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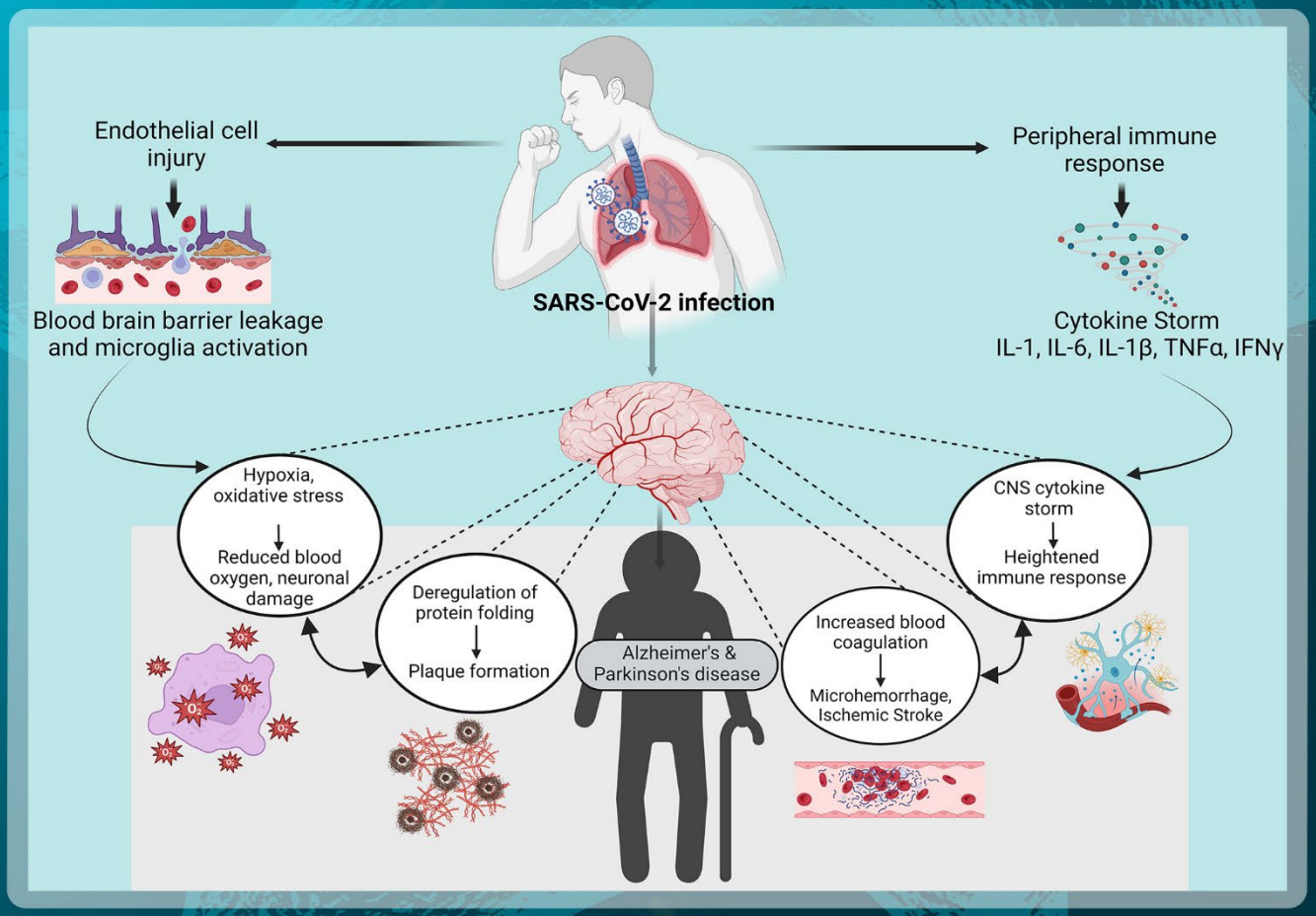

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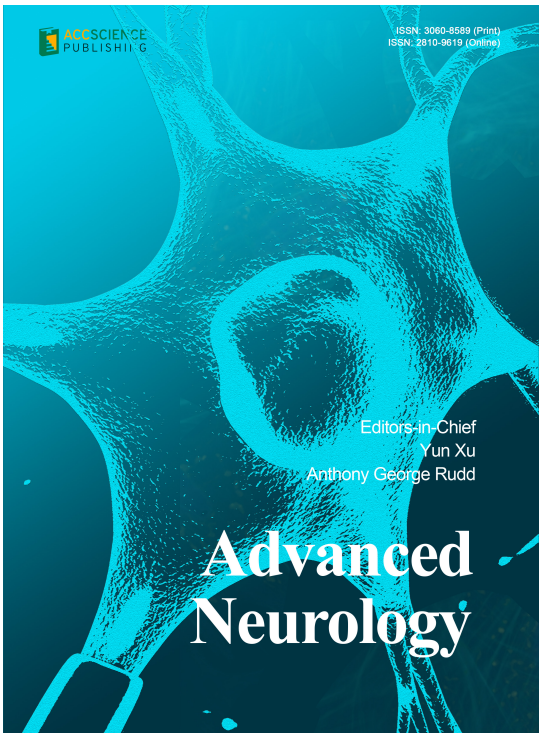

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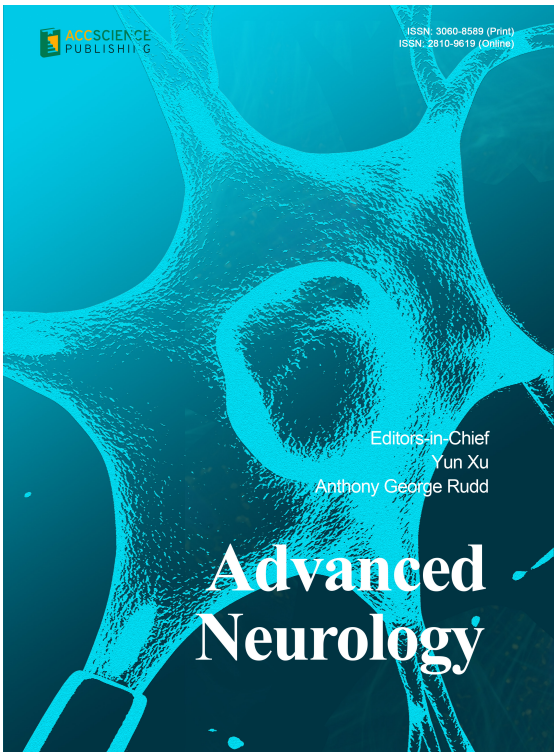

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REVIEW ARTICLE

Incidence and prevalence of Lewy body dementia in India: A systematic review

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Abstract

With increasing life expectancy in India, the prevalence of age-related disorders, such as dementia has also increased. Health and social care resources for each state are allocated based on their inhabitants' age, sex, education, and urban/rural status but not on the dementia subtype, which can significantly influence prognosis, healthcare utilization, and quality of life. Herein, we aimed to systematically review studies investigating the prevalence of the Lewy body dementia (LBD) subtype in India. We conducted a systematic review of EMBASE, MEDLINE, and APA PsychINFO databases on June 22, 2023. Two independent reviewers performed screening and full-text review, with a third reviewer resolving any disputes. Quality was assessed for each extracted paper. Of 1372 identified studies, full-text reviews were conducted on 399 and data were extracted from 4. Two studies included prevalence data on dementia with Lewy bodies (DLB), one on Parkinson's disease dementia and one on LBD. DLB or LBD has been reported to represent 1.0 – 8.9% of dementia diagnoses. Methodological heterogeneity was characterized by study design, access to biomarkers, diagnostic criteria, and use of cognitive tools. No studies reported incidence data. A paucity of research on LBD epidemiology in India is compounded by methodological heterogeneity, poorly representative cohorts, and varying access to biomarkers. Consensus guidelines may support data harmonization and the creation of multisite consortia, which could redress the under-representation of Central Asian data in epidemiological and genetic LBD studies.

Keywords: Lewy body dementia; Dementia with Lewy bodies; Parkinson's disease dementia; Prevalence; Incidence; India

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1. Introduction

India, with a population of over 1.4 billion, surpassed China as the world's most populous country in 2023.[1] The life expectancy in India has consistently increased over the past six decades, from 42.9 years in 1960 to 70.4 years in 2020.[1] Because of the increasing age of the Indian population, age-related disorders, such as dementia, are expected to increase.[1] In 2010, the Alzheimer's and Related Disorders Society of India estimated that 3.7 million Indians are living with dementia,[2] but recently published nationally representative data

from the Longitudinal Aging Study in India (LASI) reported that over 8.8 million people aged ≥60 years (7.4% of this population) are living with dementia.[3]

In 2019, dementia cost the global economy 1.3 trillion USD, half of which was related to informal care from family and friends.[4] Care partners spend an average of 5 h daily on providing care, which impacts their physical and emotional wellbeing.[4] The expenses associated with caring for an individual with dementia in India differ based on the extent of the condition. The annual costs can range from ₹The expenses associ(545 – 2423 USD) in cities and from ₹20,300 to ₹66,025 (242 – 791 USD) in rural areas.[5] The costs increase as the disease advances.[5]

The LASI study highlighted variations in all-cause dementia prevalence across different Indian states and reported that the differing influence of sex, age, education, and urban/rural status required policy and resource allocation tailored to each state's needs.[3] However, it did not investigate the prevalence of different dementia subtypes, which are associated with variations in demographic characteristics and disease burden.[6]

Lewy body dementia (LBD) is neurodegenerative dementia comprising Parkinson's disease dementia (PDD) – in which dementia emerges in the context of established PD – and dementia with Lewy bodies (DLB) – wherein dementia occurs within a year of Parkinsonian symptoms.[7] DLB is clinically characterized by the symptom tetrad of visual hallucinations, spontaneous parkinsonism, rapid eye movement (REM), sleep behavior disorder, and fluctuations in cognition with variations in alertness.[7]

Although believed to be driven by the same neuropathological process – the aggregation of α-synuclein into the Lewy bodies and Lewy neuritis – DLB and PDD are thought to represent two distinct but converging clinical phenotypes.[8] LBD is associated with more frequent rates of hospitalization,[9] a worse prognosis,[10] and increased caregiver stress compared to Alzheimer's disease.

Although neuropathological evidence of LBD was found in >25% of people with dementia who underwent postmortem,[11] it was diagnosed in <5% of cases antemortem, with significant variation observed between different geographical areas.[12] Of individuals with PD, 20 – 30% are estimated to have PDD, but significant variation between cohorts is observed.[12,13] Accurate LBD diagnosis is crucial, as it directs clinicians toward appropriate treatment strategies.[14] Critically, these strategies include the avoidance of antipsychotic agents, as patients with LBD can exhibit severe sensitivity to them.[7]

With the increasing incidence and prevalence of dementia in India, the number of people living with LBD is expected to increase. This systematic review aimed to review published literature investigating the incidence and prevalence of LBD in Indian populations. As secondary aims, we sought to determine the designs, diagnostic criteria, and cohort classification (LBD, PDD, or DLB) adopted by the included studies, as each could influence prevalence and incidence. Finally, we determined the use of biomarkers and cognitive tools in each study, acknowledging their influence on detection and, consequently, on epidemiology.

2. Methods

2.1. Search strategy

This systematic review was conducted as part of HPK's Masters in Public Health program at Queen's University Belfast. We devised a search strategy (Appendix 1) and were assisted by a medical research librarian. The search was conducted on June 22, 2023, and included all observational studies involving an adult Indian population, published in English after 1996, when the diagnostic criteria for LBD were first established.[15] The exclusion criteria comprised papers published in languages other than English, case series, and case studies.

Recognizing that epidemiological studies may not have mentioned LBD prevalence in their titles, abstracts, or aims, we included the names of other forms of dementia in our search strategy: "Alcohol-related brain injury," "Alzheimer's disease," "Frontal lobe dementia," "Frontotemporal dementia," "Mixed dementia," "Korsakoff psychosis," and "Vascular dementia." Parkinson's disease was also included as a search term to avoid excluding studies that included PDD prevalence. The search criteria included both anglicized and native names of Indian states, union territories, and capitals. The search included EMBASE, MEDLINE, and APA PsychINFO databases. We used the Covidence platform (*covidence.org*), which differs from manual extraction by automating and organizing many systematic review tasks, such as citation screening, full-text review, and data extraction, thus reducing time, minimizing errors, and enhancing collaboration among reviewers. HPK and NM screened the title and abstract and also conducted full-text reviews where appropriate. JK cast the deciding vote when disagreements emerged between HPK and NM.

2.2. Quality assessment

We used the Joanna Briggs Institute's (JBI's) quality assessment tool and JBI's critical appraisal checklist for studies reporting prevalence data to evaluate the quality and risk of bias for each study.[16] Quality assessment was tracked using a bespoke Microsoft Excel spreadsheet.

2.3. Data analysis

When prevalence was not reported by individual studies, we calculated DLB prevalence as the percentage of DLB cases among the total number of dementia cases. When PDD prevalence was not reported using other methods, it was calculated as the number of PD cases diagnosed with dementia divided by the entire PD population during the screening period.

When confidence intervals were not identified, we used the *Epitools (epitools.ausvet.com.au)* online resource and the Wilson calculation method to estimate these. This method provides more accurate intervals for small sample sizes, which we anticipated would be represented in the included studies. Unlike the symmetric normal approximation interval, the Wilson score interval is asymmetric, avoiding the issues of overshoot and zero-width interval that affect normal intervals.[17]

3. Results

3.1. Search results

Of the 1372 studies identified by our search, 399 were subjected to full-text reviews (Figure 1) and data were extracted from 4.

3.2. Prevalence and incidence

All four studies discussed the prevalence of either DLB, PDD, or LBD, and none reported the incidence of DLB, PDD, or LBD. Two studies described DLB[18,19] cohorts: one PDD[20] cohort and one study reported on an LBD[21] cohort but did not specify the number of subjects with PDD and DLB (Table 1). The mean age of participants varied; some studies had an age range of 38 – 50 years, whereas others included participants >50 years. The average proportion of females across the four studies was 37%.

3.3. Study design

Studies reporting DLB and LBD adopted a cross-sectional survey design, whereas the study investigating PDD (Table 1) adopted a prospective cohort.[18-21] Table 1 shows the setting and recruitment for each study.

3.4. Diagnostic criteria

Two studies investigated DLB populations. One study[18] cited the third report of the 2005 international consortium criteria for defining DLB,[22] whereas the other[19] used separate criteria, also published in 1996 for "diffuse LBD".[23] The studies investigating PDD[20] and LBD[21] used the Diagnostic and Statistical Manual of Mental Disorders 3rd edition–Revised (DSM-III-R)[24] and Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV),[25] respectively.

3.5. Cognitive scales and biomarkers

Only one study[19] included the Mini-Mental State Examination (MMSE).[26] Table 1 shows the details of the language of administration and each version's validation status. The remaining study[19] used the Kolkata Cognitive Screening Battery (KCSB),[27,28] which comprises verbal fluency, calculation, visuospatial, and both immediate and delayed recall tasks adapted from the Consortium to Establish a Registry for Alzheimer's Disease neuropsychological battery.[27] Although the language in which the KCSB was administered was not specified by the study, KCSB has been validated in both Bengali, the most commonly spoken language in Kolkata, and Hindi.[27] Other scales included the Addenbrooke's cognitive examination-revised version,[29] the scale for the outcome of PD-Cognition,[30] and the Frontal Assessment Battery.[31] One study used single photon emission computed tomography and positron emission tomography imaging in the subtype diagnosis,[18] but specific biomarkers were not discussed.

3.6. Quality assessment

We conducted a quality assessment for each study to ensure the reliability of this systematic review. This helped identify gaps in evidence, enhanced transparency and reproducibility, and minimized the waste of valuable resources. A quality assessment was essential to achieve a rigorous and reliable systematic review. Hence, to guarantee the dependability of this research, the JBI[32] quality assessment was used because it has checklists for different study designs, and it was performed using an acritical appraisal tool.[32] This appraisal aimed to thoroughly evaluate the methodological quality of a particular study.[32] It also comprehensively assessed the entire research process, including the design, conduct, and analysis. In addition, the appraisal aimed to determine the extent to which the study has considered the possibility of bias and implemented measures to address it appropriately.[32] The quality assessment checklist for each study type was filled out in a Microsoft Excel spreadsheet (Appendix 2). The assessment quantitatively scored papers on a scale of 1 for "Yes" and 0 for "No." Although assigning quantitative scores for "unclear" and "not applicable" is possible, this approach could lead to the arbitrary exclusion of papers. Consequently, the inclusion of papers would be subjective rather than based on a quantitative scoring system.[16] Subsequently, HPK customized the data extraction tool to suit the specific requirements of the study (Appendix 3) by modifing the existing JBI's prevalence and incidence extraction tool[33] (Appendix 4). The studies' information was extracted from the papers and compiled into a Microsoft Excel spreadsheet.

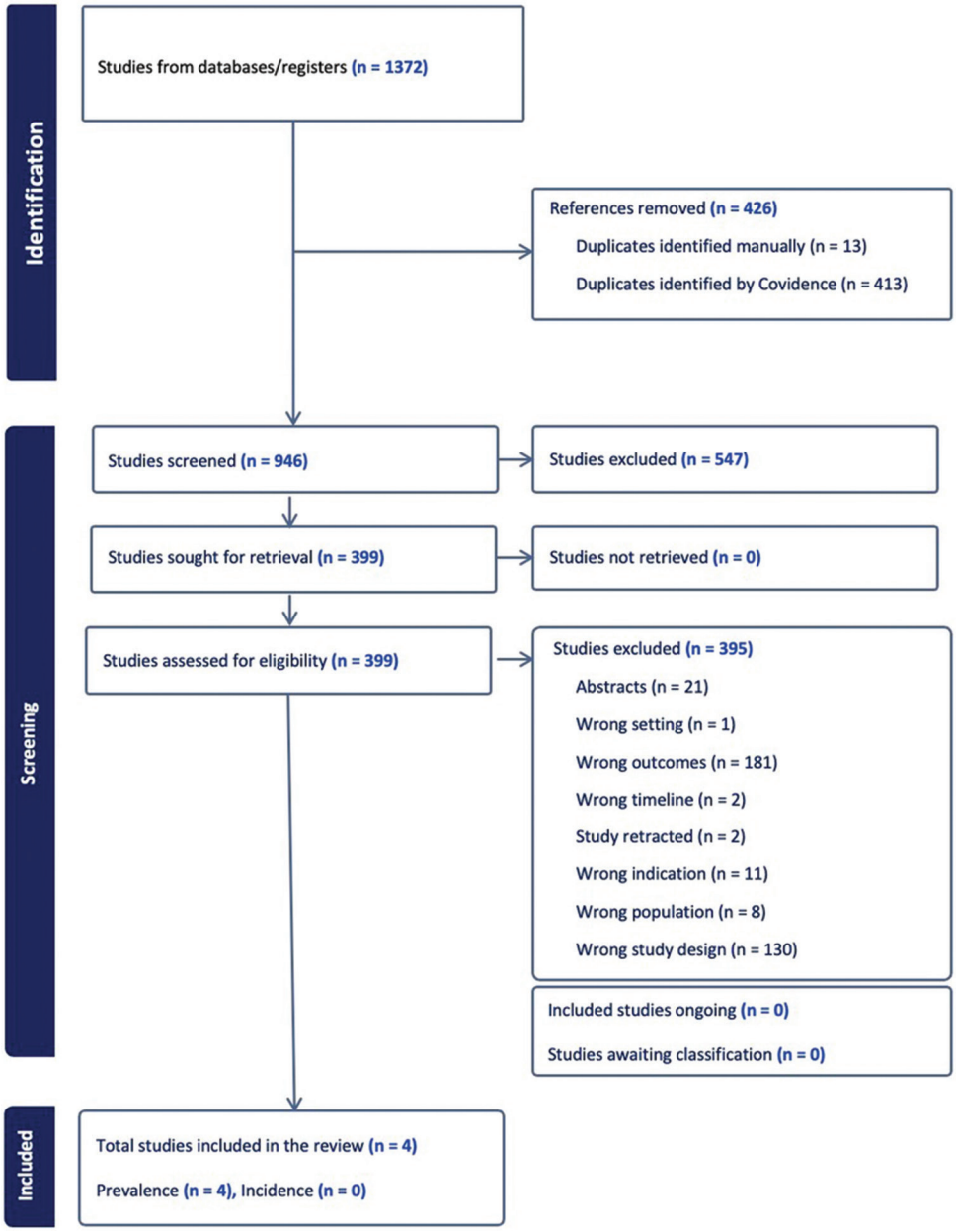

Figure 1. PRISMA flow chart

4. Discussion

Our systematic review identified four studies reporting the prevalence of LBD in India, which included 103 participants with LBD. We used a range of study designs, settings, and diagnostic criteria, and no two studies adopted the same diagnostic criteria for defining DLB, PDD, or LBD. Furthermore, the tools used to support diagnosis – notably psychometric scales and biomarkers – differed greatly between the two studies, precluding comparison. One unifying characteristic was that all studies were conducted in urban populations.

We observed a difference in the prevalence between the three studies reporting DLB or LBD prevalence. Alladi *et al.* reported that DLB represented 8.9% of patients with dementia in a memory clinic in Hyderabad;[18] Banerjee *et al.* reported that DLB comprised 1.0% of subjects with dementia in a community-based survey in Kolkata;[19] and Patel reported 2.4% of subjects in a clinical population in Karamasad.[21] However, this variation in prevalence is not uncommon; a 2014 systematic review of DLB prevalence[34] identified studies reporting that DLB comprised between 0.0%[35] and 24.0%[36] of all dementia cases. The same systematic review also identified that

Table 1. Studies show the identified prevalence of DLB, PDD, and LBD in India.

Studies reporting DLB prevalence							
Author	**DLB *n*/dementia *n***	**% DLB (95% CI)**	**Age $\bar{x}$ (±SD)**	**% Female**	**Setting location**	**Cognitive scales Language of administration**	**Diagnostic criteria**
Alladi *et al.*[18]	31/347	8.9% (6.4 – 12.4%)	70.2 (±8.0)	34	Patients attending a memory clinic Hyderabad	MMSE,[26] *"Participant's native language"* ACE-R[29] Hindi, Telugu	McKeith *et al.*, 2005
Banerjee *et al.*[19]	1/103	1.0% (0.2 – 5.3%)	71.7	46.7	Community-based survey Kolkata	KCSB[27,28] Bengali, Hindi	Mega *et al.*[23]
Studies reporting PDD prevalence							
Author	**PDD *n*/PD *n***	**% PDD (95% CI)**	**Age $\bar{x}$ (±SD)**	**% Female**	**Setting location**	**Cognitive scales Language of administration**	**Diagnostic criteria**
Sanyal *et al.*[20]	68/250	27.2%^ (22.1 – 33.0%)	57.9 (±12.1)	35.2	Patients attending neuromedicine clinic Kolkata	MMSE[26] Not specified SCOPA-COG[30] Not specified	DSM-III-R[24]
Studies reporting LBD prevalence							
Author	**LBD *n*/dementia *n***	**% LBD (95% CI)**	**Age $\bar{x}$ (±SD)**	**% Female**	**Setting location**	**Cognitive scales Language of administration**	**Diagnostic criteria**
Patel *et al.*[21]	3/125	2.4% (0.8 – 6.8%)	73 (±0.04)	40.0	Prescription data from neurology, medicine, and psychiatry outpatient clinics Karamasad, Gujarat	MMSE[26] Not specified FAB[31] Not specified	DSM-IV[25]

Notes: ^ -27.2% was given in the study; hence, number with PD was calculated.
Abbreviations: ACE-R: Addenbrooke's Cognitive Examination-Revised version, DSM-III R: Diagnostic and Statistical Manual of Mental Disorders-3rd ed..ition–revised, DSM-IV: Diagnostic and Statistical Manual of Mental Disorders-4th ed..ition, FAB: Frontal Assessment Battery, KCSB: Kolkata Cognitive Screening Battery, MMSE: Mini-Mental State Examination, SCOPA-COG: Scale for Outcome of PD-Cognition; DLB: Dementia with Lewy bodies; PDD: Parkinson's disease dementia; LBD: Lewy body dementia; SD: Standard deviation; CI: Confidence interval.

studies utilizing consensus diagnostic criteria for DLB[22] and studies in clinical settings rather than community-based settings had a higher prevalence, which was consistent with our observation (8.9%).[18]

Notably, the prevalence reported by Allaidi *et al*. was higher than most studies of this type;[12,34] the highest prevalence study of this type found that DLB comprised 4.6% of dementia cases in two English cohorts, which used the same diagnostic criteria as Allaidi *et al.*[12] Although a smaller sample size might have contributed to Allaidi *et al.*'s reported value, it could also be explained by improved clinical detection[37] or the possibility that DLB is more common in Indian populations.

In contrast, the findings of the single study on PDD prevalence in our review, where Sanyal *et al*. reported dementia in 27.2% of patients with PD,[20] closely aligns with the pooled frequency of 26.3% reported in a large recent meta-analysis.[38]

Only one study[18] recorded the use of imaging techniques. Although no details were provided for whether these included the use of supportive DLB biomarkers, such as ^{18}F-fluorinated N-3-fluoropropyl-2-beta-carboxymethoxy-3-beta-(4-iodophenyl)nortropane or cardiac I^{131}-metaiodobenzylguanidine, their presumed use may have supported a high clinical sensitivity in the DLB cohort.

We observed significant methodological heterogeneity for the cognitive scales adopted. Although MMSE was commonly used, only Allaidi *et al*. specified that this was administered in the subject's native language, and of the languages in which MMSE was administered, only the Hindi translation has been validated.[26] Other cognitive scales (Addenbrooke's cognitive examination-revised version and KCSB) were administered in Bengali, Hindi, and Telugu, the local languages of the respective geographical areas. These observations highlight two major issues for future research: harmonization and evidence synthesis of LBD studies in India. First, India is a highly linguistically diverse country, with 121 major languages identified in the 2011 census.[39] Even if validated for the most common languages spoken, this might contribute to a recruitment

or diagnostic bias that disadvantages or underestimates minority or marginalized groups. Second, although the global use of MMSE might make it a suitable candidate for wider validation, its lack of sensitivity in capturing some cognitive deficits may support an alternative scale, such as Montreal Cognitive Assessment (MoCA).[40] An exercise in establishing a core outcome set for DLB, informed by all stakeholder groups and at an international level, may help support a consensus in future directions for validating cognitive scales in India.[41]

Another important factor in promoting harmonization and evidence synthesis is the common use of diagnostic criteria. All four studies included in the present study adopted different diagnostic criteria. The international consensus criteria, revised in 2017,[7] were partly designed to promote such harmonization and support a consistent approach to diagnosis and nomenclature. This was also highlighted by our manuscript; LBD and DLB are often erroneously used interchangeably, and the absence of discussion of PDD and DLB in Patel *et al.*'s paper suggests that DLB represented a more precise definition of findings compared to the LBD used throughout.[21]

We were unable to identify any studies investigating LBD incidence in India. Understanding the relationship between the incidence and prevalence is critical in public health. Although the prevalence and incidence of all-cause dementia are increasing globally, the age-adjusted incidence is decreasing, probably due to advances in cardiovascular healthcare.[42] The modifiable risk factors for dementia evolve throughout the lifespan,[43] and amelioration of such factors in India in recent decades, such as child literacy[44] and visual impairment,[45] may similarly diminish the age-adjusted dementia incidence in the country. Therefore, we hope that the Longitudinal Aging Study in India-Diagnostic Assessment of Dementia will provide some insight into these risk factors by assessing 3000 LASI participants aged ≥60 years.[46]

Several factors limit our finding's generalizability. First, the sample sizes of the included studies were relatively low. Recruiting large LBD samples is challenging at any single site, and the global DLB research community supports the adoption of multicenter consortia in aggregating sufficient data to support thorough analysis.[47] Second, the setting for each study was urban and reflected specialist clinics in three of four studies. This recruitment bias fails to provide insight into rural or underserved communities, which is compounded by the infrastructure of Indian healthcare, including government and private facilities. Approximately 80% of India's population receives outpatient care at private facilities because of the perceived higher quality of care.[44] However, not everyone can afford private care. Thus, the variation in hospital infrastructure between the private and government sectors may affect the diagnosis and treatment of dementia, resulting in significant differences in care across different regions.[48] Third, any analysis of LBD prevalence must consider routine LBD detection, which is inevitably influenced by access to education, training, and biomarkers.[49,50] Our study identified only one study leveraging biomarkers in LBD diagnosis. Access to diagnostic biomarkers is associated with better detection and a higher clinical prevalence;[37] any epidemiological study of LBD in India must therefore consider access to biomarkers as a significant factor.

