influx and glutamate efflux under physiological conditions.[48] It consists of a solute carrier family 7 member 11 (SLC7A11) light chain, which mediates its function, and a solute carrier family 3 member 2 (SLC3A2) heavy chain, which facilitates its transport and membrane expression.[47,49] Excessive glutamate buildup due to ischemia-reperfusion injury or other insults inhibits system x_c^-, triggering a decrease in intracellular cysteine.[50] This has been identified as an essential mechanism in ferroptosis, as counteracting inhibition of system x_c^- attenuates erastin-mediated cell death.[31,46,47,51] Cystine is necessary for producing glutathione (GSH), an antioxidant: cystine is reduced to cysteine and incorporated into glutamate-cysteine ligase catalytic subunits.[52] Therefore, intracellular GSH levels decrease in response to system x_c^- inhibition.[52,53] By depleting GSH through system x_c^-, erastin indirectly inactivates glutathione peroxidase-4 (GPX4), a protein that protects against oxidative stress by preventing membrane lipid peroxidation.[45,54] GPX4 is indirectly inactivated by erastin through system xc^- inhibition, and directly inactivated by RSL3. In both cases, GPX4 inactivation promotes ferroptosis by stimulating ROS production.[45] GPX4 has been deemed an essential regulator of ferroptosis, with several studies demonstrating that GPX4 downregulation renders cells increasingly susceptible to RSL-mediated death, whereas GPX4 upregulation confers increased resistance to it.[14,41,45,55] The system x_c^-/GSH/GPX4 pathway has been identified as the central mechanism of ferroptosis regulation.[31,45]

Several other signaling molecules modulate the activity of this central pathway.[55-59] Nuclear factor erythroid 2–related factor 2 (NRF2) is a transcription factor that restores redox balance under oxidative stress. In addition to its role in regulating mitochondrial dysfunction and iron metabolism to protect against ferroptosis, it directly acts on SLC7A11 and GPX4 to upregulate their activity.[56,57] Consequently, NRF2 knockdown increases susceptibility to RSL-mediated cell death, whereas NRF2 overexpression protects against it.[56] The mechanistic target of rapamycin complex 1 (mTORC1), which consists of the protein kinase mTOR and regulatory proteins, also alters activity of this central pathway. On activation by cystine, mTORC1 promotes the synthesis of GPX4, protecting against ferroptosis. Therefore, mTORC1 is dependent on system x_c^- activity. mTORC1 inactivation increases the susceptibility of cells to ferroptosis by reducing GPX4 production.[55] The alpha subunit of hypoxia-inducible factor-1 (HIF-1α) – a transcription factor that mediates protective cellular adaptations to hypoxia – also regulates this central pathway. HIF-1α upregulates system x_c^-, GSH, and GPX4 activity to protect against ferroptosis.[47,58,59] These proteins represent notable examples, not an exhaustive list, of agents that modulate system x_c^-/GSH/GPX4 signaling.

Another key process in ferroptosis is iron-dependent lipid peroxidation, which occurs through two mechanisms: fenton-like reactions, where ferrous iron (Fe^{2+}) catalyzes the production of hydroxyl radicals ('OH) from hydrogen peroxide (H_2O_2), and iron-dependent lipoxygenase (LOX) enzyme activity.[60] Therefore, proteins involved in iron metabolism help regulate ferroptosis. These include transferrin (TF), which acts through the transferrin receptor (TFR), to transport inactive ferric iron (Fe^{3+}) into the cell, where it is modified into Fe^{2+}, the active form. Ferritin, which stores excess iron, and ferroportin, which facilitates iron efflux, are also important proteins.[35,60,61] In addition, mitochondrial and nicotinamide adenine dinucleotide phosphate oxidase (NOX) enzyme activity is important for ROS production in ferroptosis. Mitochondria also play a key role in iron metabolism.[27,31] Voltage-gated anion channels (VDACs) are mediators of erastin-induced mitochondrial dysfunction. Both VDAC and NOX inhibitors attenuate ferroptosis.[31,34,35] Other agents that indirectly modulate mitochondrial and NOX activity have also shown promise in regulating ferroptosis.[37,62,63] The details of these mechanisms are beyond the scope of this review.

The activities of acyl-coenzyme a synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) are also important because they promote ferroptosis by acylating polyunsaturated fatty acids, such as arachidonic acid, and incorporating them into the cell membrane, respectively, thereby increasing the quantity of membrane lipids available for peroxidation. Deletion of these enzymes suppresses ferroptosis.[64,65] Additional aspects of ferroptosis signaling have been reviewed by Feng *et al.*[66] and Peeples *et al.*[67] Further research is required to establish the complex interactions between these diverse mechanisms.

3. Establishing the role of ferroptosis in neonatal HIBI and HIE

Several studies, dating back to before ferroptosis was described by name, have supported its putative role in neonatal HIBI (Table 1). As the Stockwell group.[31] stated, excitotoxic neuronal death has long been established to be oxidative and iron-dependent. Prior studies revealed that excessive glutamatergic NMDA receptor activation promotes iron accumulation in the brain and that this iron catalyzes free radical production, resulting in oxidative stress.[68-70] Consistent with this, the group showed that Fer-1, an iron chelator, and a positive control NMDA receptor antagonist all inhibit excitotoxic cell death to the same degree in organotypic hippocampal slice cultures.[31] This finding further implicated ferroptosis as a mechanism underlying excitotoxic cell death and demonstrated the therapeutic potential of ferroptosis inhibitors in treating neurological diseases.[31] Such excitotoxic cell death is a well-established mechanism in neonatal HIBI.[16,71,72] The role of iron-catalyzed ROS production in the pathophysiology of neonatal HIBI has also been described for decades. Early work revealed that neonatal animals experience iron overload in response to HIBI, the degree of which positively correlates with susceptibility to damage, and that iron chelators counteract this.[10,11,13] Further supporting these findings, a study in human neonates identified a positive correlation between the level of non-protein-bound iron (NPBI) in the plasma of newborns with asphyxia at birth and the severity of their neurodevelopmental deficits at 1 year of age. The levels of thiobarbituric acid reactive species, a marker of lipid peroxidation, were also elevated in infants with asphyxia compared to controls; although this effect was not statistically significant.[73] Another study found that NPBI is detectable in the cerebrospinal fluid (CSF) of neonates with HIE but not in unaffected controls. The levels of *ortho*- and *meta*-tyrosine, markers of ROS-mediated protein oxidation, were also notably elevated in the CSF of affected neonates compared to healthy controls.[74] Other studies have demonstrated that the neonatal brain is hypersensitive to the effects of iron overload. This can be explained by increased iron concentrations, which are highest at the time of birth due to iron's key role in developmental processes, coupled with incomplete development of iron processing and antioxidant mechanisms.[75-78] Ferroptosis may, therefore, play an even more important role in neonatal HIBI than in adult conditions of neurological ischemia-reperfusion.

To the best of our knowledge, the first study that directly investigated the role of ferroptosis, since it was described by name, in neonatal HIBI was performed by Wang *et al.*[44] in 2016. They found that anemia, induced by controlled blood loss, attenuates signs of brain damage after subsequent HIBI in neonatal rats. HIBI was modeled via bilateral carotid artery ligation, followed by exposure to hypoxic conditions. This result suggests that anemia may be neuroprotective against HIBI in neonatal rats. The authors then treated a subset of the anemic rats with iron, starting at 2 time points. Early iron treatment (beginning 1 day after HIBI) increased signs of brain damage, while late treatment (beginning 7 days post-HIBI) reduced them. The latter may be explained by the essential role of iron in neurodevelopment. Although these findings do not suggest high therapeutic potential of anemia in neonatal HIBI, the study supports involvement of ferroptosis in the pathophysiology of neonatal HIBI.[44] Another group provided more direct evidence of this role by measuring characteristic features and mediators

Table 1. Key primary articles on establishing the role of ferroptosis in neonatal HIBI

Paper	Models	Key findings	Contribution to establishing the role of ferroptosis in neonatal HIBI
Groenendaal *et al.*[10]	7.5-day-old lambs with severe hypoxia-ischemia	• Post-HIBI administration of DFO increased Na^+, K^+-ATPase activity, potentially indicating reduced free radical production as this ATPase is highly vulnerable to lipid peroxidation by free radicals. • Na^+K^+-ATPase activity was positively correlated with electrocortical brain activity, with higher levels in DFO- versus placebo-treated animals.	Suggests that HIBI in neonatal animals is associated with iron-mediated oxidative stress
Palmer *et al.*[13]	7-day-old rats with R–V method-induced HIBI	• Post-HIBI injection of DFO, which was previously determined to inhibit iron-mediated free radical activity, considerably reduced ipsilateral brain hemisphere water content and atrophy in neonatal rats.	Suggests that HIBI in neonatal animals is associated with iron-mediated oxidative stress
Dixon *et al.*[31]	OHSCs	• Fer-1, the iron chelator ciclopiroxolamine (CPX), and the NMDA receptor antagonist MK-801 all similarly inhibited excitotoxic cell death in OHSCs when cotreated with glutamate.	Demonstrates that ferroptosis is a mechanism underlying excitotoxic cell death
Wang *et al.*[44]	3-day-old rats with R–V method-induced HIBI	• Anemia attenuated signs of brain damage post-HIBI in neonatal rats. • Early iron treatment increased signs of brain damage, whereas late iron treatment reduced them in anemic rats.	Implicates iron-dependent ferroptosis as a mechanism underlying HIBI in neonatal rats
Tan *et al.*[51]	7-day-old rats with R–V method-induced HIBI	• Brain iron content was substantially elevated, accompanied by mitochondrial atrophy. • An iron-dependent increase in MDA and decreases in SLC7A11 and GPX4 levels were detected. • These effects were supported by *in vitro* experiments.	Strongly implicates ferroptosis as a key mechanism underlying neonatal HIBI
Cheah *et al.*[68]	Yeast two-hybrid analysis, rat pheochromocytoma (PC12) and human embryonic kidney 293 (HEK293T) cells, nNOS-knockout mice, primary cortical neurons	• Dexras1, a Ras protein, was activated by NMDA receptor–mediated activation of nNOS and promoted iron uptake by stimulating PAP7 to bind to the iron import channel DMT1. • Treatment with an iron chelator, salicylaldehyde isonicotinoyl hydrazone, eliminated NMDA receptor-mediated increases in hydroxyl free radicals and protected against neuronal death.	Suggests that NMDA receptor activation promotes iron accumulation, contributing to oxidative stress
Yu *et al.*[69]	Organotypic rat spinal cord cultures	• Threohydroxyaspartate (THA), an agent that induces excitotoxicity by inhibiting postsynaptic glutamate uptake, promoted iron accumulation and increased TFR and divalent metal ion transporter 1 expression, while decreasing ferritin expression in rat spinal cords. • Treatment with the iron chelator DFO blocked THA-mediated degeneration of motor neurons.	Provides evidence of iron accumulation-dependent glutamatergic excitotoxicity

(Cont'd...)

Table 1. ***(Continued)***

Paper	Models	Key findings	Contribution to establishing the role of ferroptosis in neonatal HIBI
Dorrepaal *et al.*[73]	Newborn human infants (healthy, moderate asphyxia, and severe asphyxia groups)	• Plasma NPBI levels were elevated in moderate and severe asphyxia groups, with more notable elevations in the severe asphyxia group compared to healthy controls. • Increased NPBI concentrations 0 and 8 h after birth were strongly correlated with neurodevelopmental deficits at 1 year of age. • TBARS levels were higher in the moderate and severe asphyxia groups than healthy controls.	Shows that neonatal asphyxia is associated with increased NPBI levels and oxidative stress, suggesting that the two may be related
Ogihara *et al.*[74]	Newborn human infants with HIE, healthy controls	• NPBI was detected in the CSF of infants with HIE, with its concentrations significantly correlating with the infants' Sarnat's clinical stages, but not in that of healthy controls. • Levels of *ortho*- and *meta*-tyrosine were also elevated in the CSF of infants with HIE, indicating oxidative stress.	Demonstrates that neonatal HIE is associated with detectable NPBI and oxidative stress, providing additional support for a potential link
Lin *et al.*[79]	7-day-old rats with R–V method-induced HIBI	• Signs of mitochondrial damage characteristic of ferroptosis, but not of other cell death types, were identified post-HIBI. • HIBI was associated with decreases in GSH, GPX4, and SLC7A11 levels and increases in ROS, TF, TFR, FLC, and FHC.	Provides strong evidence for the role of ferroptosis in neonatal HIBI

Abbreviations: CSF: Cerebrospinal fluid; DFO: Deferoxamine mesylate; FHC: Ferritin heavy chain; FLC: Ferritin light chain; GSH: Glutathione; GPX4: Glutathione peroxidase-4; HIBI: Hypoxic-ischemic brain injury; HIE: Hypoxic-ischemic encephalopathy; MDA: Malondialdehyde; MDA: *N*-methyl-D-aspartate; nNOS: Neuronal nitric oxide synthase; NPBI: Non-protein-bound iron; OHSC: Organotypic hippocampal slice culture; ROS: Reactive oxygen species; R-V: Rice-Vannucci; SLC7A11: Solute carrier family 7 member 11; TBARS: Thiobarbituric acid reactive species; TF: Transferrin; TFR: Transferrin receptor.

of ferroptosis in neonatal rats subjected to the Rice-Vannucci (R-V) method.[79] This is a well-established technique for modeling neonatal HIBI in rodents, which involves subjecting them to unilateral common carotid artery ligation on post-natal day 7, followed by hypoxia.[80] Using electron microscopy, the authors detected signs of mitochondrial damage, namely, atrophy and cristae loss, which are hallmarks of ferroptosis but not of other types of cell death. Furthermore, morphological changes consistent with these other types of cell death were absent. The authors also found that GSH, GPX4, and SLC7A11 levels were considerably reduced 72 h post-HIBI, while ROS levels were notably elevated. In addition, they noted HIBI-mediated elevation in TF, TFR, ferritin light chain (FLC), and ferritin heavy chain (FHC) protein expression. Overall, their study provides strong evidence for the role of ferroptosis in mediating HIBI in neonatal rats.[79]

Recently, an additional study helped establish this role.[51] The R-V method was again used to induce HIBI in neonatal rats.[80] This substantially increased brain iron content, which was associated with observable characteristics of ferroptosis, including mitochondrial atrophy, decreased SLC7A11 and GPX4 expression, and increased levels of malondialdehyde (MDA), a marker of lipid peroxidation.[51] Iron overload, induced by treatment with iron dextran, potentiated the increase in MDA, while treatment with deferoxamine mesylate (DFO), an iron chelator, attenuated the increases in MDA, indicating that the observed MDA elevation was iron-dependent. Iron dextran also exacerbated the observed HIBI-mediated decrease in SLC7A11. Furthermore, inhibition of ferroptosis through administration of either DFO or Fer-1 reduced observable brain damage on 2,3,5-triphenyltetrazolium chloride and Nissl staining, while iron dextran treatment increased it. These results were corroborated *in vitro*. Oxygen and glucose deprivation (OGD) reduced survival of cultured cells and increased MDA and ROS levels while decreasing levels of SLC7A11, GSH, and GPX4. All these effects were exacerbated by ferric ammonium citrate but inhibited by DFO or Fer-1. In addition, overexpression of SLC7A11, induced through plasmid transfection, suppressed ROS production and increased cell survival under OGD conditions, indicating inhibition of ferroptosis.[51] Overall, this literature strongly suggests an essential role of ferroptosis in neonatal HIBI, directly and indirectly. This role has also been discussed in reviews by Feng *et al.*,[66] Peeples *et al.*,[67] and Huo *et al.*[81] Thus, there is significant

therapeutic potential for antiferroptotic agents to treat HIBI and HIE in neonates.

4. Modulating activity of mechanistic targets of ferroptosis in neonatal HIBI

Several groups have investigated the utility of putative antiferroptotic agents in combatting neonatal HIBI *in vivo* and *in vitro*. NRF2 signaling has been a target of interest in three studies to date. One study[43] assessed the effects of resveratrol (Res), an agonist of silent information regulator factor 2–related enzyme 1 (SIRT1), in neonatal HIBI based on a previous report that SIRT1 upregulates NRF2 to protect against traumatic brain injury.[27] Consistent with other studies, the authors noted a decrease in GPX4 expression and an increase in iron and MDA levels in rats 24 h after HIBI was induced using the R-V method.[80] They also observed detrimental structural changes and characteristic signs of ferroptosis-related mitochondrial damage in hippocampal CA1 neurons. Functional impairments on the Morris water maze (MWM) were detected in association.[43] As expected, all these consequences were attenuated by intracerebroventricular (ICV) injection of Fer-1 30 min before HIBI. ICV injection of Res conferred similar neuroprotective effects: it increased SIRT1 and NRF2 levels, counteracted the HIBI-mediated decrease in GPX4, and reduced iron accumulation in response to HIBI. Furthermore, Res-treated animals showed reduced brain atrophy and structural damage, along with improved MWM performance. These findings suggest that Res inhibits ferroptosis by activating SIRT1/NRF2/GPX4 signaling. Overall, the study highlights the potential efficacy of this indirect NRF2 activator as a neuroprotectant against neonatal HIBI. There may also be utility in investigating other agents that upregulate SIRT1/NRF2/GPX4 signaling.[43] Another study investigated the impact of farrerol (FA), an anti-inflammatory and antioxidant agent used in traditional Chinese medicine, alone and in combination with ML385, a specific NRF2 inhibitor, on HIBI in neonatal rats.[12] The neonatal HIBI was again modeled using the R-V method.[80] FA decreased post-HIBI infarct volumes, as well as brain edema, neuron death, nucleus atrophy, and cytoplasmic abnormalities. It also reduced iron buildup and ROS and MDA levels, while increasing GSH-Px and superoxide dismutase (SOD) antioxidant enzyme levels. Furthermore, FA counteracted the HIBI-mediated decreases in SLC7A11 and GPX4 and increased levels of NRF2 and heme oxygenase-1 (HO-1),[82] which exerts antioxidant effects on NRF2 activation. FA also reduced behavioral signs of neurological deficits. The protective effects of FA were attenuated, and in some cases eliminated, by ML385, suggesting that they are mediated by NRF2/HO-1 signaling-induced suppression of ferroptosis. This study provides support for a potential role of FA and other agents that promote NRF2/HO-1 signaling in preventing neonatal HIBI.[12] The third study assessed the effects of Vitamin D on ferroptosis in rats with R-V method-induced HIBI.[83] The authors showed that Vitamin D reduces HIBI-associated edema, neuron loss and damage, and mitochondrial damage. It also prevents the post-HIBI increase in MDA and decreases in SOD, GSH, NRF2, and HO-1. These results were supported in cultured cells under OGD conditions as Vitamin D increased GSH expression and SOD activity, while preventing an increase in ROS. The authors used quantitative reverse transcription polymerase chain reaction on cells subjected to OGD to further investigate their hypothesis that Vitamin D upregulates NRF2/HO-1 signaling. They detected increased NRF2, HO-1, and GPX4 messenger RNA (mRNA) expression in Vitamin D-treated cells. Vitamin D also decreased the release of proinflammatory cytokines under OGD conditions. Overall, the authors concluded that Vitamin D inhibits ferroptosis in HIBI by activating NRF2/HO-1 signaling. It is noteworthy that although the focus of the study was neonatal HIBI, the rats used were 10 – 12 weeks old.[83] Further investigation is, therefore, necessary to determine the relevance of these findings in the neonatal setting. Overall, these studies highlight NRF2 as a promising target for therapeutic development.

Another study's findings centered on system x_c^- signaling. The authors compared the effects of ginsenoside Rb1 (GsRb1), an active ingredient in ginseng, to those of liproxstatin-1 (Lip-1), a known ferroptosis inhibitor, in neonatal rats with R-V method-induced HIBI.[47,80] Both agents decreased brain atrophy, liquefaction, cerebral infarct volume, and pathological structural changes 72 h later. They also similarly attenuated HIBI-induced increases in ionized calcium-binding adaptor molecule 1 and glial fibrillary acidic protein, markers of microglia and astrocytes, respectively; and reduced signs of mitochondrial damage. Furthermore, they counteracted decreases in SLC7A11, SLC3A2, glutathione synthetase (GSS, which helps catalyze GSH production), GSH, and GPX4, indicating protection against system x_c^- inhibition.[47] GsRb1 and Lip-1 also counteracted the decrease in HIF-1α, implicating HIF-1α activation as a potential starting point for these effects, due to the role of HIF-1α in upregulating system x_c^- expression.[47,58,59] The authors reproduced these results *in vitro* using cells subjected to OGD. Overall, the study illustrates the potential therapeutic utility of GsRb1 in protecting against ferroptosis-induced oxidative stress and inflammation in neonatal HIBI. Its antioxidant effects focus on the system x_c^- signaling pathway and may commence with HIF-1α activation.[47] Another study also implicated system x_c^- as a therapeutic target, while emphasizing the

contribution of inflammatory signaling to ferroptosis.[14] The authors assessed the impact of TAK-242, a specific antagonist of Toll-like receptor 4 (TLR4). TLR4 plays a key role in activating proinflammatory signaling in the nervous system and is, thus, activated in response to hypoxic-ischemic insult. The authors had previously identified TLR4 as a promising therapeutic target for neonatal HIBI and demonstrated the efficacy of TAK-242 in treating it.[84] In this study, they established a link between this effect and ferroptosis. Consistent with other groups, they used an R-V rat model[80] and an OGD cell model. In the rat model,[85] TAK-242 resisted HIBI-induced increases in TLR4, MDA, proinflammatory signaling molecules, and tumor protein p53 (a transcription factor that downregulates SLC7A11 expression). TAK-242, also, counteracted decreases in SLC7A11, GSH, GPX4, and SOD expression. Furthermore, it reduced signs of mitochondrial damage and mRNA expression of ferroptosis-related genes. It also improved performance on the MWM, indicating improvement of neurobehavioral outcomes. These results were verified *in vitro* as similar protective effects were observed with TAK-242 treatment before OGD in cells. SB203580, an inhibitor of the proinflammatory molecule p38 mitogen-activated protein kinase (MAPK), also demonstrated antiferroptotic effects *in vitro* by counteracting OGD-mediated decreases in SLC7A11 and GPX4 expression. This supports the authors' proposed mechanism that TAK-242 antagonizes TLR4, inhibiting p38 MAPK, which then suppresses p53, leading to SLC7A11 activation.[14,85-87] TAK-242 and other agents that inhibit inflammatory signaling and activate system x_c^- should be further investigated in this regard.[14]

A recent study explored the utility of modulating the lipid peroxidation component of ferroptosis.[88] The authors studied the effects of 7-dehydrocholesterol (7-DHC), a Vitamin D precursor, in primary neurons subjected to OGD and in neonatal mice with R-V method-induced HIBI.[80,88] Of all lipids, 7-DHC is most susceptible to oxidation; therefore, introducing it was intended to conserve the much less reactive membrane lipids. The authors used cariprazine (CAR), an agent that increases 7-DHC levels by inhibiting 7-DHC reductase (DHCR7), as it promoted cell survival with the highest potency among three such agents tested. CAR administration 48 h before or 30 min after OGD initiation enhanced primary neuron survival. *In vivo*, pre- and post-HIBI treatment with CAR increased brain levels of 7-DHC and reduced those of MDA. CAR treatment also prevented the HIBI-mediated decrease in viable brain tissue, but interestingly, this effect was only statistically significant with post-HIBI administration. These findings implicate CAR and other agents that inhibit lipid peroxidation by elevating 7-DHC as potential therapeutic candidates.[88]

In addition, the effects of Lip-1 as a ferroptosis inhibitor in neonatal HIBI were recently further evaluated in an R-V neonatal rat model.[80,89] Lip-1 stimulated signs of healing against HIBI-induced brain damage and counteracted the HIBI-related decreases in SLC7A11, GSH, and GPX4 and the increase in ROS. Moreover, it prevented the reductions in HIF-1α and NRF2 and the elevations in TFR, FHC, and FLC. These findings suggest that multiple mechanisms may be responsible for the antiferroptotic effects of Lip-1.[89]

Taken together, these studies suggest that NRF2, system x_c^-, and members of their signaling cascades, along with regulators of lipid peroxidation and iron metabolism, are key mediators of ferroptosis in neonatal HIBI and, therefore, therapeutically relevant targets. The potential mechanisms of action of the novel ferroptosis inhibitors in neonatal HIBI models are summarized in Figure 2. Research is still required to establish these mechanisms and determine the efficacy and safety of the relevant agents in treating HIBI in human neonates. However, these preliminary *in vivo* and *in vitro* experiments show that ferroptosis inhibitors may represent the necessary class of mechanistically distinct therapeutic alternatives to TH.

5. Future directions

These preliminary findings open up numerous avenues for future research. First, the agents studied and mechanistically related ones should be investigated *in vitro* and *in vivo* to gain greater insight into their therapeutic potential. Ideally, they may then be progressed to clinical trials to help address the gap in available therapeutics. Challenges in therapeutic development may include off-target effects, blood-brain barrier impermeability, and plasma instability. However, the diverse nature of agents already tested creates hope that some may overcome these challenges. In addition, ferroptosis inhibitors currently in clinical trials for the treatment of other conditions should be considered for preliminary testing in neonatal HIBI models. These include bardoxolone methyl, an NRF2 activator being studied in patients with diabetes-associated chronic kidney disease, and mTORC1 inhibitors INK128 and AZD8055, which are being tested for pancreatic cancer and glioma, respectively. Such agents were reviewed by Scarpellini *et al.*[90] There may also be utility in testing antiferroptotic agents which have shown early-stage efficacy against other conditions. For example, in preliminary experiments, a Tat-conjugated selenocysteine peptide, an inhibitor of 15-LOX, and a spiroquinoxalinamine derivative protect against stroke, myocardial ischemia-reperfusion, and hepatic ischemia-reperfusion, respectively, all of which have been linked to ferroptosis inhibition.[39,41,91]

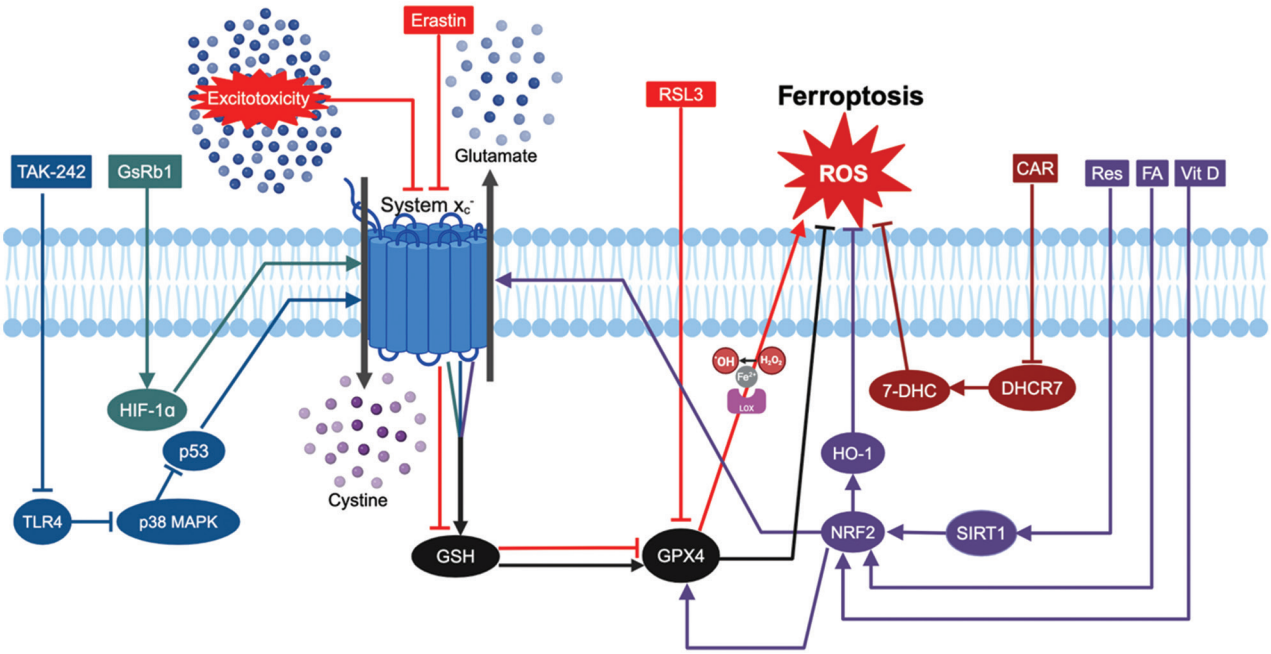

Figure 2. Potential mechanisms of action of ferroptosis inhibitors tested in neonatal HIBI models to date. GsRb1 activates system x_c^-/GSH/GPX4 signaling to suppress ferroptosis and increases HIF-1α levels, suggesting that HIF-1α may be its initial target. Res activates NRF2 signaling via SIRT1, and FA and vitamin D directly activate NRF2, inhibiting ferroptosis through activation of system x_c^-, GPX4, and HO-1. TAK-242 inhibits the TLR4/p38 MAPK/p53 signaling cascade, which typically suppresses system x_c^-/GSH/GPX4 signaling to promote ferroptosis; TAK-242-mediated inhibition protects against this. CAR suppresses ferroptosis by inhibiting DHCR7 to increase 7-DHC levels, reducing membrane lipid peroxidation. Novel inhibitors are shown in rectangles, while members of relevant signaling pathways are shown in spheres. Arrows represent activation, and blunt-ended lines represent inhibition. Notably, these potential mechanisms have not yet been well-established, and this is a simplistic representation of their effects. The central pathway of ferroptosis is also shown, in which excitotoxicity, erastin, or RSL3 inhibit system xc–/GSH/GPX4 signaling, promoting membrane lipid peroxidation through iron-dependent Fenton-like reactions and LOX enzyme activity. This results in ROS production and ferroptotic cell death. Figure was created with BioRender.com.

Abbreviations: 7-DHC: 7-dehydrocholesterol; CAR: Cariprazine; DHCR7: 7-dehydrocholesterol reductase; FA: Farrerol; GSH: Glutathione; GPX4: Glutathione peroxidase-4; GsRb1: Ginsenoside Rb1; HIBI: Hypoxic-ischemic brain injury; HIF-1α: Alpha subunit of hypoxia inducible factor-1; HO-1: Heme oxygenase-1; LOX: Lipoxygenase; MAPK: Mitogen-activated protein kinase; NRF2: Nuclear factor erythroid 2–related factor 2; Res: Resveratrol; ROS: Reactive oxygen species; RSL: Ras-selective lethal; SIRT1: Silent information regulator factor 2-related enzyme 1; system xc–: Cystine/glutamate antiporter.

6. Conclusion

Ferroptosis, a novel form of cell death involving iron-dependent ROS accumulation, plays an essential role in neonatal HIBI. This finding may explain the limited efficacy of TH – the standard of care – which potentially targets apoptosis.[8] Ferroptosis occurs through complex signaling mechanisms, including the central system x_c^-/GSH/GPX4 pathway and pathways that regulate iron metabolism and lipid peroxidation. Several additional signaling molecules, including HIF-1α, NRF2, ACSL4, LPCAT3, and mTORC1, also contribute to ferroptosis regulation. Some such signaling molecules have been identified as potential therapeutic targets in preliminary experiments because altering their activity appears to protect against ferroptosis. The mechanisms and effects of the putative ferroptosis modulators should be further delineated to address the urgent need for alternative therapeutics to reduce the burden of neonatal HIBI and HIE.

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Conflict of interest

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Liquid–liquid phase separation in neurodegenerative diseases: An updated understanding

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Abstract

Liquid–liquid phase separation (LLPS), once understood merely as a physicochemical phenomenon, has emerged over the past decade as a critical player in life processes. LLPS provides a novel framework for understanding the structure, function, and spatiotemporal regulation of intracellular biomolecules. Through LLPS, biomolecules within cells can spontaneously assemble into membraneless compartments, which allow precise regulation of biochemical reactions and influence critical cellular processes such as signal transduction and gene expression. Despite its recognized significance in basic biological research, the role of LLPS in human disease is still an area worthy of continued exploration. Currently, the most studied disorders in relation to LLPS are neurodegenerative diseases and cancer. In neurodegenerative diseases, LLPS is closely linked to protein misfolding and aggregation, processes that can lead to the formation of toxic assemblies, ultimately causing neuronal damage and death. In cancer, aberrant LLPS may contribute to the dysregulation of signaling pathways, promoting uncontrolled cell proliferation and metastasis. This review highlights recent advances regarding the role of LLPS in the pathogenesis of neurodegenerative diseases, discussing its function in these pathological conditions and proposing directions for future research. As research progresses, the potential role of LLPS in other human diseases will likely be uncovered, offering new avenues for diagnosis and therapy. Therefore, further investigation into the mechanisms of LLPS and its involvement in disease pathology will be crucial for advancing our understanding of human health and disease.

Keywords: Liquid–liquid phase separation; Neurodegenerative diseases; Protein aggregation; Signal transduction; Gene mutation

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1. Introduction

Liquid–liquid phase separation (LLPS) is a ubiquitous phenomenon in biological system that drives the formation of membraneless compartments through the phase separation of biomolecules such as proteins, nucleic acids, and polysaccharides like glycogen. These compartments often referred to as condensates, arise from multivalent, weak, and transient interactions between macromolecules, including protein-protein, protein-RNA, and RNA-RNA interactions. LLPS is particularly enriched in intrinsically disordered regions (IDRs) or low-complexity domains (LCDs) of proteins, which lack a stable secondary or tertiary structure, making them highly flexible and prone to phase separation. These regions enable interactions such as π–π stacking, dipole–dipole interactions, cation–anion interactions, and π–cation interactions, which collectively facilitate the reversible formation of liquid-like droplets.[1,2]

LLPS plays important roles in neuronal signaling, regulation of transcription and translation, ribosome biogenesis, oxidative stress, and other physiological functions of the cell. Neuronal degeneration and death are characteristic features of neurodegenerative diseases. Although the pathogenesis of different types of neurodegenerative diseases varies, a growing number of studies indicate that LLPS plays a key role in the disease process. In neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), LLPS has been increasingly implicated as a key player in pathological protein aggregation and cellular dysfunction. Proteins that normally undergo LLPS to form functional, dynamic condensates can transition into aberrant solid-like aggregates under pathological conditions. This transition is driven by mutations, post-translational modifications, or environmental stress, leading to the formation of amyloid fibrils or toxic protein aggregates characteristic of neurodegenerative disorders.[1,3,4]

The development of neurodegenerative diseases from the perspective of LLPS has become an important research topic. LLPS is believed to regulate the spatiotemporal organization of signaling pathways and gene expression in neurons and its dysregulation can contribute to disease pathogenesis. Understanding how LLPS influences signal transduction, gene stability, and condensate modulation could provide new therapeutic targets for preventing or reversing the aggregation of disease-related proteins. This review aims to delve into these key areas – protein aggregation, signal transduction, gene stability, and condensate modulation – and explore how LLPS contributes to both normal cellular function and disease states, providing a comprehensive overview of the emerging role of LLPS in neurodegenerative diseases (Figure 1).

2. Protein aggregation

Irreversible protein aggregates are an important pathological feature of neurodegenerative diseases. Through phase separation, biomolecules can be reversibly separated from the solution into a dense and dilute phase. The material properties of droplets assembled by LLPS evolve over time. Usually, droplets "age" over time, characterized by a decrease in droplet dynamics, gelation, or vitrification.[5-7] There are multiple lines of evidence that aging can transform dynamically reversible droplets into irreversible solid amyloid fibrils.[8,9] The physicochemical characteristics of LLPS are highly suggestive that it could act as a key link in the pathophysiological process of protein aggregation. An increasing number of studies have demonstrated that pathological marker proteins for neurodegeneration, such as 43 kDa TARDNA binding protein (TDP-43),[10,11] fused in sarcoma (FUS),[12] Tau,[13,14] Aβ,[15] Huntingtin[16] and α-synuclein (α-Syn),[17,18] share certain similarities. LLPS is found to be a critical stage before their aggregation.[5,10-13,17,19] Through LLPS, the local concentration and volume crowding of biomolecules such as proteins and RNA within the condensate phase is significantly increased, which greatly increases the probability of nucleation and the risk of fibrillar aggregate formation. Moreover, neurodegenerative pathological mechanisms such as disease-related mutations, stress, and decreased proteostasis can also significantly affect phase separation and abnormal phase transition dynamics, which can have an impact on neuronal dysfunction and neurotoxicity.[12,20-24] For example, LLPS-mediated reversible

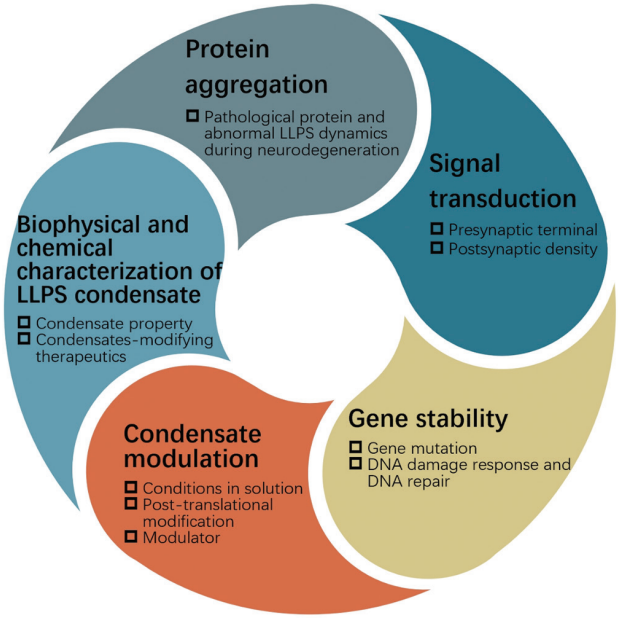

Figure 1. Updated understanding of LLPS in neurodegenerative diseases
Abbreviation: LLPS: Liquid-liquid phase separation.

droplet-like nucleosomal TDP-43 nuclear bodies (NBs) co-localize with nuclear speckles, and the formation of TDP-43 NBs in mammalian and *Drosophila* neurons attenuates TDP-43-mediated cytotoxicity. In contrast, the ALS-associated TDP-43-D169G mutant impairs lncRNA NEAT1-mediated LLPS, causing excessive cytoplasmic translocation of TDP-43 and assembling into stress granules (SGs). Dynamic impairment under prolonged stress promotes the accumulation of pTDP-43 cytoplasmic lesions.[25] ALS/frontotemporal dementia (ALS/FTD)-associated mutations S96del or G156E in *FUS* LCD alter the properties of LLPS droplets and increase the propensity for an irreversible fibrillar hydrogel state. Ribonucleoprotein (RNP) particle function is critical for intracellular transport, RNA metabolism, and local protein synthesis. The assembly of irreversible RNP granules containing mutant FUS interferes with RNP granule function, which in turn leads to neurotoxicity.[20]

3. Signal transduction

Synapses are the molecular devices for processing and transmitting signals between neurons and represent the basic units of neural networks. Higher-level activities, such as learning and memory, are inextricably linked to synaptic plasticity. Impaired synaptic plasticity is therefore closely related to neurodegenerative diseases. For example, abnormal synaptic plasticity is one of the causes of Parkinson's disease and Huntington's disease. One of the pathological features of Alzheimer's disease is the loss of synapses, which is accompanied by cognitive decline.[26-28] Synapses have very delicate and complex structures and change their molecular composition and signal transduction instantly in response to different stimuli, requiring rapid protein transport and spatiotemporal coordination of cells. LLPS mediates synaptic assembly and participates in synaptic physiological functions, thus actively participating in the regulation of synaptic plasticity and local protein synthesis.[29-31] The biophysical properties of LLPS indicate that they are sensitive to small changes in the environment and proteins, and allow rapid material exchange with solvents. An increasing number of studies have shown that synaptic structures contain various biological condensates, such as active zone condensate and post-synaptic density.

3.1. Pre-synaptic terminals

The function of the pre-synaptic active zone is crucial for rapid neurotransmitter release and well-regulated synaptic vesicle recycling.[32] LLPS is involved in the functional regulation of pre-synaptic active zone proteins. Synaptic scaffold proteins in pre-synaptic terminals (*e.g.*, liprin-α, Rab3 interacting molecule [RIM], and RIM binding protein [RIM-BP]) form active zones for neurotransmitters, which are essential in synaptic transmission. However, the exact mechanism of active zone assembly remains unclear. Protein–protein interactions enable liprin-α to act together with active zone scaffold proteins. Oligomerized liprin-α mediates ELKS and RIM/RIM-BP LLPS through protein polyvalent interaction, and determines compartmentalization of protein distribution, realizing the integrated function of active zones.[33]

Synaptic proteins crosslink synaptic vesicles and bind them to the cytoskeleton within the resting pre-synaptic terminals. Synaptic proteins can aggregate synaptic vesicles at pre-synaptic terminals through an LLPS-mediated mechanism.[34] Ca^{2+}-activated calmodulin-dependent protein kinase (CaMK) II phosphorylates synaptic proteins, reducing the interaction of synaptic proteins with synaptic vesicles and the cytoskeleton, and facilitating the process of vesicle release. The purified synaptic proteins undergo LLPS *in vitro*. Synaptic protein condensates can capture liposomes and disperse by CaMKII phosphorylation.[34] Voltage-gated Ca^{2+} channels (VGCCs) bind directly to RIM and RIM-BP through C-terminal tail mediation, and can be enriched into RIM and RIM-BP condensates. RIM and RIM-BP aggregate VGCCs into nano- or microdomains on the lipid membrane bilayer through LLPS, and locate the Ca^{2+} channels and Ca^{2+} sensors on the docking vesicles to achieve efficient and accurate synaptic transmission.[35] LLPS at the synapse may be envisaged for RIM–RIM-BP–VGCC aggregates in pre-synaptic active zones,[36] which will be a form of communication between membraneless organelles and membrane-associated organelles.

3.2. Post-synaptic density

The post-synaptic density (PSD) is located beneath the post-synaptic membrane of each synapse. The function of the PSD in receiving, amplifying, and storing pre-synaptic cellular signals is essential for neuronal activity. The PSD consists of hundreds of concentrated proteins. The main components of PSD are scaffold proteins PSD-95, Shank3, GKAP, and Homer. PSD is characterized by a dense protein component bound to the dendritic spine cytoplasm on one side of the post-synaptic plasma membrane and exposed on the other side, which are dense subcellular compartments not surrounded by lipid membranes.[36] LLPS of synaptic scaffold protein and neurotransmitter receptor interactions may be involved in the formation of PSD assembly.[37,38] Dendritic spines and PSD are highly dynamic, and the volume of dendritic spines is closely related to synaptic strength. This corresponds to the property of LLPS that the PSD condensate can be dynamically enlarged or contracted to facilitate the addition or removal of new proteins.

A number of specific protein–protein interactions mediate the organization of the PSD, constituting functional post-synapses. PSD-95 interacts with various proteins, such as the C terminus of transmembrane AMPA receptor (AMPAR) regulatory protein (TARP).[39] AMPARs are the major receptor group at excitatory synapses. TARPs are a family of auxiliary subunits of AMPARs that are essential for ion channel transport and synaptic transport. Stargazin (Stg) is the first TARP identified.[40] Stg promotes the formation of condensate from LLPS through the multivalent interaction of PDZ-binding motif and arginine-rich motif with PSD-95. LLPS is necessary for the efficient binding of AMPARs to synapses and may be a mechanism of AMPAR synaptic aggregation and capture. Charge-neutralizing mutations in the TARP C-terminal arginine-rich tail motif attenuate TARP aggregation with PSD-95, impairing TARP-mediated AMPAR synaptic transmission in mouse hippocampal neurons.[36,41]

SynGAP is a GTPase-activating protein (GAP) that is highly enriched in the dendritic spines of excitatory neurons. SynGAP catalyzes the conversion of small G proteins from GTP-binding to GDP-binding form and is an inhibitor of synaptic activity, with a profound impact on synaptic plasticity. Shortly after LTP induction, CaMKII is activated, followed by the activation of small G proteins. However, its cellular mechanism remains unclear.[42] The C-terminal domain of SynGAP-α1 contains a class I PDZ ligand sequence, which can bind to PSD-95.[43] SynGAP forms curly helix trimers that bind to multiple copies of PSD-95, and this polyvalent interaction of SynGAP/PSD-95 drives the formation of LLPS *in vitro* and in living cells. Importantly, the formation of SynGAP/PSD-95 condensates is critical for SynGAP stabilization in the PSD. This is essential to prevent neuronal overexcitation.[38] *In vitro* reconstruction reveals that after the addition of other PSD proteins (GluN2B, GKAP, Shank, and Homer),[37] the physiological concentration of spontaneous LLPS in SynGAP/PSD-95 becomes lower.

4. Gene stability

Many diseases, such as cancer or neurodegenerative diseases, are caused by genetic or epigenetic changes that lead to defects in the acquisition of functions or to the loss of functions.

4.1. Gene mutation

Defects in functional acquisition are likely to be associated with condensates, as mutations in phase-separated proteins can affect not only the protein itself but also many other proteins in the condensate, leading to changes in the overall properties of the condensate. Mutations in RNA-binding protein (RBP) associated with neurodegenerative diseases accelerate droplet aging and alter RNP particle dynamics, such as FUS, TDP-43, and hnRNPA1. Many mutations occurring at the prion-like disorder regions of these proteins may alter their conformation.[19,20,44,45] These mutations may eventually lead to amyloid aggregation by promoting protein interactions.[46,47] Disease-related mutations tend to reduce droplet mobility and accelerate fibrosis of RBP droplets, and even form irreversible pathological fibers. It has been shown that disease mutations in protein LCD generally promote phase separation and reduce droplet dynamics and that disruption of the physiological balance of this process may lead to pathological behavior of membraneless organelles associated with them.[48] However, disease-associated mutations do not necessarily alter LLPS kinetics, nor do all mutations accelerate the transformation of droplets into proteopathic aggregates.[19,49]

LLPS is a critical step before RBP aggregation. Recent studies have revealed that in addition to increased local protein concentration in the cohesive phase, irreversible aggregation can be triggered during LLPS by the key factor of RBP conformational changes; for example, Tau conformational changes may be a key factor for irreversible aggregation of pathogenic mutants such as P301L and P301S.[24] Recent studies have also shown that RING-in-between-RING-type E3 ligase TRIAD3A(RNF216) is an E3 ubiquitin (Ub) ligase of the RING-in-between-RING (RBR) class, an important gene in neurodegenerative diseases, forms droplets and accumulates Tau protein in them for autophagic degradation. Animal studies have shown that TRIAD3A reduces the accumulation of phosphorylated tau, and disease-associated mutations in TRIAD3A affect tau protein homeostasis by causing glial cell proliferation and exacerbating pathologic tau accumulation and spreading.[50] hnRNPA1 G304Nfs*3 mutation in prion-like domain (PrLD) removes several effective spatial zippers, reduces PrLD multivalency and leads to reduced fibrosis and more rapid SG disassembly.[51] hnRNPA2 tyrosine to glutamate mutation reduces the probability of spatial zipper formation. Disease mutations D290V and P298L may greatly reduce the probability of *in vitro* aggregation.[52]

Mutations in RNA-binding proteins can also lead to loss of RNA-binding protein function or altered cohesive properties by affecting protein-RNA binding. For example, it is widely observed that TDP-43 cohesions have apparently hollow cores in RNA-binding-defective mutants of TDP-43, such as the acetylation-mimicking mutant TDP-43^{K145Q,K192Q}, and three-dimensional live-cell imaging reveals that these droplets often merge into larger droplets.[53] Recent studies reveal through real-time imaging of living animals that RNA binding defects and post-translational modifications can lead to aberrant

condensate and compartmentalization alterations of TDP-43 and that these altered condensation properties may be responsible for the change of liquid condensates into solid aggregates.[54]

Mutations in genes that do not encode RNA-binding proteins are also involved in LLPS. Neuronal hypofunction and decreased numbers in neurodegenerative diseases are closely associated with decreased protein homeostasis. The ubiquitin-proteasome system is an important component of protein quality control. Disease-associated mutations can significantly affect cohesive assembly, droplet dynamics, and protein homeostasis.[55] ALS-linked genes encoding the shuttle protein ubiquilin-2 (UBQLN2) with missense mutations P497H, P497S, and P506T, among others, impair droplet dynamics and show a propensity for amyloid aggregation.[56,57]

5. DNA damage response and DNA repair

DNA damage response and DNA repair defects are closely related to neurodegenerative diseases. Progeria syndrome, for example, is caused by defects in DNA repair genes. In addition, the accumulation of DNA damage has been observed in the brains of Alzheimer's disease patients and in the spinal cord of ALS and FTD patients.[58,59] However, it is unclear whether defects in DNA repair foci are a cause or a consequence of neurodegenerative disease. Recent study has shown that the condensate formed by LLPS is important for the DNA damage response. DNA repair foci can be assembled by DNA repair proteins through LLPS. Inhibition of poly (ADP-ribose) polymerase (PARP) prevents the assembly of DNA repair lesions, leading to a neurodegenerative phenotype in motor neurons of ALS patients, such as reduced organelle transport.[60] *In vitro* reconstruction of PARP-1/PAR/DNA system, FUS and PARP1 were observed to be located at the site of DNA double-strand breaks after induced DNA damage. Mesoscale DNA repair foci can be organized by transient compartmentalization of LLPS and are rapidly dissolved after PAR glycosylation.[61] In yeast, Rad52 DNA repair protein assembles into droplets through LLPS at DNA damage sites through the action of petite DNA damage-inducible intranuclear microtubule filaments (DIMs) and fuses to repair center droplets. Larger droplets concentrate tubulin and project short aster-like DIMs. With LLPS, the repair center and the longer DIM can be connected to mediate the movement of damaged DNA toward the periphery of the nucleus for repair.[62]

6. Condensate modulation

Condensates or droplets are highly dynamic. The absence of a membrane envelope allows them to undergo rapid material exchange. The condensates can quickly respond to small changes in solution to assemble, disassemble, change material properties, etc. Small changes in temperature, pH, osmotic pressure, macromolecular crowding, and other conditions in solution can significantly modulate LLPS.[5,63-65] For example, the C-terminal low-complexity domain of TDP-43 (TDP43-LCD) drives LLPS and is an important component of TDP-43 pathological inclusions during neurodegeneration. TDP43-LCD LLPS is regulated by solution pH and salt. TDP43-LCD spontaneously forms hydrogels and continues to form irreversible amyloid aggregates at low heparin concentrations.[11]

Post-translational modifications (PTM) such as phosphorylation, methylation, acetylation, and guanylation can regulate cohesive assembly and disassembly by affecting multivalent interactions. Targeting PTM regulation may be a promising therapeutic strategy for neurodegenerative diseases. For example, serine/threonine/tyrosine phosphorylation affects charge-charge interactions. Arginine methylation alters volume, charge distribution, hydrophobicity, and hydrogen bonding potential.[63,66-68] Tyrosine phosphorylation of the ALS/FTD-associated protein hnRNPA2 glycine-rich LCD alters hnRNPA2 LLPS *in vitro*, blocks interactions with hnRNPF and ch-TOG, reduces aggregation *in vitro*, and downplays neurodegeneration in the *Cryptobacterium hidradi* model.[52]

Modulators such as RNA, proteins, metal ions, and small molecule compounds have been demonstrated to be extensively involved in LLPS regulation.[69-72] RNA has a high charge density and is an important regulator of condensates in the complex network of intracellular interactions. RNA exerts synergistic or antagonistic effects on RBP phase separation.[73] Studies have elucidated that a lower RNA: protein ratio promotes LLPS, while a higher ratio appears to inhibit LLPS.[74] The human prion protein (PrP) is associated with infectious neurodegenerative diseases. A disease-associated stop codon mutation Y145Stop in PrP residue 145 results in a highly disordered N-terminal IDR. The material properties of its spontaneously assembled condensates are regulated by RNA.[75] Low RNA concentrations promote LLPS, while LLPS is eliminated at high RNA concentrations. A recent study showed that the addition of RNA to α-Syn-PrP condensates weakens α-Syn-PrP interactions and disrupts ordered structural domains, preventing LLPS.[64] These studies suggest that the critical buffering effect of RNA on aberrant phase transitions may be a potential mechanism for inhibiting neurotoxicity. Complex interactions between RNA, PTM, and molecular crowding regulate the formation and function of Tau condensates.[76,77] RNA

can bind competitively to tau. Studies have demonstrated that the tau: RNA ratio regulates the formation of protein droplets from tau and RNA, which is a reversible and sensitive process.[78]

PARP has also been proven to be a driver of neurodegenerative diseases. PARP can synthesize the RNA-like polymer poly (ADP-ribose) (PAR) through NAD^+. PARs have been found to be enriched in condensates. A recent study revealed that PAR length determines the threshold and physical properties of protein condensates. When the PAR chain length is greater than 8 units, the extent of LLPS increases with increasing chain length. Moreover, a concentration three orders of magnitude below the concentration of RNA-induced condensation (1 μM) triggers FUS condensation.[79] 14-3-3ζ is an isoform of the 14-3-3 protein family. There is growing evidence that 14-3-3 proteins are involved in Alzheimer's disease pathology. A recent study has revealed a modulatory role of 14-3-3ζ on LLPS. 14-3-3ζ promotes tau LLPS through electrostatic and hydrophobic interactions between the proline-rich domain and the microtubule-binding domain of tau, whereas the interaction between the disordered C-terminal tail of 14-3-3ζ and the N-terminal domain of tau negatively regulates tau LLPS.[80]

7. Biophysical and chemical characterization of LLPS condensate

Biophysical and chemical properties of LLPS condensates, such as viscosity, polarity/solvation, interfacial tension, and redox state, are critical to understanding their function within the cell and are increasingly being discussed in the context of disease relevance. Viscosity is a defining feature of LLPS condensates. These condensates can range from highly viscous, gel-like states to more dynamic liquid states. This property is crucial as it influences the condensates' ability to regulate intracellular reactions, allowing selective retention or diffusion of molecules. The dynamic behavior of condensates also enables rapid assembly or disassembly in response to environmental changes, which is crucial in processes such as stress response and gene regulation.[81] Polarity and solvation properties determine the affinity of the condensate for different molecules and affect the accumulation and release of molecules in the condensate, allowing the molecules to form transient, reversible interactions that lead to phase separation. Polar and hydrophobic interactions govern the assembly of biomolecules within the condensate.[82,83] The formation of condensates is influenced by interfacial tension, which results from variations in solubility and molecular composition between the condensed and surrounding phases. High interfacial tension establishes a clear boundary between the phases, impacting the way condensates interact with other cellular structures. For instance, interfacial tension can affect the merging of droplets or their interactions with membrane-bound organelles.[84,85] While the redox state may affect the activity and interaction of proteins in the condensate and interactions, the occurrence of oxidative stress, which is common in neurodegenerative diseases, impacts phasic behavior.[86] The properties of *in vivo* condensates vary widely and are affected by the surrounding environment. The state of the condensate is constantly changing. In neurodegenerative diseases, LLPS droplets can transition to a more solid state over time or due to mutations. Therefore, it is important to investigate whether it is possible to reverse this process by altering the material properties of the condensate. Several theoretical models describe and predict LLPS, but these need to be supported by empirical evidence.[87]

Condensates in cells have various functions, such as regulating transcription, signaling, and maintaining protein quality. Abnormalities in condensates are linked to a wide range of diseases. Therefore, new therapeutic approaches can be developed by altering the properties of condensates. For instance, using small molecule drugs or other biomolecules to control the formation and dissolution of condensates can impact the progression of a disease. This method has the potential to precisely target disease-related biomolecular condensates without affecting similar condensates in the cell, thus reducing potential side effects. A recent study utilized optogenetics to manipulate the material properties of transcription factor condensates, discovering that the material properties of condensates influence their ability to activate target promoters. Transcription factors in relatively liquid condensates were found to be associated with increased gene expression, while those in relatively solid condensates had the opposite effect.[88] The strength of intermolecular interactions in LLPS is intricately linked to the thermodynamic stability and viscoelasticity of the condensate. Researchers have demonstrated that by manipulating the phase behavior of light-activated assembly, they can precisely tune the composition and material properties of the condensate. When the condensate solidifies, it temporarily inhibits the translational activity of mRNA. In addition, modulation of condensate-based translational repression in neuronal cells can effectively influence downstream cellular activity. For example, isolating β-actin mRNA from neurons inhibits spine enlargement during chemically-induced prolonged enhancement.[89] This evidence emphasizes how the material properties of condensates modulate function and the potential intervenability of such modulatory mechanisms.

Biomolecular condensates bring a comprehensive perspective to developing new drugs and new therapeutics, especially in the therapeutic area of neurodegenerative diseases. Drug discovery strategies targeting LLPS condensates-modifying therapeutics (c-mods) are becoming a hot research topic. With the help of protein neighborhood tagging, advanced microscopy techniques, and computational methods, it is increasingly possible to explore the possibility of modulating the biological properties of specific biomolecular condensates to discover drugs for preventing or reversing disease. First, this approach requires accurate primary phenotypic screening models to assess condensate changes during disease processes. Second, intervenable c-mods need to be developed, a process that involves the specificity of this biological process. Ultimately, the target is detached or retained in the condensate by the material properties of the LLPS for functional intervention. Abnormal LLPS is often associated with protein aggregation in neurodegenerative diseases, and these aggregates may lead to cellular dysfunction or even cell death. By altering the scaffold, composition, conformation, and interaction landscape of condensates, their functions can be affected, thus providing new avenues for treating related diseases. For example, altering the scaffold of condensate can change the stability of the condensate, and some small-molecule drugs have been shown to interfere with structural interactions or protein-nucleic acid interactions to affect condensate assembly.[90,91]

The novelty and potential therapeutic efficacy of c-mods provide new directions for future drug discovery. During neurodegenerative pathologies, LLPS frequently exhibits some paradigm shifts, suggesting the significance and feasibility of our potential intervention through LLPS. However, this modification is also challenging, as disease development involves complex genetic and environmental factors. For specific disease mutations, intervention by targeting aberrant LLPS processes is a potentially powerful therapeutic tool, although it faces both great opportunities and challenges. Condensates are very sensitive to changes in the expression levels of their components and regulators, as well as to environmental changes, which poses challenges for phenotypic monitoring.[91] Effective phenotypic monitoring requires the maintenance of strict control over experimental and therapeutic conditions to ensure reproducibility of the observed effects. Any changes in condensate behavior, including their kinetics, material properties, or the functional outcomes of therapeutic interventions, must be carefully assessed to avoid inconsistent or misleading results. Furthermore, because condensates function at the interface of cellular signaling and metabolism, even minor shifts in their composition or physical properties may have profound implications for cellular function. This underscores the importance of developing robust and reliable monitoring systems that can accurately capture these subtle changes in real-time during therapeutic trials. While the therapeutic potential of c-mod strategies holds great promise, the path forward involves addressing these challenges to realize the full potential of targeting LLPS in disease management.

8. Summary

The newly discovered phenomenon of LLPS has gained significant attention as it integrates insights from a diverse range of disciplines, including medical biology, physics, chemistry, and engineering. This interdisciplinary approach has fostered a rapidly expanding body of research, revealing that LLPS plays a critical role in a variety of cellular pathophysiological processes. LLPS creates dynamic, membraneless droplets that can quickly exchange components with the surrounding nuclear or cytoplasmic environment. This ability for rapid assembly and disassembly in response to minor intracellular changes suggests that LLPS may serve as a key spatiotemporal regulatory mechanism for cellular components and biochemical reactions within the cell.

In the context of neurodegenerative diseases, current research predominantly focuses on factors such as aging, neuroinflammation, oxidative stress, mitochondrial dysfunction, brain metabolic imbalances, and impaired signal transduction pathways. Recent studies have uncovered that the multivalent interactions driving LLPS are widespread within cells and profoundly influence neuronal function on multiple fronts.[92-97] These include the regulation of gene expression, maintenance of chromatin structure and genomic stability, signal transduction processes, mitochondrial function, apoptosis, and the division of neural stem cells. These multifaceted roles position LLPS as a central player in maintaining neuronal health and underscore its potential significance in the pathogenesis of neurodegenerative diseases. Investigating how LLPS impacts disease mechanisms could open up new, innovative pathways for regulating and intervening in disease progression through the modulation of biomolecular condensates.

However, our understanding of LLPS is still in its infancy. Due to the differences between *in vivo* and *in vitro* environments, criteria for the validation of LLPS *in vivo* have not been established, and the biological role of LLPS *in vivo* has not yet been proven. There are also questions about the mechanisms regulating biomolecular

condensates, how disease-related mutations or epigenetic modifications affect the physical properties of condensates, and how LLPS can be regulated to achieve the desired effect in the treatment of neurodegenerative diseases. Currently, there is no universally accepted definitive test or "gold standard" for conclusively confirming that a process is driven by LLPS, and some methods for visualizing LLPS *in vitro* are prone to artifacts.[98-100] The use of high concentrations of *in vitro* is particularly problematic as many proteins will form LLPS in the presence of crowders at unphysiologically high concentrations.[101,102] Some molecular behaviors, such as short-lived, non-specific binding interactions, can also produce effects that appear like LLPS but arise from physical mechanisms that are inherently different in nature.[98] Still, the phase state of water inside and outside the cell is controversial.[103] The experimental conditions in the current study are highly biased and do not exactly match the physiological conditions. Therefore, the conclusions drawn from these studies may not be fully consistent with the function of *in vivo* phase separation. Further methods and tools for LLPS research should be developed in the future.

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Conflict of interest

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Cognitive assessment in acute care following traumatic brain injury: A scoping review of performance-based tests

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Abstract

It is recommended that the assessment of cognitive function by occupational therapists is best conducted by observing the performance of everyday tasks in real-life environments. Given the constraints of acute care settings, selecting the appropriate cognition assessment for patients with traumatic brain injury (TBI) can be challenging. A scoping review was used to explore the evidence for occupation-based assessments of cognition for use with patients with TBI and explore their clinical applicability for the acute setting. Assessments were included if they focused on performance-based tests using real-life or simulated activities of daily living (ADL) or instrumental ADL tasks. From 29 identified articles, 18 occupation-based assessments of cognition were reported for use in patients with TBI. They varied in terms of time and resources required to administer, complexity, and variety of assessments. This review highlights a range of assessments of cognitive function available to patients with TBI in acute care, which support occupational therapists to use an occupation-centered approach. Issues faced by occupational therapists assessing cognitive function in the acute care setting include time and environmental constraints. A small number of portable, contemporary, and performance-based assessments relevant to younger adults warrant further investigation to determine their feasibility for use in acute care.