Our study investigated LBD epidemiology in the world's most populous country, contextualizing our findings in the broader field. Our broad search strategy included both anglicized and native names and followed robust quality assessment and reporting guidelines. The drawbacks of our study include limiting the papers to the English language and not including some less commonly used names for LBD, such as "diffuse Lewy body disease," in our search strategy. Nevertheless, this did not preclude extracting a paper using these terminologies.[19]

Our work highlights the paucity of published research on LBD epidemiology in India. Further epidemiological work is required to determine the disease's impact on communities, healthcare systems, and individuals, particularly in a larger and more representative cohort than what is currently available.

5. Conclusion

Our comprehensive review underscores a notable deficiency in the epidemiological comprehension of LBD in India. The scarcity of studies, methodological disparity, and diverse diagnostic criteria present challenges in determining the precise prevalence rates. DLB accounted for 1.0 – 8.9% of dementia cases, indicating considerable variability that may be influenced by study design and context. The insufficiency of incidence data further emphasizes the necessity of further research.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Harshini Priya Kirushnakumar, Joseph P.M. Kane
Writing – original draft: Harshini Priya Kirushnakumar
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

All data are obtained from publicly available databases. The authors created all images included in this manuscript and, therefore, reserve the right to use them.

Further disclosure

This project was submitted as a part of a Masters in Public Health dissertation (2022 – 2023) at Queen's University Belfast, Northern Ireland.

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Appendix

Appendix 1. Search strategy

We primarily aimed to determine the most appropriate search terms that would help obtain pertinent and reliable information. Thus, HPK did extensive background reading and identified a set of search terms. These were then refined with the help of the medical research librarian and supervisor. The search was conducted on June 22, 2023, using the final search terms.

1. *Incidence* OR Prevalence* OR Epidemiology* OR Diagnosis rate**
2. *Lewy body Dementia or dementia with Lewy bodies or Parkinson's disease dementia or Lewy body disease or dementia subtypes or dementia* or Alzheimer disease or vascular dementia or frontotemporal dementia OR frontal lobe dementia OR Korsakoff psychosis OR alcohol related Brain injury OR alcohol-related dementia OR Mixed dementia*
3. *India**
4. *Andhra Pradesh OR Arunachal Pradesh OR Assam OR Bihar OR Chhattisgarh OR Goa OR Gujarat OR Haryana OR Himachal Pradesh OR Jharkhand OR Karnataka OR Kerala OR Madhya Pradesh OR Maharashtra OR Manipur OR Meghalaya OR Mizoram OR Nagaland OR Odisha OR Punjab OR Rajasthan OR Sikkim OR Tamil Nadu OR Telangana OR Tripura OR Uttar Pradesh OR Uttarakhand OR West Bengal OR Ladakh OR Jammu & Kashmir OR Puducherry OR Lakshadweep OR Delhi OR Chandigarh OR Dadra and Nagar Haveli and Daman & Diu OR Andaman and Nicobar Islands*
5. *Amaravati OR Itanagar OR Patna OR Raipur OR Panaji OR Gandhinagar OR Chandigarh OR Shimla OR Ranchi OR Bengaluru OR Thiruvananthapuram OR Bhopal OR Mumbai OR Imphal OR Shillong OR Aizawl OR Kohima OR Bhubaneswar OR Chandigarh OR Jaipur OR Gangtok OR Chennai Hyderabad OR Agartala OR Dehradun OR Bhararisain OR Kolkata OR Port Blair OR Daman OR New Delhi OR Srinagar OR Jammu OR Kavaratti OR Pondicherry OR Leh*
6. *3 OR 4 OR 5*
7. *1 AND 2 AND 6*
8. *Limit 7 to human*
9. *Limit 8 to English Language*
10. *Limit 9 to yr="1996 -Current"*

We thoroughly researched all states, union territories, and corresponding capitals to ensure comprehensive and accurate data. The names of many states and cities were historically anglicized to reflect the UK's colonial history in India (*e.g.*, Madras, Bombay, Orissa, Pondicherry, and Bangalore). Since the turn of the millennium, the use of Indian names has become more common (e.g., Chennai, Mumbai, Odisha, Puducherry, and Bengaluru, respectively) when referring to these locations. To ensure that studies were not omitted based on naming conventions, both Indian and anglicized names were included in the search.

The Lewy body dementia (LBD) data are provided in other dementia studies. LBD was also discussed in other dementia subtype studies. Therefore, several other dementia subtypes, such as Parkinson's disease, Lewy body disease, dementia subtypes, Alzheimer's disease, vascular dementia, frontotemporal dementia, frontal lobe dementia, Korsakoff psychosis, alcohol-related brain injury, alcohol-related dementia, and mixed dementia, were also incorporated into the search terms to ensure no missing data on DLB and PDD.

Appendix 2. Quality assessment for the four studies included in the systematic reviews (primary aim)

Table 1. Quality assessment of three cross-sectional studies included in the review

Cross-sectional studies (Study name, year) ➢	Alladi *et al.*, 2011	Banerjee *et al.*, 2017	Patel *et al.*, 2015
Questions ▼			
1. Were the criteria for inclusion in the sample clearly defined?	Yes	Yes	Yes
2. Were the study subjects and setting described in detail?	Yes	Yes	Yes
3. Was the exposure measured in a valid and reliable manner?	Yes	Yes	Not applicable
4. Were objective, standard criteria used for measurement of the condition?	Yes	No	Yes
5. Were confounding factors identified?	Yes	No	Yes
6. Were strategies to deal with confounding factors stated?	Not applicable	Not applicable	Not applicable
7. Were the outcomes measured in a valid and reliable manner?	Yes	Yes	Yes
8. Was appropriate statistical analysis used?	Yes	Not applicable	Not applicable
Overall appraisal:	Include	Include	Include

Table 2. Quality assessment of one cohort study included in the review

Cohort studies Study name (year) ➢	Sanyal *et al.* (2014)
Questions ▼	
1. Were the two groups similar and recruited from the same population?	Yes
2. Were the exposures measured similarly to assign people to both exposed and unexposed groups?	Yes
3. Was the exposure measured in a valid and reliable manner?	Not applicable
4. Were confounding factors identified?	Yes
5. Were strategies to deal with confounding factors stated?	Yes
6. Were the groups/participants free of the outcome at the start of the study (or at the moment of exposure)?	No
7. Were the outcomes measured in a valid and reliable manner?	Yes
8. Was the follow-up time reported and sufficient for outcomes to occur?	Yes
9. Was follow-up complete, and if not, were the reasons for the loss to follow-up described and explored?	Yes
10. Were strategies to address incomplete follow-up utilized?	Yes
11. Was appropriate statistical analysis used?	Yes
Overall appraisal	Include

Appendix 3. JBI modified the data extraction tool for this study

Reference - Munn *et al.*[16]

Study details/Methods

- Study ID/record number is a numeric code to identify the study from which the effect size estimate was obtained.
- Study title – the full title of the study
- Author – This is an alphabetic or character code that is usually the first few characters of the primary study's author name, which serves as an easy way to identify the study in the bibliography.
- Year of publication
- Journal – the journal in which the article was published.
- Type of study design
- Aims of the study – as stated in the report.
- Setting – may refer to a hospital, community, or aged care facility; rural/urban; primary/secondary/tertiary.
- Method of data analysis
- Follow-up or study duration – any details on the study duration or follow-up of the participants

Results

- Demographics – age at diagnosis, sex, state/city, sample size
- Cognitive score (MMSE, MoCA, ACE, etc.)
- DLB clinical symptoms
- What type of prevalence was measured?
- Diagnosis methods/criteria used.
- Screening methods
- Prevalence n/N (%) – Living with dementia, LBD, and non-LBD
- Proportion and 95% confidence intervals
- Incidence n/N (%) – Newly diagnosed dementia, LBD, and non-LBD

Number of individuals with PD diagnosed with PDD
- Proportion and 95% confidence intervals
- Findings

Appendix 4. JBI data extraction tool (prevalence and incidence)

Reference - Munn *et al.*[16]

Study details

- Reviewer – Mostly includes details or ID of the primary reviewer.
- Study ID/record number – is a numeric code to identify the study from which the effect size estimate was obtained.
- Date – the date when this data extraction form was filled.
- Study title – the full title of the study
- Author – This is an alphabetic or character code, which is usually the first few characters of the primary study's author name, which serves as an easy way to identify the study in the bibliography.
- Year – year of publication
- Journal – the journal in which the article was published?

Study method

- Aims of the study – as stated in the report
- Setting – may refer to hospital, community, aged care facility, or rural/urban, etc.
- Study design – briefly describes the type of the study design. For example, if it is a randomized controlled trial or a quasirandomized controlled trial
- Subject characteristics – include age, sex, country/location, sample size, diagnosis, and other relevant characteristics.
- Dependent variable
- Outcomes – primary outcome measured, and where relevant, includes associated secondary outcomes.
- Outcome measurements – describe the scales or tools used to measure the outcomes. For example, a standardized pain scales to measure pain.
- Method of data analysis

Results

- Prevalence n/N (%)
- Proportion and 95% confidence intervals
- Incidence n/N (%)
- Proportion and 95% confidence intervals and duration of recruitment or the study
- Cognitives
- Authors' comments

ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Unraveling the thrombin–Alzheimer's connection: Oral anticoagulants as potential neuroprotective therapeutics

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Abstract

In Alzheimer's disease (AD), toxic amyloids formed by amyloid-β (Aβ) proteins and tau are implicated in the development of inflammatory, vascular, and neurodegenerative brain disorders. Thrombin has also been recognized as a proteopathic factor involved in Aβ-induced neurovascular dysfunction. Vascular Aβ activates the contact system in the blood, stimulating the production of inflammatory bradykinin and procoagulant thrombin. Thrombin, in turn, triggers inflammation, platelet activation, and the formation of fibrinolysis-resistant, Aβ-containing fibrin clots, leading to Aß-type cerebral amyloid angiopathy and associated neuropathology. Targeting thrombin with oral anticoagulants can normalize proinflammatory and prothrombotic states, counteracting the neurovascular consequences of AD. Pre-clinical studies have shown that such interventions preserve vascular and blood–brain barrier integrity, improve cerebral blood flow and brain perfusion, and reduce parenchymal accumulations of toxic Aβ, tau, fibrin(ogen), and thrombin. These effects mitigate neuroinflammatory and neurodegenerative processes, ultimately preserving cognitive functions for a longer period. Recent observational clinical studies in patients with atrial fibrillation (AF) demonstrated that treatment with direct oral anticoagulants (DOACs) or vitamin K antagonists (VKAs) reduced the risk of dementia by up to 48% compared to non-users. The anti-dementia effects were most prominent in elderly patients but were also observed in individuals with low AF risk or newly diagnosed AF. In addition, DOACs reduced the risk of intracranial hemorrhage by approximately 50% compared to VKAs. The current review highlights the potential neuroprotective role of DOACs in AD. By preventing excessive thrombin generation caused by Aβ pathology, DOACs could protect vascular and neuronal functions, thereby slowing cognitive decline. DOACs, such as dabigatran, apixaban, and rivaroxaban, warrant further clinical investigation for their potential repurposing as disease-modifying therapeutics in AD.

Keywords: Amyloid-β proteins; Thrombin; Neurovascular dysfunction; Disease-modifying therapeutics; Direct oral anticoagulants; Alzheimer's disease

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1. Introduction

Alzheimer's disease (AD), the most widespread neurodegenerative disease and leading cause of dementia, was first described in 1906 at a conference in Tübingen, Germany.[1] On this occasion, psychiatrist and neuropathologist Alois Alzheimer

attributed symptoms of mental confusion, memory loss, and personality deterioration in a patient to protein deposits found post-mortem in her atrophic brain. In addition, he reported vasculopathic changes in the AD brain.[1] Since then, extensive research has revealed that abnormal brain inclusions of amyloid proteins, which adopt specific pathogenic structures, are the defining features of a multitude of neurodegenerative diseases, including Parkinson's disease and AD.[2-4] In AD, a bundle of neurodegenerative, inflammatory, vascular, and hemostatic abnormalities is typically observed, mainly in neocortical and hippocampal brain regions. Pathological evidence increasingly points to amyloid accumulation as the main trigger of the disease.[2-7] Hallmarks of AD, in their sequential appearance, include deposits of parenchymal and vascular amyloid-β (Aβ) proteins, intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, glia-induced neuroinflammation, and brain atrophy. Eventually, synaptic and neuronal loss occurs, leading to cognitive decline.[2-5] Aβ-triggered vasculopathies are also early hallmarks of AD, contributing to the disease pathology.[6-10] The resulting pathophysiological consequences include disruption of vascular and blood–brain barrier (BBB) function, reduction in cerebral blood flow (CBF), and decline of brain perfusion and nutrient supply, all of which lead to neuronal and cognitive dysfunction.

At present, approximately 40 million people worldwide – 60 – 80% of all dementia patients – suffer from AD. As a major cause of mortality, its prevalence is expected to rise with the aging population.[5,9,11] The majority of AD patients, over 65 years of age, are diagnosed with late-onset AD, also known as sporadic, idiopathic, or senile AD.[5,11] Less than 10% of patients develop early-onset AD, with symptoms manifesting before 65 years of age, often due to genetic causes.[12] This type of AD is known as familial or presenile AD.[12] In addition to AD, vascular dementia is the second most common dementia subtype, sharing many pathophysiological features, symptoms, and risk factors with AD.[9,13] Vascular dementia affects 5 – 10% of dementia patients and arises from ischemic, hemorrhagic, or hypoxic brain damage.[9,13] Cardiovascular disease is well recognized as a key contributor to vascular dementia, with pathological manifestations including small vessel disease (SVD) of the brain's microvasculature, damaged BBB, impaired CBF, and reduced brain perfusion.[9,13]

Despite advances in understanding AD pathology, current "symptomatic" drugs for standard-of-care treatment are only able to alleviate some symptoms, improve overall condition, and slow mild cognitive impairment. However, these treatments do not target the underlying causes of the disease.[5,11,14] Acetylcholinesterase inhibitors, such as donepezil, galantamine, rivastigmine, and tacrine, have been the standard-of-care drugs for more than two decades.[5,14] In moderate and severe AD, acetylcholinesterase inhibitors are often combined with memantine, a glutamatergic N-methyl-D-aspartate receptor antagonist, which is also applied as monotherapy.[5,14] Lifestyle modifications, nutritional changes, and reduction of cardiovascular risk factors, including high blood pressure, obesity, diabetes, hypercholesterolemia, alcohol and tobacco consumption, and physical inactivity, are believed to have protective or delaying effects on disease progression.[6] Increasing evidence suggests that AD and cardiovascular disease are interconnected, sharing several disease mechanisms.[6] Over 90% of AD patients exhibit signs of vascular brain disorders,[8] including cerebral microbleeds, lacunar and cortical infarcts, micro-infarcts, intracranial atherosclerosis, arteriolosclerosis, Aβ-type cerebral amyloid angiopathy (Aβ-CAA), and a procoagulant state.[8] This procoagulant state is characterized by accumulations of thrombin and fibrin(ogen), key players in the blood clotting cascade.[6,7,15,16] AD and associated vascular disorders also share several pathological features, including an extended subclinical phase, age-dependency, Aβ accumulation, and a genetic predisposition linked to apolipoprotein E (APOE), the strongest genetic risk factor for late-onset AD.[6,7,12]

Overall, for the growing elderly population, there is an urgent need to identify disease-modifying drugs for AD that target its causes, prevent its onset, delay progression, or slow down its course.[11,12,14] Although the precise mechanism underlying AD is not yet elucidated, one of the main triggers for the disease is believed to be the generation of toxic aggregates of Aβ in the brain caused by the misfolding of Aβ.[2-5,11,14] This "amyloid hypothesis" posits that misfolded Aβ accumulates into toxic, extracellular deposits in the brain as the disease progresses. These deposits are thought to initiate a cascade of molecular events that ultimately lead to dementia. Based on this mechanism, the first disease-modifying therapies for AD, using the anti-Aβ antibodies aducanumab and lecanemab, have recently been approved in the United States (US).[2,11,14,17] These therapies are envisaged for patients with mild cognitive impairment due to early symptomatic AD. Data have demonstrated that these antibodies reduce Aβ levels and provide modest clinical benefits.[11,14,17] Besides Aβ pathology, pharmaceutical research in AD is increasingly focused on addressing neurodegenerative processes driven by toxic tau protein.[5] In addition, there is growing interest in targeting cardiovascular risk factors that impair the integrity and function of the brain vasculature, as both sporadic and familial AD exhibit vasculopathic changes.[5-10]

This vasculopathy is particularly evident from recent transcriptome analyses of human vascular cells, which identified 30 of the top 45 AD risk genes as being linked to the vascular system and dysregulated CBF.[18] Therefore, drugs that preserve vascular integrity, normalize blood pressure and procoagulant states, and maintain CBF and brain perfusion are believed to offer beneficial effects on neurovascular function and cognitive abilities in AD patients.[5-10,15,16] Approved cardiovascular drugs, due to their known safety profiles and established efficacy, are attractive candidates for repurposing as AD therapies, potentially offering significant savings in developmental time and costs.[19] For example, antiplatelet drugs[20] and diuretic bumetanide[21] have been explored as repurposed treatments for AD. Anticoagulants are also increasingly discussed as potential candidates for targeting vascular-driven neuronal dysfunction in AD.[15,16,22-27] These drugs have been used in antithrombotic therapies for decades.[15,16,28] As early as 60 years ago, there was speculation that anticoagulants could also be used to combat dementia.[29-31] Mechanistically, thrombin-inhibiting anticoagulants are particularly well suited to counteract key factors driving neurovascular dysfunction in AD.[15,16,22-27] Recent findings suggest that these anticoagulants may mitigate the proinflammatory and prothrombotic states present in the blood vessels of the AD brain, which are caused by Aβ-induced thrombin and fibrin production.[6,15,16,25,27]

This review provides an update on the potential of oral anticoagulants (OACs), specifically direct OACs (DOACs), as a disease-modifying therapy for early AD. The focus is on preventing excessive thrombin production caused by Aβ pathology and addressing its associated vascular, neuronal, and cognitive sequelae.

2. Hemostasis, thrombosis, and the benefit of anticoagulant drugs

Hemostasis is a complex biochemical process involving the blood vessel wall, platelets, and various blood factors from the coagulation and fibrinolytic systems.[15,28] Its primary function is to form a temporary plug to seal vessel injuries, arrest bleeding, and facilitate wound healing. In addition, hemostasis regulates the removal of clots once the healing process is complete.[15,28] Hemostasis involves three interrelated biochemical pathways: the extrinsic, intrinsic, and common coagulation cascades, all of which ultimately lead to fibrinolysis.[15,16,28]

2.1. Blood coagulation and fibrinolytic system

The extrinsic coagulation pathway is activated by the subendothelial tissue factor (TF), which is exposed to the blood only after vessel injury.[15,16,25,28] TF forms a complex with protease factor VII (FVII), activating it to FVIIa. This TF-FVIIa complex initiates a protease cascade by activating factor IX (FIX) and factor X (FX), ultimately leading to the generation of thrombin, a key serine protease in the coagulation process. The intrinsic coagulation pathway is triggered by damage to the cell surface, leading to the activation of the serine protease factor XII (FXII) into FXIIa as part of the plasma contact system.[15,16,25,28] FXIIa then drives the intrinsic protease cascade by activating factor XI (FXI) and subsequently FIX, also culminating in the production of thrombin. Both the extrinsic and intrinsic coagulation pathways converge at the activation of FIX to FIXa, which, through the subsequent activation of FX to FXa, forms the prothrombinase complex. This complex cleaves prothrombin into thrombin. Numerous coagulation factors require vitamin K and calcium for their activation and function.[15,16,25,28,32]

Hemostasis is tightly regulated by various inhibitors, including antithrombin III, which inactivates thrombin, and alpha-2-antiplasmin, which inhibits plasmin in the fibrinolytic system.[15,16,28] Collectively, thrombin synthesis through the coagulation cascade ensures the generation of active thrombin in the blood. Thrombin production is further amplified by a thrombin-driven positive feedback loop in the coagulation cascade involving factors V, VIII, and XI.[15,16,28] Procoagulant thrombin plays several critical roles, including the activation of factor XIII (FXIII) and platelets, as well as the conversion of soluble fibrinogen into insoluble fibrin protofibrils. Together, these processes lead to the formation of a blood clot or thrombus. A thrombus primarily consists of cross-linked fibrin, platelets, and red blood cells, forming the final product of the blood coagulation process in hemostasis.[15,16,28] At the same time, the fibrinolytic system initiates the cessation of thrombus growth and its subsequent degradation to support wound healing. This process is mediated by the activation of the serine protease plasmin, although plasmin activation occurs relatively slowly. The release of plasminogen activators, such as urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), induces the conversion of plasminogen into fibrin-degrading plasmin.[15,16,28] For a detailed illustration of the coagulation cascade and the fibrinolytic system, reference is made to additional sources.[16,25,28]

Disruption of the hemostatic process can have fatal consequences. In individuals with hemophilia, characterized by coagulation factor deficiencies, there is an increased risk of uncontrolled bleeding.[15,28] On the other hand, excessive thrombus formation can occur in individuals who experience major trauma or have conditions that lead to slow blood flow, such as vascular disorders, heart arrhythmias, or physical inactivity.[15,28]

When occlusive thrombi form inside blood vessels, blocking blood flow, life-threatening arterial or venous thrombosis may develop. If a thrombus detaches from the vessel wall and travels through the vascular system, it may result in pulmonary embolism, myocardial infarction, or ischemic stroke.[15,28]

2.2. Anticoagulant drugs for the prevention of thromboembolic incidents

To prevent fatal thromboembolic events, anticoagulants have been used in prophylactic and therapeutic cardiovascular medicine for decades.[15,16,28,32] They interfere with the coagulation cascade and prevent the formation of thrombotic fibrin clots through various mechanisms.[15,16,28,32] Parenteral heparins (e.g., enoxaparin) activate antithrombin III, which inhibits coagulation factors, such as FXIIa, FXIa, FXa, FIXa, thrombin, and kallikrein in the inflammatory bradykinin pathway. Small-molecule OACs include classical vitamin K antagonists (VKAs), such as warfarin (Coumadin®) and phenprocoumon (Marcumar®). They block the vitamin K-dependent synthesis of various coagulation factors, including FX, FIX, FVII, and FII, as well as the proteins prothrombin, C, and S. VKAs are derivatives of the plant substance dicumarol, originally isolated from spoiled sweet clover hay.[15] In addition to VKAs, a newer class of OACs, known as DOACs, acts independently of vitamin K by directly inhibiting key serine proteases in the coagulation cascade. These DOACs, introduced in the 2010s, include the thrombin inhibitor dabigatran (Pradaxa®) and the FXa inhibitors rivaroxaban (Xarelto®), apixaban (Eliquis®), edoxaban (Lixiana®), and betrixaban (Bevyxxa®).[15,16,28] A further class of DOACs is currently under development, targeting FXIa in the intrinsic coagulation pathway.[33] These inhibitors, such as asundexian and milvexian, are believed to have a reduced risk of bleeding since FXIa appears to be essential for thrombosis but less crucial for hemostasis.[33]

DOACs are used temporarily in situations such as acute vein thrombosis and thromboembolism prevention in patients hospitalized for medical illness or after total hip or knee replacement.[28] Permanent anticoagulant therapy is indicated in cases where long-term prevention of thromboembolic events is required,[28] such as in patients with recurrent deep vein thrombosis, pulmonary embolism, heart arrhythmias like atrial fibrillation (AF), or cardiovascular risk factors such as heart failure and hypertension.[28] Patients with AF particularly benefit from anticoagulant use due to their increased risk of thromboembolic events. Their likelihood of experiencing a heart attack or ischemic stroke leading to disability or death is five times higher than in the general population.[28] AF is also a major risk factor for dementia related to heart abnormalities (cardiogenic dementia), alongside conditions such as coronary artery disease, heart failure, and myocardial infarction.[28] However, the use of anticoagulants carries a risk of intracranial and gastrointestinal bleeding.[15,16,28,32] In Germany, DOAC treatment is currently prescribed to nearly 2 million patients, who are predominantly over the age of 70. In contrast, the use of VKAs has been declining, with approximately 1 million patients currently using them.[15,28] A similar trend is observed in France, where DOACs are more frequently prescribed than VKAs for ambulatory individuals aged 80 years and older.[34] This preference also extends to AD patients at risk of thrombosis, as DOACs offer a more favorable benefit-risk profile compared to VKAs.[34] The primary advantages of DOACs include consistent therapeutic efficacy, a more favorable safety profile with a lower risk of intracranial hemorrhage, and the absence of vitamin K-related complications.[15,22,28,32] Recently, there has been growing interest in whether DOACs could serve as disease-modifying therapies in AD, where procoagulant and proinflammatory states contribute to neurovascular dysfunction.[15,16,22-24,27] Emerging studies suggest that DOACs could be an appropriate therapeutic option for repurposing in AD.[15,16,19,22-24,26,27] This idea seems plausible, given the extensive clinical experience with antithrombotic drugs and the current lack of effective treatments for AD.