Keywords: Traumatic brain injury; Functional cognition; Occupational therapy; Cognition; Assessment; Acute care

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1. Introduction

A traumatic brain injury (TBI) can be a life-changing event, affecting performance in multiple domains, including cognitive function with impairments commonly seen in attention, memory, and executive functions.[1] For patients who have had a severe TBI, difficulties in instrumental activities of daily living (IADLs), such as preparing a meal, using transport, house cleaning, and completing laundry, were reported to persist at 2 years post-injury for 90% of people.[2] IADLs learned in early adulthood require the integration of complex cognitive functions and are more likely to be affected by TBI.[3] Impaired IADL performance as a result of TBI negatively impacts a person's ability

to return to previously valued life roles as a worker or student and impacts community participation.[4] Therefore, observation of IADL performance can provide a useful means of assessing cognitive function.[2]

Some consider accurate assessment of cognition in acute care to be the starting point for rehabilitation, with the end goal of community participation.[5] However, cognitive impairments are often invisible, making them difficult to detect in acute stages post-injury.[6] An occupational therapist can infer cognitive impairments from performance errors observed during IADLs and make recommendations for rehabilitation.[7] Without occupational therapy assessment, functional implications of cognitive impairments may not be detected until a patient leaves the hospital and has difficulty with more complex activities in a less supportive environment. Therefore, one of the purposes of assessing cognition in acute care is to predict an individual's real-world functioning and consequently facilitate early rehabilitation to maximize rehabilitation.[8] This scoping review aims to explore occupation-based approaches for cognition assessment that may have clinical applicability for acute care TBI settings to provide clinicians with evidence-based assessment options.

2. Literature review

In occupational therapy practice, performance-based testing (PBT) refers to using an occupation-based approach for assessing actual performance against known or standard criteria to evaluate competency.[9] PBT can be an observation of an actual activity that a patient needs to perform in their daily life, such as using a phone to obtain information, or a simulation of a real-world task that may or may not be directly relevant to their current situation, such as filling a pillbox with medications for independent medication management. PBT allows a patient to respond to novel situations or interruptions, using whatever strategies they have available to them, as in real life.[10] PBTs include a range of assessments from brief screening tests to comprehensive evaluations, which measure a patient's occupational performance across various activities in home environments. Occupational therapists use PBTs to analyze and infer a person's functional cognition based on task performance, errors observed, and strategies used,[9] thereby informing rehabilitation plans.

When it comes to the assessment of cognitive function using PBT, the acute care environment presents additional challenges due to time constraints and other organizational or practical issues. Acute patients may not be able to leave the ward or even their bed, their medical status may fluctuate, and they may require a high level of nursing care,[11] making occupational performance assessment difficult or high-risk. The scope of occupational therapy practice in acute care settings can also be limited by organizational factors, such as funding models, which dictate the type and amount of occupational therapy provided. Occupational therapy in acute care practice may also be limited by access to physical resources, such as kitchens available for assessment purposes.[11] Therefore, the identification of appropriate PBTs for patients with TBI in acute care settings may be useful for clinicians.

A systematic review of cognitive assessments used in TBI research identified 728 assessments.[12] This large number makes it difficult for clinicians to decide which assessment to use with a patient.[12] A scoping review of assessments of executive functioning identified 12 tools suitable for acquired brain injury (ABI) patient populations, summarizing psychometric data, applicability, and the components of executive function that each tool addresses.[13] This study concluded that clinicians' decision-making should be based on information provided by research evidence, but may be affected by external pragmatic factors due to resource requirements.[13] A systematic review identified 21 assessments of functional cognition for older adults and found that evidence of their psychometric properties was lacking and that further research on existing assessments is warranted.[7] Similarly, a systematic review of PBT for TBI patients evaluating their measurement properties found that most instruments had limited or unknown evidence for validity and reliability.[14] While systematic reviews evaluating psychometric properties assist occupational therapists in making informed decisions, further information about clinical feasibility was recommended by authors to determine which assessments are recommended for clinicians.[14]

Clinical applicability refers to practical considerations of an assessment that impact on assessment choice for a clinician.[15] Applicability includes pragmatic qualities of assessments and is differentiated from commonly used terms, such as clinical utility, which includes psychometric properties (e.g., validity and reliability).[15] For the purposes of this review, applicability focused on assessment tool information available in the data, primarily relating to examiner burden, such as assessment availability, cost, specific resources, environmental requirements, and administration time. Descriptions of cognitive domains addressed, categorization of assessments, and information on underlying models or approaches all provide additional important information for clinicians.[15] Explicit information on an assessment's content and applicability may add further considerations for clinicians and enable comparison of assessments and informed decision-making. In addition

to the evidence on assessments of psychometric strengths, clinical applicability is an important factor to consider when evaluating assessments and is especially relevant to the acute care setting.

3. Methods

A scoping review was considered to be the most appropriate choice to facilitate an overview of the variety of cognitive assessments and associated concepts, describing existing research in the area and highlighting opportunities for future research. This scoping review followed the five-stage guidelines outlined by Arksey and O'Malley[16]: (i) identifying the research question, (ii) identifying relevant studies, (iii) selecting studies, (iv) charting the data, and (v) summarizing the results. Consideration of the psychometric properties of each assessment was not included in this review, as the aim was to conduct a broad overview of occupation-based assessments of cognition in acute care, PBTs, and pragmatic issues relevant to acute care.[17] Reviews of the psychometric properties of cognitive assessments for patients with TBI,[14] executive function, and ABI,[13] as well as performance-based assessments for older adults,[7] have been conducted previously. Although ratings of methodological quality are not typically completed in scoping reviews,[16] we included a quality appraisal tool, the quality assessment with diverse studies (QuADS), as it allows for appraisal of a broad range of study designs included in the scoping review.[18] The purpose of the QuADS tool is to offer supplementary information on the evidence presented, prompting further discussion by the reader.

3.1. Stage 1: Identifying the research question

Two main research questions were identified: (i) What cognitive assessments are available for occupational therapists to use in an acute care setting with patients who have had a TBI using performance-based approaches?; and (ii) What are the characteristics of available performance-based tests in relation to clinical applicability for acute care settings?

3.2. Stage 2: Identifying relevant studies

A comprehensive search of the following databases was initiated in September 2019, with no limits on date range imposed: PubMed, EMBASE, CINAHL, Cochrane Library, and PsycINFO. An example of the search strategy and search terms for PubMed is shown in Table 1. Manual searching of reference lists from included articles was also completed. The search strategy sought to identify research articles and grey literature, including textbooks, reference books, and theses published in English. The search was repeated in May 2020.

3.3. Stage 3: Selecting studies

Table 2 details the inclusion and exclusion criteria developed by the research team. Quantitative, qualitative, and mixed methodology studies were eligible for inclusion. Conference abstracts, posters, and critical appraisal articles were excluded from the study. Titles and abstracts of articles were screened in the first instance by the first author. Articles were included if they: Focused on PBT, used an occupation-based approach, were suitable for use with adults with TBI, and were able to be completed within the physical constraints of an acute hospital ward. Following a discussion between the first and second authors and a review against inclusion criteria, the full text of the studies was reviewed by the first author. Discussions were conducted between the first and second authors with full-text articles until a final agreement on the articles to be included was reached. Articles were included if the assessments they documented were suitable for use with adults with TBI and were focused on PBT using real-life or simulated ADL or IADL tasks. Where it was unclear from the article whether an assessment was designed for use with patients with TBI, the author of the assessment was contacted for further information to determine eligibility.

3.4. Stage 4: Charting the data

Data on descriptive characteristics of the assessments were extracted from the articles by the first author and included: the name and origin of the assessment; a description of the assessment task; characteristics of the participant sample; proportion of participants with TBI; cognitive domains assessed; and where documented, any underlying approaches or models for each assessment. Assessments were charted against fundamental pragmatic factors, which included the availability of an assessment manual; cost and resources required; formal training required; and documented time required to administer the assessment.[15] The time required to score an assessment and document the findings was included, where published, by assessment authors. Data on descriptive characteristics were entered into a table by the first author and then checked by the second and third authors. Descriptive characteristics included the methodological quality of the heterogeneous articles reporting on each of the 18 assessments, using QuADS.[18] Data on each assessment's potential clinical applicability for acute care was interpreted by the first author initially, then reviewed by the second and third authors, with final adjustments made through discussion within the research team.

3.5. Stage 5: Collating, summarizing, and reporting results

Collated data were examined to identify commonalities and differences across assessments. Three assessment

Table 1. Example of search strategy for PubMed

Search strategy	Search terms
#4	((("functional cognition" OR "everyday cognition" OR "cognitive capacities" OR "cognitive impairment" OR "cognitive impairments" OR "cognitive deficits" OR "cognitive function") OR "Cognition"[Mesh]) AND ("assessment" OR "observation" OR "observational" OR "screening" OR "performance-based testing" OR "occupational therapy" OR "occupational therapists" OR "activities of daily living" OR "ecological assessment") AND ("Brain Injuries, Traumatic"[Mesh]) OR ("traumatic brain injury" OR "TBI" OR "head injury")))
#3	(("functional cognition" OR "everyday cognition" OR "cognitive capacities" OR "cognitive impairment" OR "cognitive impairments" OR "cognitive deficits" OR "cognitive function") OR "Cognition"[Mesh])
#2	("assessment" OR "observation" OR "observational" OR "screening" OR "performance-based testing" OR "occupational therapy" OR "occupational therapists" OR "activities of daily living" OR "ecological assessment")
#1	(("Brain Injuries, Traumatic"[Mesh]) OR ("traumatic brain injury" OR "TBI" OR "head injury"))

Note: No limits applied.

Table 2. Inclusion and exclusion criteria for articles

Inclusion	Exclusion
– Participants in the study to be aged 18 and over – Studies published in English – Evidence of published studies of assessment use with adults who have experienced TBI – Assessment able to be completed in an acute ward – Focus on performance-based testing of a real-life simulated ADL or IADL task to assess cognitive function – Assessment focused on functional cognition not isolated cognitive domains	– Assessments for patients with progressive neurological conditions or mental illness – Questionnaires, carer reports, clinician reports, or self-reports – Assessments unable to be used by occupational therapists – Global screening tests of cognition, for example, pencil-and-paper screening tests or electronic versions of global screening tests – Assessments that need to be conducted in the home environment or community setting, for example, requiring shops – ADL assessments where the focus is on ADL performance and not underlying cognitive deficits contributing to performance – Virtual-reality simulations of real-life ADLs or IADLs

Abbreviations: TBI: Traumatic brain injury; ADL: Activities of daily living; IADL: Instrumental activities of daily living.

groupings were identified based on the composition of the assessments: Those that were a single prescribed task; those with multiple prescribed tasks; and those that consisted of a variable number of tasks, where the assessment was individualized. Assessments were categorized according to their level of complexity and focused on either ADL or IADL tasks or both. Data were organized into tables, and assessments were documented against applicability criteria, underlying approach/model, cognitive domains addressed, and methodological and reporting quality of studies (QuADS). The QuADS tool consists of 13 criteria, with a possible rating score of 0 – 3 for each and a maximum total score of 39. Criteria of the QuADS include whether the study includes a discussion of theories and concepts that underpin the research, whether sampling was appropriate to achieve the aims of the study, and whether research strengths and limitations were critically discussed. Criteria are not weighted, and summary scores do not indicate a high or low quality of an individual study; instead, they serve to encourage discussion about the quality of reporting of individual studies,[18] in relation to the scoping review.

4. Results

The initial database search was conducted in August 2019 and supplemented by additional searches in May 2020 and hand-searching. After the removal of duplicates, articles were screened by title and abstract by the first author. Where the application of the criteria was unclear, discussion between the first and second authors occurred until an agreement was reached. Of the 120 articles selected for full-text review, 91 were excluded due to various reasons; for exmaple, if the study focused on PADLs only without reference to cognitive function, used global rehabilitation outcome measures, required access to a patient's own home/community shops, or involved tests completed in virtual reality. A total of 18 assessments (reported across 29 articles) were retained for analysis in this review. The preferred reporting items for systematic reviews and meta-analyses flow diagram is displayed in Figure 1.

The final 18 assessments included in the review are summarized in Table 3, which also features the original article for the assessment and key articles where the assessment was used relevant to patients with TBI. Summarizing the assessments in this format identified wide variation in assessments, cognitive domains, and underlying approaches. Among the 18 assessments included in this review, most were published by American authors.

Regarding participant characteristics, of the 18 included assessments, 12 had published studies where

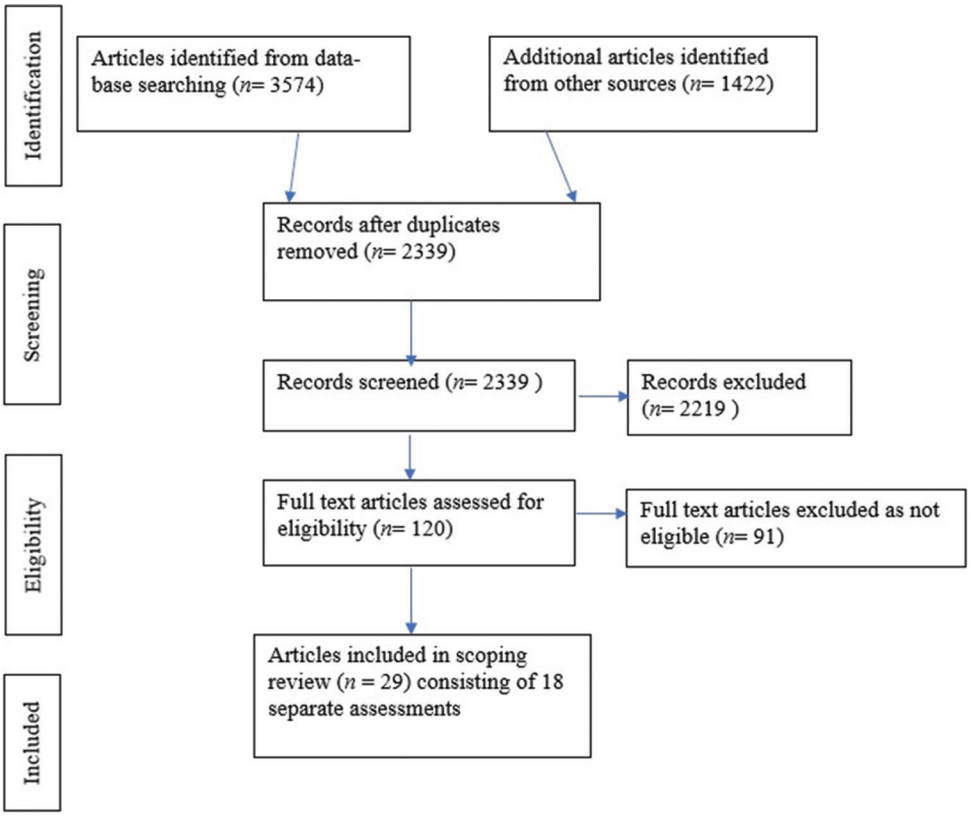

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram

TBI participants were a subgroup of a larger sample, and six studies were of TBI participants only. Information on mean ages for TBI participants is provided in Table 3 with the age ranges predominantly representing young adults. Time since injury, timing of assessment administration, and severity of TBI participants varied across the studies, making comparison difficult. TBI severity was not consistently defined or documented for all assessments, making it difficult to interpret the assessment's applicability to TBI subgroups, such as mild to moderate or severe TBI. The sample sizes were generally small, with seven of the studies with TBI participants having sample sizes of <30. The test of functional executive abilities[19] was an exception and had the largest TBI sample with 340 TBI participants.

Table 3 summarizes the results from the QuADS assessment tool, presenting details data on each study in relation to methodological and reporting quality. Each criterion in QuADS is rated from 0 to 3, with a total of 39 for each study. Scores on the appraisal tool ranged from 11 for the hotel task to 25 for the functional cognitive task. Appraisal of the included studies was intended to provide further information for the review but cannot be used to make conclusions regarding the quality of the studies.[18] As a recently developed tool, QuADS adds to the scoping review by providing a brief overview of the body of evidence for the included assessments.

Among the 18 assessments, eight were made up of a series of subtasks simulating real-world tasks. The naturalistic action test (NAT)[20] and the executive function and performance test (EFPT)[21] are examples of assessments with multiple subtasks. NAT[20] consists of three tasks; making toast with two spreads, wrapping a child's present, and packing a child's bag for school. In contrast, criterion-referenced assessments, such as the assessment of motor and process skills (AMPS)[22] and the perceive, recall, plan, perform (PRPP) system,[23] and use multiple tasks to determine an overall score or percentage. Eight of the assessments include tasks that test novel problem-solving as their means of assessment.

Factors influencing clinical applicability for acute care are summarized in Table 4. A wide range of time to administer tests was identified, with up to 3 h to administer the executive secretarial task.[24] Notably, the time to administer the assessment was not always documented, and the time to score and document assessments was not reported for any assessments. All assessments in the review had manuals available, some by contacting the assessment

Table 3. Description of included assessments

Assessment	Study relevant to the TBI population	QuADS Rating[iv] (x/39)	Description	Sample size and TBI subgroup	Mean age (SD) of the TBI subgroup	Cognitive domains assessed	Approach/model
Assessment of motor and process skills (AMPS)[29]	30	23	Evaluation of motor and process skills while the patient performs two activities from a list of 83 criterion-referenced PADL[i] and IADL[ii] tasks; sensitive to change in patients with severe TBI; able to be used during PTA[iii]	*N*=10 TBI=10 (severe to extremely severe TBI)	26.5 (6.9)	Sequencing, selecting, and using appropriate tools; adapting performance if problems encountered	Occupational therapy intervention process model[31]; WHO ICF framework,[32] model of human occupation[33]
Actual reality (AR)[34]	34	24	Assessment of patient's ability to use the internet to purchase a set item (cookies) on a pre-determined website	*N*=20 TBI=10	45.4 (9.3)	Computer skills; ability to follow instructions	Identified as a "top- down" performance-based test; no particular model identified
Executive function performance test (EFPT)[21]	35	20	Four tasks: prepare oatmeal using a stovetop, use a phone to obtain information, sort medications into a dosette box, and pay bills using checks; scoring based on the level of independence and assistance required	*N*=228 TBI=182 (mild/ moderate: 83; severe: 99)	Mild/moderate: 44.2 (2.05); severe: 34.9 (1.44)	Executive functions that mediate goal-directed activity, including initiation, execution, and completion	Person-environment-occupation-performance approach[36]
Executive function route-finding task (EFRT)[37]	37	24	Route-finding task: locating a specific office in a hospital campus as quickly and efficiently as possible	*N*=31 TBI=31	25.4 (range: 18 – 42)	Executive functioning skills, for example, problem-solving, flexibility, initiation, functional memory, self-monitoring, and error correction	Executive functioning by Lezak[38]
Executive secretarial task (EST)[39]	24	22	3-h task: work simulation of secretarial and general administrative tasks	*N*=92 TBI=35	44.4 (15.1)	Executive functioning skills, for example, self-management, initiation, organization, prioritization, and time management	Based on the multiple errands test[40]

(Cont'd...)

Table 3. ***(Continued)***

Assessment	Study relevant to the TBI population	QuADS Rating[iv] (x/39)	Description	Sample size and TBI subgroup	Mean age (SD) of the TBI subgroup	Cognitive domains assessed	Approach/model
Financial capacity instrument (FCI)[41]	42	19	Performance-based assessment of financial capacity; 18 tasks of specific and overall financial abilities (coin counting, simulated grocery purchases, and complex investment options)	*N*=44 TBI (moderate to severe) = 24	32.2 (13.4)	Evaluates cognition specific to financial capacity	No approach/ model documented
Functional cognitive task (FCT)[43]	43	25	Information gathering by phone (i.e., making phone inquiries to a council phone number); requires setup with a call center	*N*=171 TBI=46	40.68 (17.2)	Problem-solving, verbal communication, attention, memory, organization, planning, and initiation	Not documented
Functional Lowenstein occupational therapy cognitive assessment (FLOTCA)[44]	45	17	Three simulated functional tasks: navigate a map, organize tools, and plan a daily schedule; task completion and errors are recorded	*N*=50 TBI=25	25.12 (7.22)	Ability to follow directions, planning and spatial organization, time management, sequencing, prioritization, and decision-making	Cognitive functional evaluation model[46]
Functionally Simulated Technology Task (FSTT)[47]	48	17	Online bill-paying using simulated bills and credit cards to find and pay bills online; scoring for independence or cueing; a summary outcome score was obtained; this test assumes previous computer and internet competency	*N*=18 TBI=6	47 (range: 28 – 74)	Attention, memory, and problem-solving	Not described
Goal-processing scale (GPS)[49]	50	24	Choosing three activities and sourcing information on them using a phone and internet; patients are responsible for monitoring time	*N*=19 TBI=18	41.4 (12.9)	Planning, initiation, self-monitoring, sustained attention, sequencing, problem-solving, task execution, learning, and memory	No approach/ model documented

(Cont'd...)

Table 3. ***(Continued)***

Assessment	Study relevant to the TBI population	QuADS Rating[iv] (x/39)	Description	Sample size and TBI subgroup	Mean age (SD) of the TBI subgroup	Cognitive domains assessed	Approach/model
Hotel task (HT)[51]	51	11	Simulated hotel work tasks: preparing bills for guests, sorting coins, finding details on a menu, sorting name tags, and proofreading	*N*=10 TBI=9	32.1 (11.1)	Executive functioning skills, including planning, organization, self-monitoring, and flexibility	No approach/ model documented
Naturalistic action test (NAT)[20]	52	19	Four subtasks: preparing toast, making coffee, wrapping a present, and packing a child's school bag; standardized cueing and scoring; objects required for the tests are placed amongst distractor items; scoring based on steps completed and errors made	*N*=100 TBI=25	35.7 (12.7)	Comprehension of instructions, planning, problem-solving, attention, and initiation.	Developed as a simpler version of the multiple errands test[40] for use in a hospital; based on Luria's multiple object tests (MOTs),[53] modified to become the multi-level action test,[54] which later became NAT
Observed tasks of daily living-revised (OTDL-R)[55]	56	22	Three IADL tasks: answering medication questions, using a phone to source information, and paying a bill by check; provides a summary score of performance of everyday problem-solving	*N*=140 TBI=40	52.5 (17.4)	Everyday problem-solving	Based on a hierarchical model of everyday cognition[57]
Perceive, recall, plan, and perform system of task analysis (PRPP)[23]	58	15	Observation of a PADL or IADL task chosen by clinician and patient; scores patient's ability, task breakdown, and influence of the environment	*N*=16 TBI=7	45.2 (14.6)	Information processing components required for task, perception, recall, response planning, and performance; these four areas of cognitive processing are interrelated in this assessment	the underlying conceptual model is the information processing model from learning new tasks in the workplace[59]
The Rabideau kitchen evaluation-revised (RKE-R)[60]	61	18	Patient asked to prepare a meal and hot drink (i.e., a cold sandwich with two fillings and a hot drink); cueing and level of assistance were scored	*N*=34 TBI=34	30.3 (6.4)	Functional sequencing; test designed to evaluate client status over time	Based on Lezak's research[62] (i.e., if an individual can synthesize their skills to complete a task) and Trombly's scoring of meal preparation skills[63]

(Cont'd...)

Table 3. ***(Continued)***

Assessment	Study relevant to the TBI population	QuADS Rating[iv] (x/39)	Description	Sample size and TBI subgroup	Mean age (SD) of the TBI subgroup	Cognitive domains assessed	Approach/model
Systematic behavioural observation of executive performance[64]	64	20	Multi-step cooking tasks (preparing brownies and making a hot drink simultaneously); microwave used for brownies	*N*=27 TBI=10	49.1 (18.5)	Attention, initiation, planning, cognitive shifting, impulsivity, error correction, and time management	Informed by the WHO/ ICF model[65] for assessments in structured, natural environments
Test of functional executive abilities[19]	19	20	Information gathering task: using a phone, phone book, and map to find a hotel's address	*N*=340 TBI (mild to moderately severe) = 340	25.71 (6.65)	Cognitive linguistic abilities, staying on task, and time management; executive functioning using a dynamic approach	Based on the EFRT[37]
University of California San Diego performance-based skills assessment brief (UPSA-B)[66]	67	15	Brief version of the assessment battery includes calling to source information for an outing and reschedule an appointment; money management and bill payment tasks using checks	*N*=50 TBI (mild to moderate) = 50	31.76 (7.27)	Executive functioning, auditory comprehension, and recall, planning, memory, and functional math abilities	No approach/ model documented

Note: [i]Personal activities of daily living; [ii]Instrumental activities of daily living; [iii]Post-traumatic amnesia; [iv]Quality assessment with diverse studies.

author. Resources required ranged from simple items, such as access to a phone, to more specific resources, such as a particular shaped table or access to a full bathroom or kitchenette, which may be difficult for clinicians in acute care. The AMPS and the PRPP assessments required formal training. Kit-based assessments also had associated costs ranging from US$140 for the weekly calendar planning task to US$650 for the University of San Diego California performance-based skills assessment brief (UPSA-B).

From the 120 full text articles reviewed, seven of the assessments did not have published articles with TBI participants, and these were excluded from the final scoping review. Further information was sought and gained from their authors on the suitability for use with patients with TBI. Authors confirmed that these assessments were developed for future use with patients with TBI. These were the: kettle test; menu task; weekly calendar planning activity; ManageMed screen; performance assessment of self-care skills; complex task performance assessment, and the pillbox test. Five of these assessments had ABI participants (not TBI), with stroke the predominant diagnosis within ABI subgroups. Two assessments, the menu task and the weekly calendar planning activity, had published data with healthy community-dwelling adults. These seven assessments with emerging potential for use with TBI populations are described in Table 5.

5. Discussion and implications

With regard to the first research question (i.e., what performance-based cognitive assessments are available for occupational therapists to use in an acute care setting with patients following TBI?), 18 assessments were identified and described. The results of this review are consistent with findings from the systematic review by Wesson *et al.*[7] of functional cognition assessments suitable for older adults. The current scoping review relied on specific inclusion and exclusion criteria to determine performance-based assessments with potential suitability for patients following TBI in acute care settings. Articles with published data on TBI participants generally had

Table 4. Clinical applicability issues of assessments

Assessment composition	Assessment	Manual available	Resources required to administer	Training required	Documented time to administer
Single prescribed task	Actual reality (AR)	Yes, in journal article	Computer	No formal training required	Not documented
	Executive function route-finding task (EFRT)	Yes, in journal article	Hospital grounds with signs	No formal training required	Not documented
	Functionally simulated technology task (FSTT)	Yes, from university website or authors	Computer	No formal training required	Not documented
	Test of functional executive abilities	Yes, in journal article	Access to a phone	No formal training required	Not documented
	Functional cognitive task (FCT)	Yes, in journal article	The Montreal access card was used in the original test	No formal training required	15 min to complete
	Goal processing scale (GPS)	Yes, in journal article	Computer, phone, office supplies	No formal training required	40 min to complete
	The Rabideau kitchen evaluation-revised (RKE-R)	Yes, in journal article	Kitchenette facility	No formal training required	Not documented
	Systematic behavioral observation of executive performance	Yes, assessment procedures available from the author on request	Kitchenette facility with access to a microwave	No formal training required	<30 min
Multiple prescribed subtasks within one assessment	University of California San Diego performance-based skills assessment brief (UPSA-B)	Yes, kit-based	US$650 for a kit	No formal training required	15 min to complete a brief version of this test
	Hotel task (HT)	Yes, in journal article	Office items	No formal training required	15 min allowed to complete as much of the assessment as possible and then ceased
	Executive secretarial task (EST)	Yes, in journal article	Office items	No formal training required	3 h required to complete this test
	Functional Lowenstein occupational therapy cognitive assessment (FLOTCA)	Yes, kit-based	Map, tools, and toolbox	No formal training required	30–60 min to complete the test
	Naturalistic action test (NAT)	Yes, in journal article	Present, wrapping materials, a school bag, and a lunch box for a child; a U-shaped table	No formal training required	15 min to set up assessment; 45 min to complete
	Observed tasks of daily living-revised (OTDL-R)	Manual and scoring available as a free download	Medications, checkbook	No formal training required	25 min on average to complete
	Financial capacity instrument (FCI)	Yes, in journal article	Grocery items required for simulated shopping	No formal training required	Approximately 45 min to complete
	Executive function performance test (EFPT)	Instructions and scoring free to download	44 standard items required; electric stovetop cook plate required as a portable option	No formal training required	Not documented
Variable number of tasks, able to be individualized	Perceive, recall, plan, and perform (PRPP) system of task analysis	Yes, manual provided as part of training	Items required dependent on the task chosen; for example, bathroom for ADL tasks, kitchen for IADL tasks	PRPP level 1 course consists of 5 days face-to-face training; US$950 to complete level 1; PRPP is a two-level course	Not documented

(Cont'd...)

Table 4. ***(Continued)***

Assessment composition	Assessment	Manual available	Resources required to administer	Training required	Documented time to administer
	Assessment of motor and process skills (AMPS)	Yes, manual provided as part of training	Items required dependent on the task chosen; for example, bathroom for ADL tasks, kitchen for IADL tasks	AMPS training consists of 5 days face-to-face course at US$995 (online course also available); certification is awarded after rater calibration is assessed	30 – 40 min to complete

Note: The documented time to administer excludes scoring/documentation time.
Abbreviations: ADL: Activities of daily living; IADL: Instrumental activities of daily living.

Table 5. Emerging assessments without published evidence with TBI populations

Assessment	Related studies with ABI and community-dwelling adults	Description	Sample size	Mean age (SD) of participants	Cognitive domains assessed	Approach/model
Complex Task Performance Assessment (CTPA)[68]	69	Simulated library work (calculation of fines and replacement costs for books; listening and responding to recorded phone messages) and two prospective memory tasks are added to the test; scoring based on rule breaks, inefficiencies, and task failures	*N*=34 Stroke=14	52.93 (9.52)	Multi-tasking, attention, problem solving and memory.	Multiple errands test approach, which is based on the supervisory attention system theory[40]
Kettle test (KT)[70]	71	Preparation of a hot drink for themselves and a clinician; includes novel everyday problem solving; score based on 13 steps	*N*=36 stroke patients	19.3 (5.8)	Working memory, problem-solving, safety, and judgment	"Top-down" approach, based on executive function performance test (EFPT)[21] and assessment of motor and process skills (AMPS)[29]; scoring based on routine task inventory[72], derived from Allen's cognitive disabilities model[73]
ManageMed screen (MMS)[74]	75	Assessment of safety and judgment in relation to medications: answering questions about medication bottles and sorting three medications into a dosette/pillbox	*N*=5 stroke patients	72.6	Safety, judgment, attention, and working memory	No approach/model documented

(Cont'd...)

Table 5. ***(Continued)***

Assessment	Related studies with ABI and community-dwelling adults	Description	Sample size	Mean age (SD) of participants	Cognitive domains assessed	Approach/model
Menu task (MT)[5]	76	Screening tool assessment: Patients are required to make choices from a simulated menu, adhering to seven rules; initiation and completion of task scored	*N*=114 community-dwelling adults	69 (9.85)	Executive function skills, such as inhibition, initiation, and decision-making; ability to recall and follow rules	Based on the multiple Errands test and variations of the test[40,77,78]
Performance assessment of self-care skills (PASS)[79]	80	Consist of 26 tasks and 163 sub-tasks, including functional mobility, basic PADLs, and physical and cognitive IADLs; not necessary to administer all 26 tasks with a patient, and the number of tasks can be individualized; cognitive IADL tasks include simulated shopping tasks and stovetop tasks (e.g., using sharp knife and small home repairs); assesses tasks required for community living; rates a patient on independence, safety, and adequacy; assesses safety and independence as separate constructs	*N*=941 clinical database datasets Stroke=114	Not provided	Safety and adequacy of task performance	Based on the dynamic-interactive assessment approach and the work of Vygotsky[81]
Pillbox test (PT)[82]	82	Medication sorting test: reading medication labels, organizing a pillbox/dosette box, and following rules; errors of omission and commission are scored	*N*=120 Stroke=18	68.63 (8.08)	Error detection, self-correction, and comprehension of tasks; problem-solving, attention, and concentration	Based on four factors of Lezak's executive functioning model[62]
Weekly calendar planning activity (WCPA)[10]	83	Multi-step task scheduling appointments on a calendar; the clinician chooses from three difficulty levels to suit the patient; the patient is asked to evaluate their performance; strategy use is encouraged and scored by the clinician	*N*=808 community-dwelling adults	Detailed age breakdown in article	Attention, memory, and problem-solving	Based on the dynamic interactional model[84]

Abbreviations: ABI: Acquired brain injury; IADL: Instrumental activities of daily living; PADL: Personal activities of daily living; PTA: Post-traumatic amnesia; Quads: Quality assessment with diverse studies.

small sample sizes and mixed participant samples, where TBI patients were not clearly differentiated. These factors should be considered when concluding the generalizability of assessment tools and comparability of assessments. The time required to complete assessments was documented for a minority of assessments, which limited comparison between assessments. While this review documented all available data on participant samples and assessment details, the variability in studies makes it difficult to make clear recommendations about the assessments' clinical utility for acute care TBI populations. Therefore, given the heterogeneity and complexity of TBI presentations, clinicians are encouraged to use an individualized approach to assessment selection.

The quality of the included studies was mixed; eight studies scored between 10 and 20 and 10 studies scored 20 – 30 on QuADS, where the maximum possible score was 39. Although intended to provide a brief overview of reported assessment methodological quality, appraisal scores may have been impacted by a lack of published methodology detail, which may not be an accurate reflection of the individual study's overall quality.

Patients who have sustained TBI have complex and varied cognitive deficits, resulting in a wide range of levels of functional ability and disability. This makes accurate assessment challenging and highlights the role of occupational therapists in investigating an individual's cognitive functioning to determine readiness for discharge home or the need for rehabilitation. The AMPS and the PPRP assessments are flexible and can be individualized to suit patients with varying levels of TBI severity. However, such assessments are a minority among those reviewed. The remainder of the 18 assessments was either single prescribed tasks or groups of prescribed subtasks. Where subtasks are grouped to form one assessment, they may form a collection of seemingly unrelated tasks. Occupational therapists work within a client-centered practice model, which encourages client-centered goals, outcomes, and assessments. Practically, this suggests choosing assessments that closely relate to an individual's occupational roles and tailoring assessments to relevant ADL or IADL tasks.

Assessments of cognitive function have emerged from a broad range of theoretical approaches within occupational therapy and neuropsychology. Models have been identified as a factor influencing clinicians' decisions to use assessments, as they can frame the way we interpret information and discuss practice.[25] As registered health professionals, occupational therapists are expected to have the skills and knowledge to decide when to apply or refrain from using an assessment or intervention with complex patients.[25] Clinicians working within a particular practice model are more likely to choose an assessment aligned with that model to guide their practice. Of the identified assessments, eight were not based on an approach or model; two were based on Lezak's model of executive functioning; and one assessment was based on the multiple errands test. Variety in theoretical approaches underlying these assessments may reflect the input of multiple professions involved in assessing cognition and their differing reasons for assessment.

Executive function assessments, including five of the 18 assessments, encompass complex individual cognitive domains and make a substantial contribution to estimates of a patient's cognition. In a review of the most frequently assessed cognitive domains following TBI, 34 of 40 studies assessed executive function.[26] Executive function deficits correlate with impairment in IADLs, highlighting interconnections between executive function, IADLs, and cognitive function in everyday tasks.[8] Although many of these assessments were not designed to assess the impact of cognitive deficits on real-world functioning, tasks simulating real-life ADLs or IADLs provide valued information on an individual's cognitive function.

Simulating the multi-faceted demands of everyday adult life from within an acute hospital ward is difficult. This is particularly relevant to clinicians assessing cognitive function in the acute care setting, as international incidence data have indicated that most TBIs occur in adult males.[27] There are few age-appropriate, challenging assessments for young to middle-aged adults. These adults may be running a household, working, studying, raising children, and caring for aging family members simultaneously. Three recently developed assessments, including the menu task, actual reality test, and weekly calendar planning activity, attempt to assess the real-life demands of young to middle-aged adults using tasks that may be of relevance to this group. Of the assessments reviewed, the menu task, actual reality test, and weekly calendar planning activity present good options for PBTs that can assess the ability to use cognitive function, as their content approximates real-life tasks for young to middle-aged adults. Emerging PBTs not developed specifically for use in the acute care setting require further investigation to determine if they are feasible to implement in acute care in their current form, or need to be adapted to suit acute care settings.[28] Emerging assessments, such as the menu task, actual reality test, and weekly calendar planning activity, may be easy to use practically in an acute care setting at the bedside and warrant further investigation to determine psychometric strengths or weaknesses for use with patients with TBI.

With regard to the second research question (i.e., the clinical applicability of the assessments for acute care), the

review found a large variation in the pragmatic aspects of the assessments, such as time required for administration and physical environments required such as kitchens or bathrooms. For qualified health professionals, the choice of assessments should not be based solely on practical issues of individual assessments, nor the usual practices in the organization, but on the best available research evidence. However, a systematic review of the measurement properties of PBTs for patients who had TBI could not recommend specific assessments based on psychometric data and recommended further consideration of feasibility factors affecting assessment use.[14] This scoping review attempted to summarize factors relevant to acute care occupational therapists when comparing assessments of cognitive function with patients following TBI. PBTs that are quick to administer, such as the menu task, actual reality test, and weekly calendar planning activity, have good potential for use in acute care, as they use a degree of novel problem-solving, are inexpensive, can be used at the bedside, and present alternatives to traditional kitchen-based assessments. This review was limited to assessments that met inclusion and exclusion criteria as defined for this study. Additional assessments may exist that are used by acute care occupational therapists but were excluded from this review due to their apparent limited application for use on an acute care ward (e.g., assessments requiring access to community shops).

It is important to test patients with items relevant to their context and occupational history to ensure patient motivation and valid outcomes. Assessments have the potential for gender and cultural bias based on their content. An individual's performance may be unduly affected by their work history or educational background. Where an assessment requires a prescribed task to be administered, maintaining the relevance of task items becomes an issue. Checkbooks are present in three included assessments; while they remain a common financial practice in America and Canada, many patients in Australia do not use checks. The NAT was modified to suit the Korean population, with the modified version substituting assessment tasks such as "wrapping a present" with "folding laundry." Although assessments may be modified to suit the cultural norms of the country where they will be used, this process can be time-consuming and limit future comparisons with the original tests' psychometrics.

Comprehensive assessments, such as the PRPP, AMPS, and performance of self-care skills, undoubtedly provide client-centered, occupation-based evaluations of an individual's cognition. However, the time required to complete and score comprehensive assessments may prohibit their use on an acute ward, despite valid information gained. Time pressures in acute care will lead some occupational therapists to choose brief screening tests. For example, assessment using a microwave or an electric cook plate may be clinically indicated but is not always safe or possible in an acute ward. This may lead occupational therapists to use shorter screening tests that are performance-based. The risk of using a shorter test is that occupational therapists may fail to detect mild impairments of cognition, which may impact subsequent support or rehabilitation access. An alternative option may be to develop shorter versions of existing comprehensive assessments to suit time constraints in an acute care setting.

This review has several limitations. Assessments designed for use with populations other than patients who have had a TBI were excluded, and relevant assessments may have been missed. The search strategy was targeted to include articles with patients with TBI, and as such, assessments designed for use with stroke or acquired brain injury populations may have been missed. This review focused on a defined range of clinical applicability criteria available in the assessment articles, which is a limitation. It is acknowledged that an assessment's applicability to patients encompasses other criteria not reported in the data, and these were beyond the scope of this review, such as respondent burden, format compatibility, and assessment popularity.[13,15] Assessments were excluded if they required geographical access to home or community shops; however, assessments were not excluded based on time taken to administer, and, arguably, an assessment that requires an hour or more is not practical in acute care. The methodological quality of assessments was not included in this review, as this was the subject of earlier systematic reviews.[7,13,14] In their systematic review, Kristensen *et al.*[14] highlighted that there are limited studies of psychometric properties for PBTs of cognitive function, which restricts the ability to make recommendations about which assessments to use. Attempts were made to comment on the quality of reporting and methodology of the included articles referencing 18 assessments. To supplement the findings of the scoping review, the QuADS appraisal tool was used; however, its effectiveness is dependent on published methodology data. Virtual reality (VR)-based assessments were excluded and are an area for separate review and further research, especially regarding comparison to real-world environment observations and examination of the ecological validity of VR. This review has highlighted the need for a wider exploration of assessments developed for other populations and their potential suitability for patients following TBI.

6. Conclusion

Providing evidence to estimate a patient's cognitive functioning in real-world environments following discharge

is an important role of occupational therapists working in acute care with patients following TBI. Using performance-based approaches for assessment, occupational therapists can integrate everything they know about a patient's occupational role, environment, and task performance and make recommendations for rehabilitation following TBI. The challenge faced by occupational therapists in acute care is choosing which standardized assessment is the best fit for each patient, within acute care constraints. Clarity of assessment findings enables prediction of real-world functioning and appropriate and timely rehabilitation to commence. Having increased awareness of assessments to choose from may improve evidence-based occupational therapy assessment practices and facilitate critical reflection on how occupational therapists currently assess cognition.

Assessment choice ultimately depends on the experience of the occupational therapist in applying clinical reasoning to determine the best assessment or combination of assessments for each patient who has had a TBI. This review provides a list of assessments that have potential for use in acute care within defined parameters of inclusion and exclusion criteria and should be viewed in conjunction with existing psychometric evidence. It remains unclear how occupational therapists currently choose assessments, and it is suspected that assessment choice has a high variability between clinicians, organizations, and internationally. Further research on issues influencing the decision-making of occupational therapists working in acute TBI settings is required. When choosing a cognitive assessment with patients following TBI, it is recommended that occupational therapists use an occupation-based approach to ensure that they are providing evidence-based assessment.

There are several performance-based assessments of cognition available for occupational therapists to use with patients with TBI. PBT has potential clinical applicability in the acute care setting. This scoping review may provide a reference for occupational therapists seeking to conduct performance-based tests of cognitive function in acute care settings by outlining the clinical applicability of published assessments.

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ACCSCIENCE PUBLISHING

Advanced Neurology

PERSPECTIVE ARTICLE

Thirty years of the South London Stroke Register

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Abstract

Since the beginning of the South London Stroke Register (SLSR) in 1995, stroke care has undergone major transformations and the SLSR adapted alongside. Recruitment strategies changed in line with patient pathways, and data collections were updated to reflect clinical practice and provide clinicians and policymakers with the most impactful data. Our Stroke Research Patient and Family group was pivotal to define the most relevant care and outcome measures for stroke survivors. The SLSR has published numerous studies on epidemiological trends and the implementation of care interventions. By providing real-world data, the SLSR has contributed to shaping local and national stroke policies, such as the UK's National Audit Office reports 2005 and 2010, the reconfiguration of London's stroke services and national stroke guidelines. Linking SLSR data with routinely collected health data might further address many unanswered questions around stroke as a long-term chronic condition in ageing populations.

Keywords: Stroke; Epidemiology; Cohort study; Population-based register

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1. Introduction

Population-based registers, such as the South London Stroke Register (SLSR), are designed to estimate the epidemiology of stroke and to evaluate the implementation of evidence-based care in real-world settings.[1] While routinely collected health data are now widely available, population-based registers hold advantages, such as near-complete case ascertainment based on multiple overlapping sources of notification,[2] rather than, *for example,* relying solely on hospital admissions, and a level of relevant detail and consistent, disease-specific outcome data not contained in routine care records.

The SLSR was established in 1995 recording all first-ever strokes in inner-city London and more than 8600 participants have since been recruited. The register has provided data for over 400 peer-reviewed research papers, demonstrating improvements, shortcomings and inequalities in stroke care and outcomes. Data from the SLSR underpinned national reports, stroke strategies and clinical guidelines.

We describe the challenges of running a population-based stroke register, its key findings and its impact on the delivery of effective stroke care locally, nationally and internationally.

2. Recruitment and follow-up – and its changes and challenges over time

London as a location for a population-based register brings distinct advantages. The SLSR study area comprises a geographic area of South London (defined by electoral wards) with a multi-ethnic population and areas of significant deprivation. Its study population grew from 234,533 in 1996 (72% White ethnic group)[3] to 398,555 in 2021, while becoming ethnically highly diverse (52% White ethnic group, 25% Black ethnic group, 23% from other ethnic backgrounds)[4] and neighbourhoods on average less deprived.[5] Figure 1 shows the increase in ethnic minority participants within the SLSR.

Ethnic and socio-economic diversity of participants is vital to be able to investigate health inequalities and to ensure findings of health research are applicable in diverse communities. Particularly, ethnic minority groups have been reported to be under-represented in research, especially clinical trials.[6]

Near-complete case ascertainment is central for population-based studies.[7] Participants are identified by 'hot pursuit',[8] which includes daily visits to all relevant hospital wards. To ensure milder, non-admitted cases are identified, outpatient clinics seeing stroke or transient ischaemic attack (TIA) patients are screened regularly and all brain imaging reports from hospitals treating stroke patients within the study area are checked for evidence of stroke or previous strokes that might have been missed before. This notification source typically also identifies patients with rapidly fatal strokes or patients with other health issues and therefore not admitted to a stroke ward.

Case ascertainment strategies have changed significantly over time, in line with changing clinical pathways and diagnostic procedures. In earlier years, notifications included community- and primary care-based sources, such as general practitioners, community therapists and coroners. Now, in the UK, all patients with stroke or TIA symptoms are expected to undergo brain imaging and be referred to specialist hospital services. Case ascertainment has therefore shifted further towards hospital-based sources. Apart from hospitals within the study area, those neighbouring the study area have been established as secondary recruitment sites to ensure outlying patients are included.

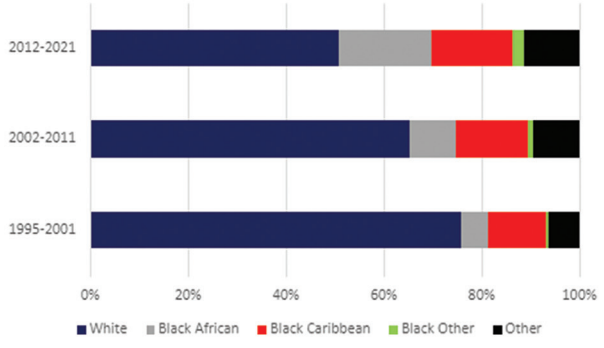

Figure 1. Ethnic composition of the study population by year of stroke. Image created by the authors.

Running the SLSR involves multiple logistical challenges, including conducting detailed follow-up interviews, in some years with over 1300 participants. The majority of follow-ups were conducted face-to-face, through time-intensive home visits by fieldworkers. The difficulty of maintaining long-term contact with participants, some of them followed up for over 25 years, is exacerbated by the mobility of a relatively young, urban and ethnically diverse population. Multiple attempts, including evening and weekend calls, and various methods are employed to locate and follow-up participants, such as contacting the next of kin and searching hospital records for any updated phone numbers. Despite this, 20% of participants cannot be reached at each follow-up time point. In recent years, emphasis was placed on securing email addresses to facilitate ongoing contact, but electronic response rates proved disappointing. Since the COVID-19 pandemic, the proportion of telephone follow-ups has increased, which has improved overall follow-up rates, but some outcome scales are difficult to complete by telephone with the typically elderly cohort of stroke survivors.

Language barriers in patients who do not speak English as a first language, but also those with speech and language difficulties or cognitive impairments following stroke, further impact the completion of complex follow-up measures. This can lead to a significant proportion of data either being collected through a carer or not possible to collected at all.

3. Measuring the impact of stroke

Over the years, data items collected have evolved (Table 1). Integrating new classifications and scales has ensured the register remains current and allowed for inter-study comparisons, for instance, through the National Institutes of Health Stroke Scales. An intensive programme of the patient and public engagement takes place in parallel to the data collection to best understand the data needs of stakeholders and how to measure under-explored domains such as fatigue or informal care. Newer data items include:

Table 1. Data collected and changes over time

Time point	Measurement
Baseline	• Socio-demographic characteristics (ethnic categories shaped in parallel with ONS census categories, educational attainment since 2004) • Risk factors and medications before stroke (BMI since 2000) • Stroke severity measures (GCS, swallow assessment, incontinence and other acute impairments collected since 1995; NIHSS introduced in 2000) • Interventions and resource use during admission (thrombolysis since 2004) • Newly diagnosed risk factors, medications on discharge and Early Supported Discharge (since 2022) • Stroke classification (OCSP classification since 1995, TOAST classification since 1998)
Follow-up assessments	• Living conditions • Therapies, such as physio-, occupational speech-and-language therapy • Other resource use (including visits to doctors and help from friends, family or social services) • Social networks (since 1999) • Newly diagnosed risk factors and current medications (since 1995) • Barthel index (level of disability; since 1995) • Modified Rankin Scale • Mortricity Index (1995 to 1998) • Frenchay Activities index (since 1995) • Hospital anxiety and depression scale, since 1998 • Cognition: MoCA cognitive score (since 2022); Abbreviated Mental Test (since 2000), Mini–Mental State Examination (before 2000) • Health related quality of life: SF12; EQ5D-5L (since 2022)

Abbreviations: ONS: Office for National Statistics, UK; BMI: Body mass index; GCS: Glasgow Coma Scale; NIHSS: National Institutes of Health Stroke Scale; OCSP: Oxford Community Stroke Project; TOAST: Trial Org 10172 in Acute Stroke Treatment classification; MoCA: Montreal Cognitive Assessment; SF12: 12-Item Short form survey; EQ-5D-5L: 5-level EQ-5D.

(i) the Montreal Cognitive Assessment, which will provide insights into mild cognitive impairment amongst stroke survivors; (ii) the EQ5D-5L, a measure of health-related quality of life with improved sensitivity and reduced ceiling effects and (iii) a fatigue severity scale, which was selected in close cooperation with our patient engagement group.

Participants also emphasised the importance of limiting the length of follow-up interviews. Therefore, for scales added, other items were removed, which has to be balanced against the need for consistency of data collection over time for the purpose of calculating trends, an important aspect in this long-running study.

In 2022, the SLSR moved from the World Health Organization (WHO) International Classification of Diseases (ICD)-10 to the ICD-11 definition of stroke, which focusses on neuroimaging for diagnosis and reflects the increased availability of brain scanning (near universal in the study population). The inclusion of milder but detectable cerebrovascular disease will lead to an increase in the reported number of strokes. The extent of this increase and parallel reduction in stroke severity and post-stroke impairments are still unknown and estimates are part of ongoing work.

4. Patient and public involvement, collaboration and data linkage

Patient and public involvement has been at the centre of the SLSR and shaped its many transformations over time. The King's College London Stroke Research Patient and Family Group was established in 2005 and brings together stroke researchers and people affected by stroke. The group has input at all stages, including setting study priorities, introducing new data items and informing funding applications and ethical aspects of the research. To encourage further patient involvement, a newsletter has been sent out regularly to each participant since 2006, summarising current and planned research projects in lay terms and inviting participants to contribute.

The SLSR has collaborated with several established stroke registries, including the European Registers of Stroke Collaboration, which led to insights into the delivery of stroke care, costs and outcomes across Europe.[9] In its current 5-year programme, collaboration is ongoing with the other long-running UK register, the Oxford Vascular Study, for the purpose of data validation by comparing incidence and outcome data and improving generalisability.

A data linkage with Lambeth DataNet, providing primary care data for part of the register participants, has been established and we are currently preparing a linkage with the Sentinel Stroke National Audit Programme, the national register of acute stroke. Combining the SLSR's rich socio-demographic and outcome data with SSNAP's detailed data on acute care will allow further analyses of the real-world effectiveness of acute care interventions outside clinical trial settings and socio-demographic inequalities in care.

Linkage of research data collected in SLSR to routine health records and administrative databases presents significant further possibilities to make use of all available data in the most efficient way and avoid duplication of data collection.

5. Core academic output – the changing epidemiology of stroke and stroke care

The epidemiology of stroke is at the centre of this population-based register. Since its first key study,[3] the

SLSR has reported a decline in the incidence of first[10-12] and recurrent stroke.[13] Potentially contributing to this decline, SLSR analyses showed improved but not yet optimised management of vascular risk factors, with some of them becoming more prevalent.[14] Incidence rates and their decline varied significantly by ethnicity, age and sex (Figure 2).

SLSR analyses showed a significant improvement in post-stroke survival since 1995, likely due to a combination of improved risk factor control and acute interventions, such as the rise in thrombolysis and stroke unit admission.[15,16] However, 20 – 30% of stroke survivors were found to have poor functional outcomes up to 10 years after stroke (Figure 3).[17] Post-stroke depression affected

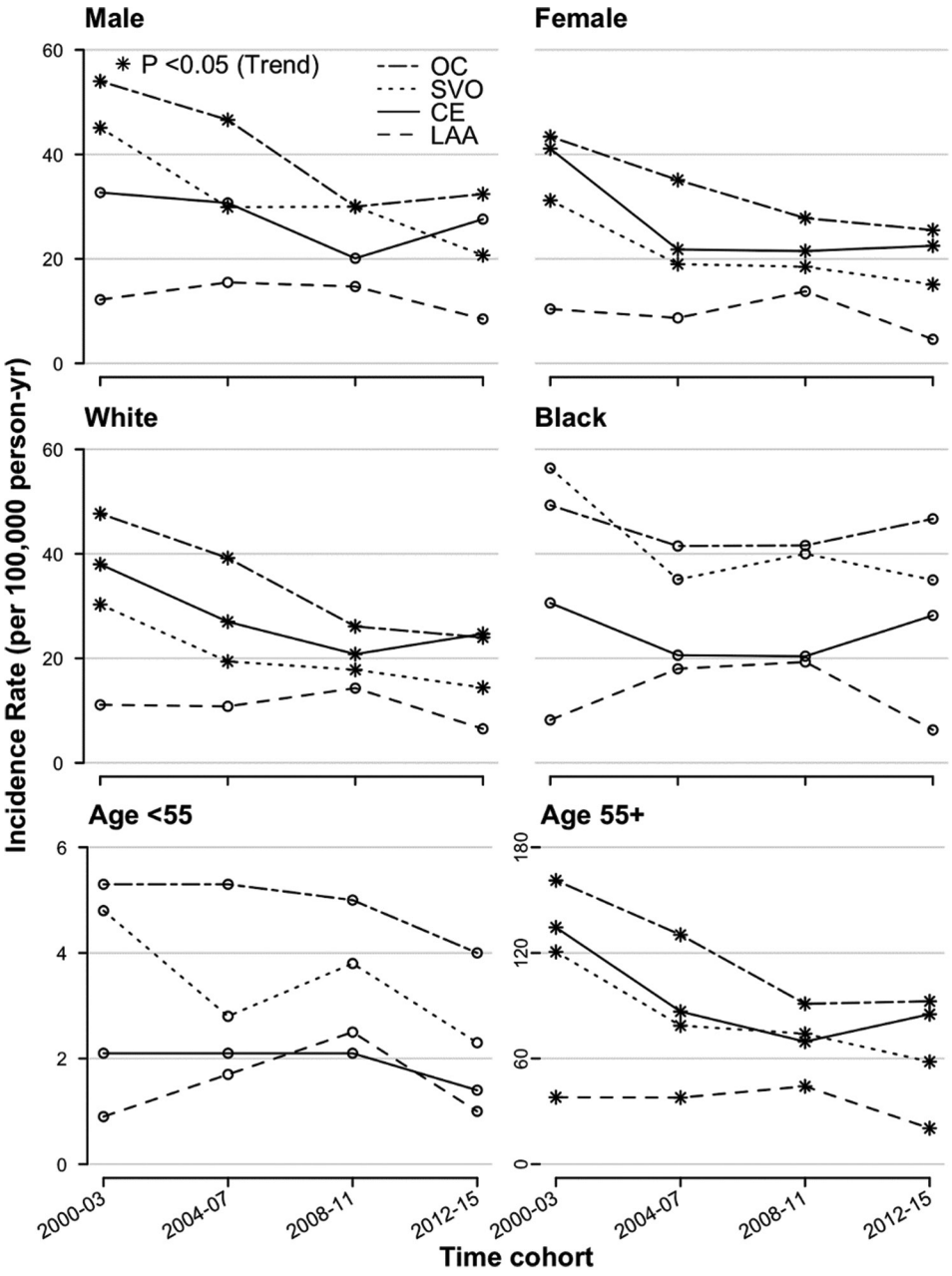

Figure 2. Trends in the age-standardised** annual incidence per 100,000 per year for first-ever ischaemic strokes by sex, ethnicity, and age[12] (Open Access Source). Notes: **to the 2011 population of England and Wales, *P*-values were obtained from the Cochran-Armitage test for trend. *denotes significant trends ($P < 0.05$).
Abbreviations: CE: Cardioembolism; LAA: Large-artery atherosclerosis; OC: Other causes; SVO: Small-vessel occlusion.

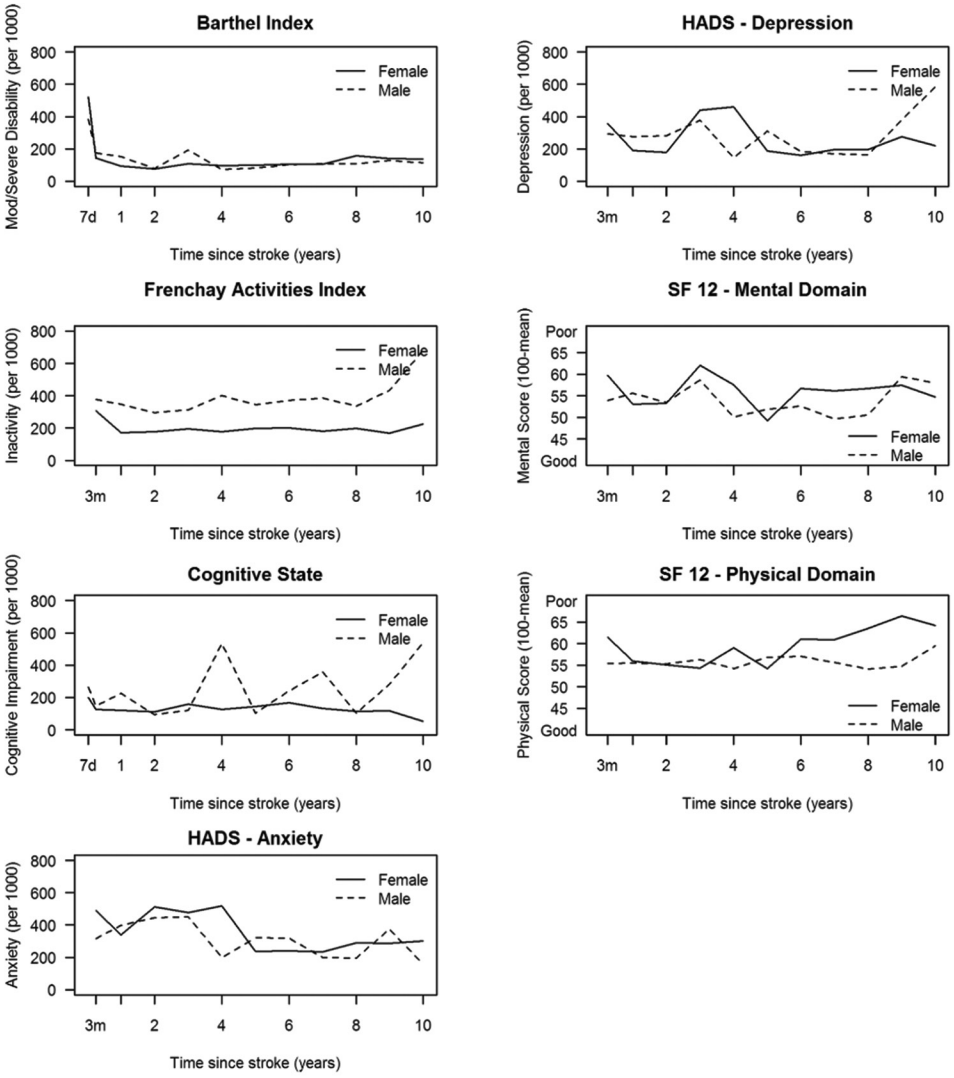

Figure 3. Age-adjusted rates of outcome per 1000 stroke survivors, with 95% pointwise confidence intervals[17] (Open Access Source).
Abbreviation: HADS: Hospital Anxiety and Depression Scale; SF 12: Short Form-12 (measure of health-related quality of life).

around 30% of stroke survivors[18] and was associated with lack of family support, inability to work and functional dependence.[19] Over 20% of stroke survivors experienced cognitive impairment,[20] but appropriate vascular risk management reduced this risk.[21]

The SLSR has provided real-world, population-based data on the provision and effectiveness of acute care, reporting substantial improvements: brain imaging rates increased from 85.2% in 1995 – 1997 to 99.6% in 2007 – 2009, whereas stroke unit admission increased from 18.9% to 78.4% and median length of hospital stay decreased from 21 to 31 days.[22] Thrombolysis rates reached 13.9% in 2005 – 2015.[23] SLSR analyses showed an association between evidence-based acute interventions, such as stroke unit admission, acute-phase aspirin and thrombolysis and improved functional outcome and long-term survival.[23,24]

Finally, the SLSR cohort served as a sampling frame for clinical trials and social science research. A randomised controlled trial provided evidence that outcomes following early supported discharge (ESD) were comparable to conventional care, whereas length of hospital stay was substantially reduced.[25] ESD has since become a cornerstone of evidence-based stroke management.

6. Translating research into changes in guidelines, policy and service provision

Data and analyses from the SLSR have yielded a significant impact by effectively translating into both local and national policies for stroke and stroke survivors.