3. Pathogenic proteins in AD

It was not until the last three decades that research confirmed Alois Alzheimer's conclusion that AD is a proteopathic neurodegenerative disease.[1-5,11,20] During the progression of AD, extracellular amyloid plaques, composed of misfolded Aβ, are formed in the brain parenchyma, followed by intraneural deposits of NFTs, which are composed of hyperphosphorylated tau protein. The presence of these pathogenic proteins is associated with the initiation of oxidative stress, inflammation, neurodegeneration, and subsequent loss of memory and cognitive functions.[2-5,15,20,35-38] The progression of AD can be traced using biomarkers, such as Aβ or neurofilament light chain protein – a marker derived from neuron death – which reveals that the pathogenesis begins 10 – 20 years before cognitive impairment becomes evident.[39,40] In particular, studies using mouse models have made significant progress in elucidating the proteopathic mechanisms underlying AD.[2-5,12,15,16,25,41] These animal models provide a detailed systems biology perspective on the genetic, biochemical, structural, and physiological processes involved in AD. The models often carry human risk genes, which replicate the Aβ- and tau-associated parenchymal and vascular pathologies observed in the human brain.[12,41] In addition, advances in cryo-electron microscopy have

enabled the determination of the atomic structures of Aβ and tau amyloids in human brain inclusions.[4] Both types of misfolded proteins exhibit self-replicating conformations, characteristic of prions, which allow their self-perpetuating spread and deposition throughout the brain.[2-4,37] Thus, AD can be considered a double-prion proteopathy.[3,4,37] In the AD brain, the generation and deposition of Aβ are typically the first major pathogenic event associated with inflammation, neurovascular dysfunction, synapse loss, neuronal death, and cognitive decline.[2-6,10,11,14,15,27] An imbalance between Aβ production and clearance, leading to the accumulation of Aβ in brain tissue, is believed to be crucial in the pathology of Aβ.[2,3,5,35-37] The occurrence of NFTs, predominantly observed in the hippocampus and secondarily in the cortex, usually follows Aβ accumulation and signals the onset of neurodegeneration and brain atrophy, which strongly correlates with cognitive decline.[5]

3.1. Aβ pathology

In a healthy brain, native Aβ is believed to contribute to proper nervous system function, the integrity of the BBB, and pathogen defense.[14,15] However, in the diseased brain, Aβ monomers are increasingly generated by the proteolytic cleavage of the membranous Aβ precursor protein (APP) through the amyloidogenic pathway.[2-5,10,15,35,38] In this process, APP is sequentially cleaved by β-site APP-cleaving enzyme 1 (BACE1) and the γ-secretase complex, generating Aβ, which is released into the extracellular space. Mutations, especially in the Aβ region of the *APP* gene or in presenilin genes that encode γ-secretase subunits, result in misfolded Aβ proteins that adopt a self-replicating, β-sheet-rich oligomeric structure.[2-4,42] This APP processing through the amyloidogenic pathway occurs in microglia, astrocytes, oligodendrocytes, and particularly neurons,[2,3,20,38,43] as well as in the platelets of AD patients.[44] Aβ's prion-like feature triggers the misfolding of the additional native protein,[2-4,37] perpetuating its spreads in the brain spatiotemporally through Aβ seeds, which consist of small amounts of misfolded Aβ.[3,4,37] Aβ monomers polymerize into various assemblies, including soluble oligomers and protofilaments, which deposit as amyloid filaments between neurons, eventually forming insoluble Aβ plaques or senile plaques.[2-4,15,38,43] Cryo-electron microscopy studies have revealed that Aβ plaques contain a mixture of filaments, some of which are branched, forming parallel and lattice-like structures[43] interspersed with other brain materials.[43] Among the more than 100 possible Aβ isoforms – exhibiting variable lengths and chemical modifications – the isoforms with 40 (Aβ40) and 42 (Aβ42) amino acids are the predominant neurotoxic types in AD.[3,4,20,38,43] Filament structures derived from Aβ consist of two identical protofilaments, which exhibit two distinct filament types that differ mainly in their protofilament packing.[4] In sporadic AD, type I filaments are predominant, while type II filaments dominate in familial AD.[4] Aβ filaments are present in both parenchymal amyloid plaques, which are enriched with Aβ42, and in deposits within blood vessel walls, where Aβ40 is more prevalent.[4,43] Oligomers of both Aβ40 and Aβ42 form small, soluble, and non-fibrillar assemblies, which interact with the membranes of synapses, neurons, and glial cells, contributing to their pathogenicity within the brain parenchyma.[43] The early formation of toxic Aβ oligomers is thought to originate in the thalamus, from where they may spread to other brain regions, particularly the neocortex and hippocampus, as the disease progresses.[45] These brain parts are key centers of information processing for cognitive, behavioral, and motor skills.[46] During the early stages of AD, both vascular and parenchymal Aβ deposition, as well as neuronal hyperactivity, synapse and neuron loss, and the decline of cognitive abilities, are concentrated in these brain areas.[36,43,46]

Around parenchymal Aβ plaques, which grow in size and number outside neurons over the course of years, significant neuronal changes occur.[47] The highly aggregated Aβ that forms the plaque core is surrounded by parenchymal areas where synaptic links between neurons have been lost, and axons display abnormal swellings known as dystrophic neurites.[47] Each amyloid plaque contains hundreds of axons with dystrophic neurites, which serve as hotspots for the intracellular accumulation of tau and APP.[47] Time-lapse imaging of single axons in live mice has revealed that dystrophic neurites impair axonal transport between the neuron's cell body and synaptic terminals, disrupting the propagation of action potentials.[47] As a result, long-range axonal connectivity is compromised, leading to dysfunction within the nervous system in the affected areas.[47] One of the earliest predictors of cognitive decline in AD is synaptic dysfunction, which involves reduced plasticity in forming new synaptic connections and synapse loss.[2,5,14,36] The presence of Aβ plaques is also closely linked to neuroinflammatory events, as the association between Aβ plaque formation and microgliosis is strong, while the correlation with neurodegenerative changes is relatively weak.[20,38,42,47] This observation is consistent with recent studies in mouse models and in familial AD,[42] where Aβ aggregation has been shown to be kinetically decoupled from neurotoxicity. Neurodegenerative events appear to occur after Aβ seeding activity has reached saturation but before Aβ deposition reaches critical (half-maximal) levels.[42] This temporal dissociation suggests a prion-like molecular mechanism for Aβ, similar to the bi-phasic progression observed in prion diseases.[3,37,42,48]

In addition to Aβ pathogenesis in the brain parenchyma, Aβ is also implicated in cerebrovascular dysfunction in AD.[6,7,10,15,16,25,35,49] Aβ is transported from the parenchymal tissue through interstitial fluid (ISF) along blood vessel walls to the meningeal cerebrospinal fluid (CSF) and lymphatic vessels.[50] This pathway is particularly important for the elimination of parenchymal Aβ, which moves from the ISF across the vascular BBB into the bloodstream –a process known as perivascular Aβ clearance.[50] As Aβ accumulates in the blood, oligomers and amyloid filaments, primarily composed of Aβ40, begin to accumulate around and within the walls of leptomeningeal and cortical blood vessels.[7,10] Aβ40, being shorter and more soluble compared to Aβ42, is believed to diffuse more easily along the perivascular drainage route into the bloodstream.[10] On the other hand, the lower solubility of Aβ42 tends to keep this subtype within the parenchymal tissue, promoting the formation of insoluble plaques.[10] In addition, blood platelets are an important source of Aβ synthesis.[44] Studies have shown that activated platelets from AD patients produce more Aβ than those from healthy individuals, contributing to an elevated procoagulant state.[16,44] Aβ itself can activate the coagulation cascade, triggering a prothrombotic state in the blood.[6,16,25,51-53] This activation of the coagulation cascade boosts thrombin and fibrin production, resulting in the formation of fibrin clots, especially Aβ-containing fibrin clots, that are resistant to fibrinolysis.[6,15,16,25,44,51-53] These clots, along with oligomeric Aβ deposits, accumulate in cerebral blood vessels, contributing to vasculopathies such as Aβ-CAA. These conditions are associated with vascular dysfunction, inflammation, and neurodegenerative sequelae.[6,7,10,15,16,35,49,50] Aβ-induced vasculopathies also impair BBB function, including perivascular clearance of Aβ. This clearance process is further hampered by the reduction in the size of meningeal lymphatics, which limits Aβ outflow. As a result, Aβ increasingly accumulates in the parenchymal tissue.[6,7,10,15,35,49,50] Despite the significant role of vascular Aβ pathology in AD, this aspect of the disease has long been underappreciated in AD research. Notably, vascular amyloid extracted from the brains of AD and Down syndrome patients served as the starting material for the first structural analysis of Aβ nearly 40 years ago.[6-10,20,49]

3.1.1. Evidence for a causative role of Aβ

Recent research has revealed that misfolded Aβ plays a key role in neuronal and vascular dysfunction in AD, ultimately leading to cognitive decline.[2-11,14-16,25,35,36,38,43,49] Several lines of evidence support this conclusion:

(i) Data from a cohort study of cognitively normal participants showed that approximately one-third of individuals over the age of 65 have parenchymal amyloid deposits in the brain.[11,54] Among these individuals, more than 85% developed AD symptoms within the subsequent 10 years.[54] In the few people with amyloid plaques who remained asymptomatic, lower total levels of Aβ, as well as Aβ filaments with different structural and biochemical properties, were detected on average compared to those who developed AD.[55]

(ii) The most critical gene variants associated with a high risk of AD are implicated in the generation, aggregation, and clearance of Aβ, as well as in the innate immune system, including glial function.[2,12] Each patient with inherited AD shows excessive Aβ accumulation in the brain.[4] In particular, genotypes associated with mutations in the *APP*gene are causally linked to dominant inherited forms of AD. These mutations affect the cleavage of APP, the production of Aβ, and the structure and binding properties of Aβ.[12] Likewise, mutations in the *PSEN1* and *PSEN2* genes, which encode the presenilin component of γ-secretase, and *SORL1*, which encodes an endosomal recycling receptor for cell-surface proteins like APP, are recognized as AD risk factors.[2,12,56] In addition, multiplications of the *APP* gene, such as those seen in individuals with Down syndrome, trigger amyloid and NFT deposition in the brain,[2,12,37] often leading to dementia by age 40 – similar to early-onset AD. Mutations that increase the Aβ42/Aβ40 ratio or promote Aβ42 accumulation and assembly into filaments are also associated with a heightened risk of AD.[43] Moreover, mutations in genes involved in the innate immune system, especially those affecting glial cells, can increase Aβ deposition and contribute to AD risk. One of the strongest and most widespread genetic risk factors is the E4 allele of the lipid carrier protein *APOE4* gene, which influences Aβ deposition and impairs its clearance from the brain.[2,48,57-61] Other potential risk factors include gene variants encoding microtubule-associated protein tau, which is highly expressed in neurons, and variants in the gene encoding triggering receptor expressed on myeloid cells (*TREM2*), which functions in microglial activation.[62,63]

(iii) Recent studies have suggested a prion-like mechanism driving Aβ pathology in AD.[2-4,37,42,48] Misfolded Aβ adopts a self-replicating, oligomeric structure that can induce misfolding in native proteins. These misfolded Aβ seeds can then spread throughout the brain, aggregating into filaments that form Aβ plaques.[2-4,37,42,48]

(iv) Mechanistically, misfolded Aβ disrupts neural transmission and brain connectivity by interfering with neurotransmitter receptors, synaptic sodium-

potassium pumps, glutamatergic neurons, and glutamate uptake in astrocytes.[5,14,36] Prolonged disruption leads to nerve hyperactivation and synaptic dysfunction, both of which are closely correlated with the death of neurons and synapses and, ultimately, cognitive decline.[2,5,14,36]

(v) In addition to parenchymal Aβ pathology, Aβ accumulation in cerebral vasculature can cause vascular and BBB dysfunction, contributing to neuronal and cognitive impairment.[6-10,35,49,64] This vascular dysfunction is triggered by Aβ-induced vasoconstriction and vasculopathies, such as Aβ-CAA, which is found in over 80 – 90% of patients with both sporadic and familial AD.[35,65] Aβ-CAA is associated with ischemic, cellular, and hemorrhagic lesions.[6-10,35,49] The severity of vascular Aβ deposition correlates with a decline in CBF and a quantitative loss of brain perfusion.[66] In addition, this condition is linked to an Aβ-induced procoagulant state, resulting in increased thrombin production.[6,16,25] Studies using AD mouse models have demonstrated that peripherally administered Aβ inoculates can trigger Aβ-CAA in cortical and hippocampal blood vessels.[67]

(vi) Passive immunotherapy with Aβ-targeting antibodies has been shown to reduce brain Aβ deposition and alleviate cognitive impairment in both AD patients and mouse models, as reported in several studies.[2,11,17,68,69]

3.1.2. Immunotherapies focused on Aβ

The current treatment targeting Aβ-induced pathology in AD offers the most promising opportunity to slow, halt, or prevent disease progression.[2,5,11,12,14] One of the first passive immunotherapies developed for AD patients was aducanumab, a humanized monoclonal antibody that targets Aβ oligomers.[2,11,68] Intravenous infusions of aducanumab block the second phase of Aβ aggregation into filaments and their clumping into plaques.[2,11,68] This treatment was shown to reduce Aβ plaque load and modestly slow AD progression.[11,68] Approved by the US Food and Drug Administration (FDA) in 2021 under the name Aduhelm®, aducanumab is used to treat mild cognitive impairment caused by mild AD or AD dementia.[11] Several other Aβ-binding monoclonal antibodies targeting different aspects of Aβ pathology are currently under clinical investigation.[11,17,69] Among them, solanezumab inhibits the initial phase of Aβ aggregation, lecanemab binds to soluble Aβ protofilaments, gantenerumab prevents filament elongation, and donanemab targets existing plaques.[11] In patients with early-stage AD, these antibodies have been shown to clear large amounts of parenchymal amyloid from the brain, as confirmed by positron emission tomography imaging.[11,17,68,69] Likewise, levels of Aβ and phosphorylated tau in the CSF and blood were reduced.[11,17,68,69] In addition, in placebo-controlled, 18-month phase III trials, both lecanemab[17] and donanemab[69] were shown to slow cognitive and physical decline in early symptomatic patients by 27% and 35%, respectively. However, there is ongoing debate regarding whether this modest clinical benefit, which falls short of a curative effect, justifies the associated risks.[11,70] About 20 – 40% of participants treated with aducanumab, lecanemab, donanemab, or gantenerumab experienced adverse side effects, most of which were mild, but some of which were severe.[11,17,69,70] These side effects, referred to as amyloid-related imaging abnormalities (ARIA),[2,11,17,69,70] include localized brain swelling (ARIA-E, for edema in brain parenchyma or sulcal effusion), brain volume loss, and increased brain ventricle size. More serious side effects involve microhemorrhages (ARIA-H, for cerebral hemorrhage). Although ARIA symptoms are typically mild, severe consequences, including large intracerebral hemorrhage in <1% of antibody-treated participants (about 900), have been observed, leading to deaths in both the lecanemab and donanemab trials.[17,69] Fatal brain bleeding occurred predominantly in AD patients with significant Aβ-CAA and inflammation, or in those receiving anticoagulation therapy for stroke risk associated with AF.[17,69,70] One proposed explanation is that anti-amyloid antibodies may not effectively distinguish between structurally different parenchymal and vascular Aβ deposits.[4] As a result, antibody treatment could weaken cerebral blood vessels and increase the risk of bleeding by clearing amyloid deposits in vessel walls, potentially leading to glial-mediated vessel inflammation, leakage, and swelling.[70] Due to these risks, the FDA advises special caution when administering lecanemab, particularly in elderly patients with amyloid buildup in blood vessels.[17,70] Moreover, antibody therapy should be discontinued if a medical condition requiring anticoagulation arises.[17,70] Individuals carrying the E4-allele of the *APOE* gene also face a three- to sixfold higher risk of developing antibody-induced ARIA.[17,70] Despite these concerns, the FDA granted approval for lecanemab (Leqembi®) in 2023 as the first AD drug shown to clearly slow cognitive decline, and donanemab (Kisunla®) was approved in 2024.[70]

A key lesson from the clinical trials with Aβ-directed therapies is that treatment should begin as early as possible, ideally in the symptomless stage, to achieve the greatest benefit.[11,14,17,42,45,69,70] The optimal window for Aβ therapies is believed to be from pre-amyloid stages up until just before half-maximal Aβ deposition in the brain is reached.[42] For example, blocking BACE1-mediated Aβ production in pre-amyloid stages in AD mice has been shown to prevent the onset of neurodegenerative processes.[42]

3.2. Tau pathology

Intraneural accumulation of misfolded tau proteins in the brain is a pathological hallmark of AD and is strongly correlated with the severity of neurodegeneration and cognitive impairment.[5,15,58,71] Tau pathology, also referred to as tau proteinopathy or tauopathy, occurs not only in AD but also in a variety of dementias and movement disorders.[5,15,58,71] As a result, the search for anti-tau drugs, either as standalone treatments or in combination with anti-Aβ therapies, is a major focus of intensive pharmaceutical and clinical research.[14,71]

Tau is a small protein that usually attaches to microtubules and is primarily found in the microtubule-rich axons of the nervous system.[5,71] In a healthy brain, tau stabilizes and bundles microtubules, which is vital to fundamental functions, such as axonal transport, cell signaling, and neuron structural maintenance.[4,5,71] In contrast, in the AD brain, six tau isoforms are present, which form oligomers and progressively aggregate into abnormal assemblies of tau filaments.[4,5,71] Key triggers for tau aggregation include tau cleavage, disrupted cellular transport, and, in particular, aberrant tau phosphorylation by kinases, which begins in the entorhinal cortex and spreads to the hippocampus.[5,71] Hyperphosphorylated tau proteins adopt conformations that induce misfolding in naïve tau through a prion-like mechanism, enabling the spread of tau aggregates within neurons and between neurons through neuronal synapses.[4,37] In neurons, hyperphosphorylated tau dissociates from axonal microtubules, modifying their structure, and relocates from the axon to the somatodendritic compartment.[5,71] Here, hyperphosphorylated tau assembles into two types of unbranched filaments – straight filaments and paired helical filaments – which cluster in parallel to form NFTs.[4,5,43,71] Both types of tau filaments comprise two protofilaments sharing a specific conformation known as the "Alzheimer tau fold," a structural feature also observed in inherited forms of AD caused by *APP* mutations.[4] NFTs are usually observed in the most advanced stages of AD, where they are closely associated with neuronal cell death and brain atrophy.[5,71] Pathologically, the accumulation of misfolded tau in the brain disrupts synaptic and neuronal function, contributes to neuroinflammation, and interferes with neuron-glial interactions.[5,71] Tau, along with Aβ, also causes axonal myelin sheath loss and cellular deficits, such as neurotransmitter shortages and the activation of apoptotic processes, which contribute to synapse and neuron death.[5,71] Furthermore, immune responses through activated microglia and T cells appear to be involved in the neurodegeneration associated with tau pathology.[72] Studies have shown that Aβ pathology precedes tau accumulation to toxic levels and is involved in triggering tau pathology.[2,14,57,73] Oligomeric Aβ has been shown to promote tau hyperphosphorylation by activating tau kinases and inactivating tau phosphatases.[2,14,57,73] In addition, variants of the *APOE* gene, which are associated with late-onset AD, have been linked to impaired tau uptake by neurons, as well as intensified tau hyperphosphorylation and aggregation.[57] Vascular dysfunction, such as the disruption of smooth muscle cells and declines in CBF and brain perfusion, also appears to be involved in tau hyperphosphorylation.[6,64,71,74] Conversely, tau-related pathological alterations contribute to cerebrovascular dysfunction.[75]

3.3. Inflammation and glial reactions

In addition to Aβ- and tau-related therapeutic approaches, the current drug research in AD also focuses on addressing inflammatory and degenerative changes that contribute to synapse and neuron loss, ultimately leading to cognitive decline.[2-5,15,16,27,38,76,77] While inflammation typically serves as a protective tissue response in the early stages of AD, as the disease progresses, systemic inflammation, chronic inflammation, and oxidative stress reactions occur in the brain parenchyma, exacerbating both Aβ and tau pathologies.[3,57,76-79] Inflammatory reactions in AD are primarily driven by activated glial cells (e.g., microglia, astrocytes, and oligodendrocytes) as well as the release of proinflammatory proteins (e.g., cytokines and chemokines), complement proteins involved in immune defense, and reactive oxygen species (ROS) and nitric oxide (NO) species.[3,27,57,76-79] Beyond the brain parenchyma, progressive inflammation in AD also affects the cerebrovascular system and its function, which has been largely overlooked in therapeutic research.[6-10,13,15,16,20,27,35,38,76,77] In the microvasculature of the AD brain, elevated levels of inflammatory inducers – including cytokines, interleukins (ILs), chemokines, tumor necrosis factor-alpha, transforming growth factor-beta, Aβ, thrombin, and fibrin(ogen) – are found.[6,7,9,13,15,16,27,77] Collectively, these factors create an inflammatory and procoagulant environment in the cerebrovascular system, which further accelerates neurodegeneration in AD.[6,7,9,13,15,16,27,77]

In the central nervous system (CNS), astrocytes (the most abundant cell type and a key component of the tripartite synapse) and microglia (resident phagocytosing cells) form the first line of defense in the immune system.[38,57,76,78,79] This "firewall" is indispensable for the proper functioning of neurons and synapses, neural connectivity, and maintaining extracellular homeostasis in the brain.[38,57,76,78,79] Myelinating oligodendrocytes are another type of glial cells[38,76] that produce the insulating sheath around axons, which facilitate neuronal transmission

of electrical signals and support axoglial metabolism while detoxifying oxidative radicals. In AD, Aβ plaque formation, neuroinflammation, and oxidative stress are accompanied by decreased myelin production due to oligodendrocyte dysfunction,[38,57,76] resulting in myelin defects and impaired neuronal signaling, both of which contribute to cognitive decline. Astrocytes also play a crucial role in supporting the vascular and nervous systems.[38,76] They are an integral part of the BBB, contributing to its function and ensuring proper blood flow within the vasculature.[38,76] Astrocytes further participate in mediating the transport of metabolites and neurotransmitters between the CNS and the periphery, including the elimination of waste products such as toxic Aβ from the CNS through the glymphatic and blood systems.[38,76] Microglia, on the other hand, are engulfing and degrading cerebral debris and foreign substances through phagocytosis, and they also promote the repair of damaged tissues.[38,76,79,80] In addition, a network of microglial cell-surface receptors and intracellular signaling pathways regulates the production and secretion of both pro-inflammatory and anti-inflammatory proteins, as well as the oxidative stress reactions through the release of ROS and NO.[38,76,79,80]

The response of glial cells changes when brain tissue is injured, diseased, or during advancing age.[38,76,78] In these cases, the gradual loss of physiological function and chronic hyperactivation in the brain leads to excessive activation of astrocytes and microglia, shifting them into reactive and neurotoxic phenotypes.[38,57,76,78] Activation of glial cells has been observed in the brains of AD patients, closely linked to increased inflammation and disease progression. Astrocytes can undergo gliosis, adopting different reactive states characterized by distinct morphology, gene expression, and function.[38,76] In these reactive states, astrocytes can compensate for microglial dysfunction, taking on key roles such as interacting with and phagocytosing Aβ.[38,76] In addition, reactive astrocytes respond to proinflammatory proteins, such as cytokines, and promote inflammatory and neurodegenerative processes.[38,76] In the known as microgliosis, activated microglia become highly mobile, secrete proinflammatory proteins, and migrate to the affected brain tissue.[38,79,80] They phagocytose various components, such as misfolded proteins (e.g., Aß and tau), debris from damaged cells, defective neurons and synapses, and pathogens, such as bacteria and viruses.[38,79,80] In the early phases of AD, as Aβ accumulates, microglia are initially activated to the M2 phenotype, which enhances both the expression of proinflammatory proteins and the phagocytosis of Aβ.[5,38,76,79,80] However, as the disease progresses, chronic activation shifts microglia to the M1 phenotype, which promotes Aβ pathology and induces neurotoxic effects.[38,76,80] This is particularly evident when Aβ plaques form, and microglia surround the plaques and NFT-containing neurons, attempting to remove them but ultimately lacking the sufficient phagocytic capacity to do so.[5,38,76] Oligomeric Aβ has been proven to be a potent activator of microglia.[2,5,38,76] Likewise, Aβ plaques can directly stimulate glial cells to release a variety of toxic agents into the brain parenchyma, including ROS, NO, proinflammatory cytokines, chemokines, and complex protein mediators.[38,76,79,81] These toxic products recruit more microglia and astrocytes to the sites of Aβ deposition, generating an environment of inflammation, oxidative stress, and neuronal cell death.[76,78,79,81] This inflammatory condition reduces the brain's capacity to eliminate Aβ, promoting further accumulation and, ultimately, accelerating AD progression.[76,78,79,81] In addition, cytokines, such as ILs, are able to promote Aβ synthesis and spreading in the brain. Certain cytokines can induce the expression of inflammasome multiprotein complexes, such as interferon-induced transmembrane protein 3 (IFITM3), in neurons and astrocytes.[81,82] IFITM3 enhances Aβ production by modulating γ-secretase.[82] On the other hand, certain cytokines like IL-3, secreted by astrocytes, can activate microglial cells to cluster around Aβ and tau aggregates and eliminate them through phagocytosis.[83] In contrast, IL-17 has been implicated in Aβ-induced neuroinflammation, cognitive decline, systemic inflammation, peripheral vascular dysfunction, and a prothrombotic state.[84] Microglial cells detect Aβ through a range of cell surface receptors and respond through multiple signaling cascades.[5,38,76] These receptors include triggering TREM2 and pattern recognition receptors, such as formyl peptide receptors.[5,38,76] Antibodies that bind to and modify microglia-activating receptors are currently promising candidates for the treatment of neuroinflammation in AD, potentially in combination with anti-Aβ therapies.[5,38,76] Among these receptors, TREM2 is considered a key receptor on the surface of microglia.[62,63,76] TREM2 promotes the phagocytic activity of microglia and regulates their signaling response to inflammation, Aβ, and tau in AD.[62,63,76] Loss of TREM2 function has been found to increase amyloid pathology and the risk of AD, particularly by impairing Aβ clearance through phagocytosis.[62] On the other hand, excessive phagocytic activity can cause the loss of healthy neurons and synapses, contributing to neurodegeneration.[63] Collectively, the microglial response to Aβ and associated disease progression appear to be context-dependent.[12] Microglia can switch from a protective role, characterized by Aβ phagocytosis, to a disease-promoting role, marked by proinflammatory protein expression and associated neurotoxic effects.