SLSR data informed the 2005 National Audit Office report 'Reducing Brain Damage: faster Access to Better Stroke Care', which detailed the poor state of stroke care in the UK,[26] leading to the National Strategy for Stroke 2007, devised by the Department of Health.[27] The subsequent 2010 National Audit Office report 'Progress in Improving Care' again used SLSR data to demonstrate improvements in care and cost-effectiveness, specifically the provision of stroke unit care and ESD.[28]

SLSR data underpinned the successful 2010 reconfiguration of stroke services in London, which involved a centralisation from 30 hospitals into eight Hyper Acute Stroke Units covering the first 72 h of stroke care. SLSR data were used to estimate the expected number of stroke patients and to model the most cost-effective way to deliver stroke services, *that is*, the number and allocation of Acute and Hyper Acute Stroke Unit beds. A subsequent evaluation of the reconfiguration estimated an additional 96 lives saved per year.[29]

SLSR data contributed to the 2020 evidence review 'Stroke pathway – Evidence Based Commissioning', commissioned by NHS England, summarising current understanding and knowledge gaps across the stroke pathway, and informing service providers and national policymakers of what needs to be achieved to provide high-quality services.[30] Finally, evidence based on SLSR data regularly informed the development of the National Clinical Guidelines for Stroke.[31]

To further bridge the divide between research findings and policymaking, the current programme is running a series of 'Policy Labs', an innovative model that brings together key decision-makers, professionals, stroke survivors and carers to address how research findings can be put into policy and practice.

7. Conclusion

Since the beginning of the SLSR in 1995, stroke care and services have been profoundly transformed in London and elsewhere. Data and evidence provided by the SLSR were central for stakeholders to drive this change forward, improving the lives of stroke survivors and their families.

Among the many unanswered questions for future research are the implications of the new WHO ICD-11 definition of stroke; the trend of stroke incidence in the face of demographic, environmental and economic challenges; the management of stroke as a long-term chronic condition as well as multimorbidity in an ageing population, with a focus on vascular health more broadly; the drivers of inequalities in stroke risk and outcomes between socio-demographic groups and the approaches used to improve outcomes for those disadvantaged. While routinely collected health data will continue to answer some of these questions, labour-intensive population-based research has a unique role to play due to its low recruitment bias and detailed, consistent and disease-specific data. Population-based registers can serve as a suitable sampling frame for trailing digital and other interventions. Future directions of research might include artificial intelligence methods research, *for example*, in relation to brain imaging, and further linkages between different types of datasets, to extract the most impactful insights for improvements in stroke prevention, care and outcomes.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

Advanced Neurology

ORIGINAL RESEARCH ARTICLE

Neuroprotective effects of *Tetrapleura tetraptera* fruit extract on hippocampal histological features in pentylenetetrazol-induced kindling in Wistar rats

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Abstract

Seizures are a hallmark of epilepsy and often lead to cognitive impairment. However, most antiepileptic medications have limited efficacy in this context and are associated with negative consequences with prolonged use. *Tetrapleura tetraptera*, an antioxidant-rich plant known for its anticonvulsant properties, prompted this study to examine its role in hippocampal protection after pentylenetetrazol (PTZ)-induced kindling. Phytochemical screening and median lethal dose assessments of the *T. tetraptera* fruit extract (TFE) were conducted. Forty-nine male Wistar rats (150 – 200 g, *n*=7 per group) were assigned as follows: Control, TFE (500 mg/kg), PTZ alone (40 mg/kg), and PTZ (40 mg/kg) following sodium valproate (200 mg/kg) or TFE at low (250 mg/kg), intermediate (500 mg/kg), and high (1,000 mg/kg) doses over 21 alternate days. PTZ was administered intraperitoneally, while all other treatments were given orally. On day 22, spontaneous alternation behavior (SAB) was assessed, followed by euthanasia and histological analyses. Phytochemical analysis of TFE identified phenols, alkaloids, and flavonoids as key constituents, and its oral median lethal dose was greater than 5,000 mg/kg. Pre-treatment with TFE showed suppressed seizures with quantal protection ($p<0.05$) compared to the PTZ group. While SAB was not significantly different, hippocampal Nissl expression was mildly affected, and neuron-specific enolase (NSE)-positive cells and glial fibrillary acidic protein (GFAP)-positive cells significantly decreased ($p<0.05$) compared with the control group. Therefore, oral administration of TFE is safe in Wistar rats with protection against PTZ-induced seizure and mortality. While short-term memory remained unaffected, the hippocampal Nissl substance, NSE, and GFAP were preserved, particularly in the low and intermediate TFE dose groups.

Keywords: Hippocampus; Pentylenetetrazol; *Tetrapleura tetraptera*; Sodium valproate; Immunohistochemistry; Nissl substance

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1. Introduction

Seizures are a major characteristic of epilepsy, occurring recurrently due to an imbalance in excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA) neurotransmitters.[1,2] This disorder could be inherited or acquired from conditions,

such as hippocampus sclerosis, trauma, intracranial infections, and exposure to toxic agents, among others.[3,4] The incidence of epilepsy is reported as 50/100,000 in developed countries and twice as high in developing countries.[5,6] Anxiety, depression, and low self-esteem are often associated with this disorder.[7,8]

Epilepsy is more common in adults and frequently leads to cognitive impairment, particularly affecting the hippocampus, which is crucial for memory retention.[9,10] This memory function of the hippocampus can be studied through spontaneous alternation behavior in a T- or Y-maze, which helps identify impairments.[11] Cognitive impairment associated with epilepsy may have deleterious effects, and the majority of antiepileptic medications exhibit limited efficacy in this regard.[12,13] Individuals with epilepsy also face a higher risk of death than the general population.[14,15] Histological changes in the hippocampus are involved in epilepsy, with extensive pyramidal cell loss in the cornu ammonis 1 and 3 (CA1 and 3) regions.[9,10] Evaluation of hippocampal changes in epilepsy can include histological analysis of Nissl substance and the regulation of neuronal and astrocytic markers, such as neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP), among other immunohistochemical or biochemical methods.[16-18]

Most antiepileptic drugs act by blocking sodium or calcium channels, reducing the release of excitatory glutamate and increasing inhibitory GABA levels.[19,20] However, GABA can also have pro-convulsive effects, either directly or indirectly.[19] Epilepsy treatment is often long-term, sometimes extending throughout a patient's life. Aside from this limitation, the use of these drugs is also associated with various adverse reactions.[21,22] Valproic acid, a carboxylic acid antiepileptic drug, or its sodium salt, sodium valproate, has a long history of efficacy in seizure management,[23-25] and serves as a standard antiepileptic drug control in this study.

Plants have been reported to offer medicinal and nutritive constituents. One such plant, *Tetrapleura tetraptera* Taub., belonging to the *Fabaceae* family,[26] is used in the management of various diseases and ailments in Nigeria and other African countries.[27-31] In addition, some local ethnic groups have reported using the plant for epilepsy management.[27,32]

T. tetraptera is known for several medicinal properties, including antimalarial, antibacterial, anti-inflammatory, antioxidant, hypoglycemic, central nervous system depressant, hypothermic, and antiepileptic effects in experimental animals.[31,33-42] The plant also exhibits anticonvulsant, analgesic, and hypothermic properties.[27,28] These properties make *T. tetraptera* a prospective treatment option for epilepsy and its associated neuronal deficits. Establishing the optimal dosage of *T. tetraptera* extract is crucial to maximize its efficacy in epilepsy protection while minimizing potential side effects. However, it is essential to assess the potential toxicity of the extract, which can be done through a median lethal dose test to prevent adverse outcomes upon usage.[43]

In experimental models, epilepsy is often induced in rodents using chemicals, such as pentylenetetrazol (PTZ), with the resulting seizure activity scored according to the Racine scale.[44,45] This research investigated the effects of *T. tetraptera* plant extract on PTZ-induced kindling and its potential neuroprotective effects on the hippocampus in a rat model.

2. Material and methods

2.1. Animal handling

Forty-nine male Wistar rats, 9 – 10 weeks old and weighing 150 – 200 g, were utilized in this study. The rats were obtained and housed in the animal facility of the Faculty of Basic Medical Sciences at the university. Each rat was cared for and handled following the guidelines for the use of laboratory animals set forth by the United States National Institute of Health.[46] This research was approved by The Faculty of Basic Medical Sciences Research and Ethical Committee (approval reference: UU_FBMSREC_2022_001).

The rats were maintained throughout the experiment at a room temperature of 25 – 28°C with a 12:12 h natural light/dark cycle. They were given *ad libitum* access to a standard pelletized rat diet (Grand Cereals, Nigeria) and drinking water. The rats were divided into seven groups (n=7), designated as control and test groups.

2.2. Preparation of *T. tetraptera* extract

Fresh, mature fruits of the *T. tetraptera* Taub., commonly known as Uyayak by the Ibibio ethnic group of Nigeria, were obtained from a local market in Calabar, Nigeria. A taxonomist identified the fruit, which was assigned specimen number UUPH/A32(f) and deposited at the Pharmacognosy Herbarium of the Faculty of Pharmacy, University of Uyo, Nigeria. The fruits were chopped into pieces after the seeds were removed. The fruits were first washed and air-dried before being pulverized into a smooth paste. A total of 1,342 g of the paste was macerated with 80% ethanol for 72 h, filtered through Whatman No. 1 filter paper. The resulting filtrate was concentrated at 45°C in a water bath and subsequently stored at 4°C until further use.

2.3. Preparation of PTZ and sodium valproate solutions

All drugs were prepared daily. PTZ (Sigma-Aldrich, Germany) was dissolved in distilled water, and 40 mg/kg

body weight was administered intraperitoneally to the rats.[45] Sodium valproate (Sanofi-Aventis, United Kingdom) tablets (200 mg) were dissolved in distilled water, and a dose of 200 mg/kg body weight was administered orally.

2.4. Phytochemical screening of *T. tetraptera* fruit

Qualitative and quantitative analyses of the phytochemicals present in the ground *T. tetraptera* fruits were conducted following standard methods.[47]

2.5. Determination of median lethal dose of *T. tetraptera*

The ethical approval for the lethal dose testing on mice was also granted by the Faculty of Basic Medical Sciences Research and Ethical Committee. To assess the toxicity of the *T. tetraptera* fruit extract, an oral median lethal dose was carried out on 25 CD-1 mice, following the Organization for Economic Cooperation guidance document. The up-and-down procedure was used to determine the median lethal dose.[43] Briefly, the 25 CD-1 mice were assigned to five groups (n = 5). After overnight fasting, the mice were administered the extract, with water provided ad libitum. Two hours after administration, the mice were allowed access to food.[43] In Group 1, *T. tetraptera* was administered at 1,000 mg/kg, and the mice were observed for signs of toxicity. After 48 h, with no deaths recorded, the procedure was repeated for the second through fifth groups at doses of 2,000, 3,000, 4,000, and 5,000 mg/kg, respectively. As no signs of toxicity or death were recorded during the experiment, the dosing procedure was discontinued, and the lethal dose was estimated.

2.6. Administration of *T. tetraptera*, sodium valproate, and PTZ

The *T. tetraptera* fruit extract (2 g) was dissolved in 20 mL distilled water. Calculated doses of 250 (5%), 500 (10%), and 1,000 (20%) mg/kg of the median lethal dosage (LD_{50}) of *T. tetraptera* fruit extract were administered. In addition, 200 mg/kg sodium valproate was administered orally, 1 h before the PTZ intraperitoneal injections, which were given on alternate days (every 48 h) until day 21. The administration schedule is shown in Table 1.

2.7. Kindling induction and determination of seizure scores

To induce kindling, 40 mg/kg PTZ was administered intraperitoneally every 48 h. Kindling was achieved on alternate day 21. Following the administration of PTZ, seizure scores, and mortality rates were observed and recorded for up to 30 min. The seizure stage and accompanying behaviors were noted using the following Racine scale: Stage 0 – No response; stage 1 – Ear and facial twitching; stage 2 – Myoclonic jerks; stage 3 – Myoclonic jerks with rearing; stage 4 – Forelimb clonus; stage 5 – Seizures with rearing, rolling over to the side, and generalized clonic-tonic seizures; stage 6 – Death. The rats were monitored for 24 h after the last administration to check for mortality. The percentage of quantal protection against seizures and death was calculated. Quantal protection was determined as the number of live rats per group divided by the total number of rats in the group.[45]

2.8. Spontaneous alternation behavior test

On day 22, a spontaneous alternation behavior test was conducted in a T-maze. Briefly, the rats were acclimated in the test room for one h before the test commenced. The T-shaped maze consisted of two short, goal arms (left and right) and one long, start arm. Gates were placed at the junctions of the arms and were opened when the rat was placed in the start arm. The rats were allowed to choose between entering either of the short arms.

Each animal underwent five successive trials, each lasting 60 s. When all four paws of the rat entered one of the short arms, the gate was closed, and an arm entry was recorded before the rat was returned to the holding cage. If a rat did not enter a goal arm within the allotted time, the trial was scored as blank, and the rat was returned to the holding cage. After each trial, the T-maze arena was cleaned with 70% ethanol. The frequency with which the rats alternated between the short arms and the total trial duration were recorded. The percentage alternation was then calculated.[11,48]

2.9. Termination of the experiment

Ketamine hydrochloride (Rotex Medica, Germany) was administered intraperitoneally as an anesthetic agent at a dose of 50 mg/kg. The thoracic cavity of each rat was opened, and a cannula was inserted into the left ventricle of the heart for perfusion fixation using 10% buffered formalin. The entire brain was removed and post-fixed in 10% buffered formalin for 48 h, followed by processing for paraffin wax sectioning for histological and immunohistochemical analysis.

2.10. Nissl substance and immunohistochemistry protocols

Briefly, the left dorsal hippocampal brain region of the rats was routinely dehydrated using a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Serial sections (10 μm thick) were mounted on slides and were rehydrated for both Nissl substance staining and immunohistochemistry. For the Nissl substance study, the sections were stained with 1% cresyl violet, supplemented with a few drops of acetic acid, for 10 min.

Table 1. Schedule of groupings and administration protocol

Groups (*n*=7)	Drugs	Dosages (mg/kg)	Duration (alternate days)
Group 1	Control, distilled water	5 mL/kg	11
Group 2	*Tetrapleura tetraptera*	500 mg/kg	11
Group 3	PTZ	40 mg/kg	11
Group 4	Sodium valproate pre-treatment then PTZ	200 mg/kg then 40 mg/kg	11
Group 5	Low-dose *Tetrapleura tetraptera* pre-treatment then PTZ	250 mg/kg then 40 mg/kg	11
Group 6	Intermediate-dose *Tetrapleura tetraptera* pre-treatment then PTZ	500 mg/kg then 40 mg/kg	11
Group 7	High-dose *Tetrapleura tetraptera* pre-treatment then PTZ	1,000 mg/kg then 40 mg/kg	11

Notes: Pentylenetetrazol was administered intraperitoneally, while *Tetrapleura tetraptera* fruit extract and sodium valproate were administered orally. All administrations were carried out every alternate day for 21 days (a total of 11 alternate days).
Abbreviation: PTZ: Pentylenetetrazol.

The excess stain was then washed off with distilled water. For the immunohistochemical study, antigen retrieval was performed in a citrate buffer solution (pH 6.0) in a microwave oven for 5 min, followed by protein blocking with 3% hydrogen peroxide for 10 min. Sections were then pre-incubated in 2% normal goat serum for 30 min and incubated with either a monoclonal mouse anti-NSE (22C9, 1:100, Leica Biosystems, United States) or monoclonal mouse anti-GFAP (NCL-L-GFAP-GA5, 1:100, Leica Biosystems, United States) for 2 h. Afterward, sections were incubated for 1 h with goat anti-mouse secondary antibody (1:100). The reaction was detected using the avidin-biotin complex with diaminobenzidine as the chromogen. Finally, sections were counterstained with Harris hematoxylin.

All stained sections were dehydrated in increasing concentrations of alcohol, cleared in xylene, and cover-slipped with dibutylphthalate polystyrene xylene. Processed slides were examined under a light microscope, and photomicrographs were captured using a computer-assisted digital microscope camera.

2.11. Hippocampus cell counts

The populations of Nissl substance-stained Cresyl violet and NSE- and GFAP-immunopositive cells in the hippocampus were quantified manually using ImageJ® software (United States of America). Images of the three layers of the hippocampal cornu ammonis 3 (CA3) regions were acquired from three brain sections per animal, with three animals per group. Each image was mapped randomly using the ImageJ® gridlines as a guide. Manually counting was conducted using a tool to select Nissl, NSE, or GFAP-stained cells located on the upper and right borders of each mapped area.

2.12. Statistical analyses

One-way and repeated measures analyses of variance were employed to compare the calculated means and percentages. Subsequently, Tukey's or Bonferroni's *post-hoc* tests were performed using GraphPad Prism software (version 5.0; United States of America) to determine statistical significance at $p \leq 0.05$. All results are presented as mean ± standard error of the mean or standard deviation.

3. Results

3.1. Phytochemical analysis of *T. tetraptera*

Qualitative screening of *T. tetraptera* fruit extract revealed the presence of phenols, flavonoids, alkaloids, saponins, tannins, terpenoids, steroids, phytate, anthraquinones, oxalates, cardiac glycosides, and hydrogen cyanide. The quantitative analysis of *T. tetraptera* fruit extract showed that phenols (7.80 g/100 g) had the highest concentration, followed by alkaloids (6.20 g/100 g), flavonoids (5.30 g/100 g), saponins (3.40 g/100 g), tannins (1.20 g/100 g), and phytate (0.40 g/100 g). Oxalates, cardiac glycosides, and hydrogen cyanide were present in trace amounts.

3.2. Oral median lethal dose

Mice that received oral administration of *T. tetraptera* fruit extract at doses ranging from 1,000 mg/kg to 5,000 mg/kg showed no signs of death or toxicity. Hence, the median lethal dose of *T. tetraptera* ethanol fruit extract was determined to be greater than 5,000 mg/kg.

3.3. Antiseizure activity of *T. tetraptera*

Repeated administration of PTZ at a sub-convulsive dose of 40 mg/kg for 11 alternate days resulted in a steady increase in seizure scores, leading to generalized clonic-

tonic seizures. A repeated measure one-way analysis of variance revealed a significant difference (F = 20.78, p<0.001). Bonferroni's *post-hoc* test further indicated that the seizure scores increased significantly from days 1 to 11 (alternate days) (Table 2).

A one-way analysis of the variance of the seizure scores between groups on an alternate day 11 revealed a significant difference (F = 8.52, p<0.001). The seizure scores in the PTZ group were significantly higher (p<0.05) compared to the group pre-treated with 250 mg/kg and 500 mg/kg of *T. tetraptera*. However, no significant difference was observed between the 1,000 mg/kg *T. tetraptera* and sodium valproate groups. In addition, the group pre-treated with sodium valproate exhibited significantly higher (p<0.05) seizure scores compared to the 500 mg/kg *T. tetraptera* group (Table 3).

3.4. Quantal protection action of *T. tetraptera*

The percentage quantal protection for the groups pre-treated with sodium valproate (85.71%), low-dose *T. tetraptera* (71.42%), or intermediate-dose *T. tetraptera* (85.71%) was higher compared to the PTZ group (57.14%). However, there was no significant difference between the PTZ group and the high-dose *T. tetraptera*-pre-treated group (57.14%) (Table 4).

Consistently, the mortality rates in the groups pre-treated with sodium valproate (14.29%), low-dose *T. tetraptera* (28.58%), and intermediate-dose *T. tetraptera* (42.86%) were lower than those in the PTZ group (57.14%), which did not differ from the high-dose *T. tetraptera*-pretreated group (42.86%) (Table 4).

3.5. Spontaneous alternation behavior

A repeated measures analysis of variance and Tukey's *post-hoc* tests revealed no significant difference (p = 0.1475, F = 1.751) in spontaneous alternation behavior between the test groups: *T. tetraptera* alone, PTZ alone, pre-treated sodium valproate, and pre-treated *T. tetraptera* at low, intermediate, and high doses (Figure 1).

3.6. Cresyl violet-stained cells

In the control group, the CA3 region of the hippocampus exhibited distinct and intensely stained Nissl substance across all three layers: molecular, pyramidal, and polymorphic (Figure 2A). Similarly, the CA3 region of the hippocampus in the *T. tetraptera* fruit extract group showed distinct and intense Nissl staining in these layers, resembling the staining pattern of the control group (Figure 2B).

The PTZ group exhibited less prominent neuronal nuclei and a slightly weaker Nissl substance staining intensity in the CA3 layers of the hippocampus compared

Table 2. Daily seizure scores of the experimental groups after pentylenetetrazol administration

Alternate days	1 (n=35)	2 (n=35)	3 (n=35)	4 (n=35)	5 (n=35)	6 (n=35)	7 (n=35)	8 (n=35)	9 (n=35)	10 (n=35)	11 (n=35)
Seizure scores	0.0±0.0	1.03±1.98	1.37±2.00*	2.49±2.24***	0.5±0.78*	2.03±2.38***	2.83±2.20***	3.20±2.44***	3.31±2.75***	3.29±2.80***	3.14±2.79***

Notes: Repeated measures analysis of variance and Bonferroni's *post-hoc* tests were performed. Values are expressed as mean±standard deviation. F=20.78, p<0.001. *Indicates a significant difference from day 1 at P<0.05. ***Represents a significant difference from day 1 at p<0.001.

Table 3. Seizure score differences on alternate day 11 between experimental groups after pentylenetetrazol administration

Groups	PTZ (*n*=7)	PTZ+SV (*n*=7)	PTZ+TT (LD) (*n*=7)	PTZ+TT (ID) (*n*=7)	PTZ+TT (HD) (*n*=7)
Seizure scores	4.29±1.60	3.50±1.76	1.20±0.84**	1.00±0.63***[b]	3.25±0.96

Notes: One-way analysis of variance and Bonferroni's *post-hoc* tests were performed. Values are expressed as mean±standard deviation. F=8.52, p<0.001. **Indicates a significant difference from the PTZ group at p<0.01. ***Represents a significant difference from the PTZ group at p<0.001. [b]Significantly different from the PTZ+SV group at p<0.05.
Abbreviations: HD: High dose; ID: Intermediate dose; LD: Low dose; PTZ: Pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera*.

Table 4. Quantal protection and mortality rate of the experimental groups after pentylenetetrazol administration

Groups	Quantal protection	Percentage protection (%)	Mortality rate	Percentage mortality (%)
PTZ (*n*=7)	4/7	57.14	3/7	42.86
PTZ+SV (*n*=7)	6/7	85.71	1/7	14.29
PTZ+TT (LD) (*n*=7)	5/7	71.42	2/7	28.58
PTZ+TT (ID) (*n*=7)	6/7	85.71	1/7	14.29
PTZ+TT (HD) (*n*=7)	4/7	57.14	3/7	42.86

Abbreviations: HD: High dose; ID: Intermediate dose; LD: Low dose; PTZ: Pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera*.

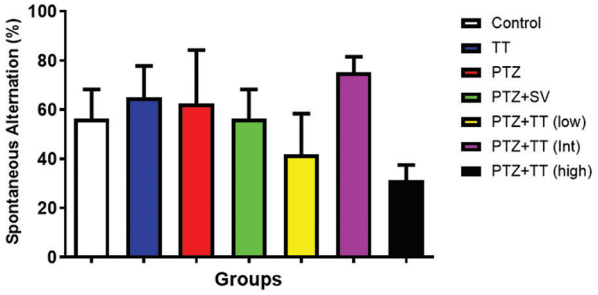

Figure 1. Spontaneous alternation behavior test. A repeated measure analysis of variance and Tukey's *post-hoc* tests. No significant differences were observed among the groups (F=1.751; p=0.1475). Notes: Values are expressed as mean ± standard error of the mean. Sample size per group: Control=5; TT=5; PTZ=4; PTZ+SV=4; PTZ+TT (low)= 4; PTZ+TT (int)=5; PTZ +TT (high)=4.
Abbreviations: PTZ: Pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera*.

to the control group (Figure 2C). The hippocampal CA3 layers in the groups pre-treated with sodium valproate or high-dose *T. tetraptera* showed no apparent Nissl substance staining intensity compared to the control group (Figure 2D and G). Similarly, the CA3 region in the low-dose *T. tetraptera*-pretreated group showed no apparent difference in Nissl substance staining intensity compared to the control group (Figure 2E). The CA3 region of the intermediate-dose *T. tetraptera*-pretreated group also showed no apparent difference in Nissl substance staining intensity compared to the control group (Figure 2F).

A repeated measure analysis of variance and a Tukey's *post-hoc* test revealed no significant difference (p>0.05) in the number of cresyl violet-stained pyramidal cells between the test groups and the control group, or among the test groups. However, the number of other cresyl violet-stained cell types in the sodium valproate and intermediate-dose *T. tetraptera*-pretreated groups was significantly lower (p<0.05) compared to the control group (Figure 3).

3.7. Neuron-specific enolase immunoreactivity

Expression of NSE was observed in the hippocampal CA3 region of the control group (Figure 4A). The hippocampal CA3 regions of the *T. tetraptera*-alone and the low-dose *T. tetraptera*-pretreated groups showed increased NSE expression intensity throughout the layers compared to the control group (Figure 4B and E). The CA3 region of the PTZ group also exhibited increased NSE expression intensity throughout the layers compared to the control group (Figure 4C). In contrast, the CA3 regions of the sodium valproate and the intermediate- and high-dose *T. tetraptera*-pretreated groups showed reduced NSE expression intensity in some neurons of the layers compared to the control group (Figure 4D, 4F, and 4G).

A repeated measures analysis of variance and *post-hoc* tests revealed that the number of NSE-positive pyramidal cells was significantly higher (p<0.05) in the *T. tetraptera* group, but significantly lower (p<0.05) in the PTZ group and the intermediate- and high-dose *T. tetraptera*-pre-treatment groups compared to the control group. There were significantly fewer (p<0.05) NSE-positive pyramidal cells in the PTZ group compared to the *T. tetraptera*-alone and sodium valproate-pretreated groups, with no significant differences (p>0.05) when compared to the low, intermediate, and high-dose *T. tetraptera* groups (Figure 5).

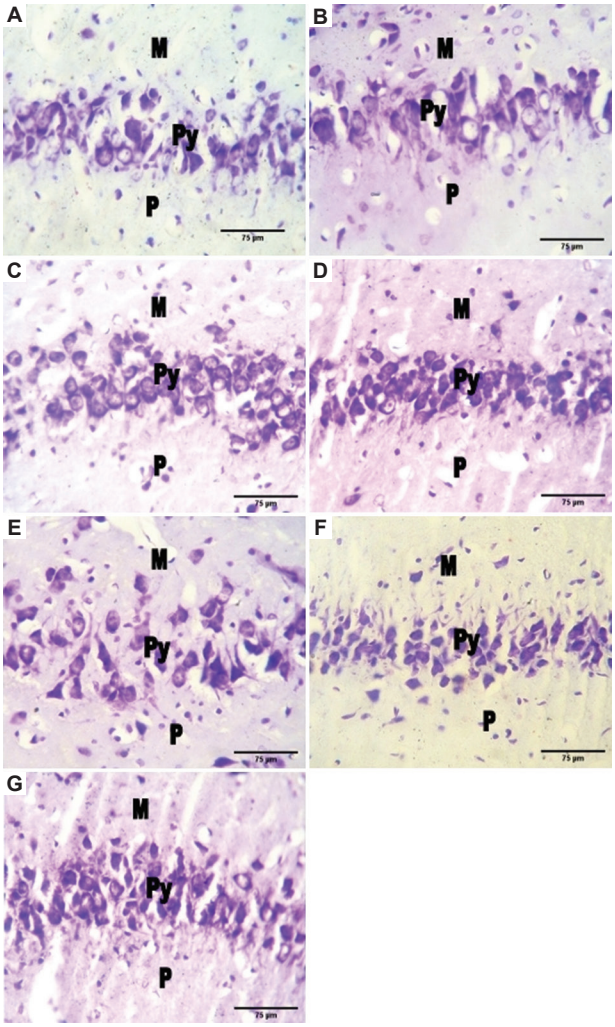

Figure 2. Sections of the hippocampal cornu ammonis 3 region stained with cresyl fast violet (scale bars: 75 µm; magnification power: ×400). (A) Control group showing deeply stained Nissl substance. (B) *Tetrapleura tetraptera* group with strongly stained Nissl substance. (C) Pentylenetetrazol group showing the reduced staining intensity of Nissl substance. (D) Sodium valproate pre-treatment group with moderately stained Nissl substance. (E) Low-dose *T. tetraptera*-pretreatment group showing less intense Nissl substance staining. (F) Intermediate-dose *T. tetraptera*-pretreatment group with intensely stained Nissl substance. (G) High-dose *T. tetraptera*-pretreatment group showing moderately stained Nissl substance.
Abbreviations: M: Molecular; P: Polymorphic layers; Py: Pyramidal layer.

Other NSE-positive cell types were significantly fewer (p<0.05) in the test groups compared to the control group. However, the intermediate-dose *T. tetraptera* group had significantly (p<0.05) more NSE-positive cells than the PTZ group (Figure 5).

3.8. Glial fibrillary acidic protein immunoreactivity

Glial fibrillary acidic protein was expressed in the hippocampal CA3 regions of the control and *T. tetraptera* groups (Figure 6A and B). The PTZ group exhibited slightly reduced GFAP expression in the hippocampal CA3 region compared to the control group (Figure 6C). In contrast, the hippocampal CA3 regions of the groups pre-treated with sodium valproate, as well as the low, intermediate, and high doses of *T. tetraptera*, showed less GFAP expression compared to the control group (Figure 6D-G).

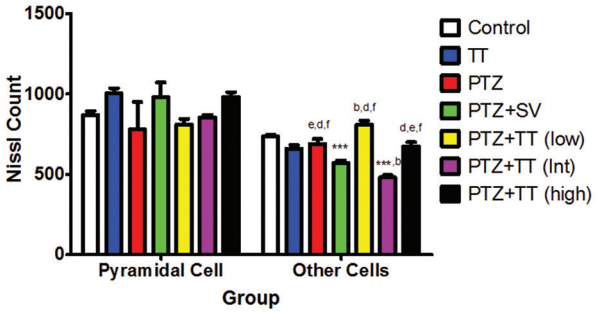

Figure 3. Nissl-stained cell count of the cornu ammonis 3 region of the hippocampus. A repeated measures analysis of variance and *post-hoc* tests were performed. Values are expressed as mean±standard error of the mean. Notes: ***Indicates significant difference from the control group at p<0.001. [b,d,e,f]Significantly different from TT, PTZ+SV, PTZ+TT (low), and PTZ+TT (int), respectively, at p<0.05. Sample size per group: Control=3; TT=3; PTZ=3; PTZ+SV=3; PTZ+TT (low)=3; PTZ+TT (int)=3; PTZ+TT (high)=3.
Abbreviations: PTZ: pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera*.

A repeated measures analysis of variance and *post-hoc* tests revealed that the number of GFAP-labeled cells was significantly lower (p<0.05) in the *T. tetraptera*-alone group, as well as in the sodium valproate, intermediate-dose, and high-dose *T. tetraptera*-pretreatment groups, compared to the control group. However, there was no significant difference (p>0.05) in the GFAP-labeled cells between the PTZ group and the low-dose *T. tetraptera*-pretreated group, in contrast to the control group. The number of GFAP-labeled cells was significantly higher (p<0.05) in the PTZ group compared to the sodium valproate and the intermediate- and high-dose *T. tetraptera*-pretreated groups (Figure 7).