4. Amyloid β-induced cerebral vasculopathies and neuronal sequelae in AD

In AD, Aβ deposits are found not only in the brain parenchyma but also in cerebral blood vessels.[2-10,15,16,35,49,64,85,86] These deposits trigger both neurodegenerative and vasculopathic changes, which collectively participate in cognitive decline. Recent studies in AD mouse models have shown that microvascular damage in cortical and hippocampal brain areas occurs before the formation of parenchymal Aβ plaques and deposition of NFTs.[75,87] Similarly, in AD patients, age-related vasculopathic changes in the brain are aggravated, including increased vessel resistance, thickening of vessel walls, and reduced wall elasticity.[6,49,88] In fact, vascular abnormalities in the AD brain, such as Aβ-CAA, have long been recognized as early and typical manifestations of the disease.[1,6-10,35,49,65,88] Aβ-CAA is a vasculopathy associated with occlusive, cellular, and hemorrhagic lesions, leading to vascular and BBB dysfunction.[6-10,35,49,64,65,88] Aβ-induced damage primarily affects the inner endothelial cell layer of the vessel walls and the surrounding structures of the BBB, which form the critical interface between the blood and brain tissue.[6,7,35,49,64,86] In addition to damaging vessel walls, Aβ induces capillary constriction,[64,89] further impairing CBF, brain perfusion, and neuronal and cognitive functions.[6,35,49,88] In particular, ischemic and hypoxic conditions in the brain cause a marked reduction in cerebral metabolism, which is associated with a self-reinforcing cycle of increased Aβ generation.[6,7,35,49,64,86] This vasculopathic aspect of Aβ pathology has only gained significant attention in AD research over the past 15 years.[6,7,15,35,49,64,86] More recently, the procoagulant activity of Aβ has been found as a key factor in triggering vasculopathic, inflammatory, and neurodegenerative changes in AD.[6,16,25,51-53] Aβ promotes a procoagulant state in the blood, leading to increased production of proinflammatory thrombin and fibrin(ogen). This process results in the formation of Aβ-containing fibrin clots, which are resistant to degradation and are involved in the development of vasculopathies, such as Aβ-CAA.[6,16,25,27,53] Consequently, the involvement of Aβ-induced vasculopathies in cognitive decline presents novel therapeutic strategies for AD. Particularly, anticoagulants could be suitable agents for normalizing the Aβ-induced procoagulant state in AD and counteracting the associated neurovascular and cognitive dysfunction.[15,16,19,22-24,26,49,64,90]

4.1. Aβ-CAA

Cerebral SVD is a major contributor to cognitive impairment in vascular dementia and also represents a significant vasculopathy in AD.[9] Among the several types of SVD, CAA and non-amyloid SVD are the most common.[9] CAA is a cerebrovascular disorder frequently observed in the elderly and is categorized according to the type of amyloid protein involved.[7,9,10,15,35,65] In AD, vascular deposits of misfolded Aβ lead to Aβ-CAA,[9,10,15,35,65] in which Aβ oligomers and filaments accumulate within the walls of cerebral blood vessels.[6,7,9,10,35] While parenchymal Aβ deposits in AD are primarily composed of the longer Aβ42 subtype, Aβ40 is the predominant form found in vessel walls in Aβ-CAA. The occurrence of Aβ-CAA is closely related to age and an increased risk of dementia with rapid cognitive decline.[6,7,9,35,49,65] Aβ-CAA is especially prevalent in the vascular systems of the neocortical and hippocampal brain regions, which are also primary sites of parenchymal Aβ deposition in AD. In Aβ-CAA, congophilic material composed of Aβ is deposited in the walls of small- to medium-sized leptomeningeal and cortical brain vessels.[6,7,9,35,49,91] These vascular lesions are associated with an increased risk of brain hemorrhage (e.g., primary lobar hemorrhage, cortical microhemorrhage, and cortical superficial siderosis) and ischemic damage (e.g., cortical microinfarcts and ischemic changes in the white matter).[35] As a result, vascular and BBB function are impaired, leading to decreased cerebral perfusion. This cascade of events contributes to inflammatory and neurodegenerative changes in the brain, which eventually culminate in cognitive impairment in AD.[6,7,9,10,15,49,85,91] Studies in AD mouse models suggest that Aβ is the key trigger for Aβ-CAA and its associated sequelae.[67] For instance, peripherally applied Aβ-containing inoculates have been shown to induce Aβ-CAA in cortical and hippocampal blood vessels.[67] Moreover, individuals who received cadaver-derived pituitary growth hormone contaminated with Aβ in childhood developed Aβ-CAA, parenchymal Aβ pathology, and AD-like dementia.[92,93] On the other hand, tau deposits observed around Aβ-loaded cerebral vessels are considered to play a minor role in the development of Aβ-CAA.[10] Collectively, Aβ-CAA and other Aβ-induced vascular abnormalities are increasingly recognized not merely as symptoms of AD but as integral components of Aβ pathology in the disease.[6-10,15,49]

In fact, Aβ-CAA is a common phenomenon in AD. Clinical studies have found that 82 – 98% of AD patients suffer from Aβ-CAA.[35,65,91] In both sporadic and familial forms of Aβ-CAA, elevated prion-like infectivity and spreading of Aβ have been detected in postmortem brain tissue.[48] Familial Aβ-CAA often manifests more severely and at an earlier age than sporadic Aβ-CAA.[9] In familial cases, mutations in the *APP* gene, duplications of *APP*, or mutations in *PSEN1* and *PSEN2* – genes involved in the γ-secretase complex – are frequently observed.[6,7,9,35,49]

Death in these patients usually occurs around the age of 50, often without significant tau accumulations.[48] The *APOE4* genotype, which is associated with a higher risk of late-onset AD, is linked to both Aβ and tau pathologies,[57,59] as well as conditions such as type 2 diabetes, atherosclerosis, and sporadic Aβ-CAA.[9,57,59] Sporadic Aβ-CAA is subdivided into two types: type 1 is more frequently associated with *APOE4* and is characterized by Aβ deposition in and around the walls of cortical capillary, larger vessels, and leptomeningeal vessels and type 2, less strongly associated with *APOE4*, exhibits Aβ deposition in arteries, arterioles, veins, and venules, but not in capillaries.[7,9,35,49,91] In the CNS, the carrier protein APOE is secreted primarily by glial cells, especially astrocytes.[57,60] APOE transfers cholesterol and phospholipids in the form of APOE-containing, high-density lipoprotein-like particles to neurons, supporting neuronal plasticity, synaptogenesis, and synapse growth.[57,60] However, in individuals carrying the *APOE4* allele, APOE4 activity is associated with increased Aβ deposition, impaired Aβ clearance, increased BBB permeability, and abnormal cholesterol accumulation in oligodendrocytes, which disrupts myelin synthesis.[2,48,57-61] An insulating myelin sheath around axons is necessary for the efficient axonal transmission of electrical impulses.[58] Therefore, reduced axon myelination slows neuronal transmission of electrical signals, impairing cognitive function.[58] In addition, the accumulation of cholesterol can increase the interaction of APP with β- and γ-secretases, thereby enhancing Aβ production in neurons.[60]

Arteriolar Aβ-CAA is first diagnosed in the peripheral extracellular matrix of the tunica media, extending to the adventitia, which includes the Aβ seeding sites at the outer regions of the vessels.[7,9,50,92] Depending on the severity, Aβ deposition can progress through all layers of the arterioles and small arteries, gradually replacing all vascular smooth muscle cells (VSMCs) and other tissue elements within the vessel wall, except for the endothelial cells.[7,9,35,50,91,92] This process progressively debilitates the vessel wall and disrupts both vascular and BBB function. Consequently, Aβ-CAA is associated with vascular disorders such as fibrinoid necrosis, microaneurysms, concentric splitting or hyaline thickening of the vessel wall, and arteriolar degeneration.[7,9,35,50,91,92] In capillary Aβ-CAA, Aβ deposition occurs in the basement membrane, which is a component of the BBB formed by endothelial cells, pericytes, and astrocytic endfeet.[9,10,35,91,92] This process leads to degeneration of the endothelial cells, loss of tight junctions, and a decline in pericytes, eventually culminating in BBB breakdown, a condition often observed in severe AD. Moreover, vascular deposits of Aβ tend to infiltrate the adjacent brain parenchyma, which has been associated with neuritic Aβ plaque formation, hyperphosphorylated tau, and neuroinflammation.[9,35,91] Aβ-CAA can also provoke vessel inflammation, which is subtyped into inflammatory angiitis or Aβ-related angiitis.[9]

Ultimately, Aβ-CAA and its associated disorders manifest in severe cerebral vascular pathology, leading to vascular and BBB dysfunction and a series of pathophysiological sequelae.[7,35,64,66,88] These include reduced CBF, brain hypoperfusion, and a resulting nutrient deficiency in cerebral tissue. Vascular defects also impair the perivascular clearance of Aβ, further increasing parenchymal Aβ load.[6,7,10,35,50,94] In addition, the pathophysiological effects of Aβ-CAA are exacerbated by a reduction in capillary diameter, caused by Aβ-induced vessel constriction.[64]

4.2. Aβ-induced capillary constriction

Vascular resistance in the brain is particularly influenced by capillaries rather than arterioles and venules.[64] Consequently, the regulation of CBF through changes in vascular diameter is mainly controlled by pericytes on the outer wall of capillaries, with less involvement from VSMCs around arterial vessels.[10] Oligomeric Aβ deposits in the capillary walls have been found to decrease capillary diameter and, as a consequence, reduce CBF by triggering localized vessel constrictions through pericyte contraction.[64,89] Capillary constrictions develop early in AD, even before significant pericyte loss due to Aβ-CAA, and are closely correlated with the extent of Aβ deposition in capillary walls.[64,89] Studies in both AD mouse models and human brain biopsies have demonstrated that capillary vasoconstriction is caused by the contraction of contractile pericytes on the vessel wall.[89] Mechanistically, pericyte contraction is initiated by Aβ-triggered ROS signaling, which promotes the release of endothelin-1 (ET) release. ET then activates contractile pericyte ET receptors,[89] leading to vasoconstriction. This process may also affect cerebral arterioles and the middle cerebral artery.[64] Additional pathological processes impairing CBF include the formation of fibrin and Aβ-containing fibrin clots that are resistant to degradation as well as aggregates of platelets and neutrophil extracellular traps within blood vessels.[15,16,64,95] Collectively, these factors contribute to thrombotic vessel occlusions in AD.

4.3. Aβ-induced procoagulant and proinflammatory states

A notable pathological feature of Aβ is its procoagulant activity, which leads to the upregulation of the protease cascade in the plasma contact system and coagulation pathways.[6,16,25,51,52,96] Recently, this function of Aβ, which induces a procoagulant state with increased thrombin production, has been demonstrated in AD mouse models, individuals with mild cognitive impairment at high risk

of AD, and AD patients (Figure 1). After Aβ transitions from the brain parenchyma into the bloodstream or is synthesized by platelets,[44] vascular Aβ activates FXII to FXIIa in the contact system. FXIIa initiates both the inflammatory kallikrein-kinin pathway and the intrinsic coagulation pathway, leading to increased thrombin generation through the activation of FXI (Figure 1).[6,16,25,52,96] Thrombin converts soluble fibrinogen into insoluble fibrin, activating platelets and FXIII, which together form cross-linked fibrin clots that can occlude blood vessels in the brain (Figure 1).[6,16,25,51,52,96,97] Blocking Aβ binding to FXII has been shown to prevent Aβ's procoagulant activity, reducing both vascular pathology and cognitive impairment in AD.[25] In addition to FXII activation, Aβ also binds to and modulates other coagulation factors, such as thrombin, fibrinogen, and FXIII, further amplifying the procoagulant state and promoting the formation of occlusive fibrin clots.[16] This thrombotic milieu is exacerbated by the downregulation of the fibrinolytic system, including the reduced activity of plasmin and tPA, which are responsible for the degradation of fibrin clots.[16,96] Beyond their roles in fibrinolysis, plasmin, its precursor plasminogen, tPA, and uPA are also believed to be involved in proper brain functioning, especially in synapse regulation and Aβ clearance.[96] Dysregulation of the fibrinolytic system has been associated with impaired BBB function, increased inflammation, and enhanced Aβ plaque formation in the brain.[96] In addition, fibrin(ogen) has the ability to form cross-linked fibrin aggregates with Aβ, which are resistant to fibrinolytic degradation and can lead to persistent thrombi in the vasculature (Figure 1).[6,16,25,53,96,98,99] In fact, vascular deposition of Aβ-containing fibrin (fibrin-Aβ) clots is suggested to be causally involved in Aβ-induced vasculopathies and their associated neuronal and cognitive sequelae in AD (Figure 1).[6,16,25,53,96-98]

An additional consequence of Aβ-induced FXII activation in the contact system is the stimulation of bradykinin synthesis through the inflammatory kallikrein-kinin pathway (Figure 1).[25,96] FXIIa activates prekallikrein (PK) to form kallikrein, which cleaves high-molecular-weight kininogen (HK) to release the vasoactive peptide bradykinin (Figure 1).[14,25,51,96,100] Elevated plasma kallikrein levels can increase the risk of hemorrhage in AD, while enhanced bradykinin production can cause peripheral inflammation, brain swelling (edema), vasodilation, and

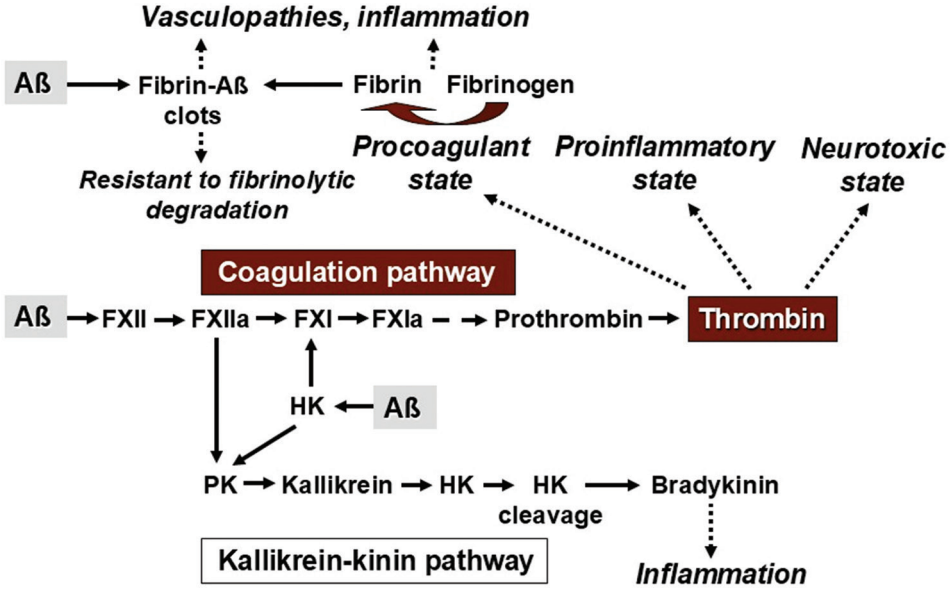

Figure 1. Amyloid-ß (Aß) proteins trigger vasculopathies, inflammation, and neurovascular dysfunction in Alzheimer's disease by dysregulating the plasma contact system. Vascular Aβ activates factor XII (FXII) to factor XIIa (FXIIa) in the contact system. FXIIa initiates both the coagulation and inflammatory kallikrein-kinin pathways. In the latter, FXIIa cleaves prekallikrein (PK) to form kallikrein, which catalyzes the cleavage of high-molecular-weight kininogen (HK) to release the proinflammatory, vasoactive peptide bradykinin. Intrinsic coagulation occurs when FXIIa activates factor XI (FXI) to factor XIa (FXIa), leading to thrombin synthesis through prothrombin cleavage. Aβ also activates HK, which is essential for both the thrombotic and inflammatory pathways, as FXI and PK must bind HK to be activated by FXIIa. Thrombin triggers procoagulant, proinflammatory, and neurotoxic states. Thrombosis occurs when thrombin cleaves fibrinogen into fibrin and activates platelets and factor XIII (not shown). Interaction of Aβ with fibrin(ogen) forms Aβ-containing fibrin (fibrin-Aβ) clots, which are resistant to fibrinolytic degradation. These clots can trigger vasculopathies such as Aβ-type cerebral amyloid angiopathy, leading to inflammation, vessel occlusion, and hemorrhagic lesions, resulting in vascular and blood–brain barrier dysfunction, reduced cerebral perfusion, and neurological and cognitive disorders. Extravasated thrombin and fibrin(ogen) in the brain parenchyma can further induce cerebral inflammation and neurotoxicity.

BBB disruption.[25,96,100] In addition to FXII, Aβ also activates HK, which is essential for both the intrinsic coagulation (through FXI activation by FXIIa) and the inflammatory kallikrein-kinin pathway (through PK activation by FXIIa). Both FXI and PK must bind to HK to be activated by FXIIa (Figure 1).[25,96]

4.3.1. Evidence for a causative role

In recent years, studies have provided evidence of the importance of the Aβ-induced activation of the contact system in generating procoagulant and proinflammatory states in AD.[6,16,25,51,52,96,100] Collectively, these investigations have yielded the following findings:

(i) In patients with sporadic and genetic AD, as well as in AD mouse models, accumulations of FXII, HK, bradykinin, thrombin, fibrinogen, and fibrin were found to be associated with the activation of the plasma contact system by Aβ (Figure 1). The pathological sequelae observed included inflammation, neurovascular dysfunction, neurodegeneration, and cognitive impairment.[6,25,27,51-53,61,96-100]

(ii) Biochemical studies suggest a causal connection between Aβ-triggered FXII activation and the upregulation of bradykinin synthesis (Figure 1).[16,25,96] The severity of cognitive impairment in AD patients correlated with elevated plasma levels of FXIIa, increased kallikrein activity, cleaved HK levels, and bradykinin levels, while FXII levels decreased.[25,51,52,100] In wild-type mice, the injection of Aβ increased plasma kallikrein activity and HK cleavage.[25,51] Similarly, normal human plasma treated with Aβ42 responded with FXII activation to FXIIa, HK cleavage, and bradykinin synthesis.[25,51] Antibody blockage of HK prevented HK cleavage and bradykinin synthesis, as well as HK-mediated FXI activation in Aβ-induced intrinsic coagulation.[16,25]

(iii) Additional biochemical studies suggest a causal connection between Aβ-induced FXII activation, subsequent FXI activation, and increased thrombin production in the coagulation cascade, leading to thrombosis (Figure 1).[16,25,52,96] In the plasma of AD patients, increased FXIIa and decreased FXI levels (suggesting enhanced FXI activation to FXIa) were found alongside elevated fibrin levels,[25,51,52,96] indicating increased activation of the intrinsic clotting system. The addition of Aβ42 to *in vitro* protein systems or normal human plasma triggered the production of thrombin and fibrin.[16,52] In FXII-deficient human plasma, these effects were abolished,[52] indicating that Aβ-induced FXII activation triggers thrombin synthesis. Blocking FXII activation, either with antibodies in plasma or with anti-FXII oligonucleotides in AD mice, decreased plasma levels of thrombin and fibrin(ogen).[25,52] In AD mice, anti-FXII oligonucleotide treatment also reduced vascular damage, neuroinflammation, neuronal loss, and cognitive impairment.[25]

(iv) Inhibition of thrombin and fibrin production by anticoagulants normalized procoagulant and proinflammatory states, reducing disease progression and associated sequelae in AD mouse models.[15,16,25,90,96,101-103] Long-term thrombin blockage was able to slow cerebrovascular and parenchymal disease progression and cognitive decline.[90,101-103] Similarly, anticoagulant treatment in patients with AF was found to benefit the cerebrovascular and neuronal systems, reducing the incidence of dementia by up to 48%.[15,16,27,104]

4.3.2. Thrombin as a proteinopathic factor

A substantial body of evidence supports the role of the multifunctional protease thrombin as a key mediator of neuroinflammatory and neurotoxic processes in AD and other neurodegenerative diseases, such as Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis.[15,27,105] In both sporadic and familial AD, Aβ-induced proinflammatory and procoagulant states, characterized by increased thrombin production, are implicated in vasculopathic and neurodegenerative events (Figure 1).[6,16,25,27,52,53,61,77,97-99] In the brain of AD patients, elevated levels of thrombin, prothrombin, and protease-activated receptors (PARs) involved in thrombin signaling have been detected, particularly in microvessels, neurons, and glial cells.[27,76,77,80,105] Thrombin has also been identified in senile Aβ-plaques and NFTs in AD patients. Similarly, increased thrombin expression levels have been found in the brain vasculature.[27,76,105] These findings have led to the suggestion of a key role for thrombin in the pathological interactions between coagulation, vascular disease, inflammation, and neurotoxicity (Figure 1).[27,76,77,105] Recent studies have demonstrated that elevated thrombin levels, directly and indirectly, trigger inflammatory, vasculopathic, and neurodegenerative changes in cardiovascular disease and AD.[27] Both diseases are multifactorial and exhibit long asymptomatic phases, sharing vascular risk factors, such as CAA, atherosclerosis, diabetes, and traumatic brain injury.[27,42] In AD, thrombin is now considered a proteinopathic factor alongside Aβ and tau.[15,16,27,77,105]

(a) Thrombin's role in generating a prothrombotic state

Aβ has been shown to trigger FXII activation in the plasma contact system (Figure 1).[6,16,25,51,52,96] The resulting dysregulation of the coagulation cascade leads to elevated thrombin levels, which enhance fibrin production and inflammation.[6,15,16,25,27,53,96,105-108] Fibrin contributes to the

formation of thrombotic fibrin clots and inflammatory reactions in cerebral vessels, working in conjunction with Aβ to promote the development of vasculopathies, such as Aβ-CAA (Figure 1). Moreover, thrombin activates procoagulant factors V, VIII, and XI, further amplifying its own production. Thrombin also activates fibrin-stabilizing FXIII and, in conjunction with fibrin(ogen) and platelets, promotes the formation of cross-linked fibrin clots containing platelets and erythrocytes.[16,27,28,44,107,109] In both AD patients and mouse models, elevated platelet activation, adhesion, and aggregation have been found in the early stages of AD, correlating with disease progression.[44] The prothrombotic state induced by thrombin is further aggravated by its ability to stabilize existing fibrin clots through the activation of thrombin-activatable fibrinolysis inhibitor and FXI.[16,27,28]

(b) Thrombin's role in generating a proinflammatory state

Beyond its procoagulant activity, thrombin, a multifunctional protease, activates a diverse array of signaling pathways that mediate systemic inflammatory responses and affect vascular and neuronal functions in the brain (Figure 1).[15,27,76,77] Through signaling through PARs, thrombin acts on multiple cellular targets, including blood platelets, vascular endothelial cells, pericytes, as well as parenchymal glial cells and neurons.[27,107] PARs, which are G-protein-coupled receptors, regulate important cellular processes such as platelet activation, cell adhesion and migration, angiogenesis, inflammation, and neurotoxicity.[27,107] In the vasculature, thrombin can elicit inflammatory reactions that affect vessel diameter, endothelial cell morphology, and BBB permeability.[27,77] Elevated thrombin levels, often seen in AD and under hypoxic conditions, have been associated with the onset of cerebrovascular inflammation and dysfunction.[27,105] Pathologically, thrombin can induce endothelial cells to adopt a proinflammatory phenotype, releasing inflammatory mediators, such as cytokines, ROS, NO, and additional thrombin, resulting in damage to the endothelial cell layer and BBB.[27,76,77] Cytokines can further upregulate the extrinsic coagulation pathway through TF activation,[27,105] which promotes additional thrombin production, exacerbating the proinflammatory and procoagulant state. In the CNS, thrombin also contributes to neuroinflammation, particularly due to increased BBB permeability, allowing blood-borne proteins, such as thrombin, prothrombin, and fibrin(ogen), to enter and accumulate in the brain parenchyma.[7,27,78,80,105] Through PAR involvement, parenchymal thrombin can convert astrocytes and microglia into an activated, proinflammatory state, leading to the release of cytokines, ROS, and the activation of nucleotide-binding oligomerization domain-like receptor family, pyridine domain containing 3 inflammasome, a key component of the innate immune system.[7,15,27,78,80,105] Ultimately, blood-borne proinflammatory mediators, such as thrombin, can damage healthy neurons in the brain parenchyma, resulting in neuronal structural loss and functional impairment, as observed in AD and other neurodegenerative diseases.[27,105]

(c) Thrombin's role in triggering neurotoxicity

In addition to its procoagulant and proinflammatory activities, thrombin has been found to be an important trigger for neurotoxic effects (Figure 1).[14,27,110] Through multiple signaling pathways, including those mediated by PARs, thrombin can induce neuronal cell death, which is associated with cognitive decline.[14,27] Thrombin has been shown to disrupt CA3 hippocampal neurons, leading to epileptic and cognitive dysfunction. In addition, thrombin can indirectly cause neuronal damage by activating astrocytes and microglia.[27,105,110] In microglia, thrombin has been found to induce oxidative stress by activating NADPH oxidase, a response linked to the death of hippocampal neurons *in vivo*.[110] Thrombin's role in hippocampal neurodegeneration is also related to both Aβ and tau pathologies.[27,76,80] Recent studies in neuronal cell cultures have revealed that thrombin enhances the expression and activity of key AD markers, including Aβ, APP, BACE1, tau, and the pro-apoptotic mediator caspase 3.[111]

4.3.3. Fibrin(ogen) as a proteinopathic factor

In the final phase of the coagulation cascade, thrombin converts soluble fibrinogen into fibrin dimers and activates platelets and FXIII, resulting in the formation of cross-linked fibrin clots containing aggregated platelets and other components (Figure 1).[28,44] Beyond its role in blood clotting, fibrinogen also plays an important role in inflammation and tissue repair.[106,109] Fibrinogen is a fibrous glycoprotein comprised of two sets of disulfide-bridged polypeptide chains (Aα, Bβ, and γ),[28,109] and it is expressed and secreted into the blood by platelets, endothelial cells, and hepatocytes in response to tissue injury.[28,109]

In a variety of neurodegenerative diseases, fibrinogen has been found to infiltrate from the blood into the brain parenchyma. There, it can interact with various proteins and cells, such as neurons, astrocytes, and microglia, which have been associated with neuronal and cognitive dysfunction.[106,109] In AD, enhanced deposition of fibrin(ogen) has been observed particularly around and within cerebral vessel walls, colocalizing with Aβ, especially in small capillaries and arterioles, often in association with Aβ-CAA.[6,27,53,61,96-99,106,109] In addition, fibrin(ogen) deposits have been detected in the perivascular spaces of the brain parenchyma.

These accumulations of fibrin(ogen) frequently co-occur with increased vascular endothelial permeability, pericyte loss, elevated levels of activated microglia, and the formation of amyloid plaques and dystrophic neurites. While fibrin(ogen) in cerebral vessels disrupts vascular and BBB function, in parenchymal tissue, fibrin(ogen) synergistically exacerbates neuroinflammation and Aβ pathology.[25,53,80,106,108,109,112,113] Parenchymal fibrin(ogen) has been associated with glial cell activation and increased expression of cytokines, chemokines, and ROS.[25,53,80,106,108,109]

A prospective clinical study has demonstrated that high plasma fibrinogen levels are linked with an increased risk of AD and vascular dementia.[112] Mechanistically, fibrin(ogen)'s γ-chain has been found to specifically bind to CD11b/CD18 and CD11c/CD18 receptors on microglia and brain-infiltrating macrophages.[25,106,109] This interaction activates microglia, as observed in AD mouse models, initiating a signaling cascade that causes dendritic atrophy and spine loss in neurons. This microglial activation and signaling cascade results in synaptic defects, disintegration of neuronal networks, neuroinflammation, and cognitive decline.[25,106,109]

Injection of fibrinogen in the brains of mice produced similar damage, which was exacerbated in the presence of Aβ.[25] However, genetic or antibody-mediated blockage of the fibrinogen domain responsible for binding glial receptors inhibited microglial activation in AD mouse models,[25,113] reducing inflammation, neurodegeneration, and memory decline without affecting fibrinogen's procoagulant properties.[25,113]

Fibrin(ogen) can also activate neutrophils, causing excessive immune and inflammatory responses.[114] Importantly, a recently discovered interaction between fibrinogen and Aβ has established a causal link between the pathologies of Aβ, fibrin(ogen), and thrombin, contributing to the prothrombotic state in AD (Figure 1).[6,53,96-99,108] Fibrinogen's β-chain has a binding affinity for Aβ, leading to the formation of abnormal, degradation-resistant blood clots. These clots can cause persistent cerebral vessel occlusion, ischemic conditions, and neurovascular dysfunction.