4. Discussion

The study investigated the effects of *T. tetraptera* fruit extract on spontaneous alternation behavior, Nissl substance staining, and NSE and GFAP immunoreactivity in PTZ-induced epileptic Wistar rats. Phytochemical screening of *T. tetraptera* fruit extract revealed high concentrations of phenols, alkaloids, saponins, tannins, flavonoids, terpenoids, and steroids, while cardiac glycosides and anthraquinones showed weak positive reactions. Plants, due to the presence of these phytochemicals, play an active role in medicine and

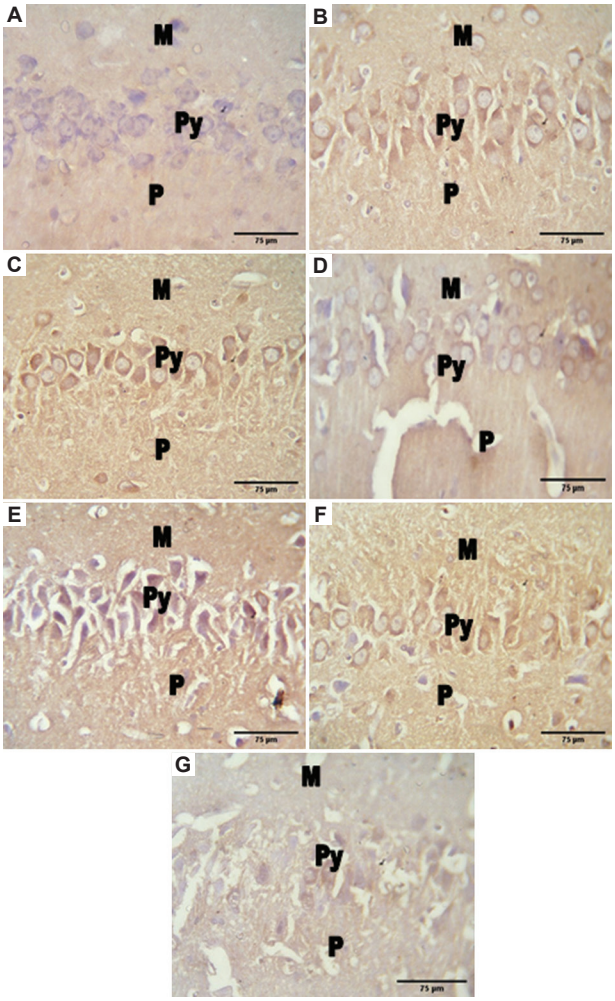

Figure 4. Sections of the hippocampal cornu ammonis 3 with neuron-specific enolase (NSE)-positive cells (brown staining; scale bars: 75 µm; magnification power: ×400). (A) Control group with NSE-positive cells. (B) *Tetrapleura tetraptera* group showing increased intensity of NSE expression. (C) Pentylenetetrazol group showing increased intensity of NSE expression. (D) Sodium valproate-pretreated group showing reduced intensity of NSE expression. (E) Low-dose *T. tetraptera*-pretreatment group showing slightly increased NSE expression. (F and G) Intermediate- and high-dose *T. tetraptera*-pretreatment groups showing reduced NSE expression intensity.
Abbreviations: M: Molecular; P: Polymorphic layers; Py: Pyramidal layer.

nutrition, offering antioxidant properties that help protect cells from oxidative stress, among other benefits.[49-51] The combination of these phytochemicals contributes to the diverse range of beneficial physiological activities associated with *T. tetraptera*.[28,31,52] The present results corroborate previous findings[52,53] that reported the presence of similar phytochemical profiles in both the aqueous and ethanol extracts of *T. tetraptera*.

The safety of *T. tetraptera* was assessed through acute toxicity testing. The result indicated an oral LD_{50} of

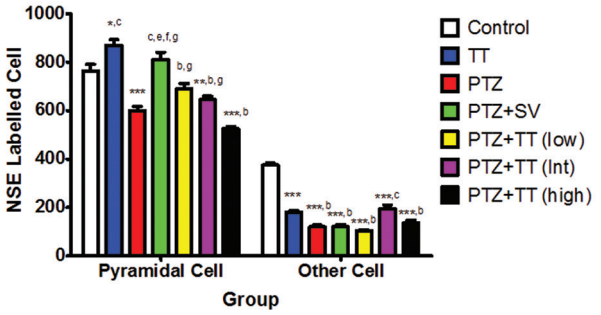

Figure 5. Neuron-specific enolase-positive cell count in the hippocampal cornu ammonis 3 region of the experimental groups. A repeated measures analysis of variance and Tukey's *post-hoc* tests were performed. Values are expressed as mean±standard error of the mean. Notes: Sample size of per group: Control=3; TT=3; PTZ=3; PTZ+SV=3; PTZ+TT (low)=3; PTZ+TT (int)=3; PTZ+TT (high)=3. *,**, and ***Indicate significant differences from the control at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. [b,c,e,f,g]Significantly different from TT, PTZ, PTZ+TT (low), PTZ+TT (int), and PTZ+TT (high), respectively, at $p<0.05$.
Abbreviations: PTZ: pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera*.

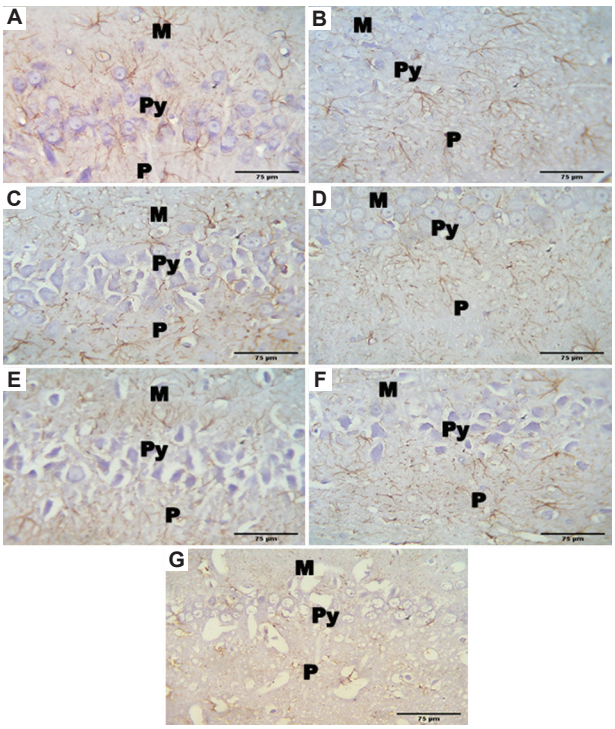

Figure 6. Sections of the hippocampal cornu ammonis 3 showing glial fibrillary acidic protein (GFAP)-positive cells (brown staining; scale bar: 75 µm; magnification power: ×400). (**A** and B) Control and *Tetrapleura tetraptera* groups showing expression of GFAP. (C) Pentylenetetrazol group showing decreased expression of GFAP. (D and E) Sodium valproate and low-dose *T. tetraptera*-pretreated groups showing decreased expression of GFAP. (F and G) Intermediate- and high-dose *T. tetraptera*-pretreated groups showing decreased expression of GFAP.
Abbreviations: M: Molecular; P: Polymorphic layers; Py: Pyramidal layer.

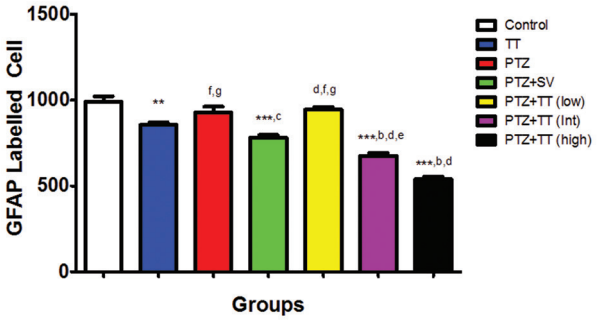

Figure 7. Glial fibrillary acidic protein (GFAP)-labeled cell count of the hippocampal cornu ammonis 3 region of the experimental groups. A repeated measures analysis of variance and Tukey's *post-hoc* tests were performed. Values are expressed as mean±standard error of mean. Notes: Sample size per group: Control=3; TT=3; PTZ=3; PTZ+SV=3; PTZ+TT (low)=3; PTZ+TT (int)=3; PTZ+TT (high)=3. ** and ***indicate significant differences from the control at $p<0.01$ and $p<0.001$, respectively. [b,c,d,e,f]Significantly different from TT, PTZ, PTZ+SV, PTZ+TT (low), PTZ+TT (int), and PTZ+TT (high), respectively, at $p<0.05$.
Abbreviations: PTZ: pentylenetetrazol; SV: Sodium valproate; TT: *Tetrapleura tetraptera.*

over 5,000 mg/kg in mice, supporting the safety of the extract, as this concentration is typically the end-point of LD_{50} determination.[54] These findings align with those of a previous study,[55] which reported an LD_{50} of over 10,000 mg/kg body weight in a mouse model.

Kindling is a recognized experimental model for human epilepsy, induced through sub-convulsive doses of chemicals or electrical stimulation.[56,57] PTZ, a chemical convulsant, exerts its effects by inhibiting GABA activity at the $GABA_A$ receptor, thus mimicking epilepsy.[58] In the present study, PTZ administration resulted in increasing seizure scores and kindling, which progressively intensified with each administration. Increased seizure scores, as assessed using the Racine scale, denote the worsening seizure severity, consistent with previous reports.[45,57] Sodium valproate, a standard antiepileptic drug, failed to suppress seizures in this study, as the seizure scores in this group did not differ significantly from those in the PTZ group. This result contrasts with previous studies that reported antiseizure effects of sodium valproate at doses of 200 and 300 mg/kg,[44] which were also used in the present study. While sodium valproate is known to attenuate PTZ-induced seizures,[59] this contrasting result may be due to a spike in glycine levels, as sodium valproate also interferes with glycine synthesis, potentially leading to increased seizure frequency.[60]

T. tetraptera attenuated PTZ-induced kindling, with significant reductions ($p<0.05$) in seizure scores observed in the low-, intermediate-, and high-dose *T. tetraptera*-pretreated groups compared to the PTZ group. These findings suggest that the administered doses of *T. tetraptera* possess anticonvulsant activity, potentially reducing the severity of PTZ-induced seizures, a result consistent with a previous report.[36] Although the exact mechanism underlying *T. tetraptera*'s anticonvulsant effect remains to be elucidated, it is likely mediated through GABAergic transmission. This hypothesis is supported by a prior study indicating that alkaloids and flavonoids,[61] which are also constituents of *T. tetraptera*, enhance GABA transmission.

Pentylenetetrazol administration resulted in mortality, with the PTZ group exhibiting a lower percentage of quantal protection, suggesting toxicity, as previously reported.[62] The groups pre-treated with sodium valproate, intermediate doses of *T. tetraptera*, and intermediate doses of *T. tetraptera* demonstrated greater protection and lower mortality compared to the PTZ group, supporting previous findings.[36] Sodium valproate, an established antiepileptic drug (54), exhibited expected antiseizure activity, whereas *T. tetraptera's* effect may be attributed to its rich phytochemical constituents, which have been shown to be safe in the present study. Notably, the high-dose *T. tetraptera*-pretreatment group showed no significant difference in quantal protection or mortality when compared to the PTZ group, suggesting that high doses of *T. tetraptera* may be unnecessary or potentially harmful, either exacerbating or having no effect on PTZ toxicity.

The spontaneous alternation behavior test is commonly used to assess short-term memory and identify memory impairments associated with various disease conditions.[11,48] The present results revealed no significant difference ($p>0.05$) between the test and control groups, suggesting that the treatment regimens did not influence spontaneous alternation. This lack of effect may be attributed to the treatment's inadequacy in influencing spatial brain functions. Given that spontaneous alternation is related to learning and memory,[48] this study found no impairment in these functions. Chemical kindling models, including those using PTZ, have previously reported cognitive, learning, and memory impairments.[63] The discrepancy between these findings and the present study could be due to the animal model used. Furthermore, the current results contrast with another study in which PTZ kindling resulted in learning and memory deficits in mice,[57] which may be attributed to the differences in behavioral paradigms tested.

Nissl substance, present in all neurons, is a rough endoplasmic reticulum component essential for protein synthesis[64] and frequently disintegrates during chromatolysis.[16] PTZ also affects this macromolecule.[9,56] In the current study, the CA3 hippocampal region of the

PTZ group demonstrated disintegrated and dispersed Nissl substance, which appeared slightly less stained, indicating PTZ toxicity. This observation aligns with a previous report.[56] The cellular population in the Nissl-stained pyramidal and other cell types was not significantly different in the PTZ group compared to the control, suggesting that the cellular population was not adversely affected, and no immediate loss of protein synthesis function occurred.

On the other hand, the *T. tetraptera*-alone group demonstrated high Nissl stain intensity in most pyramidal neurons, while no apparent difference was observed in the sodium valproate, pre-treated group or in the low-, intermediate-, and high-dose *T. tetraptera* groups. These observations indicate that Nissl substance distribution was not adversely affected. A previous report suggests that sodium valproate provides neuroprotection,[65] which may explain the current results in this drug-pre-treated group. The findings in the *T. tetraptera*-pre-treatment groups may be attributed to its rich phytochemical composition, which is known to offer neuroprotective benefits.[66,67] The cellular population in the Nissl-stained pyramidal and other cell types was not significantly different in these test groups compared to the control, further indicating that the cellular populations remained unaffected.

Neurons can also be identified immunohistochemically by their expression of, or immunoreactivity to, anti-NSE. This anti-NSE binds to NSE, a cytoplasmic glycolytic enzyme, and its reactivity reflects the metabolic state of the neurons. Increased NSE expression suggests high metabolic activity.[17] In the present study, there was increased intensity of NSE expression in the hippocampus of the PTZ group, with the population of NSE-positive cells significantly lower compared to the control. This observation indicates increased neuronal activity, potentially accompanied by injury to the hippocampal neurons. In response to injury, NSE expression and activity are markedly upregulated,[68] and neurotoxic agents have been associated with increased NSE expression.[69] Increased NSE levels may result from dysfunction in neuronal metabolic activity and synaptic connections, which may have been induced by PTZ.

Decreased intensity of NSE expression was observed in the hippocampus of the sodium valproate, intermediate, and high-dose *T. tetraptera*-pretreated groups compared to the control group. Although the number of NSE-positive pyramidal cells was not significantly different in the sodium valproate pre-treated group, it was significantly lower in the intermediate- and high-dose *T. tetraptera*-pretreated groups compared to the control. These results indicate an increase in neuronal metabolic activity in these groups, which may not be harmful. Sodium valproate, known for its neuroprotective properties,[65] may explain the intensity of NSE expression observed in this group. *T. tetraptera* has also been reported to have neuroprotective effects,[36] likely due to its rich phytochemicals, as demonstrated in the present study. *T. tetraptera* may exert its effect by influencing ionotropic GABA receptors and inhibiting acetylcholinesterase, thereby increasing GABA levels while antagonizing N-methyl-D-aspartate.[66,67,70] This neuroprotective effect of *T. tetraptera* corroborates findings from another study,[71] which reported similar effects of *Moringa oleifera*, a plant containing comparable phytochemicals, on this glycolytic enzyme.

In the low-dose *T. tetraptera* group, there was increased intensity of NSE expression, although the number of NSE-positive pyramidal cells did not significantly differ from the control. This observation suggests increased neuronal activity in this group. Given that the NSE-positive cell population was not significantly altered, it may indicate a non-adverse effect of the *T. tetraptera* pre-treatment.

Astrocytes are glial cells found in the hippocampus, as well as in other regions of the brain. They can be identified immunohistochemically through the expression of GFAP, an intermediate filament protein that provides mechanical strength to astrocytes.[18] Increased GFAP expression indicates astrocyte activation. In the present study, the PTZ group showed a slight decrease in GFAP expression in the CA3 region of the hippocampus compared to the control, which may indicate neurotoxicity, although the difference in population was not statistically significant. Following injury and astrogliosis, GFAP is typically expressed at much higher levels,[72] which contrasts with the findings in the present study. Decreased GFAP expression is also commonly reported in neurodegenerative or other detrimental conditions,[73] which may have been the case in the present study.

The CA3 regions of the hippocampus in the *T. tetraptera* group showed a similar GFAP expression to the control, although with a significantly smaller population. This observation suggests little to no adverse effect from the plant, as previously reported in the study. The CA3 regions of the hippocampus in the sodium valproate, and low, intermediate, and high doses of *T. tetraptera*-pretreatment groups exhibited decreased GFAP expression, with a significantly smaller ($p<0.05$) population. These findings may indicate a protective effect, as these pre-treated substances are known to offer neuroprotection. In this context, decreased GFAP expression could be due to either cytoskeletal destabilization or a loss of GFAP antigenicity.[74]

The hippocampus, particularly the CA3 region, plays a key role in cognitive processes such as learning and memory and is highly susceptible to trauma. Any alteration in the

normal regulation of its cellular and structural components can adversely affect or alter its functions,[10] as observed with the PTZ treatment. In contrast, the functions and structure of the hippocampus appear to be protected with *T. tetraptera* pre-treatment in the present study, with the mechanism of action likely involving the elevation of the GABAergic pathway.

In summary, PTZ-induced kindling in rats, possibly through GABAergic inhibition and glutamatergic stimulation, led to mortality from excitotoxicity, Nissl substance dispersal, NSE upregulation, and GFAP downregulation in the hippocampal CA3. Pre-treatment with *T. tetraptera* doses may have modulated both the GABAergic and glutamatergic pathways to suppress kindling, resulting in high quantal protection and minimal effects on Nissl substance, NSE, and GFAP.

5. Conclusion

This study demonstrated that PTZ-induced seizures may result in rat mortality, along with histological and immunohistochemical alterations in the hippocampal CA3 region. Oral administration of *T. tetraptera* fruit extract, a safe and phytochemical-rich plant, provided protection against PTZ-induced seizures and mortality in Wistar rats. In addition, the extract preserved hippocampal Nissl substance, NSE, and GFAP expression. The low (250 mg/kg) and intermediate (500 mg/kg) doses of *T. tetraptera* fruit extract offered the greatest protection, even in comparison with sodium valproate. While further research is needed to clarify its mechanism of action, this plant extract may hold promise for seizure management.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Clementina F. Iniodu, Moses B. Ekong
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Investigation: Clementina F. Iniodu
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Ethics approval and consent to participate

This study involved the use of animals. Ethical approval for the research was obtained from the Faculty of Basic Medical Sciences Research and Ethical Committee, with approval number: UU_FBMSREC_2022_001.

Consent for publication

Not applicable.

Availability of data

Not applicable.

Further disclosure

Part of this set of findings has been presented in a conference and has been published as an abstract.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

TDP43 negatively regulates TBK1-mediated IFN1 production through IRF7 pathway in neurodegenerative diseases

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Abstract

Mutations in the genes encoding TAR DNA-binding protein 43 (*TDP43*) or TANK-binding kinase 1 (*TBK1*) have been strongly associated with neurological disorders, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia. TDP43 is a key component of pathological protein aggregates found in more than 90% of ALS cases, while TBK1 plays a critical role in innate immune signaling and autophagy. Despite these associations, the precise molecular mechanisms linking TDP43 or TBK1 dysfunction to neurodegeneration remain poorly understood. The present study examined the impact of TDP43 on TBK1-mediated type I interferon (IFN1) production in HEK-293T cells. The findings demonstrated that co-expression of TDP43 and TBK1 resulted in a dose-dependent reduction in TBK1 and interferon regulatory factor (IRF) 7 protein levels. In addition, it led to decreased phosphorylation of IRF3 and TBK1. Interestingly, TDP43 knockout cells displayed elevated IRF7 protein levels. Moreover, co-expression of TDP43 and TBK1 significantly suppressed the IFN1 inductions and associated pro-inflammatory cytokines, a suppression reversed by IRF7 overexpression. Further, mechanistic analysis demonstrated that TDP43 facilitates TBK1 degradation through autophagy, resulting in diminished IFN1 induction. These findings uncover a new pathway through which TDP43 disrupts TBK1-mediated signaling through IRF7, potentially contributing to neurodegeneration. Overall, the disrupted TBK1-IRF7-IFN1 axis may therefore represent a critical pathway in TDP43-associated neurodegenerative diseases, offering potential targets for therapeutic intervention.

Keywords: Amyotrophic lateral sclerosis; TAR DNA-binding protein 43; TANK-binding kinase 1; Type I interferons; Interferon regulatory factor 7

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of upper and lower motor neurons, leading to muscle weakness, atrophy, paralysis, and ultimately respiratory failure.[1,2] A hallmark of ALS is the abnormal cytoplasmic mislocalization and aggregation of TAR DNA-binding protein 43 (TDP43), observed in over 90% of ALS cases.[1] TDP43, a ubiquitously expressed protein, plays a crucial role in maintaining cellular survival under normal physiological conditions. Studies have shown that TDP43 disrupts RNA metabolism, endocytosis, the ubiquitin-proteasome system (UPS), and mitochondrial function.[3-6] Furthermore, autophagy-related proteins, such as light chain 3 (LC3) and p62, have been found to colocalize with TDP43 aggregates, and TDP43 downregulation reduces LC3 and p62 levels,[7,8] indicating a reciprocal regulatory relationship between TDP43 and autophagy. Recent findings suggest that TANK-binding kinase 1 (TBK1) overexpression promotes the autophagic degradation of endogenous TDP43.[9] In addition to TBK1, other ALS-associated genes, such as ubiquilin-2 (*UBQLN2*), *C9orf72*, and profilin-1 (*PFN1*), have also been implicated in regulating TDP43 dynamics.[10-12]

Most ALS cases are sporadic, with *TBK1* mutations identified as a significant contributing factor in approximately 4% of ALS and frontotemporal dementia (FTD) patients.[9] FTD refers to a group of neurological disorders caused by neurodegeneration in the brain, leading to shrinkage of these regions. This condition can impact behavior, personality, and motor functions. TBK1, an innate immune kinase, activates interferon regulatory factors (IRF) including IRF3 and IRF7, which, in turn, promote the production of type I interferons (IFN1). In $TBK1^{\Delta/\Delta}$ mice, pathological findings include hyperkeratosis, necrosis, hyperplasia, inflammatory cell infiltration, and edema in the skin at 2 weeks of age. In comparison to $TBK1^{+/-}$ and $TBK1^{+/\Delta}$ mice, these mice show heightened vulnerability to lipopolysaccharide (LPS)-induced lethality at 3 months, marked by elevated levels of pro-inflammatory cytokines in serum, including tumor necrosis factor-alpha, interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), and keratinocyte chemoattractant. The heightened immune response and cytokine production in $TBK1^{\Delta/\Delta}$ mice are attributed to an expanded circulating mononuclear cell compartments and elevated immune cell activation.[13,14] On activation, TBK1 facilitates IRF3 and IRF7 homodimerization and nuclear translocation, enabling them to function as transcription factors for IFN1 (specifically IFN-α and IFN-β) and associated cytokines.[13-15] In humans and mice, interferon-producing cells (IPCs) comprise 0.2% – 0.8% of peripheral blood mononuclear cells and selectively express toll-like receptor (TLR) 7 and toll-like receptor 9. On viral stimulation, IPCs secrete large quantities of IFN1, which activate B-cells, natural killer cells, T-cells, and myeloid dendritic cells during antiviral immune responses.[16] Although IRF3 and IRF7 share structural and functional similarities, their roles are distinct and essential. IRF7, a critical regulator of IFN1-dependent immune responses, plays a more prominent role than IRF3 in systemic IFN induction. Studies with IRF7-deficient ($IRF7^{-/-}$) mice have demonstrated that IRF7 is indispensable for inducing IFN-α/β expression. These mice show a significant reduction in serum IFN levels and heightened susceptibility to viral infections, emphasizing the importance of IRF7 in innate and adaptive immunities.[16,17] While IRF3 is ubiquitously expressed across various cell types, IRF7 is highly expressed in plasmacytoid dendritic cells. Despite this, IRF7 remains essential for regulating IFN1 production.[18]

TBK1 has been widely studied for its role in interacting with autophagy receptors, including p62 and optineurin, which are essential in regulating selective autophagy. By phosphorylating these receptors, TBK1 enables the targeting of damaged proteins and organelles to autophagosomes for degradation, serving as a crucial component in the cellular protein degradation pathway. Autophagy, a vital cellular mechanism for eliminating toxic protein aggregates and unwanted materials, is often dysregulated in ALS/FTD, implicating its role in disease progression. For instance, *TBK1* mutations result in the accumulation of autophagic markers LC3 and p62 (SQSTM1), impairing autophagic induction.[13,19-23] Similarly, mutations in *UBQLN2*, another ALS-associated gene, impair TBK1's binding to p62, further disrupting autophagic induction.[15] TBK1-deficient mice show elevated LC3 and p62 levels, reinforcing the link between TBK1 dysfunction and impaired autophagy.[14,15] In addition, mutations in *p62* are linked to ALS/FTD, emphasizing that autophagic dysfunction plays a critical role in the pathogenesis of neurodegeneration.

The present study explored the effect of TDP43 on TBK1, revealing that TDP43 overexpression significantly reduced TBK1 expression, accompanied by decreased IRF7 and IFN1 production. However, overexpression of IRF7 alleviated these reductions. These findings indicate that TDP43 negatively regulates TBK1-mediated IFN1 production through IRF7, and disruption of this pathway may be a key factor in TDP43-associated neurodegeneration.

2. Materials and methods

2.1. Reagents

Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132), chloroquine (CQ), and bafilomycin A1 (A1) were obtained from Sigma-Aldrich (USA). HEK-293T cells were treated with MG132 (10 μM), CQ (100 μM), or A1 (100 μM) for 16 h, followed by collection 24 h post-transfection.

2.2. Plasmid construction

Plasmids were generated as described in previous protocols.[15,24] Human TDP43 was tagged with a 3x FLAG tag at its N-terminus, and a Myc tag was fused to the C-terminus using the pcDNA3 vector. Sequencing was performed to confirm the open reading frame of all plasmids before use. IRF3 and IRF7 plasmids, each harboring a FLAG tag, were obtained from GenScript (NJ, USA).

2.3. Cell culture and transfection

HEK-293T cells were purchased from ATCC, USA and maintained in Dulbecco's Modified Eagle Medium (DMEM) at 37°C in a CO_2 incubator. The culture medium contained 10% fetal bovine serum and antibiotics (ampicillin and streptomycin). Plasmid transfections were performed using Lipofectamine-2000 (Thermo Fisher Scientific, USA) when the cells reached 70% confluency in 10-cm dishes. Cells were collected for RNA extraction 24 h post-transfection and for immunoblot analysis 48 h post-transfection.

For dose-dependent experiments, HEK-293T cells were divided into five groups. The control group was transfected with 2 μg of TDP43 plasmid alone. TBK1 and TDP43 plasmids were co-transfected in the remaining four groups (TBK1: 2 μg; TDP43: 0, 0.2, 0.5, and 1.5 μg). Cells were collected for immunoblot analysis 48 h post-transfection.

2.4. CRISPR-Cas9-mediated knockout (KO) cells

TDP43 KO HEK-293T cells were generated by CRISPR-Cas9 technology (Addgene, USA). The Cas9 vector was modified to include a guide RNA targeting the *TDP43* gene. Twenty-four hours post-transfection, 500 cells were counted and cultured in 10-cm dishes for 1 week. Single clones (48 in total) were expanded and screened by immunoblotting with a TDP43 antibody, followed by further confirmation through DNA Sanger sequencing.

2.5. SDS-PAGE electrophoresis

The harvested cells for immunoblot analysis were lysed in a protein extraction buffer on ice (1% Triton X-100). The buffer contained 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 150 mM NaCl. The lysates were incubated for 30 min and then centrifuged for 10 min at 16,000 × g. The supernatant, which contained the total protein solution for each sample, was collected. The protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Ten micrograms of total proteins were loaded and resolved on 10% SDS-PAGE gel, and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free milk, following established protocols.[15] Accordingly, each transferred membrane was probed with a primary antibody overnight at 4°C. The following antibodies were used for immunoblot analysis: rabbit anti-TBK1, rabbit anti-p-TBK1^{s172}, rabbit anti-IRF3, and rabbit anti-p-IRF3^{s386} (1:1000 dilution; Abcam, UK), rabbit anti-IRF7 (1:1000 dilution; Cell Signaling Technology, USA), rabbit anti-p62 (1:2000 dilution; Proteintech, USA), rabbit anti-TDP43 (1:1000 dilution, Proteintech, USA,), and mouse anti-GAPDH (1:5000 dilution, Proteintech, USA).

The primary antibody solutions were removed on the following day, and the membranes were washed. Secondary antibody was then applied to each membrane. Chemiluminescent detection was performed using ECL reagents (Cat. No. AR1196; Boster Biological Technology, USA), and imaged with a Tanon 5200 imaging system (Tianneng Technology Co. Ltd, China). Band intensities were measured by Image J software (NIH, USA).

2.6. RNA extraction and quantitative polymerase chain reaction (qPCR)

Myc-TBK1 and FLAG-TDP43 were co-transfected into HEK-293T cells. Twenty-four hours post transfection, cells were collected for total RNA extraction using Trizol reagent (Thermo Fisher Scientific, USA). Reverse transcription was performed using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, USA). One microgram of RNA was used for each reaction. SYBR Green was used for qPCR analysis, which was conducted on an Applied Biosystems Real-Time PCR Instrument (Thermo Fisher Scientific, USA). The mRNA levels of the targets were normalized based on *GAPDH* expression. The following primers (5' – 3'; forward and reverse, respectively) were used: IFN-β (GACTTACAGGTTACCTCCGAAA, and CATATGCAGTACATTAGCCAT); IFN-α (TGACAGAGAAGAAATACAGCC, and ATTGTTT TCATGTTGGACCAG); IL6 (TGTGCAGATG AGTACAAAAGTCCT, and ATGTCCTGCAGC CACTGGTTC); IL8 (GCCAACACAGAAATTAT TGTAAAGC, and CTGGCATCTTCACTGATTCT TG); ISG54 (CTTCCCAGTCTATCATCAACCTT, and CCGTCGCTTCTAGCTATGTATCT); ISG56 (T CATCAGGTCAAGGATAGTC, and CCACACTGTA TTTGGTGTCTAGG); IRF3 (GTGCATCCTGCCGT

AGGCCGTGCTT, and ACCTGTGCCGTGGCCCGTG AGAA); and IRF7 (ACACCTGACCGCCACCTAACTG CC, and CGAGTTATCCCGCAGCATCA CGA).

2.7. Dual-luciferase reporter assay

The assay was performed according to the protocol from the company, using Dual-Luciferase® Reporter Assay System (Promega, USA). In HEK-293T cells, IFN-β co-transfected with TBK1 and TDP43. IFN-β was tagged with Firefly luciferase, and Renilla luciferase was used for TK reporter, both luciferase reporters were purchased from Addgene, USA. Twenty-four hours post-transfection, the luciferase activity of IFN-β was detected using the Promega™ GloMax® Plate Reader (Promega, USA). Finally, Firefly luciferase activity was normalized based on Renilla luciferase activity.

2.8. Statistical analysis

Quantification of histological samples was performed independently by two blinded experimenters. Statistical analyses were conducted with GraphPad Prism (San Diego, California, USA). Student's t-test was used to evaluate comparisons between two groups. One-way ANOVA was applied for multi-group comparisons. Results with $P < 0.05$ were considered statistically significant. The data are presented in bar graphs as the mean ± standard deviation.

3. Results

3.1. TDP43 negatively regulates TBK1-mediated IFN1 production

Activated TBK1 phosphorylates IRF3, promoting the induction of IFN1. In our study, it was observed that TBK1 overexpression did not affect the *IRF3* mRNA level in HEK-293T cells, but it resulted in elevated IFN1 and associated pro-inflammatory cytokines induction (Figure 1A). Subsequently, the impact of TDP43 co-expression on both IFN1 and cytokines in TBK1 expressed HEK-293T cells was investigated. The mRNA levels of *IRF3*, *IRF7*, *IFN1*, and cytokines were also examined. TDP43 co-expression did not cause any change in *IRF3* mRNA levels (Figure 1A). However, quantitative PCR analysis revealed a significant decrease in the mRNA levels of *IRF7*, *IFNB*, *ISG54*, and *ISG56* on co-expression of TDP43. In addition, the mRNA levels of *IFNA*, *IL6*, and *IL8* showed a slight decrease (Figure 1A). Furthermore, a luciferase reporter assay was conducted to evaluate IFN-β expression. The results confirmed that IFN-β production was decreased by TDP43 overexpression, and the inhibitory effect was more pronounced with increasing levels of TDP43 expression (Figure 1B). These findings implicate that TDP43 negatively regulates TBK1-mediated induction of IFN1 and related cytokines.

3.2. TDP43 down-regulates TBK1-induced IRF7 expression

A number of studies have reported that TBK1 regulates IFN1 signaling through the phosphorylation of IRF3 and IRF7 and induces the autophagic degradation of endogenous TDP43.[9] In this study, the impact of TDP43 on TBK1-mediated IFN1 production was investigated. HEK-293T cells were transfected with TBK1 and TDP43 plasmids. The protein expressions of phosphorylated TBK1 (p-TBK1), TBK1 (Myc), p-IRF3, IRF3, and IRF7 were assessed through immunoblot analysis (Figure 2A, Figure S1). The results revealed reduced expression of phosphorylated TBK1, IRF3, and total IRF7 in the co-transfection group of TBK1 with TDP43 compared to the TBK1-only transfection group (Figure 2B). These findings indicate that TDP43 may compromise TBK1 stability, leading to reduced levels of p-IRF3 and IRF7. To further explore the effect of TDP43 on TBK1 expression, TBK1 and varying amounts of TDP43 were co-transfected into HEK-293T cells. As depicted in Figure 2C and Figure S2, the levels of TBK1 and IRF7 exhibited a correlation with the changes in TDP43 expression. Moreover, increasing amounts of TDP43 resulted in progressively decreased expression of p-TBK1 and p-IRF3. These findings implicate that TDP43 plays a crucial role in the stability of the TBK1 protein in cells and may promote TBK1 degradation.

3.3. Overexpression of IRF7 mitigates the inhibitory effect of TDP43 on TBK1-mediated IFN1 production

To investigate whether overexpression of IRF3 or IRF7 can alleviate the inhibitory effect of TDP43 on IFN1 production, quantitative PCR analysis was performed. The results revealed that the mRNA levels of *IL6*, *IL8*, *IFNA*, *IFNB*, *ISG54*, and *ISG56* were significantly increased in the IRF7-transfected group. However, the mRNA expression of *IFNA*, *IFNB*, *IL6*, and *IL8* was not affected by IRF3 overexpression. In HEK-293T cells, only *ISG54* and *ISG56* mRNA levels were increased in the IRF3-transfected group. These findings indicate that enhanced overexpression of IRF7 alleviates the inhibition of TBK1-mediated IFN1 and related cytokine production caused by TDP43 (Figure 3A). Immunoblot analysis demonstrated that overexpression IRF3 or IRF7 had no effect on the expression levels of p-TBK1 and TBK1 proteins (Figure 3B). Furthermore, the dual-luciferase reporter assay confirmed a progressive increase in luciferase activity with increasing amounts of IRF7 (Figure 3C, Figure S3). These findings suggest that IRF7 overexpression plays a key role in mitigating TDP43-mediated inhibition of TBK1-induced IFN1 signaling.

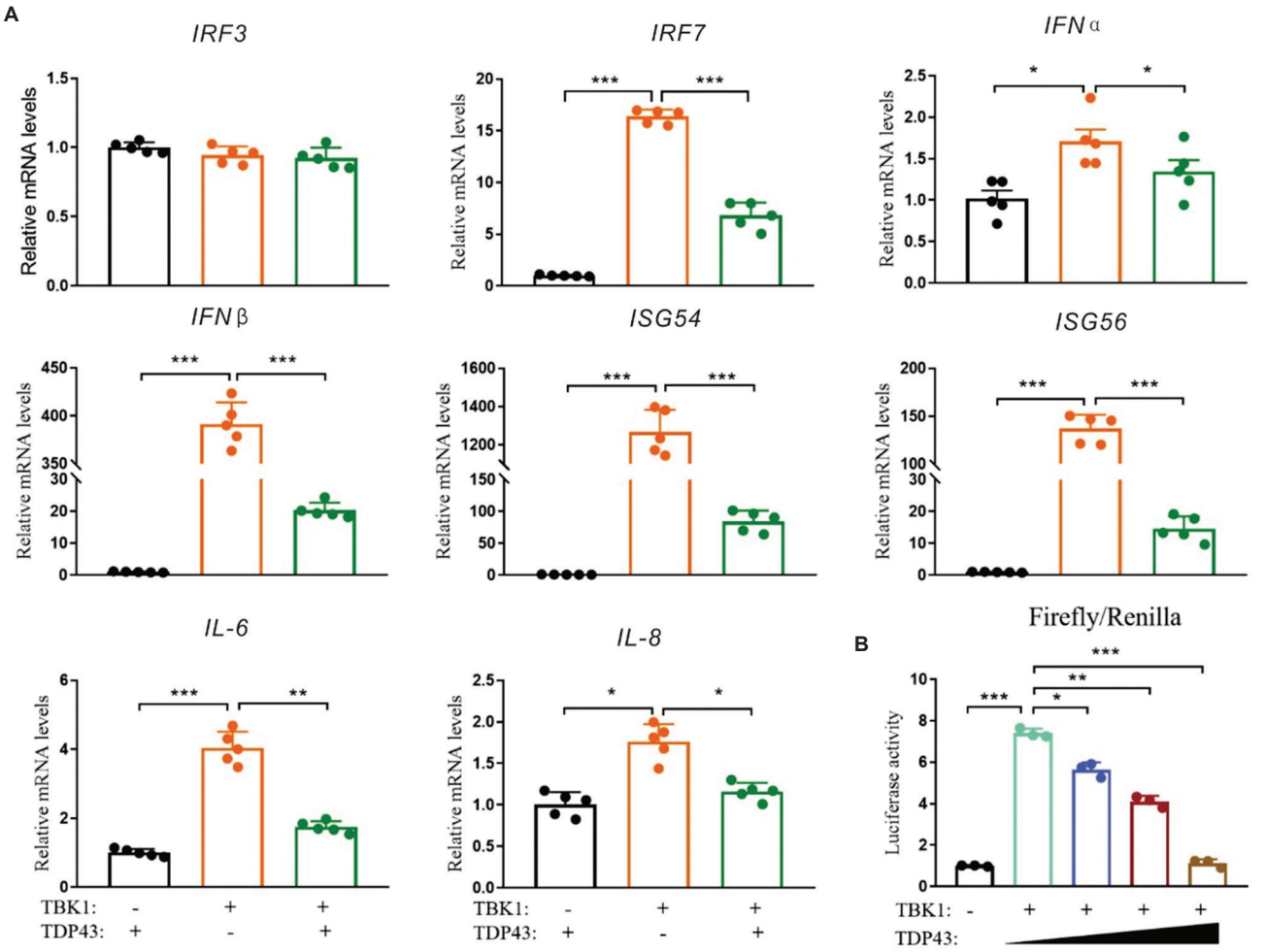

Figure 1. TDP43 suppresses TBK1-mediated IFN1 production. (A) Quantitative PCR analysis of *IRF3*, *IRF7*, *IFN1*, and related cytokine mRNA levels. (B) IFN-β activity measured by dual-luciferase reporter assay. The data are shown as mean ± standard deviation (n = 5 per group). *P < 0.05, **P < 0.01, and ***P < 0.001.

Abbreviations: TBK1: TANK-binding kinase 1; TDP43: TAR DNA-binding protein 43; IRF3: Interferon regulatory factor 3; IRF7: Interferon regulatory factor 7; IFNA: Interferon-alpha; IFNB: Interferon-beta; ISG54: Interferon-stimulated gene 54; ISG56: Interferon-stimulated gene 56; IL6: Interleukin-6; IL8: Interleukin-8.

3.4. Depletion of TDP43 upregulates IRF7 expression

To explore the effect of TDP43 depletion on IRF7, TDP43 KO HEK-293T cells were generated using CRISPR-Cas9 technology. Immunoblot analysis was conducted to evaluate the expression levels of TDP43, TBK1, IRF3, and IRF7 (Figure 4A, Figure S4). The results revealed no significant difference in TBK1 and IRF3 expression between wild-type and TDP43 KO HEK-293T cells. However, IRF7 expression was increased in the TDP43-KO cells (Figure 4B). These findings suggest that IRF7 expression may be regulated by TDP43.

3.5. TDP43 promotes the degradation of TBK1 through autophagy

To further investigate the mechanism by which TDP43 inhibits IFN-β signaling, the effect of TDP43 on TBK1 regulation through autophagy was examined. HEK-293T cells were treated with MG132 (a proteasome inhibitor), chloroquine (CQ, an autophagy inhibitor), or Bafilomycin A1 (A1, an autophagy inhibitor). Immunoblot analysis was conducted to assess the expression levels of the targeted genes. The results revealed that p-TBK1 protein expression was elevated in the MG132-treated group (Figure 5A, Figure S5). Both p-TBK1 and p-IRF3 were increased in the groups treated with MG132+CQ or MG32+A1 compared to the untreated group (Figure 5A, Figure S5). In addition, both p62 and LC3-II were also increased in the groups treated with MG132+CQ or MG132+A1 compared to the untreated group. No alteration was observed in the expression of p62 and LC3-II between the MG132-only treated group and the untreated group (Figure 5A, Figure S5). Consistently,

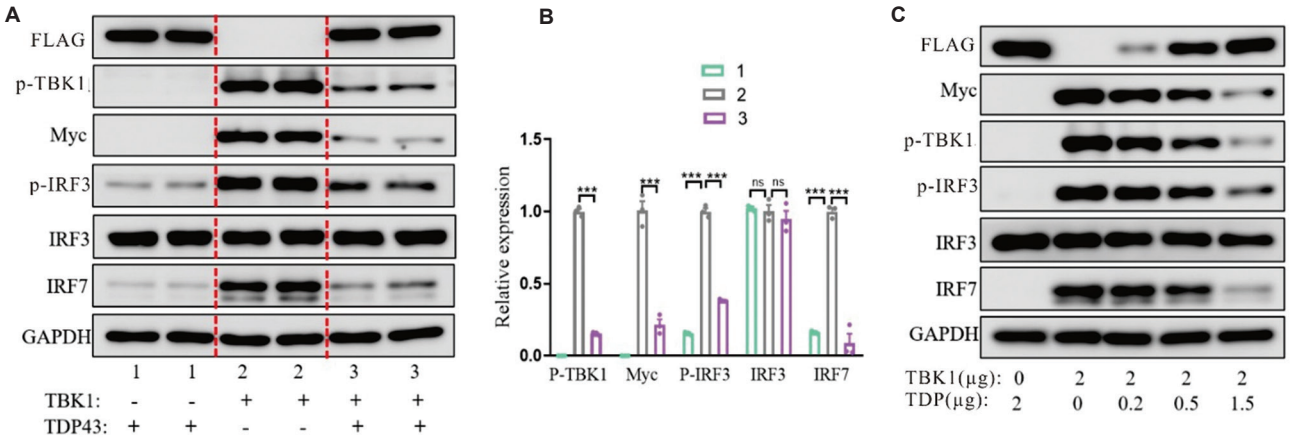

Figure 2. TDP43 reduces TBK1-induced IRF7 expression. (A) Immunoblot analysis of TBK1, IRF3, and IRF7 protein levels in HEK-293T cells transfected with TDP43 and TBK1. Each lane was loaded by 10 μg of total protein, and GAPDH was served as a loading control. Proteins were detected using Myc and FLAG tags. (B) Densitometric analysis of p-TBK1, Myc-tagged TBK1, p-IRF3, IRF3, and IRF7 protein levels based on data from panel A. (C) Immunoblot analysis of Myc, TBK1, p-TBK1, p-IRF3, IRF3, and IRF7 protein levels in HEK-293-T cells co-transfected with TBK1 (2 μg) and different concentrations of TDP43 (0, 0.2, 0.5, and 1.5 μg). The data are shown as mean ± standard deviation (n = 5 per group). ***P < 0.001.
Abbreviations: ns: Not significant; TBK1: TANK-binding kinase 1; p-TBK1: phosphorylated-TANK-binding kinase 1; TDP43: TAR DNA-binding protein 43; IRF3: Interferon regulatory factor 3; p-IRF3: phosphorylated-interferon regulatory factor 3; IRF7: Interferon regulatory factor 7.

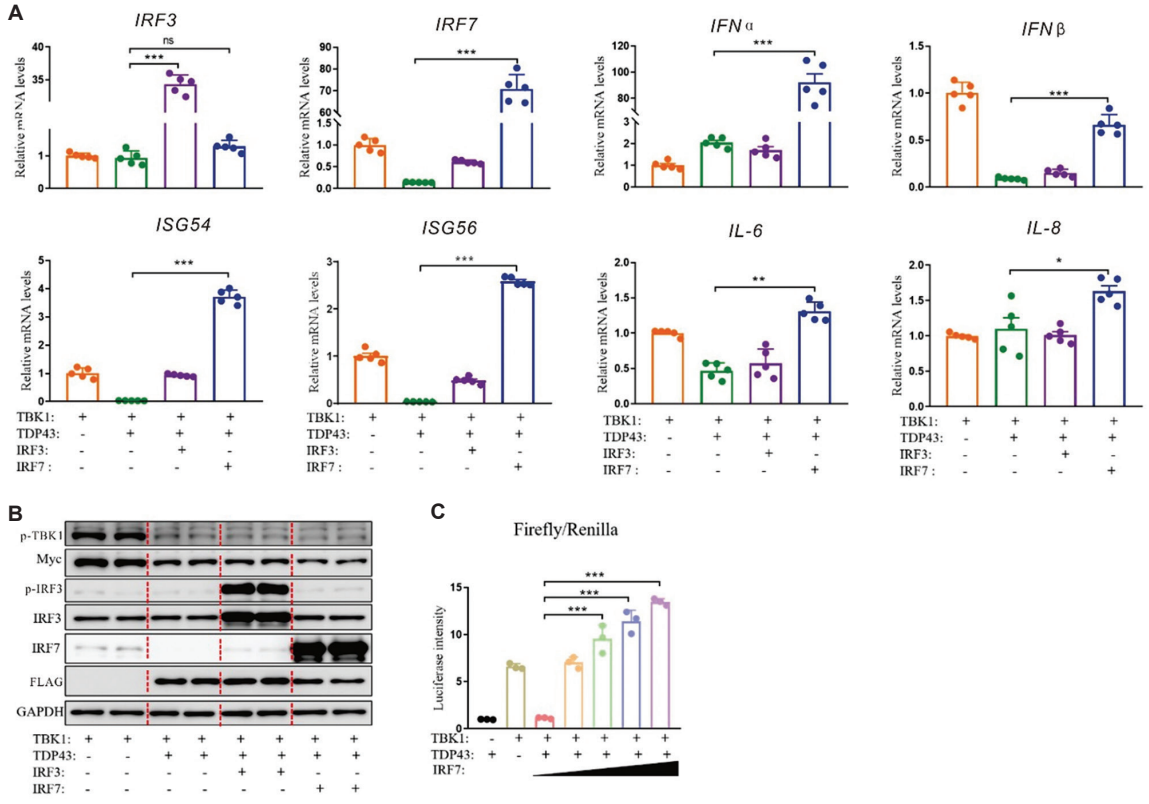

Figure 3. IRF7 overexpression alleviates TDP43-mediated IFN1 suppression. (A) Quantitative PCR analysis of *IRF3*, *IRF7*, *IFN1*, and *ISGs* (*ISG54* and *ISG56*) in HEK-293T cells co-expressing TBK1 and TDP43, along with IRF3 or IRF7. (B) Immunoblot analysis of p-TBK1, Myc-TBK1, p-IRF3, IRF3, and IRF7 protein levels. (C) IFN-β activity measured by dual-luciferase reporter assay. HEK-293T cells were co-transfected with luciferase-tagged IFN-β, Renilla-tagged TK reporter, and plasmids encoding TBK1 and TDP43, along with increasing concentrations of IRF7 (0, 0.2, 0.5, and 1.5 μg). The assay was conducted at 24 h post-transfection. The data are shown as mean ± standard deviation (n = 5 per group). *P < 0.05, **P < 0.01, and ***P < 0.001.
Abbreviations: TBK1: TANK-binding kinase 1; TDP43: TAR DNA-binding protein 43; IRF3: Interferon regulatory factor 3; IRF7: Interferon regulatory factor 7; IFNA: Interferon-alpha; IFNB: Interferon-beta; ISG54: Interferon-stimulated gene 54; ISG56: Interferon-stimulated gene 56; IL6: Interleukin-6; IL8: Interleukin-8; p-TBK1: phosphorylated-TANK-binding kinase 1; p-IRF3: phosphorylated-interferon regulatory factor 3.

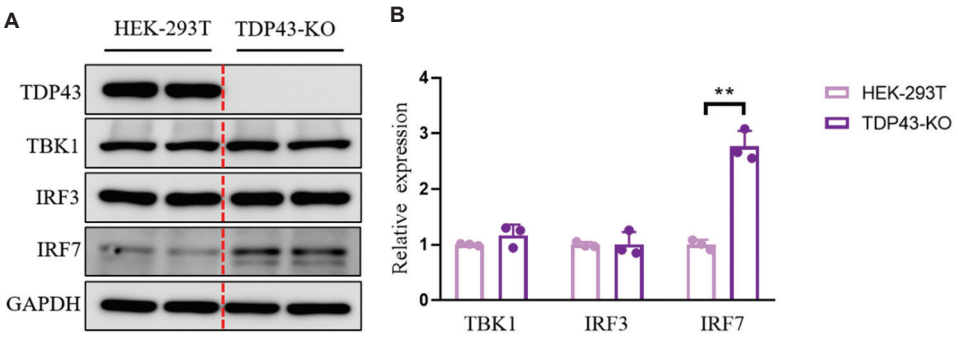

Figure 4. TDP43 depletion increases IRF7 expression. (A) Immunoblot analysis comparing TDP43, TBK1, IRF3, and IRF7 protein levels in wild-type and TDP43 KO HEK-293T cells. (B) Densitometric analysis of TBK1, IRF3, and IRF7 protein levels based on data from panel A. The data are shown as mean ± standard deviation ($n = 3$ per group). $^{**}P < 0.01$.
Abbreviations: KO: Knockout; TBK1: TANK-binding kinase 1; TDP43: TAR DNA-binding protein 43; IRF3: Interferon regulatory factor 3; IRF7: Interferon regulatory factor 7.

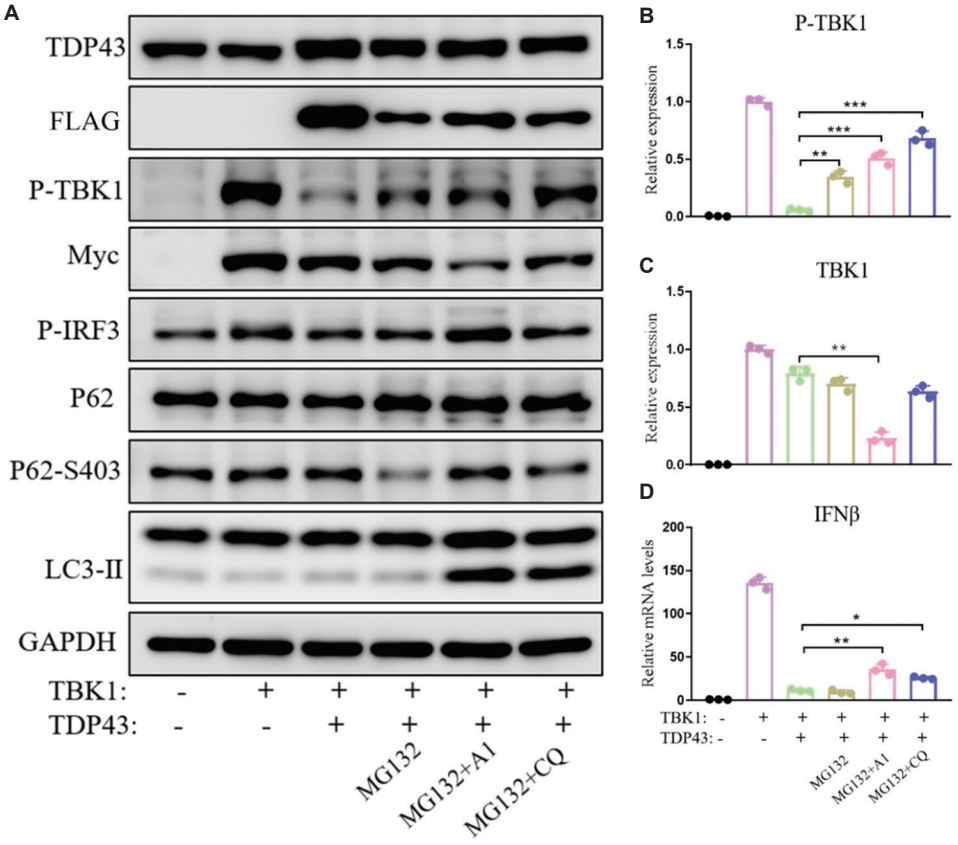

Figure 5. TDP43 facilitates TBK1 degradation through autophagy. (A) Immunoblot analysis of p-TBK1, Myc-tagged TBK1, p-IRF3, p62, p62-S403, and LC3-II protein levels in HEK-293T cells transfected with TDP43 and TBK1, followed by treatment with MG132, MG132+A1, or MG132+CQ for 16 h. (B and C) Densitometry analysis of relative TBK1 protein levels normalized to GAPDH and p-TBK1 normalized to TBK1 based on data from panel A. (D) *IFNB* mRNA levels were detected by qPCR assay. The data are shown as mean ± standard deviation ($n = 3$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
Abbreviations: TBK1: TANK-binding kinase 1; TDP43: TAR DNA-binding protein 43; IRF3: Interferon regulatory factor 3; p-IRF3: phosphorylated-interferon regulatory factor 3; LC3-II: Light chain 3-II; IFNB: Interferon beta; A1: Bafilomycin A1; CQ: Chloroquine; qPCR: Quantitative polymerase chain reaction.

densitometry analysis revealed increased p-TBK1 expression, accompanied by a progressive decrease in TBK1 protein levels (Figure 5B and C). Furthermore, qPCR analysis showed that *IFNB* was elevated in groups treated with MG132+CQ or MG32+A1 compared to the untreated group (Figure 5D). These findings collectively

indicate that TDP43 facilitates TBK1 degradation through autophagy.

4. Discussion

The ubiquitinated inclusions in neurons and glial cells are one of the hallmark features of ALS and frontotemporal lobe degeneration (FTD). These inclusions are primarily composed of TDP43.[25,26] The motor neuron degeneration in ALS leads to muscle atrophy, paralysis, and ultimately death. The pathological inclusions observed in both ALS and FTD patients consist of a 43 kDa aggregated TDP43 protein. Emerging evidence indicates that TDP43 exhibits cellular prion-like properties, which are thought to contribute to key characteristics of ALS.[26] The cytoplasmic accumulation of misfolded, hyperphosphorylated TDP43 is considered a critical factor in neurodegeneration, especially in ALS and FTD pathogenesis.[27,28] Dysfunction or mutations in *TBK1* have also been implicated in the development of ALS and FTD, although TBK1's specific role in neurodegeneration remains poorly understood. A recent study revealed that the *TBK1* p.G175S variant disrupts TBK1-mediated NF-κB signaling pathway, leading to impaired autophagy and promoting the accumulation of TDP43 protein, which is implicated in neurodegenerative diseases such as ALS and FTD. The mutation hinders the proper degradation of TDP43 through the autophagic process by disrupting TBK1's ability to activate NF-κB signaling.[9] Furthermore, Xu *et al.*[29] demonstrated that *TBK1* haploinsufficient (*TBK1*$^{+/-}$) mice exhibit hallmark ALS features, including neuroinflammation, TDP43 aggregation, loss of axons, and degeneration of neurons. These findings suggest that *TBK1* mutations or deficiencies influence TDP43 function, although the mechanisms underlying this interaction remain unclear. In this study, it was demonstrated that TDP43 overexpression impairs TBK1 function, providing new insights into the relationship between these two proteins in ALS pathogenesis.

Metastasis-associated lung adenocarcinoma transcript-1 can directly interact with TDP43 and suppress its cleavage. Its reduction following viral infection has been shown to enhance IRF3 activation and IFN1 production.[30] In THP-1 cells, overexpression of TDP43 activates cyclic GMP-AMP synthase (cGAS), subsequently leading to the release of inflammatory signals, such as NF-κB and IFN1.[31] Moreover, in Prp-TDP43$^{Tg/+}$ mice, pathological TDP43 promotes the release of mitochondrial DNA (mtDNA) into the cytoplasm. This released mtDNA activates the cGAS/stimulator of interferon genes (STING) signaling pathway, which is a key driver of innate immune responses. Interestingly, deletion of STING abolishes the upregulation of IFN1 and NF-κB in the spinal cord,[31] highlighting the critical role of STING in mediating TDP43-associated ALS/FTD pathology. In this study, co-expression of TDP43 with TBK1 in HEK-293T cells significantly reduced the expression of IFN-α and IFN-β, as well as the expression of ISG54 and ISG56. These findings suggest a strong association between TDP43-mediated neurodegeneration and IFN1 signaling. Notably, while co-expression of TDP43 with TBK1 decreased IRF7 expression, it did not affect IRF3 levels.

TBK1 activation relies on phosphorylation at serine 172 within its canonical kinase activation loop,[32] which is crucial for the transcriptional activation of the IRF family.[33] Mutations in *TBK1* underscore its importance in various human diseases, including ALS, FTD, normal tension glaucoma (NTG), and pediatric herpes simplex encephalitis (HSE). Gain-of-function mutations in *TBK1* are linked to NTG, whereas loss-of-function mutations lead to ALS/FTD or HSE, highlighting TBK1's diverse roles in neuroinflammatory diseases and emphasizing its significance in disease mechanisms.[33] Previous studies have shown that overexpression of TBK1 induces its autophosphorylation, activating the kinase. This activation enables TBK1 to phosphorylate IRF3, a crucial protein in the innate immune response pathway, which subsequently initiates the production of IFN1.[33-35] Consistently, this study revealed that TBK1 promotes its own autophosphorylation and enhances IRF3 phosphorylation. Phosphorylation of TBK1 is tightly regulated by phosphatases. For instance, protein phosphatase 1B (PPM1B) binds to TBK1 and dephosphorylates it at serine 172 following viral infection, thereby terminating TBK1-mediated activation of IRF3.[36] Similarly, the glucocorticoid dexamethasone has been shown to suppress TBK1 kinase activity, reducing phosphorylation of both TBK1 and IRF3, effectively inhibiting the signaling pathway crucial for innate immune responses, particularly against viral infections.[37] In this study, co-expression of TDP43 with TBK1 led to decreased TBK1 protein levels and reduced phosphorylation of TBK1 and IRF3, further supporting the regulatory role of TDP43 in TBK1-mediated signaling pathways.

The activation of TBK1 triggers the activation of transcription factors IRF3 and IRF7, which is crucial for initiating IFN1 production in reaction to viral infections. As a master regulator of IFN1-dependent immune responses, IRF7 interacts with N-myc and STAT interactor (NMI), which negatively regulate virus-induced IFN1 production.[18] IRF7 has been shown to stimulate the production of IFN1 (including IFN-α and IFN-β) following viral infection.[15,16] Studies utilizing IRF7-KO mice have demonstrated increased susceptibility to viral infections, characterized by impaired production of IL-1β and IFN-β during endotoxin-induced septic shock or HSV-1 infection in mouse embryonic fibroblasts.[17,18,38] The strong induction

of IFN through the activated TLR9 subfamily in plasma cell-like dendritic cells relies entirely on IRF7, which also orchestrates CD8+ T-cell responses. Whether systemic or localized, IRF7 plays a pivotal role in IFN responses during both innate and adaptive immunities.[17] In addition, IRF-7-deficient macrophages exhibit significantly reduced IFN-β induction in response to LPS stimulation.[38] These findings highlight the essential role of IRF-7 in IFN1 production in response to bacterial components like LPS, underscoring the critical importance of IRF7 expression. In this study, co-expression of TDP43 and TBK1 in HEK-293T cells led to a significant reduction in IRF7 protein levels, while IRF3 levels remained unaffected. Conversely, CRISPR-Cas9-generated TDP43 KO cells exhibited increased IRF7 expression. Moreover, overexpression of IRF7 mitigated the inhibitory effects of TDP43, resulting in elevated levels of IFN-α, IFN-β, and ISGs (ISG54 and ISG56). It was observed that both IRF7 and the phosphorylation of TBK1 and IRF3 were inversely correlated with TDP43 expression levels. These findings reveal a novel role for TDP43 in modulating TBK1-mediated IFN1 production.

A prominent pathological hallmark of ALS is the mislocalization of proteins and the presence of cytoplasmic aggregates within motor neurons. Cells rely on both UPS and autophagy-lysosomal pathways for protein degradation.[39] Autophagy induction is often assessed using p62 and LC3-II as markers. Numerous studies have highlighted the essential role of TDP43 in regulating autophagy. For example, depletion of TDP43 strongly promotes transcription factor EB (TFEB) translocation into the nucleus, mediated through its effect on the mammalian target of rapamycin complex 1 (mTORC1) component Raptor, thereby altering the expression of autophagy-related proteins.[5] In addition, TDP43 depletion disrupts the fusion of autophagosomes and autolysosomes by regulating dynactin 1 levels.[8] Furthermore, the absence of TDP43 impairs its ability to bind and stabilize autophagy related 7 (*ATG7*) mRNA, resulting in compromised autophagy and the accumulation of both p62 and LC3-II. In this study, treatment of HEK-293T cells with autophagy inhibitors MG132+CQ or MG132+A1 led to elevated expressions of p-TBK1 and p-IRF3, p62, and LC3-II. Furthermore, it was demonstrated that an increased mRNA level of *IFNB* in HEK-293T cell treated with MG132+CQ or MG132+A1, but not in the MG132-treated cells. These findings suggest that overexpression of TDP43 may promote TBK1 degradation through the autophagy-lysosomal pathway, thereby enhancing IFN-β production.