Pathogenic aggregates formed by fibrin(ogen) and Aβ have been demonstrated. Both *in vitro* (using Aβ42) and *in vivo* studies show that the β-chain of fibrinogen specifically interacts with the central region of Aβ, leading to fibrinogen oligomerization and aggregation of fibrin-Aβ clots (Figure 1).[6,53,96,98,108] This interaction results in the generation of abnormally tight fibrin networks, which are more resistant to plasmin-induced fibrin cleavage and clot lysis (Figure 1).[6,25,53,96,97,108] Notably, the Dutch- and Iowa-point mutations of Aβ, which are associated with Aβ-CAA, increase the binding affinity of Aβ for fibrinogen by a factor of 50. This enhanced affinity correlates with the severity of fibrin-Aβ clot deposition and disease progression in AD patients.[25,108]

The persistent formation and deposition of fibrin-Aβ clots, along with their associated vascular disorders, are further consequences of the Aβ-induced procoagulant state in AD.[6,25,53,61,96-98] Fibrin-Aβ deposits are typically found in and around the walls of cerebral vessels, contributing to the development of Aβ-CAA.[6,53,61,97] The increasing generation of fibrin-Aβ clots causes vascular lesions, such as vessel occlusion, damage to capillary pericytes and endothelial cells, and hemorrhage. As a result, vascular and BBB dysfunction ensues.[6,25,86,96] Pathophysiologically, this leads to a decline in CBF and hypoperfusion, creating ischemic and hypoxic conditions. These changes drive neuroinflammatory and neurodegenerative sequelae.[5-9,15,16,25,27,53,96,98,108,115] In addition, this scenario exacerbates Aβ accumulation in the brain by stimulating parenchymal Aβ synthesis and disrupting perivascular Aβ clearance due to BBB damage.[25,82,115]

Fibrin-Aβ deposits are also found in the cortical and hippocampal parenchyma, especially in regions containing dystrophic neurites and adjacent Aβ oligomers and plaques.[53,61,97,98] These deposits are associated with neuroinflammation, synapse and neuron loss, and cognitive decline.[6] The formation of parenchymal fibrin-Aβ deposits is largely attributed to BBB breakdown, which enables thrombin and fibrinogen to infiltrate the brain parenchyma.[7,97]

In summary, the accumulation of Aβ, thrombin, fibrin(ogen), and fibrin-Aβ aggregates triggers inflammatory and damaging responses in both the vascular system and brain parenchyma. This results in the activation of glial cells and the release of cytokines and ROS.[27,86,92,106,107,109,113] The generation of fibrinolysis-resistant fibrin-Aβ deposits is a hallmark of AD, emerging from the pathological interactions between Aβ, fibrin(ogen), and thrombin (Figure 1).[15,16,25] Given these insights, preventing the formation of fibrin-Aβ clots has been proposed as a novel therapeutic approach for AD.[6,116] Studies have shown that antibody or genetic suppression of the fibrinogen domain that interacts with Aβ can reduce thrombotic, inflammatory, neurodegenerative, and cognitive impairment in AD mice.[25,96,113] Similarly, small molecular inhibitors, such as RU-505, which bind to Aβ and prevent fibrin-Aβ clot formation, have been shown to reduce vascular amyloid deposition, vessel infarction, parenchymal microgliosis, and cognitive decline in AD mice.[6,96,116]

At present, therapeutic options under investigation include antisense oligonucleotides, monoclonal antibodies,

and small-molecular inhibitors of FXI, all aimed at preventing fibrin-Aβ deposition and its vascular and neuronal sequelae.[16] One advantage of these agents is that they are expected to pose a relatively low risk of bleeding due to minimal interference with hemostasis.[16] In addition, fibrin-targeting immunotherapies are being clinically evaluated for their potential to reduce neuroinflammation, neurodegenerative changes, and cognitive impairment in patients with dementia.[106]

4.4. Pathophysiological consequences

In early AD, Aβ-induced vasculopathies, such as Aβ-CAA, vasoconstriction, and procoagulant and proinflammatory states, are key drivers for occlusive, cellular, and hemorrhagic lesions, leading to vascular and BBB dysfunction (Figure 1).[6,15,16,25] Key pathological contributors in this vascular environment include Aβ, fibrin(ogen), fibrin-Aβ clots, aggregates from activated platelets, and occlusive neutrophil extracellular traps.[6,7,15,16,25,49,64] The brain's pathophysiological consequences to these factors include a decline in CBF, hypoperfusion, and a subsequent decrease in nutrient supply and metabolism.[6,7,15,16,25,49,64] In addition, BBB dysfunction enables thrombin, fibrin(ogen), and other harmful agents to infiltrate parenchymal tissue.[7] On the other hand, the release of parenchymal Aβ and other toxic products into the bloodstream for dilution and degradation becomes impaired.[7] This creates a self-reinforcing cycle of increased vascular and parenchymal Aβ accumulation, further exacerbating the spread of Aβ and associated pathology in the brain.[6,7,16,25,49,64] The resulting sequelae include chronic neuroinflammation, largely driven by an excessive glial response and neurodegeneration characterized by synaptic and neuronal loss. These processes contribute to the progressive decline in memory and cognitive function.[6,7,49,64]

4.4.1. CBF reduction, brain hypoperfusion, and cognitive decline

Chronic hypoperfusion, defined as persistent insufficient blood flow to the brain (ischemia), results from sustained reduction in CBF.[7,15,66,88,89,92,117] This condition significantly impairs cerebral metabolism and the overall functioning of the nervous system. The supply of essential blood components – such as oxygen, glucose (the brain's primary energy source), proteins, and cellular elements – becomes increasingly restricted. Chronic reductions in CBF have been documented in both AD patients[7,88,117] and mouse models.[64,66] The typical decline in CBF is in the range of 25% of normal levels, but in certain brain regions, reductions can exceed 50%.[118]

Notably, even in the early, asymptomatic stages of AD, reductions in CBF and increased vascular resistance can be detected.[64,88,119] As a result, CBF decline and hypoperfusion have been proposed as bona fide *in vivo* predictors of future cognitive disorders.[119] Clinical studies have demonstrated that circulation-promoting physical exercise can slow cognitive impairment in AD patients.[120]

Advanced brain imaging has revealed a spatial-temporal pattern of CBF reduction in individuals ranging from healthy subjects to those with mild cognitive impairment and AD. The decline of CBF begins in regions, such as the precuneus, posterior cingulate, and temporal-parietal areas before spreading to broader regions.[119] This reduction in CBF, along with the consequent deficits in oxygen (hypoxia) and glucose supply, correlates with the severity of vascular Aβ load and the associated dysfunction in the AD brain.[16,64,66,88,89,119]

The hypometabolic tissue milieu contributes to inflammatory, neurodegenerative, and cognitive sequelae.[64] This situation is further intensified by the increased metabolic demands from elevated Aβ production and amyloidosis development.[115] In fact, ischemia and hypoxia in the brain have been shown to stimulate the synthesis of Aβ from APP cleavage, driven by both the upregulation of *BACE1* gene expression and the activation of γ-secretase. Likewise, tau pathology has been found to worsen in response to declining CBF and the accumulation of parenchymal Aβ.[7,64,74]

Moreover, in AD individuals carrying the *APOE4* risk allele, the loss of capillary pericytes and BBB dysfunction have been associated with reduced CBF and are predictors of cognitive decline.[121] Pericytes are highly sensitive to ischemic conditions, and the severity of pericyte loss and BBB breakdown correlates with the vascular Aβ load.[64]

4.4.2. BBB breakdown and dysfunction

Aβ-induced vascular pathologies have a particularly damaging effect on the endothelium of blood vessels, a layer consisting of endothelial cells connected by tight junctions.[7,86] The endothelium forms the primary barrier lining the interior surface of all blood vessels and regulates substance exchange between the blood, parenchymal tissue, and ISF of the brain.[7,86] This vascular endothelium is a crucial component of the BBB, along with the outer basement membrane, mural cells (pericytes in capillaries, VSMCs in arterioles, and arteries), and surrounding astrocyte endfeet.[7]

Capillaries, the smallest and most widely distributed blood vessels in the brain, are particularly important for the selective transport of nutrients, ions, and signaling molecules.[7,86] This selective transport involves the exchange of substances across the BBB from the blood into the brain's parenchymal tissue and vice versa.[7,86] Through

this exchange, perivascular clearance of neurotoxic compounds and proteins, such as Aβ, occurs as they are transferred from the brain parenchyma into the blood. A healthy BBB ensures a favorable environment for brain metabolism, neuronal function, and protection.[7,86] In contrast, BBB breakdown is an early hallmark in AD, playing an important role in the initiation and progression of the disease. This disruption is driven by Aβ, thrombin, and fibrin(ogen) pathologies, which collectively contribute to the deterioration of BBB integrity.[6,7,86]

Studies in AD patients with the *APOE4* genotype have shown that Aβ-CAA is a major cause of BBB breakdown.[7,121] BBB breakdown in capillaries is associated with damage to the endothelial cell layer and pericytes, disruption of transporter systems, and increased endothelial bulk transcytosis.[7,86] As a result, BBB dysfunction permits an uncontrolled influx of substances from the blood into swollen perivascular areas of the brain parenchyma.[7,80,86] These substances include proinflammatory proteins (e.g., prothrombin, thrombin, and fibrinogen), neurotoxic protein aggregates (e.g., Aβ and tau), autoantibodies, ions, water, cells, and pathogens.[7,80,86]

The consequences for the brain include edema; disrupted ion homeostasis; and harmful immune, inflammatory, and neuronal responses.[7,86] This scenario is further exacerbated by impaired local ISF formation and flow, as well as disruption of normal cargo transport from the blood into the brain parenchyma.[7,86] In addition, BBB dysfunction hampers the transport of parenchymal Aβ into the blood, thereby impairing the perivascular clearance of toxic Aβ through its dilution and degradation in the bloodstream.[7,86]

Key cellular transport mechanisms affected by this dysfunction include transcytosis pathway involving endothelial phosphatidylinositol-binding clathrin assembly protein and low-density lipoprotein receptor-related protein 1, which are located in the membranes of endothelial cells, pericytes, and perivascular astrocytes.[7,86] Reduced CBF has also been found to decrease the activity of these transporter systems and reduce the removal of Aβ by glial cells.[64] A further mechanism of Aβ degradation is mediated by neuronal enzymes, such as neprilysin and insulin-degrading enzymes, both of which have altered production in AD patients.[5] Collectively, BBB dysfunction causes increased Aß accumulation in the brain parenchyma, primarily by impairing perivascular Aß clearance, but also by facilitating the re-entry of Aß from the blood.[7] In parallel, blood-borne proteins, such as thrombin and fibrinogen, can infiltrate the brain parenchyma, where they accumulate and trigger neuroinflammatory responses.[7,86]

5. Potential therapeutics with vascular targeting in AD: Thrombin-inhibiting anticoagulants

In AD, the interplay between a prothrombotic, proinflammatory blood milieu, and cerebrovascular damage forms a critical pathogenic unit.[6-10,15,16,25,27,76,105,111] This pathological condition is largely driven by Aβ-triggered thrombin formation through the coagulation cascade. Thrombin-mediated inflammation and the formation of degradation-resistant fibrin-Aβ clots contribute to vasculopathies, such as Aβ-CAA, within the brain (Figure 2). Similarly, elevated thrombin levels are believed to be implicated in the pathogenesis of conditions such as atherosclerosis, diabetes, and traumatic brain injury, all known risk factors for AD.[27] At the cellular level, excessive thrombin particularly damages vascular endothelial cells and disrupts BBB function. Once thrombin and fibrin(ogen) infiltrate the brain parenchyma, they also affect glial cells and neurons.[15,27] Therefore, inhibiting thrombin could represent a plausible idea to normalize the procoagulant and proinflammatory states in AD and prevent thrombin-mediated vasculopathies and neuronal damage (Figure 2).[15,16,22-24,26,27,76,105,111]

Thrombin-inhibiting anticoagulants, especially small-molecule DOACs, have been proposed as suitable therapeutics for AD (Figure 2).[15,16,22-24,26,27] By blocking excessive thrombin and its downstream effects, including inflammation, fibrin-Aβ clot formation, and vasculopathy development, these anticoagulants could help preserve vascular integrity and BBB function (Figure 2). Consequently, CBF, brain tissue perfusion, and the associated nutrient supply and metabolism could be maintained (Figure 2), potentially preventing chronic brain hypoperfusion, hypoxia, metabolic collapse, and the exacerbation of Aβ and tau pathology.

Furthermore, intact BBB, CBF, and brain perfusion promote perivascular clearance of Aβ and limit the accumulation of thrombin, fibrin(ogen), and fibrin-Aβ deposits within the brain parenchyma (Figure 2). This counteracts vascular-driven neuroinflammatory (e.g., through glial and oxidative responses) and neurodegenerative changes (e.g., synapse and neuron loss) (Figure 2). Collectively, inhibition of excessive pathological thrombin by DOACs could mitigate vascular-triggered neuropathogenesis and associated cognitive decline (Figure 2). Ultimately, this treatment could modify the progression of AD, potentially slowing cognitive and physical deterioration (Figure 2).[15,16,22-24,26,27,76,96,105,111] However, early intervention with anticoagulant is of primary importance to effectively address Aβ-induced procoagulant and proinflammatory states and prevent

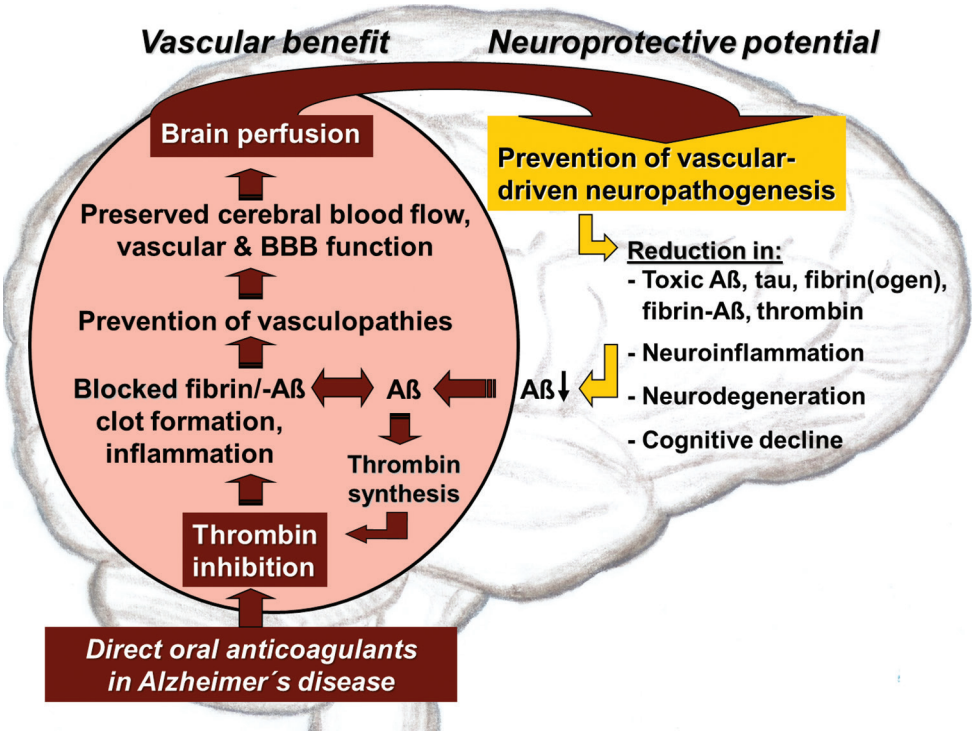

Figure 2. Therapeutic potential of direct oral anticoagulants (DOACs) in preventing vascular-driven neuropathogenesis and cognitive decline in Alzheimer's disease (AD). In hippocampal and neocortical brain regions, toxic amyloid-ß (Aß) proteins are produced by neurons and released into the bloodstream. In the blood, Aβ activates the contact system by activating blood clotting factor XII, which leads to the increased production of inflammatory bradykinin (not shown) and procoagulant and proinflammatory thrombin. Thrombin induces inflammation, platelet activation, and the conversion of fibrinogen to insoluble fibrin. Fibrin forms, together with activated platelets and Aβ, Aβ-containing fibrin (fibrin-Aβ) clots that are resistant to fibrinolytic degradation. Within cerebral vessels, these thrombotic clots, Aβ oligomers and filaments, platelet aggregates, and proinflammatory thrombin and fibrin(ogen) accumulate, contributing to vasoconstriction and vasculopathies, such as Aβ-type cerebral amyloid angiopathy. These conditions result in occlusive, cellular, and hemorrhagic lesions. Early inhibition of excessive thrombin by DOACs, through blocking its activity or production, normalizes thrombin-mediated proinflammatory and procoagulant states in AD, thus preventing vasculopathic sequelae. This preserves vascular and blood–brain-barrier (BBB) function, cerebral blood flow, and brain perfusion. An intact BBB ensures proper transport of substances between vascular and brain parenchymal compartments, promotes the perivascular clearance of Aβ, and prevents the infiltration of thrombin, fibrin(ogen), and Aβ from the blood into the parenchyma. Sufficient brain perfusion provides the tissue with oxygen and vital nutrients, preventing hypoxia/ischemia-triggered generation of Aβ and tau proteins. Collectively, the parenchymal accumulation of toxic Aβ, tau, thrombin, fibrin(ogen), and fibrin-Aβ deposits is reduced, and neuroinflammatory (e.g., glial activation and oxidative responses) and neurodegenerative (e.g., synapse and neuron loss) changes are minimized. As a result, neural network function and cognitive abilities are preserved for a longer period. Early inhibition of Aβ-induced thrombin activity by DOACs could prevent, delay, or at least mitigate vascular-triggered neuropathogenesis in AD, potentially slowing overall cognitive decline.

vasculopathic sequelae.[15,16,22-25,27] Early treatment helps preserve brain perfusion and optimize the anti-dementia effects while minimizing the bleeding risks associated with vulnerable vasculature in elderly patients.

5.1. Outcomes of preclinical studies in AD mouse models

Over the past two decades, a series of preclinical studies in AD mouse models has provided evidence that thrombin-inhibiting anticoagulants can prevent or slow the progression of vascular, neuronal, and cognitive disorders associated with AD.[90,101-103,105,111,122,123] Peripheral treatment of AD mice with the heparin-type anticoagulant enoxaparin demonstrated for the first time that inhibiting thrombin production can decrease Aβ deposition and glial activation in brain tissue while preserving cognitive abilities.[102,103] Likewise, enoxaparin has been shown to reduce Aβ-induced activation of the plasma contact system *in vitro*, attenuating both inflammatory and neurotoxic responses.[102]

In addition, treatment with the DOAC dabigatran has been found to reduce glial activation in the brains of AD mice.[122] Dabigatran also diminished the expression of inflammatory and AD-related proteins, as well as the production of ROS, as observed in vascular endothelial cells[105] and a tau-based mouse model.[123] Furthermore, in cultured human neuroblastoma cells, dabigatran reduced thrombin-induced expression of AD-related proteins,

such as Aβ, APP, BACE1, tau, the pro-apoptotic mediator caspase 3.[111]

Of particular importance, however, are recent proof-of-concept studies in AD mouse models that demonstrate the therapeutic benefits of DOAC treatments.[90,101] The data confirm the efficacy of DOACs in inhibiting thrombin-mediated vascular disorders and inflammation, and in reducing their neuronal and cognitive sequelae.[90,101] Long-term (1 year) anticoagulation with dabigatran preserved vascular and BBB function, CBF, brain perfusion, and memory abilities in AD mice.[90] Mechanistically, thrombin inhibition prevented the formation of vessel-occlusive fibrin clots. In addition, the extent of parenchymal Aβ oligomer and plaque deposition, as well as the neuroinflammatory milieu – characterized by phagocytic microglia and infiltrated T cells – was reduced.[90] Concomitantly, no intracerebral hemorrhages have been developed.[90]

Similarly, long-term studies using rivaroxaban in AD mice predisposed to severe Aβ-CAA confirmed that thrombin-inhibiting treatment can halt the progression of neurovascular disease.[101] Rivaroxaban reduced BBB dysfunction, parenchymal Aβ deposition, neuroinflammatory response, and memory decline compared to untreated or warfarin-treated AD mice.[101] Mechanistically, these effects have been linked to the inhibition of thrombin receptors PAR-1/PAR-2.[101]

Collectively, data from pre-clinical studies suggest that thrombin-inhibiting anticoagulants, such as enoxaparin, dabigatran, and rivaroxaban, offer disease-modifying benefits in AD. The focus of this therapeutic approach is to prevent prothrombotic and proinflammatory states, along with the associated neurovascular and cognitive impairments characteristics of AD.[15,16,22-24,26,27,80,90,96,101-103,111] Pathologically, Aβ-induced thrombin production triggers a cascade involving thrombin, fibrin(ogen), Aβ, and fibrin-Aβ clot formation, leading to vascular and BBB dysfunction and brain hypoperfusion. A thrombin-inhibiting therapy could counteract these pathophysiological changes in the AD brain vasculature (Figure 2), thereby preventing chronic CBF decline, cerebral hypoperfusion, hypoxia, hypometabolism, systemic inflammation, and the resulting neuronal and cognitive impairments.

5.2. Outcomes of clinical studies

Interestingly, the first clinical studies using antithrombotic therapy to treat dementia in elderly and presenile individuals date back to the 1960s.[29-31] In these small-scale, partially placebo-controlled trials, VKA-type OACs, such as dicumarol and warfarin, were shown to slow cognitive impairment and reduce morbidity and mortality in treated subjects.[29-31] However, these results did not receive significant attention at the time and did not prompt further clinical trials for several decades.

It was only in the 2010s that this approach began to regain focus. Advances in basic research and preclinical studies have provided new insights into the therapeutic potential of anticoagulants in AD, leading to recommendations for in-depth clinical studies.[15,16,22-24,26,27,90,101-103] This renewed interest stems from a deeper understanding of thrombin-triggered mechanisms that are causally related to prothrombotic, proinflammatory, and neurovascular disease progression. Furthermore, the development of novel small-molecule OACs, specifically DOACs, which target thrombin more precisely, has significantly reduced the risk of dangerous intracranial hemorrhage.[15,16,22-24,26,27,96,111]

One of the primary concerns among clinical neurologists regarding long-term thrombin-inhibiting therapy in AD, particularly in elderly patients with more vulnerable vasculature, has been the risk of bleeding.[15,16,22-27] However, it is noteworthy that the FDA has recently approved Aβ-targeting antibody therapies for AD despite concerns about the risk of cerebral bleeding and edema by ARIA.[11,17,69,70]

Over the past decade, a multitude of observational clinical studies has been carried out to assess the risk of hemorrhage associated with OAC use, especially in elderly populations. Interestingly, these studies have also revealed considerable antidementia benefits associated with OAC therapy. In the following sections, the benefit-risk profile of OAC use will be examined, comparing its anti-dementia effects against the incidence of bleeding. Collectively, these findings suggest that DOAC-type anticoagulants offer distinct advantages.

5.2.1. Anti-dementia effect of OACs in patients with AF

With the increasing use of OACs, especially DOACs, over the past two decades, a series of global observational clinical studies have focused on AF patients, examining stroke prevention and cognitive health. AF is a common cardiac arrhythmia in the elderly and is linked to multiple comorbidities, including an elevated risk of developing dementia.[124,125] The current observational clinical studies provide evidence that OACs, administered for AF, can protect individuals against cognitive impairment and dementia, including AD.[104,124-149] Below, we provide an overview of observational clinical studies reporting the effects of OACs on the incidence of dementia in AF patients, with reference to a recent review article discussing this topic.[16]

In detail, a clear benefit of OAC use (including both DOACs and VKA-type warfarin) in reducing the incidence of new dementia has been demonstrated in a large

retrospective observational study involving nearly 450,000 AF patients in Sweden (2006 – 2014).[104] In an on-treatment analysis, OAC users had a 48% lower risk of developing dementia compared to non-OAC users (hazard ratio [HR] = 0.52; 95% CI = 0.50 – 0.55).[104] Particularly noteworthy, even patients with a low risk of AF, as well as those aged over 65 years, benefited from OAC use, irrespective of their stroke risk score.[126] Likewise, retrospective cohort studies from the United Kingdom (UK) have concluded that OAC treatment (DOACs or warfarin) reduces the risk of cognitive impairment and dementia (including AD, vascular dementia, and unspecified dementia) compared to non-OAC users or patients on antiplatelet therapy.[127,128]

Comparable findings were reported in an Australia-wide retrospective study (2010 – 2018) that included nearly 19,000 patients with newly diagnosed AF and no prior history of dementia or stroke.[140] OAC users had a significantly lower incidence of dementia compared to non-users (HR = 0.59; 95% CI = 0.44 – 0.80; $P < 0.001$), with DOACs (apixaban, dabigatran, and rivaroxaban) offering greater protective effects compared to warfarin.[140] Likewise, a meta-analysis of nearly 480,000 AF patients has found that OACs significantly reduced the occurrence of cognitive impairment compared to non-users (HR = 0.71; 95% CI = 0.69 – 0.74; $P < 0.00001$).[130] Recent systematic reviews and meta-analyses of observational and controlled studies have consistently reiterated the anti-dementia benefits of OAC use.[131,132]

A number of observational studies have compared the efficacy of DOACs versus VKA in reducing dementia risk in AF patients. For instance, a retrospective US trial involving approximately 5000 elderly AF patients (2010 – 2014) has revealed that individuals on long-term anticoagulation with DOACs (apixaban, dabigatran, and rivaroxaban) had a 51% reduced risk of dementia (including AD, vascular, senile, and unspecified dementia) or subsequent stroke/transient ischemic attack, compared to warfarin users (HR = 0.49; 95% CI = 0.35 – 0.69; $P < 0.0001$).[129] No significant differences in dementia rates were found between the various DOACs.[129]

Similarly, analyses of both controlled and real-world studies in AF patients have demonstrated that treatment with DOACs was associated with a lower risk of cognitive impairment and all-cause dementia compared to VKAs or acetylsalicylic acid.[134,135] Furthermore, an analysis of US health-care databases has revealed lower rates of incident dementia in AF patients treated with DOACs (apixaban, dabigatran, and rivaroxaban) compared to warfarin,[136] with no apparent advantage for any specific DOAC.[136]

A recent historical cohort study involving nearly 40,000 AF patients aged 40 years and older from the UK (2012 – 2018) has also concluded that DOAC treatment reduced the risk of dementia.[144] In the study, first-time DOAC users (apixaban, dabigatran, and rivaroxaban) showed a 16% reduction in newly diagnosed all-cause dementia (HR = 0.84; 95% CI = 0.73–0.98; $P = 0.02$) and a 26% reduction in mild cognitive impairment (HR = 0.74; 95% CI = 0.65 – 0.84; $P = 0.009$) compared to VKA users (acenocoumarol, phenprocoumon, and warfarin).[144]

In addition, three retrospective studies conducted in elderly AF patients from Korea (2014 – 2017)[137,139] and Taiwan (2012 – 2016)[138] have demonstrated that the use of DOACs (apixaban, dabigatran, edoxaban, and rivaroxaban) was associated with a lower risk of incident dementia, including vascular dementia and AD,[137,139] compared to warfarin.

Four large systematic reviews and meta-analyses of comparative studies have also recently confirmed the superiority of DOACs over VKAs in lowering the risk of composite dementia.[141,142,145,146] Specific DOACs, such as apixaban and rivaroxaban,[141] dabigatran and rivaroxaban,[142,146] and edoxaban,[137] demonstrated particularly strong protective effects. For dabigatran users, beneficial effects on cognitive and psychological function, as well as a reduced incidence of dementia, were highlighted in a literature search in PubMed/Medline data updated through 2021.[133]

Most recently, a Belgian study of first-time OAC users, involving approximately 240,000 AF patients (2013 – 2019), assessed the risk of new-onset dementia, classified as AD, vascular dementia, or other/unspecified dementia.[143] The study found that DOAC use (apixaban, dabigatran, edoxaban, and rivaroxaban) was associated with a slightly lower risk of vascular dementia and other/unspecified dementia compared to VKAs (warfarin, acenocoumarol, and phenprocoumon). However, the risk of AD was similar between the DOAC and VKA groups.[143]

Worldwide clinical observational studies have demonstrated that the administration of OACs is beneficial for cognitive health in AF patients.[104,124-149] Individual studies have shown that the risk of dementia after OAC treatment could be reduced by up to 48% compared to non-OAC users.[104,140] When comparing DOACs to VKA-type anticoagulants, DOACs generally appeared to provide a more pronounced reduction in the risk of cognitive impairment and dementia.[128,129,134-146] However, some studies revealed similar efficiency between DOACs and VKAs in reducing dementia risk, suggesting that either treatment strategy may be acceptable.[104,124,127,147-149] These studies include recent long-term retrospective observations in incident AF cohorts of elderly patients[149] as well as prospective 24-month observational trials.[124,147]

The varying results in relation to the anti-dementia efficacy of VKAs versus DOACs may be partly attributed to methodological differences between studies, such as variations in the age range of participants, small sample sizes, short follow-up durations, and differing criteria for diagnosing dementia.[16,137,138,143]

At first glance, the beneficial effects of OACs on cognitive health in AF patients might be simplistically attributed to the reduction in stroke incidence. However, substantial meta-analyses of observational studies have revealed a link between AF and dementia that is independent of a history of clinical stroke.[150,151] Mechanisms such as brain hypoperfusion, recurrent silent ischemia, microhemorrhage, inflammation, and genetic factors have been proposed as underlying contributors.[151]

Moreover, as previously discussed, recent observational studies have evidenced that OAC treatment can significantly lower the risk of dementia, including AD.[104,124-149] This result is not surprising, as OACs directly or indirectly inhibit thrombin, thereby normalizing thrombin-mediated procoagulant and proinflammatory states in AD (Figure 2). Consequently, inflammatory and thrombotic events in the cerebral vasculature can be prevented. This preservation of vascular and BBB integrity, CBF, brain perfusion, nutrient supply, and neuronal and cognitive function is likely the primary reason for the anti-dementia effects of OACs (Figure 2). When evaluating the efficacy of different OAC types in reducing dementia incidence in AF patients, DOACs were typically associated with the greatest cognitive benefit, followed by VKAs, and then antiplatelet therapy.[15,16,127,129,134-146] A recent comprehensive literature review, which included experimental studies and meta-analyses, confirmed these findings, although antiplatelet therapy offered less benefit.[152] Few studies have made precise distinctions between different dementia subtypes, such as AD, vascular dementia, and other/unspecified dementia.[127,128,129,137,139,143,148] Most studies reported OAC effects on composite dementia.[104,124,126,131-136,138,140-142,144-147,149]

In summary, small-molecule OACs, particularly DOACs, are effective drugs against dementia, including AD, in AF patients.[104,124,125-149,152] Cognitive benefits have been particularly observed in elderly patients over 65 years of age, irrespective of their dementia or stroke history.[104,126,128,137] This applies to both low-risk AF and newly diagnosed AF individuals.[126,143] As a result, it has been hypothesized that anti-dementia benefits could be achieved if OACs are administered not only for AF or other cardiovascular diseases but also as disease-modifying therapy in AD.[15,16,22-24,26,27] Despite positive data from recent global clinical observations, the approval of OACs for use in AD remains pending. Therefore, detailed clinical investigation in the form of randomized, placebo-controlled, double-blind trials has been recommended to evaluate the potential of OACs as a novel disease-modifying therapy in AD.[15,16,22-24,26,27]

5.2.2. Reduced risk of intracranial bleeding in DOAC use

In patients with AF, the use of DOACs has been shown to significantly reduce the risk of severe intracranial hemorrhage compared to VKAs. For instance, a meta-analysis of phase III clinical studies (2009 – 2013) revealed that DOACs (apixaban, dabigatran, edoxaban, and rivaroxaban) were associated with a 52% lower risk of intracranial hemorrhage compared to warfarin (HR = 0.48; 95% CI = 0.39 – 0.59; $P < 0.0001$).[153] In addition, a significant decrease in thromboembolic events and all-cause mortality was observed with DOAC use. However, the risk of gastrointestinal bleeding increased by 25% (HR = 1.25; 95% CI = 1.01 – 1.55; $P = 0.04$), depending on the dose of DOACs used.[153] These results are consistent with those of a large US retrospective observational study (2013 – 2015),[154] as well as a cohort study of new AF patients with dementia (2011 – 2017).[155] In addition, a recent systematic review and meta-analysis assessed the overall impact of DOACs versus VKAs in AF patients over the age of 80.[156] In the DOAC group, lower all-cause mortality and a 43% reduction in intracranial bleeding were reported compared to warfarin (relative risk = 0.47; 95% CI = 0.36 – 0.60; $P < 0.001$), suggesting that DOACs represent a safe and effective therapy for elderly patients.[156]

When examining the effect of individual DOACs, dabigatran appears to lower the risk of intracranial bleeding the most compared to warfarin, followed by the FXa inhibitors apixaban, edoxaban, and rivaroxaban.[24] In a retrospective FDA study involving approximately 134,000 AF patients over 65 years old (2010 – 2012), dabigatran reduced the incidence of severe intracranial hemorrhage by 66%, from 9.6 cases per 1000 person-year with warfarin to 3.3 cases with dabigatran (HR = 0.34; 95% CI = 0.26 – 0.46; 186 vs. 60 events).[157] Furthermore, dabigatran was associated with a 14% reduction in mortality risk (HR = 0.86; 95% CI = 0.77 – 0.96) and a 20% reduction in ischemic stroke risk (HR = 0.80; 95% CI = 0.67 – 0.96). However, the risk of major gastrointestinal bleeding increased by 28% (HR = 1.28; 95% CI = 1.14 – 1.44).[157]

In addition, dabigatran demonstrated long-term safety and efficacy in elderly AF patients over a 30-month study.[129] Compared to rivaroxaban, dabigatran appeared to have a more favorable safety profile regarding both intracranial and extracranial hemorrhage, including gastrointestinal bleeding. This conclusion was drawn from a retrospective

study of elderly, new-user AF patients (2011 – 2014).[158] The higher risk of bleeding associated with rivaroxaban compared to dabigatran may be explained by rivaroxaban's greater ability to cross the BBB.[159] Physiochemical properties and pharmacological data suggest that rivaroxaban has the highest risk of BBB penetration, followed by apixaban, edoxaban, and dabigatran, which has the lowest risk.[159]

The beneficial safety properties of dabigatran were further confirmed in studies using mouse models of AD and CAA, where no increase in intracerebral bleeding or microbleeds was observed even after long-term use.[15,90] When evaluating stroke prevention, bleeding risk, and cost-effectiveness of OACs in AF patients, a systematic review and meta-analysis concluded that apixaban ranked best for most outcomes.[160] However, this ranking was dose-dependent, especially for dabigatran.[16,160] In a 2-year study involving approximately 18,000 AF patients, the lowest rates of life-threatening bleeding, intracranial bleeding, and major or minor bleeding were observed with a 110 mg twice-daily dose of dabigatran. These rates increased with the 150 mg twice-daily dose of dabigatran and were highest with warfarin use.[161] As a result, the administration of lower doses of dabigatran has been proposed to further improve its safety behavior.[153]

In addition to dabigatran, apixaban is a preferred DOAC, particularly with respect to safety profile.[16,26,160] In a double-blind study of AF patients, apixaban was superior to warfarin in reducing the risk of stroke, systemic embolism, major bleeding, and mortality.[162] Furthermore, apixaban was associated with lower rates of major bleeding and ischemic stroke in AF patients with dementia compared to other OACs (dabigatran, rivaroxaban, and warfarin).[163]

Collectively, to minimize safety concerns, particularly the risk of intracranial bleeding, dabigatran and apixaban – followed by edoxaban and rivaroxaban – are promising DOAC candidates for studying their suitability as disease-modifying therapies in AD.[15,16,22-24,26,27] For betrixaban, which was introduced only a few years ago, clinical data remain limited.

5.3. Portfolio of anticoagulants for potential use

Anticoagulants have been used for decades in clinical practice as life-sustaining, antithrombotic therapies for millions of elderly individuals, including those with AD.[15,16,22,28,32-34] Their production, application, pharmacokinetics, efficacy, and safety profiles in daily use are well established. Therefore, repurposing current anticoagulants as disease-modifying therapies in AD offers the potential for a time- and cost-efficient development and approval process. However, clinical neurologists often approach the use of anticoagulants in treating vascular-driven neuropathogenesis in AD with caution despite decades of cardiology experience showing the benefits of anticoagulants and evidence of their anti-dementia effects in elderly patients (Section 5.2). The primary concern remains the risk of bleeding, especially the risk of severe intracranial hemorrhage during long-term treatment in the elderly.[15,16,22-28]

Therefore, a detailed consideration of the pros and cons – concerning dosage form, anti-dementia efficacy, bleeding risk, and other potential side effects – is essential for determining the suitability of such therapies and identifying the best drug candidates. Recent review articles have also discussed these benefit-risk considerations.[15,16,24]

Parenteral anticoagulants, administered intravenously or subcutaneously, are typically used for short-term antithrombotic prophylaxis and acute therapy.[15,28] This class includes indirect thrombin inhibitors (e.g., heparin-type enoxaparin and fondaparinux) and direct thrombin inhibitors (e.g., hirudin, bivalirudin, and argatroban).[15,28] Although enoxaparin has shown beneficial effects on Aβ pathology, inflammation, and cognitive function in AD mice,[102,103] the long-term use of heparins is limited by several side effects.[15,28] The limitations of long-term heparin use include an increased risk of bleeding, thrombocytopenia, unpredictable anticoagulation due to non-specific plasma protein binding, and inadequate inhibition of fibrin-bound thrombin, which can lead to thrombus formation.[15,28]

Natural hirudin, while effective in inhibiting both thrombin and fibrin-bound thrombin without directly affecting platelets,[15,109] is often associated with severe bleeding complications and the development of anti-hirudin antibodies, which reduce its efficacy and may cause side effects.[15,109] Collectively, for potential long-term therapy in AD, heparins and hirudin are less suitable due to their bleeding risk, difficulties in controlling these risks, and the invasive nature of daily injections.

Small-molecule OACs are the preferred alternative to parenteral anticoagulants. This preference is based on the extensive evidence of OAC efficacy and safety in long-term use, as well as the convenience of oral administration, provided that adherence to the prescribed regimen is ensured.[15,28,32,34] OACs include VKAs, such as warfarin and phenprocoumon, which have been in use for decades, and DOACs, which have been introduced in the past 15 years.[15,28,32,164] DOACs were specifically developed to address the significant challenges associated with the non-specific VKAs.

The limitations of VKAs in stroke prevention are largely due to their pharmacological properties and variability in antithrombotic action. The main disadvantages of VKAs

include delayed onset of anticoagulation, a slow offset of effect in bleeding situations despite the use of vitamin K antidote, and interaction with other drugs and vitamin K-containing diet.[15,28,32,164] These factors necessitate close medical supervision to ensure effective antithrombotic therapy while minimizing the risk of bleeding from overdose – a particularly concerning side effect in elderly patients. Other potential side effects of VKAs include VKA-induced skin necrosis and disruption of vitamin K-dependent proteins involved in vascular and neuronal functions.[15,28,32]

However, VKAs have certain advantages. They can be used in patients with severe renal dysfunction, as they are not eliminated through the kidneys, and in individuals with mechanical heart valve implants.[28,164] In addition, the cost of VKA treatment is considerably lower than that of DOACs.[15,28,164]

5.3.1. Superior suitability of DOACs

At present, available DOACs include the direct thrombin inhibitor dabigatran and the direct FXa inhibitors rivaroxaban, apixaban, edoxaban, and betrixaban.[15,28] Unlike VKAs, these DOACs offer predictable pharmacological efficacy, with a rapid onset of action and a short half-life at fixed dosing.[15,22,24,28,32,151] DOACs are favored due to their ease of administration, improved treatment adherence, minimal variations in antithrombotic effect, and enhanced safety (with a lower incidence of stroke and intracranial bleeding). Additional benefits include an efficient antidote strategy, lack of interference with vitamin K-dependent metabolism, fewer drug–drug interactions, no dietary restrictions, and reduced need for medical supervision. These advantages make DOACs especially suitable for elderly and often vulnerable patients. However, in patients with severe renal dysfunction, DOACs require dose adjustment or may be contraindicated due to renal elimination.[15,28,164]

The bleeding risk associated with DOAC use, as reviewed in Section 5.2.2, has been assessed in a multitude of global observational studies, especially in elderly patients with AF. Collectively, these studies suggest that DOACs reliably prevent stroke and systemic thromboembolic events while reducing the risk of severe intracranial hemorrhage by approximately 50% compared to VKAs.[15,153,156,157,164] Among DOACs, dabigatran and apixaban appear to present the lowest risk of intracranial hemorrhage, followed by edoxaban and rivaroxaban. Apixaban has shown particular benefit in AF patients with dementia.[163]

On the other hand, DOACs increase the risk of gastrointestinal bleeding in a dose-dependent manner compared to VKAs.[15,16,24,153,155,157,164] Fortunately, effective antidotes are now available for most DOACs, allowing for rapid reversal of anticoagulation in bleeding emergencies.[15,28] For example, idarucizumab (Praxbind®) specifically binds dabigatran, reversing its effect.[15,28] In addition, andexanet alfa (Ondexxya®), a human FXa variant, acts as a decoy protein to intercept FXa inhibitors, such as apixaban and rivaroxaban, thereby restoring coagulation.[15,28] However, andexanet alfa is not yet approved for use as an antidote for betrixaban and edoxaban. For long-term therapy AD patients, having access to a fast-acting and efficient antidote is particularly important, given the increased bleeding risk in elderly patients due to more vulnerable vasculature.[91]

Mechanistically, dabigatran, released from its pro-drug form, directly binds to both soluble and fibrin-bound thrombin, inhibiting their activity in the blood.[15,16,24,27,28,32] On the other hand, FXa-inhibitors, such as apixaban and rivaroxaban, prevent thrombin synthesis by directly inhibiting free and prothrombinase-bound FXa in the coagulation cascade.[15],[16,24,27,28,32] Consequently, thrombin levels in the blood decrease, preventing excessive thrombin accumulation, while the activity of existing thrombin remains unaffected. Therapeutically, both types of DOACs may reduce thrombin-mediated fibrin formation, prevent degradation-resistant fibrin-Aβ clot formation, and inhibit inflammation and vasculopathic sequelae in the AD brain, as demonstrated in proof-of-concept studies in AD mouse models (Section 5.1 and Figure 2).[15,16,27,90,101,111,122,123]

With regard to preventing thromboembolism, minimizing intracranial hemorrhage, and enabling rapid reversal of bleeding through antidotes, dabigatran, apixaban, and rivaroxaban currently meet the criteria for potential use as anti-dementia agents. As reviewed in Section 5.2.1., protective effects against dementia (including AD, vascular dementia, and other/unspecific dementia) have been demonstrated for dabigatran, apixaban, rivaroxaban, and edoxaban, though no single DOAC has shown a distinct advantage over the others.

6. DOACs as potential neuroprotective therapeutics in AD

Thrombin-inhibiting OACs of the DOAC type, introduced over the past 15 years, have become the preferred anticoagulants in millions of patients for preventing thromboembolic events.[15,34] The main reason for this preference is their significantly lower risk of severe intracranial bleeding in long-term treatments compared to traditional VKAs or heparins.[15,16,129,153-160,163,164]

Beyond their antithrombotic use, clinical observational studies in AF patients have revealed that OACs can reduce the risk of dementia[15,16,104,124-149] by up to 48% in some studies.[104,140] Most study participants were elderly, over the

age of 65, but some studies also included individuals with low AF risk or newly diagnosed AF, with no prior history of dementia or stroke. This observed anti-dementia potential of thrombin-inhibiting OACs has raised the question of whether these anticoagulants, particularly DOACs, could also use to treat vascular-driven neuropathogenesis and cognitive decline in AD.[15,16,22-24,26,27,90, 96,101,111]

At present, the most comprehensive clinical data on antithrombotic efficacy, bleeding risk, and anti-dementia potential are available for the DOACs dabigatran, apixaban, and rivaroxaban.[16,104,124-149,153-164] Collectively, studies suggest a slight advantage for dabigatran and apixaban in reducing the risk of severe intracranial hemorrhage. However, when it comes to anti-dementia potential, no particular DOAC has demonstrated a clear advantage. Importantly, fast-acting antidotes are available for managing bleeding incidents with all three of these DOACs.[15,24,28]

7. Future perspectives

At present, a 7-year observational study in AF patients (BRAIN-AF) compares the effects of rivaroxaban on ischemic stroke and neurodegenerative events with those of acetylsalicylic acid in participants with vascular disease or placebo in patients without vascular disease.[165] In addition, a randomized, double-blind, placebo-controlled clinical study (BEACON) was announced in 2018 to investigate the effects of dabigatran in patients with mild cognitive impairment or AD.[166] However, this study has not yet been initiated.

A clinical intervention study investigating the benefits and risks of thrombin-inhibiting DOACs in AD patients would be a plausible step, ideally under the guidance of vascular neurologists. The aim of such treatment would be to normalize the Aβ-induced procoagulant state in AD, characterized by excessive thrombin formation, thereby counteracting the associated inflammatory and vasculopathic sequelae that impair neuronal and cognitive function (Figure 2). For such a study, a detailed analysis of each participant's procoagulant state, bleeding risk, personalized drug dosing, and close medical monitoring would be essential to prevent severe complications, particularly intracranial hemorrhage.

A postmortem study found that not all AD patients exhibit a procoagulant state.[97] As a result, DOAC treatment should not be prescribed in individuals without this procoagulant state to minimize the risk of bleeding.[16] However, it remains unclear whether the AD patients without a procoagulant state in the study[97] had been receiving anticoagulant treatment before the analysis. Special caution is also required in AD patients undergoing Aβ-targeting antibody therapy due to the associated risk of ARIA.[11,17,69,70]

For details on the prerequisites, framework conditions, and diagnostic methods necessary for a clinical trial on DOAC use in AD, reference can be made to recent review articles.[15,167] Ideally, *in vivo* staging of disease, selection of participants, and monitoring of treatment could be conducted using a range of diagnostic techniques, including imaging and biomarker tests from blood and CSF to assess amyloid, tau, and neurodegenerative changes. These assessments could be supplemented by tests evaluating procoagulant, proinflammatory, and vasculopathic states.[15,167] In addition, these tools could help track therapeutic outcomes and the emergence of side effects.[15,167]

The greatest cognitive benefit and safety would likely be achieved if DOAC treatment was initiated in individuals without cognitive symptoms, with a low bleeding risk (HAS-BLED score), and with AD and CAA biomarkers indicating an early disease stage.[10,11,14-16] In therapies targeting brain Aβ deposition, the maximum benefit has been suggested when treatment is administered during pre-amyloid stages or before half-maximal amyloid deposition is reached, which is when neurodegeneration typically begins.[42] This time window may also be appropriate for initiating DOAC treatment in AD patients to timely counteract Aβ-triggered procoagulant and proinflammatory thrombin (Figure 2). However, detailed investigations on the temporal dynamics of changes in the procoagulant state, vascular and parenchymal amyloid load, and the progression of neurovascular and cognitive sequelae in AD are still needed. Studies in AD mouse models or patients could benefit from recent advances in *in vivo* biomarker-based AD staging methods, which use computational imaging and fluid (blood and CSF)-based diagnostics.[15,42,167]

8. Conclusion

Neurodegenerative diseases, including AD, are on the rise globally, yet the availability of efficient drugs remains limited.[12,14,19,70] The newly approved Aβ-targeting antibody therapies for AD offer hope by slowing disease progression, but they cannot halt or cure the disease.[11,17,69,70] These therapies are also expensive, require elaborate screening for ARIA side effects, and present challenges in identifying patients who are most likely to benefit.[11,17,19,70] Given these limitations, other plausible approaches, particularly cost-effective drug repurposing, remain valuable therapeutic options for AD.[5,13,15,16,19,21-24,26,27] OACs, particularly DOACs, have shown disease-modifying potential for treating neurovascular dysfunction in AD, as supported by both preclinical and clinical evidence. By targeting excessive thrombin production caused by Aβ pathology, DOACs could prevent, delay, or reduce vascular-triggered

neuropathogenesis in AD (Figure 2). This therapeutic approach could slow the overall progression of cognitive and physical decline in AD. Among the currently available DOACs, dabigatran, apixaban, and rivaroxaban are well positioned for clinical investigation as potential neuroprotective drugs in AD. In addition, the FXa inhibitors edoxaban and betrixaban might also be suitable candidates, pending the approval of efficient antidote strategies and the availability of more data from observational studies. Whether used alone or in combination with anti-inflammatory agents, cardiovascular treatments, standard AD therapies, or other disease-modifying agents, DOACs could become part of a novel treatment strategy aimed at addressing neurovascular pathology in AD and its associated cognitive decline.[5,15,16,19-27,96]

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ACCSCIENCE PUBLISHING

Advanced Neurology

REVIEW ARTICLE

Long-term neurocognitive follow-up in children with traumatic brain injury: A literature review and monocentric cohort study

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Abstract

Children with mild traumatic brain injury (mTBI) may experience long-term cognitive sequelae. However, previous study results have been controversial. It remains unclear whether clinical follow-up is useful, how long patients should be followed-up, and which psychological dimensions should be investigated. Herein, we described neurocognitive evolution in a small sample of Italian children who were hospitalized for mTBI and systematically reviewed the existing evidence in this setting. In total, 15 children aged 4 – 16 (median, 9) years who were evaluated for mTBI at our institution between March 2017 and September 2018 were retrospectively enrolled. All patients underwent computed tomography or magnetic resonance imaging for clinical reasons; moreover, they underwent neurocognitive evaluation within few days from the event (T0), after 3 – 6 months (T1), and after 18 – 24 months (T2). Neuropsychological assessment included the Child Behavior Checklist, Developmental Neuropsychology Assessment II Edition, and Wechsler Intelligence Scale for Children. An electronic search was conducted to identify studies published in the past 12 years. Neurocognitive assessments revealed low scores in memory, sensorimotor, and social perception tasks at T1 and T2. Univariate analysis of neuroradiological and clinical findings revealed no risk factors for cognitive deficits. Overall, 17 studies involving 1336 children were reviewed and analyzed. Following mTBI, psychiatric disorders were frequently newly diagnosed and were associated with significant deficits in adaptive functioning and other pre-injury psychosocial risk factors. Our study findings demonstrate that children with mTBI exhibit subtle persistent cognitive difficulties that may affect academic and social functioning. Thus, follow-up using extensive neuropsychological evaluation is essential.

Keywords: Traumatic brain injury; Pediatrics; Cognition; Magnetic resonance imaging; Head computed tomography

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1. Introduction

Traumatic brain injury (TBI) annually affects >3,000,000 children worldwide[1] and is the leading cause of death and disability in the pediatric age group.[2-4] Although most children with TBI achieve full recovery, some of them with mild TBI (mTBI) or severe TBI may experience a combination of cognitive, behavioral, and emotional sequelae.[5,6] Consistently, a small number of mildly injured patients experience chronic physical, cognitive, and emotional impairments.[7] This condition, known as persistent postconcussive symptoms (PCS), is related to reduced quality of life,[8] challenges at school,[9] and poorer long-term psychological and behavioral outcomes.[10]

TBI management in acute settings has been extensively reported in several national and international guidelines.[11-14] However, there is no consensus regarding the recommendations for neuroradiological and cognitive follow-up of these patients. Furthermore, all published studies have demonstrated a high variation in neuropsychological domains and TBI severity.[15-19] The current study findings also suggested that these patients experienced deficits mainly in linguistic abilities and communication,[20,21] psychomotor skills,[15] and attentional capacity and executive functions.[22]

This study aimed to review the current evidence on the follow-up of children with mTBI to provide a systematic and comprehensive overview of integrating extensive neuropsychological and neuroradiological evaluations as part of the long-term management of these patients. Furthermore, we described the clinical and neurocognitive aspects of a group of Italian children with a history of mTBI by studying their development over time.

2. Materials and methods

2.1. Literature review

We conducted a systematic review in compliance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines.[23,24] An electronic search was conducted using PubMed and Google Scholar databases to identify articles published in English between January 1, 2012, and January 1, 2024. The following search terms were used: TBI AND/OR head trauma AND/OR brain injury AND/OR brain trauma AND/OR post-concussive syndrome AND children AND/OR pediatric AND/OR youth AND/OR adolescent AND psychosocial AND/OR cognitive AND/OR psychological AND/OR psychiatric. The reference list of each article was also reviewed to identify possible missing studies, and those eligible for the screening process were evaluated according to inclusion criteria.

2.1.1. Inclusion criteria

All articles published in English that focused on neurocognitive and psychological assessments of children aged 0 – 18 years with a history of mTBI (defined as a Glasgow coma scale [GCS] score of >8)[11] were included in the analysis. Narrative reviews, opinion and clinical commentary articles, and single case reports were excluded. First, the titles and abstracts of the articles were screened. Duplicates and articles with no available English summary were excluded. Articles reporting information on both children and adults were included only if pediatric data could be retrieved and extracted. Similarly, studies on different types of TBI were included only if the data of patients with mTBI could be separated. Two researchers (I.L. and T.Z.) independently performed the literature search and screening of eligible studies.

2.1.2. Data extraction

We developed a standardized grid to collect the extracted data. The following data were collected: first author name, date and journal of publication, country of study, study design (prospective, retrospective, or case–control), sample size, age (mean or median and range), outcome measures, main results, and conclusions. The data were organized into tables to easily compare the characteristics of the included studies.

2.2. Monocentric cohort study

2.2.1. Participants

Children aged 4 – 16 (median, 9) years who were evaluated for TBI at our institution between March 2017 and September 2018 were retrospectively enrolled in this study. We intentionally analyzed the data of children who had been followed-up before the COVID-19 pandemic to avoid potentially confounding factors. Data regarding anthropometric and demographic characteristics, comorbidities, mechanism of injury, GCS, and clinical symptoms at baseline evaluation (T0), clinical data related to admission (*e.g.* labs report, complications report, diagnostic test), and length of stay were collected.

The inclusion criteria were as follows: (a) availability of head computed tomography (CT) at baseline, (b) complete neuropsychological evaluation, and (c) good knowledge of Italian language. The exclusion criteria were as follows: (a) pre-existing neurological comorbidity or a former diagnosis of neuropsychiatric disorder and (b) parent's refusal to provide informed consent. The follow-up, mainly focused on neurocognitive assessments, was performed according to our clinical practice. Neurocognitive assessments were performed at the following time points: baseline (T0), *i.e.*, within 7 days after the event; T1, *i.e.*, 6 months after the event; and T2, *i.e.*, 18 – 24 months after the event.

All participants were individually evaluated over 2 – 3 sessions, each lasting approximately 30 – 45 min, on different days at intervals of <1 week. The timing of neuroradiological examinations (magnetic resonance imaging [MRI]) was not standardized. However, they were usually performed on the basis of the clinical status and close to the T0 or T1 neuropsychological evaluations.

The study was approved by the local Institutional Review Board (approval no.: CEUR-2020-OS-265), and written informed consent was obtained from the parents of all enrolled children.

2.3. Assessment

2.3.1. Neuropsychological assessment

Patients underwent neuropsychological evaluation using the Italian version of the Developmental Neuropsychology Assessment II Edition (NEPSY-II).[25,26] NEPSY-II is an age-based flexible instrument; some items can be administered to all age groups, whereas others are age specific. We chose to include only tasks administered to all children in the final analysis. As the NEPSY item "language function" was assessed via other tests according to the age of patients, it was excluded from the analysis.

The following domains were explored:

- Attention and executive functioning: visual attention, graphic fluency, auditory attention and response set, inhibition, clocks, and animal sorting
- Memory and learning: memory for faces, word list interference, memory for designs, list memory, memory for names, narrative memory, and sentence repetition
- Sensorimotor functions: fingertip tapping, imitating hand positions, and manual motor sequence
- Social perception: theory of mind and affect recognition
- Visuospatial processing: design copying, block construction, picture and geometric puzzles, route finding, and arrows.

The results of the NEPSY-II evaluation were expressed as age-adjusted scaled scores and compared with the normative reference values (mean = 10; standard deviation [SD] = 3).[25] NEPSY-II was administered during T1 (attention and executive functioning as well as memory and learning domains) and T2 (complete assessment) evaluations.

2.3.2. Cognitive assessment

The full-scale intelligence quotient (FSIQ) was assessed in patients aged 6 – 16 years using the Wechsler Intelligence Scale for Children IV Edition (WISC-IV)[27] and in patients aged <6 years using the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III).[28] The WISC-IV (or WPPSI-III) was administered during T1 and T2 evaluations. The FSIQ scores from WISC-IV and WPPSI-III were combined for the analyses.

2.3.3. Emotional and behavioral assessment

The emotional and behavioral aspects of the patients were assessed using the Child Behavior Checklist (CBCL).[29] The CBCL is a standardized tool that provides a parental report of emotional, social, and behavioral problems in children aged 6 – 18 years. The questionnaire comprises 113 items. Each item is scored using a three-point Likert scale (0 = "not true," 1 = "somewhat or sometimes true," and 2 = "very true or often true"). The total score is derived from several subscales, including internalizing aspects (*e.g.*, anxiety/depression, withdrawal, and somatic complaints) and externalizing aspects (*e.g.*, attention problems, rule-breaking, and aggressive behavior) together with social problems and thoughts problems scales. For the subscales of "internalizing," "externalizing," and "total" issues, a t-score of ≤59 indicates nonclinical symptoms, a t-score of 60 – 64 indicates a risk of behavioral issues, and a t-score of ≥65 indicates clinical symptoms.

The CBCL was administered during T0 (related to pre-morbid functioning) and T1 evaluations. Analysis of CBCL data was limited to patients aged 6 – 16 years.

2.3.4. MRI and CT data acquisition

Standard head CT was obtained for all children. MRI was performed for specific clinical reasons using a 1.5 T scanner (Magnetom Area, Siemens, Erlangen, Germany). The MRI protocol included conventional MRI and 3D reconstruction of T1-weighted images. The following parameters were selected for each sequence: T1-weighted (repetition time/time to echo [TR/TE] = 500/17 ms, 19 slices, thickness = 4 mm, field of view [FOV] = 180 mm, matrix size = 256), T2-weighted (TR/TE = 4000/86 ms, 20 slices, thickness = 4 mm, FOV = 200 mm, matrix size = 320), fluid-attenuated inversion recovery (TR/TE = 8500/81 ms, 20 slices, thickness = 4 mm, FOV = 200 mm, matrix size = 320), and volumetric T1-weighted (TR/TE = 2200/3.02 ms, 240 slices, thickness = 1 mm, FOV = 250, matrix size = 256).