5. Conclusion

Taken together, this study provides evidence that TDP43 negatively regulates TBK1 expression through autophagy, resulting in reduced induction of IFN1 through IRF7. In addition, it was found that overexpression of IRF7 alleviates this reduction, restoring IFN1 production. These novel findings uncover an essential role of TDP43 in the regulation of TBK1-mediated IFN1 production and offer new insights into the interplay between TDP43, TBK1, IFN pathway, and neurodegenerative diseases.

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Conflict of interest

The authors declared that they have no competing interests.

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Availability of data

All data are available from the co-authors on reasonable request.

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ACCSCIENCE PUBLISHING

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CASE REPORT

New onset of ataxic dysarthria with CSF overdrainage syndrome: A case report

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Abstract

Cerebrospinal fluid (CSF) overdrainage syndrome occurs when excessive CSF drainage from the cranial cavity results from a spinal CSF leak or overdrainage through a ventriculoperitoneal (VP) or lumboperitoneal (LP) shunt. Symptoms include severe orthostatic headache, worsening with sitting or standing but improving when recumbent. The headache is typically dull, throbbing, and bilateral and may be exacerbated by Valsalva maneuver, coughing, or straining. Additional symptoms can include dizziness, nausea, vomiting, gait disturbances, diplopia, back pain, and seizures. We present a case of overdrainage syndrome following LP shunt placement for idiopathic intracranial hypertension in a 38-year-old woman with Ehlers–Danlos syndrome (EDS). One year post-surgery, she reported worsening headaches and nausea after prolonged upright positioning. Neurological examination revealed cerebellar dysfunction, including dysdiadochokinesia, intention tremor, and ataxic dysarthria. This condition gradually emerged 6 months post-LP shunt placement and was attributed to chronic overdrainage. After 1 day of observation, the patient underwent surgery to clamp the LP shunt outflow, resulting in overnight symptom resolution, including ataxic speech. This case underscores the importance of recognizing ataxic dysarthria in conjunction with low intracranial pressure syndromes, particularly in patients with EDS. It emphasizes the need to be aware of the diverse clinical manifestations of EDS and their relationship to altered CSF pressure syndromes.

Keywords: Idiopathic intracranial hypertension; Ventriculoperitoneal shunt; Lumboperitoneal shunt; Intracranial hypotension; Staccato speech; Aphasia; Ehlers–Danlos syndrome

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1. Introduction

Idiopathic intracranial hypertension (IIH) is characterized by elevated cerebrospinal fluid (CSF) pressure of unknown cause. Symptoms include headaches, nausea, vomiting, brain fog, visual changes, memory and concentration problems, and neck pain, with photopsia linked to postural changes.[1,2] IIH is diagnosed through a lumbar puncture,

showing pressures over 200 mm of water; however, relief from this procedure is usually temporary. Treatment options include weight loss, corticosteroids, acetazolamide, and furosemide.[3]

The most common treatment for IIH and idiopathic normal pressure hydrocephalus is a ventriculoperitoneal (VP) shunt. Complications of VP shunts include infection, improper catheter placement, intraventricular hemorrhage, ventricular collapse, subdural hematoma, seizures, and shunt failure.[4] Lumboperitoneal (LP) shunts provide a minimally invasive alternative, avoiding cranium opening or ventricle puncture.[5,6] However, LP shunts can be challenging when standing due to significant pressure changes in the lumbar spine. This can be managed with a valve, such as the horizontal-vertical valve, which drains at low pressure when supine and high pressure when upright.[7] Although LP shunts do not carry risks of ventricular collapse or subdural hematoma, they have higher rates of CSF overdrainage and annual revision rates of approximately 34 – 40%.[8,9] This can lead to spontaneous intracranial hypotension, presenting as severe orthostatic headache, nausea, vomiting, posterior neck pain or stiffness, photophobia and phonophobia, muffled hearing, pulsatile tinnitus, and hearing loss. Less common symptoms include cognitive issues, gait disorders, tremors, and as in this case. ataxic dysarthria.[10] These can often be mistaken for meningitis or migraine.[2]

2. Case presentation

A 38-year-old woman with Ehlers–Danlos syndrome (EDS) and IIH presented with a severe headache, sensory loss, hyperreflexia, and lower extremity weakness. Magnetic resonance imaging (MRI) results were inconclusive. After lumbar puncture and CSF drainage, she experienced an improvement in headache and leg weakness for 3 days. Acetazolamide was prescribed to reduce CSF production and pressure, but the response was inadequate, leading to the placement of an LP shunt to manage her IIH.

Five months later, the patient presented with headache, nausea, memory issues, lower back and lower extremity pain, and leg weakness. Computed tomography (CT) of the abdomen and spine confirmed proper placement of the LP shunt. These IIH symptoms, resembling those before the shunt, were attributed to inadequate CSF drainage and increased pressure. Due to persistent symptoms, she underwent an LP shunt revision 1 month later with a H/V valve (Natus H-V lumbar valve system) at a lower opening pressure, resulting in complete symptom resolution. However, 11 months later, she reported worsening headaches after standing for >2 h, which required her to rest frequently. Two months later, she presented with upper and lower extremity tremors, coldness in the left leg, dizziness, tinnitus, blurry vision, and new-onset staccato speech (ataxic dysarthria) with stuttering followed by aphasia. Symptoms were triggered by standing and episodes of whole-body shaking were relieved by lying down. Physical examination revealed terminal intention tremors, dysdiadochokinesia, dysmetria, difficulty with the heel-to-shin test, left nystagmus, and mild leg spasticity, but cranial nerve function was normal with no other focal deficits.

Non-contrasted CT of the head/brain showed no intracranial hemorrhage, mass lesions, hydrocephalus, or midline shift. Magnetic resonance angiography and venography of the head, with and without contrast, were normal, showing no vertebrobasilar flow obstruction or anomaly. Non-contrasted brain MRI was normal; the cerebellar tonsils were low-lying but not below the foramen magnum, ruling out a Chiari malformation. However, the tonsils were thought to be surrounding the dorsum and sides of the medulla oblongata (Figure 1A). The ventricles showed mild narrowing but were still within normal limits (Figure 1B). Given the acute clinical presentation and medical history, differential diagnoses included Chiari malformation, CSF overdrainage through the LP shunt, multiple sclerosis, or stroke. The specific symptoms of postural headache, dizziness, and nausea were most suggestive of overdrainage syndrome.

The patient had a low-pressure H/V valve, which was designed to open at low pressure and was thus at risk for overdrainage. Symptoms gradually developed, indicating that overdrainage resulted from the valve's design rather than shunt malfunction. The presence of dysdiadochokinesia and other cerebellar signs suggested that the cerebellar tonsils sagged due to CSF overdrainage. Consequently, the lumbar shunt catheter was dissected

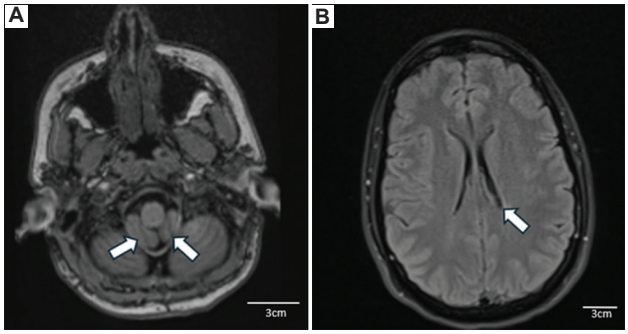

Figure 1. T1-weighted magnetic resonance imaging. (A) Axial view through the foramen magnum, showing low lying cerebellar tonsils (indicated by arrows) approximating and potentially exerting mild compression upon the medulla oblongata of the brainstem posterolaterally. A sagging effect of the cerebellar tonsils may have contributed to the mechanical distortion of the medulla oblongata. (B) Axial view showing mild narrowing of ventricles (indicated by arrows), within normal limits

and clipped just distal to the valve to stop CSF flow. On post-operative day 1, the patient showed complete resolution of positional tremors and staccato speech, with significant improvements in vision blurriness, dizziness, and headache. The staccato speech was completely resolved, with no recurrence after 6 months.

3. Discussion

The rapid improvement of dysfluent speech, headache, and dizziness supported the diagnosis of overdrainage syndrome. Ataxic Dysarthria (staccato speech and stuttering) was noted as a rare complication of this condition.

Overdrainage syndrome occurs when a shunt system excessively drains CSF, leading to spontaneous intracranial hypotension. This is typically manifested by postural headaches (holocranial, frontal, or occipital), nausea and vomiting when upright, neck pain, hearing disturbances, or tinnitus. Rarely, symptoms may include non-orthostatic headaches, gait disturbances, diplopia, upper back pain, dysarthria, and seizures as well as reduced consciousness, cognitive issues, lower back pain, photophobia, or movement disorders.[10] Symptoms can be acutely present with an extradural hematoma. Intracranial hypotension is best demonstrated through contrasted MRI, the most sensitive modality, showing diffuse venous engorgement and pachymeningeal enhancement in 73% (95% confidence interval, 67 – 80%) of patients along with distention of the transverse sinus. The pituitary gland often shows marked enhancement, and there may be sagging of the brainstem and cerebellum with a diminished prepontine cistern. Brain MRI findings can be normal in about one-fifth of patients.[2,11] Symptoms may also be absent for varying periods.

Current literature discusses the shunt siphoning of CSF, which leads to overdrainage. When a patient moves from a supine to an upright position, the pressure gradient between the ventricles and peritoneal cavity can create a negative hydrostatic effect, causing siphoning into the abdomen. This can lead to ventricular emptying and collapse, particularly in patients with post-hemorrhagic hydrocephalus, aqueductal stenosis, or white matter injury. Siphoning can result in postural headaches and subdural hematomas.[12]

The patient's neurological findings were characteristic of cerebellar motor syndrome, featuring staccato speech, tremors, dysdiadochokinesia, and coordination errors. In overdrainage syndrome, lower pressure in the subarachnoid space can create a vacuum effect, dragging intracranial structures downward and causing cerebellar sag. We believe that very low CSF pressure in the spinal canal when upright led to this sagging and mechanical compression of the lower brainstem, resulting in cerebellar symptoms.[10] Cerebellar lesions affect speech, leading to ataxic dysarthria, characterized by slow, monotonous, irregular, and staccato speech, often slurred with a nasal quality.[13-16]

In the presence of a VP shunt, overdrainage may lead to ventricular collapse, catheter tip blockage, drainage loss, and increased pressure.[12] LP shunt malfunction may also result in overdrainage and spontaneous intracranial hypotension. Programmable pressure valves, which adjust pressure based on an individual's intracranial pressure and body constitutions, are more effective than fixed valves in preventing overdrainage complications.[17] Strategies to reduce CSF flow and prevent siphoning include adjusting the pressure at which flow begins. The Medtronic Strata II valve can be externally reprogrammed to modify opening pressure, whereas the NATUS H/V valve drains at high pressure when standing and low pressure when supine, addressing pressure differences due to gravity. Anti-siphon devices can also be used to slow CSF drainage. Unfortunately, overdrainage syndrome lacks standardized clinical criteria.[12]

This patient's history was complicated by EDS, which is associated with a higher incidence of Chiari malformation type 1, IIH, and spontaneous intracranial hypotension due to spinal CSF leaks. Increased IIH risk can result from jugular vein compression, drainage sinus obstruction, or blood clot formation.[2] The tendency for CSF leaks in patients with EDS is linked to connective tissue weakness. Spontaneous leaks are most common in those with hypermobility-type EDS. Patients with EDS are often misdiagnosed; a survey of 505 individuals revealed an average of 10 incorrect diagnoses. Anxiety, depression, and migraines frequently contribute to these misdiagnoses.[18] Another study found that 56% of 414 patients with EDS received a misdiagnosis. Increased research and education about EDS are crucial, as first-line medical staff need to be more familiar with its symptoms, comorbidities, and potential diagnoses.[19]

4. Conclusion

This rare case involves a patient with ataxic dysarthria, characterized by staccato speech and stuttering, due to overdrainage syndrome. Prompt LP shunt revision resolved her symptoms. This case highlights the diagnostic challenges of overdrainage syndrome, especially in patients with connective tissue disorders, where multiple symptoms can complicate diagnosis. Overdrainage and underdrainage from LP shunt malfunction can cause headaches, nausea, vomiting, visual changes, cognitive issues, and neck pain.

Overdrainage syndrome is poorly understood, with variable clinical manifestations and no standardized

diagnostic criteria. However, advancements in shunt technology, such as programmable pressure valves and anti-siphon devices, show promise in preventing and managing CSF overdrainage complications. A comprehensive approach, including individualized treatment plans and regular monitoring, can help optimize outcomes and improve the quality of life for affected patients.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Written informed consent was obtained from the patient for the use of patient records for publication as a case report.

Consent for publication

Written informed consent was obtained from the patient for the use of patient records for publication, including test results and pertinent medical history. No protected health information/patient's confidential information was presented in this publication.

Availability of Data

Not applicable.

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CASE REPORT

A rare genetic association of one silent (*ADGRV1*) and other unquiet (*MED13L*) mutation: A case report

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Abstract

Two genes likely to cause epilepsy, one silent and the other manifesting in a patient with drug-resistant epilepsy, can be quite unusual. Herein, we described the case of a 2-year-old girl who presented with predominant language development delay, facial dysmorphism, and refractory epilepsy with normal neuroimaging and metabolic profiles, which prompted us to consider genetic etiology. Her genetic test revealed two novel likely pathogenic mutations, in Mediator complex subunit 13-like (*MED13L*) and adhesion G protein-coupled receptor V1 (*ADGRV1*). Sanger sequencing of her parents revealed an *ADGRV1* variant in her unaffected mother. The child had a phenotypic match with the *MED13L* genotype. However, only a few cases of *MED13L* have reported refractory epilepsy and the corresponding mutation was missense. To the best of our knowledge, this is the first case of frameshift mutation in *MED13L* presenting with refractory epilepsy. Although the child harbored two likely pathogenic mutations, the one inherited from her mother in *ADGRV1* did not manifest, whereas the frameshift mutation in *MED13L* had expressed as refractory epilepsy, which has not been described hitherto.

Keywords: Adhesion G protein-coupled receptor V1; Mediator complex subunit 13-like; Pediatric epilepsy; Case report

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1. Introduction

Epilepsy is one of the most common neurological disorders affecting children, with a global prevalence of approximately 0.5 – 1.0%. Approximately 10 – 20% of children can have refractory epilepsy, which can seriously impact their quality of life, development, and educational attainment. Refractory epilepsy in children has diverse etiologies. The presence of features such as facial dysmorphism, intellectual disability, and growth retardation can suggest an underlying genetic etiology for the same. It can indeed involve multiple genes, which contribute to increased susceptibility.

Mediator complex subunit 13-like (MED13L) is a component of the Mediator complex, a multiprotein complex required for gene transcription by RNA polymerase II. Multiple subunits of the complex show specificity in relaying information from

signals and transcription factors to the RNA polymerase II machinery, thereby enabling the regulation of the expression of specific genes.[1] This is important for the early embryonic development of the heart and brain. *MED13L* haploinsufficiency syndrome is characterized by moderate intellectual disability, speech impairment, and dysmorphic features, which can be accompanied with complex congenital heart defects and behavioral issues in some cases.[2] Only a few patients have been reported to develop epilepsy due to mutations in *MED13L*, specifically missense mutations rather than truncating type.[3]

The human adhesion G protein-coupled receptor V1 (*ADGRV1*) gene encodes a very large G protein-coupled receptor-1 (*VLGR1*), which is localized at synaptic junctions and acts in concert to regulate synaptic function. It is also termed the monogenic audiogenic seizures-susceptibility 1 gene, G protein-coupled receptor 98 gene, or VLGR1 gene. Three *VLGR1* mRNA isoforms, namely, *VLGR1a*, *VLGR1b*, and *VLGR1c*, are expressed in the cochlea, brain, eyes, and connective tissues. *VLGR1b*, the largest full-length isoform, has a large extracellular domain, which encompasses a signal peptide, seven epilepsy-associated repeats (i.e. epitempin repeats), and 35 calcium exchanger β (CalX-β) motifs. Variants associated with epilepsy mainly affected *VLGR1b* and *VLGR11c* rather than *VLGR1a*.[4] Variants in *ADGRV1* have been associated with audio-visual disorders, typically usher syndrome type 2, which is characterized by moderate to severe congenital sensorineural hearing loss and postnatal retinitis pigmentosa. Recent studies have identified ultra-rare *ADGRV1* missense variants in patients with myoclonic epilepsy, genetic generalized epilepsy, and atypical Rolandic epilepsy.[5-7] A recent study by Zhou *et al.* revealed that *ADGRV1* is potentially associated with febrile seizure-related epilepsy as a susceptibility gene.[4] Most of the seizures are self-limiting or can be controlled with monotherapy.

Here, we report a rare case of a child who presented with developmental delay and refractory epilepsy harboring novel variants in *MED13L* and *ADGRV1*, which represents a rare genetic combination not reported previously. This report can help us further delineate the clinical phenotype of these pathogenic variants, which can aid in genetic counseling. Understanding how these genetic mutations interact and contribute to epilepsy can provide insights into the underlying mechanisms of the disorder, which can potentially lead to more targeted therapies or interventions.

2. Case presentation

A 2-year-old girl, born of non-consanguineous parents with no perinatal insult, presented with a history of refractory seizures from 5½ months of age. She reported waking up from sleep with sudden deviation of both eyes followed by stiffening of all four limbs lasting for 10 – 20 s. The initial frequency of the events was 10 – 11 episodes per day. She was admitted and evaluated elsewhere initially and was started on anti-seizure medications (oxcarbazepine, levetiracetam, clobazam, zonisamide, and phenobarbitone); however, her seizures remained refractory. At 1 year of age, the frequency of her seizures increased up to 20 episodes per day. Her anti-seizure medications were optimized to lamotrigine, lacosamide, clobazam, and perampanel, which reduced her seizure frequency to 10 episodes daily. We also noted an increase in her seizure frequency when valproate was attempted. Although she had mild global developmental delay, she displayed a disproportionate delay in language milestones. Examination showed facial dysmorphism (Figure 1) with a depressed nasal bridge, a bulbous nose tip, low set ears, and a happy face. She had good auditory and visual regard and had no focal neurological deficits. Her complete blood count, serum electrolyte levels, thyroid profile, and metabolic profile, including her urine organic acid profile and plasma tandem mass spectroscopy findings, were all normal. Her brain magnetic resonance imaging (Figure 2), two-dimensional echocardiography, and abdominal ultrasound findings were also normal. Video electroencephalogram data revealed normal awake record with activation of left frontocentral interictal epileptiform discharges during sleep (Figure 3) and recorded events with the following semiology: (i) sudden staring with slight eye deviation to the left and behavioral arrest lasting for a few seconds, (ii) waking up during sleep with tonic posturing of all four limbs followed by brief clonic jerks and eye blinks, and (iii) sudden staring with slight head drop and abduction of both upper limbs, all with the left hemispheric ictal onset.

Based on the presence of facial dysmorphism, global developmental delay, and normal neuroimaging and metabolic profiles, we considered the possibility of a genetic etiology. She underwent chromosomal microarray,

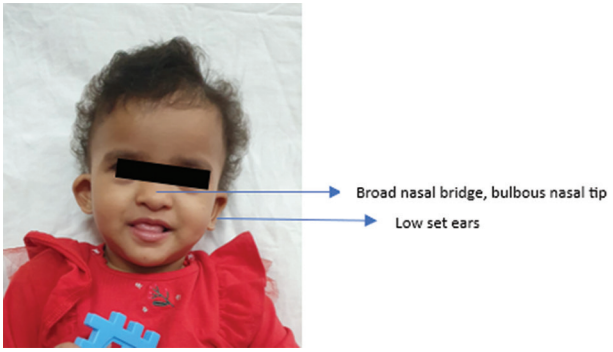

Figure 1. Facial dysmorphism in the child characterized by a depressed nasal bridge, bulbous nasal tips, and low set ears

which can back normal. Whole-exome sequencing was done, which revealed likely pathogenic mutations in two genes.

She underwent targeted gene sequencing. DNA extracted from blood was used to perform targeted gene capture using a custom capture kit. Clinically relevant mutations in both coding and non-coding regions were annotated using published variants in the literature and the following set of disease databases: ClinVar, OMIM, HGMD, LOVD, DECIPHER (population CNV), and SwissVar. Two likely pathogenic variants were detected (Table 1).

1) A heterozygous nonsense variant (c.1169dup) in exon 7 of the *ADGRV1* gene in chromosome 5 (e (chr5:g.90627707dup; Depth: 24x) that results in a stop codon and premature truncation at codon 390 (p.Tyr390Ter; ENST00000405460.9)

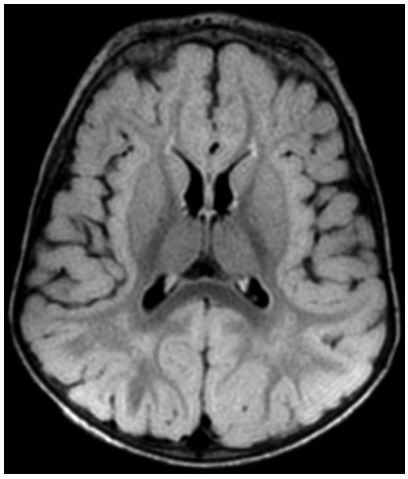

Figure 2. Brain magnetic resonance imaging of the patient

2) A heterozygous 7 base pair deletion in Exon 10 of the *MED13L* gene in chromosome 12 (chr12:g.116 009074_116009080del; Depth: 141x) that results in a frameshift and premature truncation of 38 amino acids downstream to codon 445 (p.Ser445GlnfsTer38; ENST00000281928.9).

Both of these variants have not been reported in the 1000 genomes, gnomAD v3.1, gnomdAD v2, TOPMed, and our internal databases. Subsequently, the proband sample along with samples from her parents were taken for sanger validation and segregation analysis, which revealed that the variant in the *ADGRV1* gene was found to be segregating in the heterozygous state in her unaffected mother and that the variant in the *MED13L* gene was found to be *de novo* (Table 2).

3. Discussion

MED13L-related intellectual disability is a rare syndrome caused by loss-of-function intragenic variants or whole-gene deletions in *MED13L*, with several missense variants also reported recently. Although *MED13L* had initially been believed to be a single gene cause of complex cyanotic heart disease,[8,9] it had later been shown to have a broader clinical spectrum involving intellectual disability, speech impairment, and behavioral issues.[2] The most common dysmorphic features reported are a bulbous nasal tip, open mouth appearance, low set ears, and a broad nasal bridge.[2] This characteristic facial dysmorphism along with speech impairment was noted in our patient. A review of the literature found that very few of these cases developed refractory epilepsy, all of whom had corresponding missense mutations.[3] In a study of 37 patients, Smol *et al.* found that six out of nine patients who had missense mutation had

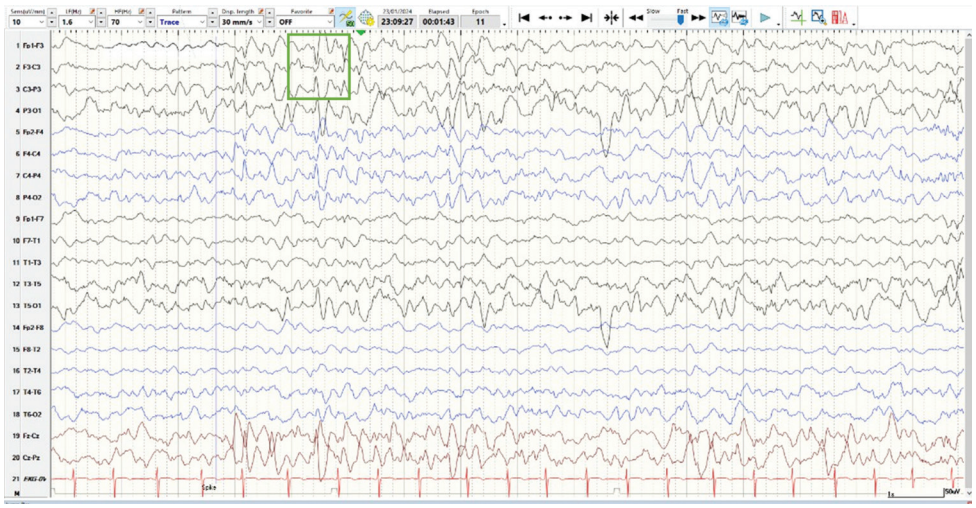

Figure 3. Electroencephalogram recorded during sleep in longitudinal bipolar montage showing left frontocentral spikes

Table 1. Whole-exome sequencing report of the proband

Gene (Transcript)	Location	Variant	Zygosity	Disease (OMIM)	Inheritance	Classification
ADGRV1 (+) (ENST00000405460.9)	Exon 7	c. 1169dup (p.Tyr390Ter)	Heterozygous	Familial febrile seizures-4 (OMIM#604352)	Autosomal dominant	Likely pathogenic (PVS1, PM2)
MED13L (−) (ENST00000281928.9)	Exon 10	c. 1333_1339del (p.Ser445GlnfsTer38)	Heterozygous	Impaired intellectual development and distinctive facial features with or without cardiac defects (OMIM#616789)	Autosomal dominant	Likely pathogenic (PVS1, PM2)

Abbreviations: *ADGRV1*: Adhesion G protein-coupled receptor V1; *MED13L*: Mediator complex subunit 13-like; OMIM: Online Mendelian Inheritance in Man; PM: Moderate evidence of pathogenicity; PVS: Very strong evidence of pathogenicity.

Table 2. Sanger sequencing of the child and parents

Gene and transcript	Variant	Location	Allele state	Inheritance	Disorder	Classification	Inherited form
MED13L NM_015335.5	c. 1333_1339d eITCTCAAC (p.Ser445GInfs*38)	Exon 10 Chr12:116009074	Heterozygous	Autosomal dominant	Impaired intellectual development and distinctive facial features with or without cardiac defects (OMIM#616789	Likely pathogenic	*De novo*
ADGRV1 NM_032119.4	c. 1169dupA (p.Tyr390Ter)	Exon 7 Chr5:90627707	Heterozygous	Autosomal dominant	Familial febrile seizures-4 (OMIM#604352)	Uncertain significance	Unaffected mother

Abbreviations: *ADGRV1*: Adhesion G protein-coupled receptor V1; *MED13L*: Mediator complex subunit 13-like; OMIM: Online Mendelian Inheritance in Man.

seizures. Reported cases had febrile seizures, late onset infantile spasms, and Lennoux–Gastaut syndrome.[3] The variant detected in our patient is novel, and to the best of our knowledge, this is the first case of a frameshift mutation in *MED13L* presenting with refractory epilepsy. Unlike non-silent mutations, which mostly include missense and nonsense mutations, frameshift mutation, as observed in this case, is considered the most damaging mutation. It leads to significant DNA damage as it completely changes the reading frame in the process of protein synthesis. Addressing the other mutated gene in our patient, *ADGRV1*, the loss-of-function mutation in this gene results in Usher syndrome characterized by sensorineural hearing deficiencies at birth and subsequent development of progressive retinitis pigmentosa. Variants in *ADGRV1* have been associated with epilepsy. In a recent study of 101 cases with febrile seizures and epilepsy with antecedent febrile seizures, *ADGRV1* variants were identified in nine unrelated cases, indicating that it is a susceptibility gene.[4] Missense variants of the gene have been linked to genetic generalized epilepsy[7] and myoclonic epilepsies,[5] with a recent study showing that biallelic variants are associated with Rolandic epilepsy.[6] In most of these cases,[4,6] the variants were inherited from asymptomatic parents, as observed in our case, which may be explained by incomplete penetrance.[10] Although further study is needed to precisely describe the phenotypic spectrum associated with *ADGRV1* variants, a review of the literature suggests that seizures in these phenotypes are mostly self-limiting or can be controlled with monotherapy.[4] The phenotype of this gene does not exactly match with that of our patient as she has refractory epilepsy with facial dysmorphism and development delay.

Regarding *ADGRV1*, the extracellular domain of *VLGR1b* contains 35 CalX-β motifs, which resemble the regulatory domains of Na^+/Ca^{2+} exchangers. In the central nervous system, Na^+–Ca^{2+} exchanges play a fundamental role in controlling changes in the intracellular concentrations of Na^+ and Ca^{2+} ions that occur in physiologic conditions, such as neurotransmitter release, cell migration and differentiation, and gene expression, as well as neurodegenerative processes.[11] Therefore, the disruption in function caused by variants in the CalX-β motif is potentially involved with epileptogenesis. Although the *MED13L* subunits relay information from temporal/spatial signals or transcription factors to the RNA polymerase II machinery, thereby controlling the expression of specific genes required for the neurodevelopment, the exact molecular mechanisms behind epileptogenesis remains unknown. The expression or activity of one gene can influence the expression or activity of another gene, often in a regulatory

or signaling context through transcription factors, signaling pathways and so on. So far, no such crosstalk between the aforementioned genes has been reported in literature.

4. Conclusion

Our patient harbored two mutations, and on verification of the literature, this rare genetic combination was found to be undocumented. Even though the child harbored a mutation in *ADGRV1*, which was inherited from her mother, both of them had not manifested the corresponding phenotype, whereas the frameshift mutation in *MED13L* presented itself in a manner not hitherto described.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Ashalatha Radhakrishnan
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Writing–original draft: Prasannakumar Surabhi
Writing–review & editing: All authors

Ethics approval and consent to participate

The parents of the patient gave written consent to participation in this study.

Consent for publication

The author has obtained the written consent from the parents to publish the case details. The identity of the child is not revealed in the manuscript.

Availability of data

Not applicable.

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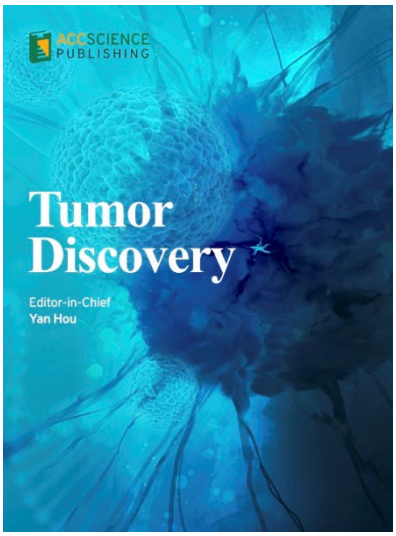

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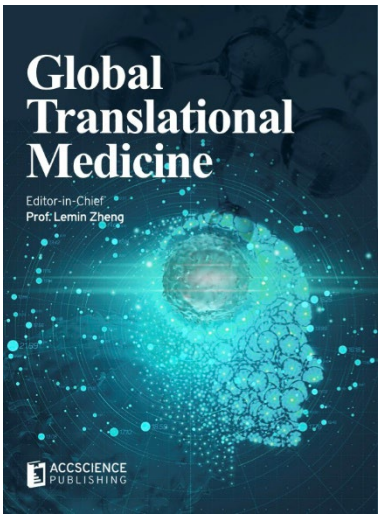

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