2.4. Statistical analysis

Descriptive analysis was performed to characterize the study population. Continuous variables were expressed as mean and SD or median with interquartile range (IQR), as appropriate. Categorical variables were expressed as percentages or frequencies. All variables were analyzed for normality distribution (D'Agostino–Pearson omnibus normality test). Differences between evaluations

conducted at different time points were analyzed using paired *t*-test or Wilcoxon matched-pairs signed rank test for nonparametric variables, as appropriate. The Fisher's and Chi-square tests were used to compare frequencies and percentages. The differences were presented as odds ratio (OR) with 95% confidence interval.

A *post hoc* power calculation demonstrated that the sample size was sufficient to reach 80% power for neuropsychological evaluations (100% for CBCL scores, 78% for NEPSY, and 81% for WISC-IV/WPPSI-III). GraphPad Prism (version 8.4.2; Boston, Massachusetts USA was used to perform all analyses. $P < 0.05$ is considered significant.

3. Results

3.1. Literature review

A total of 195 articles on pediatric mTBI published in the past 12 years were initially identified. After careful screening of titles and abstracts, 92 articles were evaluated for eligibility. Of the 29 articles that met the inclusion criteria, 17 were finally included (14 original studies[30-42] and 3 reviews;[43-45] Figure 1).

The 17 examined studies encompassed a comprehensive sample of 1336 children (790 males, 62%) who suffered from mTBI, with a weighted mean age of 12.2 (range, 2 – 17) years. Most of the studies included only school-aged children.[31-34,36-43] The timeframe for psychosocial and cognitive assessments after injury ranged from 1 week[35] to 23 months,[38] with most of them being conducted between 3 and 12 months.[31,33,34,36,37,40,43] A summary of the reviewed articles is presented in Table 1.

The cognitive and psychological outcomes were extensively assessed as primary objectives through several different assessment tools in almost all studies. Potential risk factors and effects of psychological intervention were also explored in some studies.

3.1.1. Neuropsychological morbidity

Previously published systematic reviews demonstrated that PCS was relatively frequent in children with mTBI.[44,46] However, they were mostly investigated acutely. Furthermore, higher rates of inattentive/hyperactive symptoms and mood disorder diagnoses as well as an increased risk of anxiety have been described immediately after mTBI.[45] In addition, if hospitalized, the likelihood of a substance abuse diagnosis increases by three fold. Adolescents with mTBI are more likely to have disruptive behaviors compared to younger children.[45] Moreover, mTBI may be associated with schizophrenia diagnosis in patients with a familial predisposition for the disorder (the risk would be higher if mTBI occurs before the age of 11 years).[47]

Recent findings have confirmed that psychiatric disorders frequently newly develop 6 – 12 months after mTBI. This is associated with significant deficits in adaptive functioning and other pre-injury psychosocial risk factors, such as lower socioeconomic status (SES), greater psychosocial adversity, and decline in school performance.[30] However, objective testing should be adopted to diagnose psychosocial problems because the incidence of anxiety and depression according to the caregiver's perspective may not be reliable.[38]

3.1.2. Patient-related issues

Pre-existing learning difficulties[35] and adverse behavioral functioning of the child[39] are significant predictors of PCS as well as decreased activity and social participation in the post-injury phase. Hospitalization, motor vehicle accidents, loss of consciousness, and MRI abnormality are also associated with a higher risk of poor school performance in children with mTBI.[34]

Segev *et al.* analyzed the interaction between sex and TBI injury. They found that among children with posttraumatic stress disorder, TBI significantly affected the neurocognitive performance of girls, whereas it had a reduced effect on boys.[37] On the other hand, the role of age as risk factor for negative neurocognitive outcome in children with TBI remains unclear. Taylor *et al.* reported that younger children had higher post-injury ratings on the CBCL total scale than controls.[34] However, Bernard *et al.* reported that an older age at the time of injury is a significant predictor of PCS at 1 month after injury.[35] A recent review focusing on preschool children[46] reported that there is a lack of empirical data regarding the presentation and progression of PCS in this group of patients. Therefore, evidence regarding how to optimally manage these patients during recovery is lacking.

3.1.3. Family-related issues

Most studies exploring the role of family factors demonstrated that there is a significant discrepancy between a parent's and a child's perception of PCS.[31,42] Persistent PCS can be predicted by higher levels of pre-injury parent distress.[32,35] Similarly, decreased activity and social participation among children with mTBI may be associated with adverse pre-injury family functioning.[39] However, evidence regarding the impact of familial SES on neurocognitive outcome in children with TBI is contradictory. Some authors reported an association between persistent PCS and lower parental SES,[39] whereas others reported an association between persistent PCS

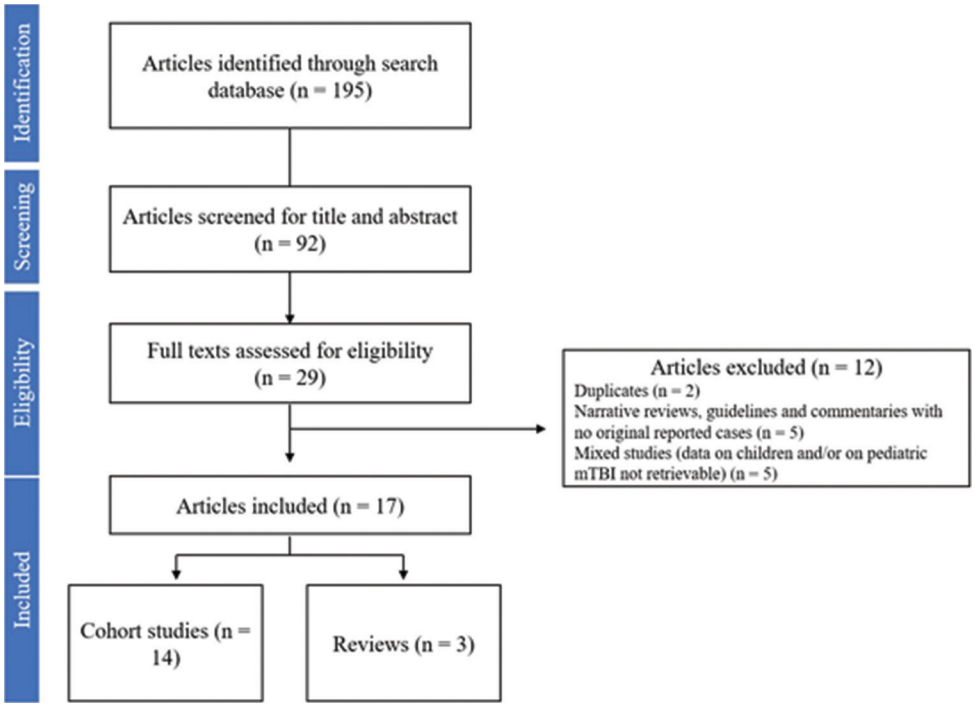

Figure 1. Flowchart of the systematic review of literature
Abbreviation: mTBI: Mild traumatic brain injury

and higher SES.[35] According to Murphy and Dodd, the association is not significant.[42]

3.1.4. Psychological intervention

Some studies have evaluated the impact of psychological intervention in children with mTBI.[36,41] Shorer *et al.* demonstrated that the patients' emotional status and their cognitive function improved after prolonged exposure to psychological support and that parental post-traumatic stress disorder was the strongest predictor of improvement.[41] Similarly, Connery *et al.* demonstrated that the identification and communication of invalid performance can promote high levels of caregiver satisfaction and reduce self- and caregiver-reported symptoms.[36]

3.2. Monocentric cohort study

3.2.1. Patient characteristics

During the study period, 892 children were evaluated at our hospital for TBI. Of these children, 36 (4%) underwent a brain CT according to the PECARN criteria.[11] Among these patients, 11 were excluded because they did not meet the inclusion criteria, and 10 were lost to follow-up (Figure 2). Thus, 15 patients (11 boys, 73%) with a median age of 9 (IQR, 5.5 – 14.0) years at the time of TBI were included in the final study population.

Most of the patients (n = 10) were involved in a motor vehicle accident. Four children reportedly fell from a height, and one child was struck on her head by a heavy object. The median GCS at the time of the first neurological evaluation was 14 (IQR, 13 – 15), and three patients were hospitalized in the intensive care unit because of the severity of other traumatic injuries. Loss of consciousness (n = 7), headache (n = 5), vomiting (n = 5), posttraumatic amnesia (n = 5), and drowsiness (n = 5) were the most frequently reported symptoms at the time of admission. The baseline characteristics of the study patients are shown in Table 2.

3.2.2. Head CT at the time of admission

All patients underwent a head CT within the first 24 h of diagnosis (T0). In 10 patients, CT revealed a specific brain injury. In the remaining five patients, no parenchymal or bone lesion was detected. A concomitant skull fracture was noted in eight patients, whereas a parenchymal lesion far from the direct site of impact was detected in four patients. A brain MRI was also performed for two patients in the acute phase. MRI confirmed the injuries detected on CT. The main findings of T0 neuroradiological examinations are depicted in Figure 3.

3.2.3. Neuropsychological assessment

All children were evaluated using the NEPSY-II scale at T1 and T2. Comparisons between T1 and T2 data, including only attention and executive functioning as well as memory and learning domains, showed no significant differences (Table 3).

Table 1. Summary of the 17 articles included in the systematic review

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
Max *et al.*[30]/2013/ US/Prospective cohort	-	79	54	33.25 (11.40)	6 and 12 months	- AIS - MRI	- Vineland Adaptive Behavior Scale - Woodcock–Johnson Revised Letter–Word Identification test - Coding and Symbol Search (WISC-III) - Rapid Automatized Naming task - CVLT–C - N-back task - Formulated Sentence task (CELF–3)		- K-SADS-PL - NRS	- Family history research diagnostic criteria - FAD	Novel psychiatric disorders observed 6 – 12 months after mTBI are associated with significant deficits in adaptive functioning and pre-injury psychosocial risk factors, including lower SES and psychosocial adversity.
McNally *et al.*[31]/2013/ US/Prospective cohort	OI	186	132	11.9 (8 – 15)	3 weeks and 12 months	- HBI - CSI - PCS-I - MISS	- WASI - WRAT-III	- CBCL		- LSSRI - FAD - BSI	Severity of brain injury predicted the parent's and child's ratings of PCS, whose contribution decreased over time. Family factors consistently predicted the parent's, but not child's, ratings of PCS.
Olsson *et al.*[32]/2013/ Australia/ Prospective cohort	-	150	108	10.9 (6 – 16)	6 and 18 months	- PCS (CBCL) - GHQ	- WASI - WISC-IV - Children's Memory Scale - Contingency Naming Test - TEA-Ch	- CHQ-PF28 - TSCC-A	-	- IES-R	Pre-injury parent distress (increased hyperarousal symptoms) were predictive of increased child PCS at 6 and 18 months after injury. Higher levels of PCS at 6 months after injury persisted up to 18 months.

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
Loher *et al.*[33]/2014/ Switzerland/ Prospective cohort	HC	13	7	7.3 (5 – 10)	2, 6, and 12 weeks		- Stroop task (inhibition task) - Complex span task (working memory) - Cognitive flexibility task (switching) - Reaction time (processing speed)				Compared with the controls, children with mTBI displayed an inferior performance enhancement in switching attention and working memory across the testing sessions in the first 6 weeks after the injury. This resulted in a delayed deficit in the executive function component of working memory 12 weeks after the injury.
Keightley *et al.*[44]/2014/ Canada/ Systematic review	HC and OI	835 (6 studies)	-	5.5	12 – 30 months				- Prevalence of PTSD -Risk of psychiatric illness and utilization of psychiatric services		Most children recover within 3 months after mTBI. After 1 year, the prevalence of post-concussion symptoms and syndrome is similar between children with mTBI and controls. The functional status of children with mTBI improved over a 30-month follow-up period.
Taylor *et al.*[34]/2015/US and Canada/ Prospective cohort	OI	176	123	11.9 (8 – 15)	3 and 12 months	-MRI	- CBCL - Teacher's report form (TRF)				Younger children with mTBI had higher post-injury ratings on the CBCL total scale than controls. Hospitalization, motor vehicle

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
											accidents, loss of consciousness, and MRI abnormality were associated with higher parent or teacher ratings in all study participants with mTBI.
Bernard *et al.*[35]/2016/ Australia/ Prospective cohort	Minor non-head trauma	46	29	7.5 (2 – 12)	1 week and 1, 2, and 3 months	- PCS-I - CSHQ				- Clinical assessment of behavior-parent - PSI-sf	Presence of mTBI was a stronger predictor of PCS in the early post-injury phase than in the late post-injury phase. Older age at injury and pre-existing learning difficulties were significant predictors of PCS at 1 month post-injury. Family factors, including higher levels of parental stress, higher SES, and Anglo-Saxon descent, were consistently predictive of greater PCS.
Connery *et al.*[36]/2016/ US/Prospective cohort	-	70	39	15 (8 – 17)	2 – 12 months	- HBI	- WASC—II - CVLT - WISC-IV - Grooved pegboard - WIAT-III - Performance validity tests: 1) Medical symptom validity test 2) TOMM 3) Rey 15-item test			- Satisfaction survey (for caregivers)	Similar high levels of caregiver satisfaction between groups and greater reduction in self-reported symptoms after feedback was observed in children with non-credible presentations than in those with credible presentations.

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
Emery *et al.*[45]/2016/US and Canada/ Systematic review	HC, OI, and minor traumas	3182 (30 studies)	-	9.34	-				Attention problems, depression and mood disorders, anxiety, oppositional defiant disorder/ disruptive behaviors, and PTSD		-Higher rates of inattentive/ hyperactive symptoms, particularly if hospitalized (3-fold increase in the likelihood of a substance abuse diagnosis) -Mood disorder diagnoses -Possible risk of anxiety immediately after mTBI. However, it was not necessarily a long-term outcome -Adolescents with mTBI were more likely to exhibit disruptive behaviors, including>1 ADHD -A study proposed that mTBI is associated with schizophrenia diagnosis if there is a familial predisposition for the disorder. This risk depends on age at the time of injury (higher if mTBI occurred before the age of 11 years)
Segev *et al.*[37]/2018/ Israel/ Prospective cross-sectional cohort	PTSD	61	38	11.9 (6 – 18)	3 months	- PCS-I	- Sorting, design fluency, and trail-making tasks (D-KEFS) - Digit and spatial spans (WISC-IV) - CPT II–V	- K-SADS-PL - TOMM - Child PTSD Symptoms Scale			There was a significant interaction between sex and TBI injury. In children with PTSD, occurrence of TBI significantly affected

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
											the neurocognitive performance of girls, whereas it had a lower effect on boys
Plourde *et al.*[38]/2019/ Canada/ Prospective cross-sectional cohort	-	33	16	14.9 (9 – 18)	23 months				- BASC (anxiety and depression symptoms) - C-DISC-IV		The agreement between methods (symptom questionnaire vs. standardized clinical interview) to assess anxiety and depressive symptoms was moderate to almost perfect in young individuals and lower in parents with anxiety and depression.
Renaud *et al.*[39]/2020/ Netherlands/ Prospective cross-sectional cohort	-	156	102	11.4 (6 – 17)	6 months	- HBI - PedsQL-fatigue - IES		- CAPE - CBCL		-FAD	Decreased activity and participation among children with mTBI can be predicted by adverse pre-injury behavioral functioning of the child, adverse pre-injury family functioning, lower parental SES, high number of stress symptoms post-injury, high number of postconcussive symptoms, and reduced resumption of activities

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Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
Bosworth *et al.*[40]/2020/ US/Prospective cross-sectional cohort	-	184	80	14.3 (7 – 17)	<12 months	- NV-MSVT	- WASI - CVLT-C - ChAMP - CPT-II - WISC-IV - D-KEFS - Trail-making test - WRAT-III (reading)				Participants who failed the NV-MSVT (15%) performed significantly worse than those who passed the NV-MSVT on measures of IQ, memory, and immediate attention/ working memory. There was no significant difference between groups in terms of processing speed, sustained attention, cognitive flexibility, or sight word reading level.
Shorer *et al.*[41]/2020/ Israel/ Prospective cross-sectional cohort	PTSD	37	25	11.9 (6 – 18)	3 months	- WHO–5 well-being index - PCS-I - C-GAS	- TOMM - RPM - D-KEFS - Digit and spatial spans (WISC-IV) - CPT II–V 5		- K-SADS - CPSS: Child PTSD scale - CDI: Children - STAI-C	- PDS	Psychological intervention after mTBI improved the children's emotional status. Children exhibited increased well-being scores and decreased ratings of PTSD, anxiety, depression, and postconcussive symptoms. Ratings of cognitive function also improved for cognitive flexibility and executive function in everyday life. Parental PTSD was the strongest predictor of improvement after intervention.

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
Murphy *et al.*[42]/2021/ US/Prospective cross-sectional cohort	-	81	37	14.9 (8 – 17)	-	- PCSS			- BASC		There was a statistically significant interaction between a parent's perception of changes in family stress and parent–child report discrepancies of internalizing symptoms, independent of postconcussive symptom severity. The impact of SES was not significant.
Yumul *et al.*[46]/2021/ Australia/ Systematic review	HC, OI, and minor traumas	2076 (from 11 studies)		4.88 (0 – 16)	<72 h – 12 months	- PCS-I - KPCQ	- Sydney PTA Scale (SYPTAS)-Starship PTA Scale				PCS was mostly investigated acutely, and data on symptom trajectory specific to preschool children were limited. Symptoms, including irritability, personality changes, anxiety, headache, and visual or auditory impairment, were observed in 6% of the preschool children 1 year after the injury.
Studer *et al.*[43]/2024/ Switzerland/ Prospective cohort	HC	64	55%	10.73	1 week to 3 – 6 months	-PCS-I	- TAP	- CASP			Parent-reported PCS is high immediately after an injury. Emotional PCS normalize within 1 week,

(Cont'd...)

Table 1. *(Continued)*

Author/year/ country/ design	Control group	Population			Evaluation time points after TBI	Outcome measure (s)					Result (s)
		N	Males	Mean age (range)/ (years)		PCS/ neurologic and neuroradiologic	Cognitive	Psychosocial	Psychiatric	Family	
											whereas cognitive and somatic PCS normalize within 3 months. Sleep-related PCS remain high even after 3 – 6 months. No difference in TAP was observed over time between the experimental and control groups. School participation was lower in patients in the 3 – 6 month follow-up period.

Abbreviations: ADHD: Attention-deficit hyperactivity disorder; OI: Orthopedic injuries; HC: Healthy control; AIS: Abbreviated injury scale, 1990 Revision; MRI: Magnetic resonance imaging; K-SADS-PL: Affective Disorders and Schizophrenia for School-Aged Children, Present and Lifetime Version; NRS: Neuropsychiatric Rating Scale; WISC-III: Wechsler Intelligence Scale for Children III edition; CVL-CT: California Verbal Learning Test-Children's Version; CELF–3: Clinical Evaluation of Language Fundamentals, 3rd Edition; SES: Socioeconomic status; HBI: Heath and behavior inventory; WASI: Wechsler Abbreviated Scale of Intelligence; WRAT: Wide Range Achievement Test; CBCL: Child Behavior Checklist; PCS-I: Postconcussive symptoms I; CSI: Concussion symptom inventory; BSI: Brief symptom inventory; LSSRI: Life stressors and social resources inventory; FAD: Family assessment device; MISS: Modified injury severity scale; CHQ-PF28: Child health questionnaire (quality of life); TSCC-A: Trauma symptoms checklist–alternate version; WISC-IV: Wechsler Intelligence Scale for Children IV Edition; TEA-Ch: Test of everyday attention–children; IES-R: Impact of event scale–revised; GHQ: General health questionnaire; TRF: Teacher's report form 6-18; PSI-sf: Parenting stress index/short form; TOMM: Test of memory malingering; D-KEFS: Delis–Kaplan executive function system; CPT II–V 5: Continuous performance test II, version 5; IES: Impact of events scale; CAPE: Children's assessment of participation and enjoyment; NV-MSVT: Nonverbal-medical symptom validity test; ChAMP: Child and adolescent memory profile; C-GAS: Children's global assessment scale; RPM: Raven's progressive matrices; CPSS: Child PTSD scale; CDI: Children depression inventory; STAI-C: State-trait anxiety inventory for children; PDS: Posttraumatic diagnostic scale; BASC: Behavioral assessment for children system; PCSS: Postconcussive symptom scale; TAP: Test of attentional performance.

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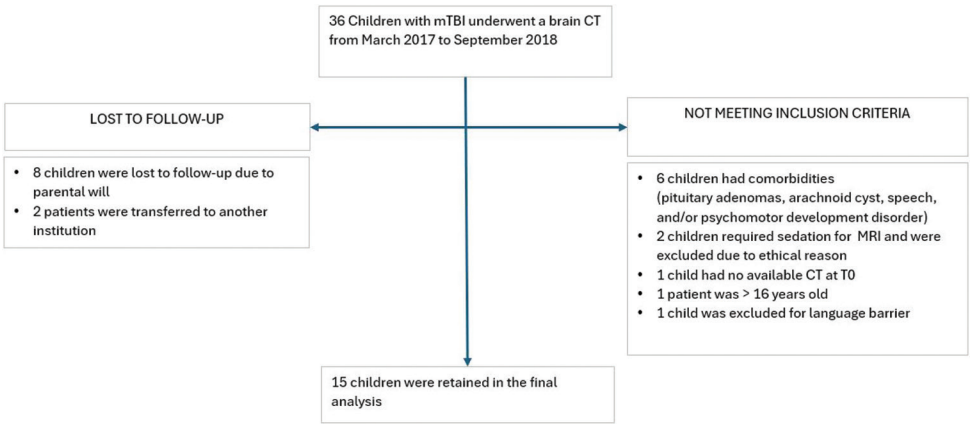

Figure 2. Eligibility and exclusion criteria for the monocentric cohort study

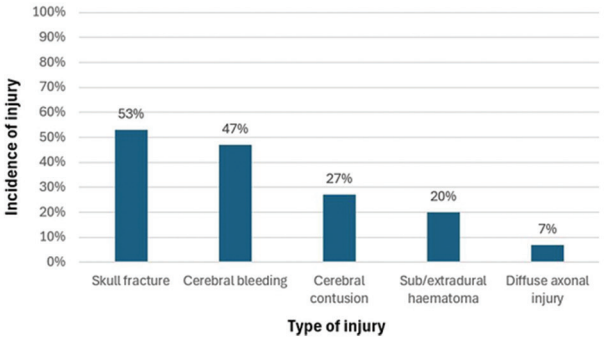

Figure 3. Neuroradiological findings obtained at the time of diagnosis (T0)

Overall, results obtained at T2 were borderline or slightly below the expected level. However, a comparison of the T2 data with reference values (one-sample *t*-test) demonstrated significantly lower scores in the memory and learning domain, sensorimotor functions (finger tapping), social perception (affect recognition), and visuospatial abilities (design copying) (Table 4).

3.2.4. Cognitive assessment

Twelve children were assessed using the WISC-IV scale at T1 and T2. However, two children were evaluated using the WPPSI-III according to age. The FSIQ results of the children were not significantly different between T1 and T2 (106.1 ± 6.9 vs. 103.5 ± 10.7; $P = 0.25$) (Figure 4). Furthermore, the T2 results were not significantly different compared with the reference values (one-sample *t*-test; $P = 0.5$).

3.2.5. Emotional and behavioral assessment

The CBCL data for children aged 6 – 18 years were obtained from the parents of eight children at T0 and T1 (Table 5). The median t-scores for total ($P = 0.898$), internalizing ($P = 0.156$), and externalizing ($P = 0.953$) behavioral issues were not significantly different between T0 and T1.

Table 2. Clinical and demographic characteristics of the 15 children who were admitted for mTBI mild-to-moderate TBI

Characteristics	*n*=15
Age at the time of TBI, years, median (IQR)	9 (5.5 – 14)
Sex, *n*	
Males	11
Females	4
Mechanism of injury, *n*	
High speed (motor vehicle accident/bicycle collision)	10
Fall from a height	4
Head struck by a heavy object (unintentional)	1
Impact site, *n*	
Occipital	2
Temporal	2
Frontal	2
Multiple	4
Unknown	4
GCS at time of first evaluation, median (IQR)	14 (12–15)
Symptoms, *n*	
Loss of consciousness	7
Headache	5
Vomiting	5
Post-traumatic amnesia	5
Drowsiness	5
Altered neurological examination, *n*	4
Admission, *n*	
General pediatric ward	10
ICU	3
Emergency department (short-stay observation unit)	2

Abbreviations: TBI: Traumatic brain injury; IQR: Interquartile range; GCS: Glasgow coma scale; ICU: Intensive care unit.

Table 3. Results of Developmental Neuropsychology Assessment (NEPSY-II) in a group of children with traumatic brain injury collected in the post-acute phase (T1) and at the end of the follow-up period (T2)

Item	T1		T2		*P**
	Mean	SD	Mean	SD	
A1 visual attention	8.6	3.5	8.7	3.6	0.916
A3 auditory attention	8.3	4.2	9.1	4.2	0.459
A4 inhibition A	9.2	2.6	9.6	3.1	0.995
A4k inhibition B	9.9	3.0	9.5	2.8	0.297
M3 memory for design	6.6	3.7	6.8	3.7	0.774
M3 memory for design (delayed)	7.5	3.9	7.2	3.5	0.703
M6 narrative memory total	8.4	3.1	8.4	3.0	0.993
M7 sentence repetition	7.7	3.7	7.2	3.0	0.423

Note: Statistical analysis was performed using the two-tailed t-test. *P<0.05. T1: 3 – 6 months after the event; T2: 18 – 24 months after the event.
Abbreviation: SD: Standard deviation.

Table 4. Results of Developmental Neuropsychology Assessment (NEPSY-II) in a group of children with traumatic brain injury obtained at T2 and a comparison of T2 data with the normal reference values

Item	T2 (n=14)			
	Mean	SD	IQR	*P**
A1 visual attention	8.7	3.6	5.8–11.3	0.221
A3 auditory attention	9.1	4.2	6.5–11.7	0.465
A4 inhibition A	9.6	3.1	8.0–11.6	0.671
A4k inhibition B	9.5	2.8	7.3–11.3	0.583
M2 word list interference (recall)	7.0	3.0	5.4–9.0	0.002**
M2 word list interference (repetition)	7.6	3.3	6.5–9.6	0.012*
M3 memory for design	6.8	3.7	5.1–9.6	0.007**
M3 memory for design (delayed)	7.2	3.5	5.3–10.4	0.011*
M6 narrative memory total	8.4	3.0	6.9–8.0	0.054
M7 sentence repetition	7.2	3.0	5.6–9.6	0.006**
SM1 tapping	8.0	2.7	6.3–9.9	0.011*
SO1 theory of mind (verbal)	9.5	3.5	8.5–12.0	0.559
SO1 theory of mind (context.)	8.8	2.8	6.7–10.6	0.126
SO4 affect recognition	5.2	2.7	3.3–7.3	<0.001**
V1 design copying	6.8	2.7	5.5–9.0	0.001*
V3 picture puzzles	8.8	2.6	6.9–11.0	0.126
V4 geometric puzzles	10.3	4.0	7.0–13.4	0.646
V6 arrows	8.3	3.2	6.3–10.6	0.073

Notes: Statistical analysis was performed using the one-sample t-test. *P<0.05; **P<0.01. T2: 24 months after the event.
Abbreviations: SD: Standard deviation; IQR: Interquartile range.

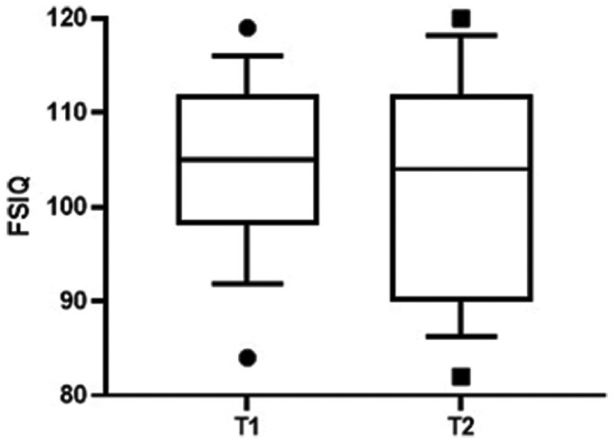

Figure 4. Comparison of the full-scale intelligence quotient between the post-acute phase (T1) and the end of follow-up period (T2)

3.2.6. Post-acute phase MRI

During the follow-up period, at least one brain MRI was performed for 11 patients (Table 6). Three children underwent two MRI examinations (at 1 and 6 months after TBI), eight were evaluated once 4 – 6 months after TBI, and one underwent MRI only 1 month after TBI. Overall, the follow-up MRI data were similar to those of the baseline head CT. MRI revealed the regular evolution of the described injuries (Table 6). In two patients (Patients 2 and 10), new abnormalities were diagnosed. In one patient (Patient 10), the corpus callosum (CC) thickness was reduced, which may be attributable to the TBI. In another patient (Patient 7), the previously reported brain injury had completely resolved (Table 5).

Four of the twelve children who exhibited signs of TBI persistence on follow-up MRI (regular evolution or new abnormalities) demonstrated several deficits on neurocognitive evaluation (Table 6). To determine potential risk factors for any type of cognitive deficits, univariate analysis included the following variables: sex, age <6 years, high energy TBI, GCS ≤14, baseline head CT data, and follow-up MRI data (for patients in whom >1 MRI was performed). No variable exhibited a predictive value (Table 7).

4. Discussion

4.1. Literature review

The current study is the most recent updated review on long-term neurocognitive follow-up in children with a history of TBI. The majority of the patients recover relatively quickly and with few sequelae.[48] However, up to 10 – 30% of them may exhibit low-performance test scores,

Table 5. Comparison of the child behavior checklist data collected from the parents of children with traumatic brain injury at T0 and T1

Patient no.	T0 (*n*=8)			T1 (*n*=8)		
	Internalizing issues	Externalizing issues	Total score	Internalizing issues	Externalizing issues	Total score
1	34	40	27	40	40	34
2	61[a]	48	57	46	47	52
3	41	39	38	41	33	42
4	50	53	54	58	59	61[a]
5	58	48	53	47	46	49
6	65b	46	52	44	40	34
7	58	43	50	44	46	46
8	44	40	40	40	43	42
Median (IQR)	54 (43.2 – 58.7)	44.5 (40 – 48)	51 (39.5 – 53.2)	44 (40.7 – 46.2)	44.5 (40 – 46.2)	44 (40 – 49.2)
P-value* (T0 vs. T1)	0.156	0.953	0.898			

Notes: [a]Risk score category; [b]Clinical symptoms category; Statistical analysis was performed using the two-tailed t-test. *P<0.05. T0: Baseline; T1: 3 – 6 months after the event.
Abbreviation: IQR: Interquartile range.

Table 6. MRI and cognitive evaluation of children with traumatic brain injury performed during the follow-up period

Patient	Baseline head CT	MRI (1 – 3 months after injury)	MRI (4 – 6 months after injury)	Cognitive outcome (up to 24 months later)
1	Left parietal fracture+fronto-orbital cerebral contusions (contrecoup)	Regular evolution of cerebral contusions	-	No deficits
2	Right fronto-temporal extradural and subdural hematoma	-	Regular evolution+non-specific hyperintense spots in bilateral semioval centers	Deficits in attention, executive functions, memory, and motor planning
3	Normal	-	Normal	No deficits
4	Normal	-	-	No deficits
5	Normal	-	Normal	No deficits
6	Minimal left frontal contusion	-	Regular evolution	No deficits
7	Right temporo-parietal fracture	-	Normal	Deficits in language and verbal memory
8	Left fronto-temporal fracture and cerebral contusion	Bilateral fronto-basal and temporo-polar contusions	Regular evolution	No deficits
9	Left fronto-basal cerebral contusions	-	Regular evolution (hemosiderin depositions)	Deficits in attention, memory, and visual perception abilities
10	Right fronto-parietal subdural hematoma+DAI	Multiple DAI (vermian, occipital, pontine, fornix, and vertex)	Regular evolution+reduced CC thickness	Deficits in memory as well as visuospatial and construction abilities
11	Cerebral contusions (contrecoup) on the tentorium	Multiple cerebral contusions (contrecoup): bilateral temporo-mesial (righ t>left), temporal lobe, CC (splenium), and left frontal involvement	Regular evolution	Deficits in attention, memory, and visual perception abilities
12	Right fronto-orbital fracture and subdural hematoma	-	Regular evolution	Deficits in memory for faces
13	Basal skull fracture	-	-	Deficits in attention and executive functions and verbal memory
14	Normal	-	-	No deficits
15	Normal	-	-	No deficits

Abbreviations: CC: Corpus callosum; CT: Computed tomography; DAI: Diffuse axonal injury; MRI: Magnetic resonance imaging.

Table 7. Univariate analysis to determine possible risk factors for any type of cognitive deficits in children with traumatic brain injury at follow-up

Variable	Risk of any cognitive deficit		
	OR	95% CI	*P**
Sex (male)	0.525	0.009 – 23.78	0.727
Age (≤6 years)	0.149	0.003 – 4.02	0.273
Cause (High energy TBI)	0.721	0.012 – 46.2	0.864
GCS (≤14)	9.33	0.669 – 127	0.119
Baseline head CT (Any injury)	-	-	-
MRI (Any injury)	1.4	0.059 – 47.5	0.83

Notes: Statistical analysis was performed using Fisher's exact test.
*$P<0.05$.
Abbreviations: CI: Confidence interval; CT: Computed tomography; GCS: Glasgow coma scale; MRI: Magnetic resonance imaging; OR: Odds ratio.

especially in the first few weeks after the injury, and these subtle deficits may persist even after several months.[45-49]

According to previous systematic reviews[44,45] and studies,[31,33,38,42,43] evidence on psychological, behavioral, and psychiatric problems following an mTBI remains controversial. This may be attributable to the fact that many results are often based only on symptom ratings (not the actual diagnosis) and are based on research protocols characterized by multiple methodological limitations, such as the use of healthy controls.[33,45] When control groups include patients with non-head injuries (*e.g.*, orthopedic fractures), statistical significance may disappear over time compared with that in analyses based on a healthy control group alone.[31,34,50]

Although the likelihood of psychological or psychiatric issues increases in the period immediately after an mTBI,[44,45] there is no evidence regarding their long-term consequences.[31,33,35] However, the time to follow-up across the studies was not standardized. Children with mTBI across different studies were assessed over a wide and heterogenous period, usually limited to 1 year after the injury[30,31,34,36,40] or after only 3 months.[33,35,37,41] Although non-injury factors are more consistently related to persistent PCS, the injury characteristics may predict the PCS in the first few months after an mTBI.[31] However, the evidence on both children-related[34,35,37,39] and family-related[31,32,39,42] factors remains unclear. Therefore, if neuropsychological morbidity impacts children with mTBI, psychological support should be a part of the follow-up in these patients and those with more severe traumas. Preliminary studies have demonstrated that a child's emotional and cognitive functions improve after prolonged exposure to psychological intervention support and with the promotion of high levels of caregiver satisfaction.[36,41]

Our results demonstrated that children with a history of mTBI may exhibit lower scores in specific neuropsychological functions and in the long-term follow-up (24 months after the event). These may impact their routine life, including school performance (when memory and visuospatial abilities are impaired) and social relationships (when affect recognition is impaired). Thus, these children should be systematically screened and evaluated for a longer period.

4.2. Neuropsychological functioning in the monocentric study

Children enrolled in our study exhibited significantly lower scores in most memory tasks compared with the normal values. Memory functioning may be highly variable in children with a history of severe TBI.[50,51] Recently, similar findings were reported in children with mTBI.[33,40] However, memory deficits may also emerge with time or be misdiagnosed in the immediate post-injury phase, as some memory functions mature later during childhood.[52]

Our study population also exhibited lower scores in visuospatial abilities compared with the normal reference values. In particular, children performed worse in design copying (aimed at evaluating visual–constructional and visual–perceptual skills). This finding is consistent with that of a recent study on the effects of concussion in adolescent hockey players.[53] In another study, young athletes with a concussion performed worse than controls in a spatial configuration task, which was specifically designed to measure their ability to form a mental representation of the spatial surrounding.[54] These abilities require an extended neural network that encompasses cortical and subcortical structures, especially bilateral parietal cortex activation.[55]

According to our study results, children with a history of mTBI may also present with subsequent difficulties in facial affect recognition. However, the ability to put oneself in the other's shoes (theory of mind), as measured by the NEPSY-II, was not compromised. Some previous studies have suggested persistent alterations in the recognition of facial emotional expressions[56-59] in children who sustained mTBI. This finding may account for the reduction in social competence in patients with TBI because the ability to perceive emotions displayed by others through non-verbal cues is crucial in shaping optimal reactions and behaviors toward others. Communication issues are also significantly and negatively associated with the ability to recognize facial emotions.[60] Although data on the neurobiological mechanisms of impairment of facial affect recognition are limited, functional neuroradiological studies in adults have revealed a correlation of impaired facial recognition ability with reduced activation in the right fusiform gyrus and medial prefrontal regions.[61]

Collectively, these data indicate that both short- and long-term memory, visual–constructional skills, and face affect recognition represent functions at greater risk for children's regular neurodevelopment after mTBI.

4.3. Cognitive functioning in the monocentric study

According to our study results, intellectual functioning was not affected in children with mTBI during the follow-up, as indicated by no significant difference in FSIQ scores compared with the normal reference values. This finding is similar to those reported in adults, in whom the IQ usually remains intact post-injury, but differs from those reported in younger children.[52,62] The study on the long-term consequences of TBI in early childhood demonstrated the persistent impact of TBI on IQ, even 10 years after the injury, in patients with a history of severe TBI that had occurred before the age of 7 years.[18] This finding may support the concept of the "*double hazard model*," which is based on the hypothesis of an interaction between age at injury and injury severity.[63-65] However, due to the specificity of our sample (which included only patients with mTBI) and the limited size of our study population, the results could not be stratified and corrected for age or other variables.

4.4. Emotional and behavioral assessment in the monocentric study

Contradictory to previous studies, the current study findings showed no difference in CBCL scores over time.[34,39] This may be attributable to the following: (1) exclusion of patients with a former diagnosis of neurodevelopmental disorder from the final analysis and (2) inclusion of only three children aged <7 years in the study. Previous studies have demonstrated that the long-term difficulties highlighted by the CBCL are often prevalent in children with a history of severe injury at an early age[61] or pre-existing attention-deficit hyperactivity disorder.[66]

Two of the eight study patients exhibited moderately high scores on the T0 internalization index, which normalized at the T1 follow-up. This reduction in the internalization index may be attributable to the development of disinhibition following a TBI with a frontal impact.

4.5. Neuroradiological examination in the monocentric study

Conventional neuroradiological examinations performed in the follow-up period did not exhibit a predictive value for cognitive outcome. However, it is largely recognized that white matter pathologies in TBI cannot be adequately visualized using standard MRI or CT.[67] Specific long-term neuroanatomical changes have been documented in pediatric patients with mTBI using advanced imaging modalities such as functional MRI and diffusion tensor imaging.[68] These changes include widespread gray and white matter losses, which may alter the white matter organization.[69] The CC is particularly vulnerable to this type of injury.[70] This is because transverse forces occurring during TBI may strain the *falx cerebri* and exacerbate the underlying CC.[71]

Advanced neuroimaging examinations are currently limited to research studies, and robust evidence is required for any future clinical application.[72,73] Moreover, during the follow-up period, children underwent MRI only on the basis of the clinical symptoms. There is no standard protocol regarding the optimal time for performing neuroimaging studies. According to our retrospective study, MRIs are usually performed immediately after (T0) or 6 months after (T1) the traumatic event. This may have introduced a bias because post-TBI structural abnormalities may develop later.

4.6. Study limitations

The current study had several limitations. First, the small sample size, even if powered for statistical analysis, prevented us from performing an advanced stratified analysis according to age and other baseline factors. The small sample size might have particularly affected the results of discriminant analyses. However, all patients were accurately and extensively assessed using a wide range of cognitive and neuropsychological items, enabling us to obtain several measures on multiple cognitive domains.

The absence of baseline neurocognitive data did not allow us to verify possible pre-existing mental conditions. We tried to control this bias by excluding all patients diagnosed with a neurodevelopmental disorder.

5. Conclusion

Children who experience mTBI may exhibit subtle memory and visual–constructional impairments and difficulties in social perception during the follow-up period. Our results demonstrate the importance of a comprehensive and long-term neuropsychological assessment, including memory assessment, to plan adequate and timely rehabilitation programs and school adaptations. Our study findings also demonstrated that psychiatric disorders are frequently newly diagnosed in the follow-up period and associated with significant deficits in adaptive functioning, especially in children with pre-injury psychosocial risk factors.

Conventional neuroradiological examinations did not exhibit a predictive value for cognitive outcome. Thus, advanced imaging techniques should be performed in these children to identify specific neuroimaging biomarkers to help tailor supportive interventions. Prospective cohort

studies that include serial neuroimaging evaluations with advanced MRI sequences that are performed at specific time points may allow the detection of subtle changes in brain structure and connectivity in children with TBI at follow-up.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study was approved by the local Institutional Review Board (approval no.: CEUR-2020-OS-265). Written informed consent was obtained from parents of all subjects enrolled.

Consent for publication

Not applicable.

Availability of data

Raw data are available from the corresponding author upon reasonable request.

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REVIEW ARTICLE

SARS-CoV-2 persistence: A potential catalyst for age-associated neurodegenerative diseases

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Abstract

The persistence of RNA viruses in the brain is increasingly recognized as a significant factor in the progression of age-related neurodegenerative diseases. This phenomenon is particularly evident in infections caused by various neurotropic and non-neurotropic viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The COVID-19 pandemic has underscored the urgent need to explore the complex relationship between viral persistence and neurological decline. Growing evidence indicates that SARS-CoV-2 affects brain structure and function, although the precise molecular mechanisms remain poorly understood. Chronic neuroinflammation induced by viral infections is thought to accelerate age-related neurodegeneration. While the immune system typically clears many viral infections in the brain, some viruses establish chronic infections, leading to restricted viral replication. These persistent infections can exacerbate neuroinflammation and contribute to ongoing neuronal damage, key drivers of age-related neurodegeneration. This review explores current knowledge on how SARS-CoV-2 infiltrates the brain, evades immune defenses, and persists within brain cells, potentially using them as viral reservoirs. As individuals age, the cumulative effects of such viral infections may accelerate cognitive decline and increase vulnerability to neurodegenerative diseases such as Alzheimer's and Parkinson's. Understanding the molecular mechanisms of viral persistence and its long-term impact on brain health is crucial for developing targeted therapies to combat these age-related diseases.

Keywords: Severe acute respiratory syndrome coronavirus 2; Post-acute consequences of COVID-19; Neuro-COVID; Neurodegenerative disease; Viral persistence; Neuroinflammation

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1. Introduction

As the world concentrates on the immediate impacts of the COVID-19 pandemic, a less visible yet increasingly alarming issue is the long-term neurological effects of the virus.[1] Despite extensive efforts to develop vaccines and therapeutic interventions, SARS-CoV-2 continues to pose a significant threat to public health and socioeconomic stability.[2] This elusive virus forges connections with the nervous system through immunological pathways or direct invasion, resulting in an array of neurological complications.[3] Alarmingly, viral persistence in the brain can trigger neurological issues in individuals without prior history or exacerbate existing conditions.[4]

The clinical manifestations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) extend well beyond respiratory symptoms; a growing number of survivors report persistent and debilitating neurological symptoms. This puzzling condition, now referred to as long COVID or neuro-COVID, is reshaping our understanding of brain health and disease.[5] Neuro-COVID, or post-acute consequences of COVID-19 (PASC), reveals a complex interplay between viral persistence, immune response, and neurodegeneration, which significantly contributes to the aging process. Symptoms such as anosmia, senility, cognitive impairment, depression, and anxiety reflect those of aging, highlighting the profound impact of this disease.[6,7]

Recent advances suggest that neuroinflammation alone can disrupt neuronal and glial cells, leading to neurodegeneration and impaired neuropsychiatric and cognitive function.[8] Beyond neuroinflammation, oxidative stress, deregulation of proteostasis, autoantibody production, and gut–brain dysregulation play critical roles in the development of neuro-COVID.[9-11] It is critical to recognize that these mechanisms, although not directly related to SARS-CoV-2 infection, collectively alter the architecture and function of distal tissues through the virus's persistent presence in host tissues. The penetration of viruses across the blood–brain barrier (BBB) has increased beyond the typical range of neurotropic viruses.[12,13] Over the past few decades, the role of viral persistence in the development of neurodegenerative diseases has gained significant global attention. The notorious SARS-CoV-2 infection has underscored the urgency of investigating the potential connection between viral persistence and the onset and progression of age-associated neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and other forms of neurodegenerative diseases.[14,15]

This comprehensive review aims to provide an overview of the current understanding of the relationship between SARS-CoV-2 infection and neurodegeneration. We focus primarily on the mechanisms by which the virus persists in the brain, the viral proteins involved in these processes, and the host pathways manipulated by the virus. In addition, we discuss recent therapeutic advances in this field and propose strategies for future improvements. Through this investigation, we aim to shed light on paths toward mitigating the long-term neurological effects of COVID-19 and improving brain health in the post-pandemic period.

2. Deciphering the enigma of PASC in the context of neurodegenerative diseases

The SARS-CoV-2 pandemic, which began in 2019, has presented unforeseen challenges to global health, extending beyond immediate respiratory impacts and significantly affecting neurological health, particularly in the context of age-associated neurodegenerative diseases. This condition, often referred to as long COVID or PASC, includes chronic fatigue, cognitive decline, and other neurological deficits that can accelerate age-related neurological symptoms.[16-18] The definition of long COVID is often debated, especially in the context of time frame (Figure 1). According to the World Health Organization, post-COVID-19 disease usually manifests 3 months after the onset of the infection, with symptoms lasting at least 2 months and not attributed to another diagnosis. Similarly, the United Kingdom (UK)'s National Institute for Health and Care Excellence describes long COVID as a multi-organ disease with debilitating symptoms that emerge during the acute or mild phase of the disease and persist for more than 4 weeks, with no alternative diagnosis.[19,20]

Statistics indicate that this debilitating illness affects at least 10% of SARS-CoV-2 cases, with over 200 symptoms impacting multiple organ systems.[17,20] Recent reports estimate that at least 65 million individuals worldwide are suffering from long COVID, with cases steadily increasing.[17] The incidence is estimated at 10 – 30% of non-hospitalized cases, 50 – 70% of hospitalized cases, and 10 – 12% of vaccinated cases.[17] Numerous biomedical findings have confirmed that long COVID can manifest in multiple organ symptoms, including cardiovascular dysfunction, thrombosis, cerebrovascular disease, and metabolic disorders. One potential cause of long COVID is the persistent presence of SARS-CoV-2 in different tissues.[21,22]

History data from previous pandemics and epidemics caused by RNA viruses in the last century provide ample evidence of acute neurological effects. However, apart from the 1918 H1N1 influenza pandemic and its associated neurological consequences, there is little information on the long-term neurological consequences following infection.[23] Neurological and cognitive symptoms are prominent features of long COVID, including cognitive impairment, sensorimotor symptoms, memory loss, paresthesia, sensitivity to light and sound, dizziness, balance issues, and autonomic dysfunction.[24] A study involving over 1.3 million people who had contracted COVID-19 reported that, while mental health issues such as anxiety and depression tended to resolve over time, increased risks of cognitive impairment, seizures, dementia, psychosis, and other neurocognitive conditions persisted for at least 2-year post-infection.[25] Interestingly, patients with prior COVID-19 infection exhibit an average decline in global cognitive performance equivalent to 10 years of aging.[26] In addition, studies have detected amyloid peptides, hallmarks of AD, in patients with long COVID.[27] Collectively, the evidence suggests altered brain

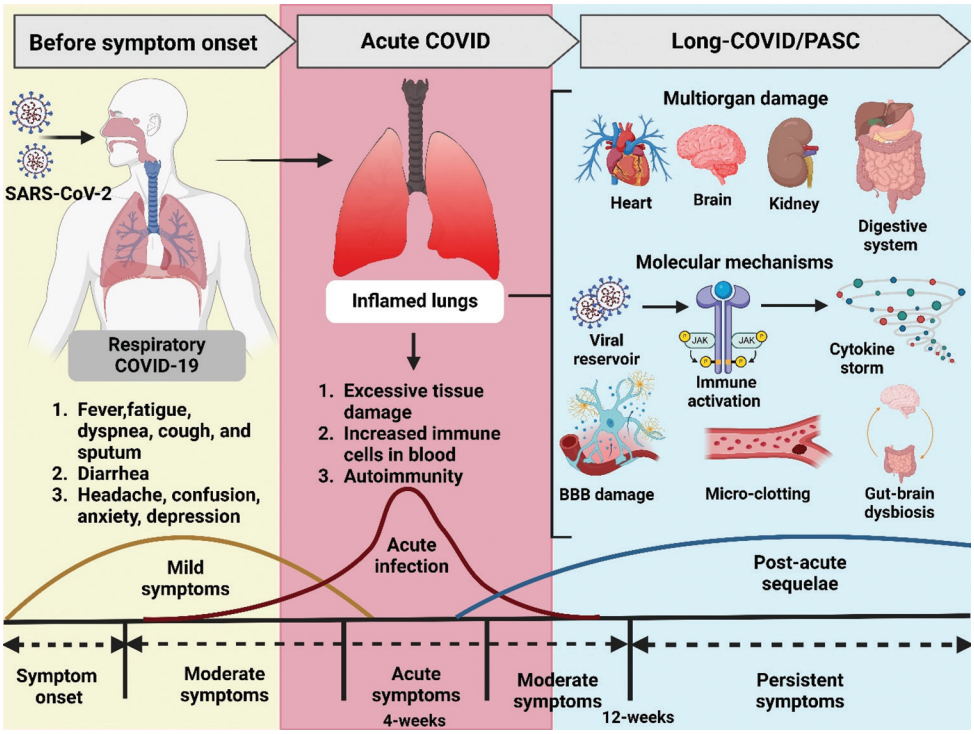

Figure 1. Deciphering the enigma of PASC in the context of neurodegenerative diseases. The time frame and symptoms associated with long COVID are illustrated. Following the infection, mild symptoms are observed, including fever, fatigue, loss of smell and taste, diarrhea, headache, and confusion. These mild symptoms coincide with the onset of acute symptoms. During acute infection, a high viral load causes excessive tissue damage and immune deregulation, which may affect multiple organs in the long term. Significant changes are observed in the heart, brain, kidneys, and digestive system, even after the acute phase of infection has resolved. These symptoms may persist for over 12 weeks post-infection. The speculated molecular mechanisms underlying these persistent symptoms include a viral reservoir in different tissues, chronic inflammation, abnormal cytokine profiles, a leaky BBB, hypercoagulation, and gut-brain dysbiosis. Image created with BioRender.com.
Abbreviations: BBB: Blood–brain barrier; COVID: Coronavirus disease; PASC: Post-acute sequelae of COVID-19.

function, with or without the presence of the viral genome. Understanding how viral persistence progressively affects individuals is critical, as is discussing recent advancements in therapeutic strategies for long COVID.

3. Traces of SARS-CoV-2 in the brain

The first case of COVID-19 was identified in Hubei province, China, in 2019.[28] However, the long-term effects of COVID-19 on various tissues have only recently been recognized. In a study by Zuo *et al.*,[29] which analyzed 317 tissue samples from 225 patients, viral RNA was detected in 16 (30%) of 53 solid tissue samples collected 1-month post-infection, 38 (27%) of 141 samples at 2 months, and 7 (11%) of 66 samples at 4 months. Viral RNA was found in 10 types of tissues, including the liver, kidney, stomach, intestine, brain, blood vessels, lungs, breast, skin, and thyroid. Subgenomic RNA was also detected in 26 (43%) of 61 solid tissue samples that tested positive for viral RNA. Two months after infection, viral RNA was present in the plasma of three (33%), granulocytes of one (11%), and peripheral blood mononuclear cells of two (22%) of nine immunocompromised patients. In contrast, viral RNA was absent in these components in 10 immunocompetent patients.[29,30] Using large sample size, multiple time points for specimen collection, and various viral detection methods, including highly sensitive digital droplet polymerase chain reaction, the study provided preliminary insights into the persistence of SARS-CoV-2 in multiple organs after COVID-19 recovery and its potential link to long COVID symptoms.[29] Importantly, the presence of viral RNA in multiple organs has been significantly associated with post-COVID conditions. A growing body of literature from independent researchers presents evidence of viral RNA in the central nervous system (CNS), including associated regions, and in the cerebrospinal fluid (CSF).[31] Subsequent studies validated the prevalence of nervous system involvement in COVID-19, with rates ranging from 22.5% to 36.4%. In addition to the CNS, there is growing evidence of peripheral nervous system involvement in COVID-19, with an incidence of 13.7%.[32]

In a retrospective, observational case series, clinical data were extracted from the electronic medical records of

214 patients, and all neurologic symptoms were reviewed by two trained neurologists. In addition to all laboratory tests (complete blood cell count, blood chemical analysis, coagulation testing, assessments of liver and renal function, C-reactive protein, creatine kinase, and lactate dehydrogenase), radiologic examinations were performed, including head and chest computed tomography scans. Patients with severe COVID-19 infections were more likely to develop neurologic manifestations, especially acute cerebrovascular disease, conscious disturbance, and skeletal muscle injury.[33] In a cohort analysis conducted by Almeria *et al*.,[34] it was suggested that patients with the acute syndrome of COVID-19 showed lower scores in memory and attention, along with a reduced global cognitive index. Similarly, in a community-based cohort study, Jacot de Alcântara *et al*., 2023[35] found that cognitive deficits following SARS-CoV-2 infection were still detectable nearly 2-year post-infection, particularly in individuals with prolonged symptoms, ongoing issues, or more severe cases of the disease. In contrast, no cognitive deficits were observed in individuals who reported full recovery from COVID-19. The impact of ongoing symptoms was assessed using multivariable linear regression models, stratified by self-perceived recovery. In addition, a longitudinal analysis was conducted to evaluate changes in cognitive performance over time.[35] Moreover, a thorough regional mapping of the olfactory nerve tracts and adjacent CNS regions in autopsy material from 33 COVID-19 patients confirmed the presence of viral RNA in the cerebellum.[36]

COVID-19-associated coagulopathy (CAC) has been identified as a significant contributor to ischemic stroke in long-COVID patients.[37] Evidence suggests that CAC arises from intricate interactions between the innate immune response, coagulation and fibrinolytic pathways, and the vascular endothelium, leading to a procoagulant state.[38] This perspective categorizes the current understanding of CAC into three main pathological mechanisms:

(i) Vascular endothelial cell dysfunction: COVID-19 induces endothelial dysfunction, impairing the normal function of blood vessels and promoting interactions between the endothelium and immune cells, such as neutrophils
(ii) Hyper-inflammatory immune response: The disease triggers a hyper-inflammatory response, marked by elevated levels of inflammatory markers such as neutrophil gelatinase-associated lipocalin, contributing to a heightened procoagulant state
(iii) Hypercoagulability: Unlike typical coagulopathy, CAC is not driven by the usual thrombin-dependent coagulation factors but is instead characterized by an abnormal coagulation response driven by SARS-CoV-2.

Although thromboembolism resulting from a "cytokine storm" is recognized as a contributor to the high morbidity and mortality associated with COVID-19, the precise mechanisms behind CAC and its role in ischemic stroke remain incompletely understood.[37,38]

In a study involving 8163 COVID-19 patients, 103 developed acute ischemic strokes, often linked to underlying conditions such as hypertension, diabetes, hyperlipidemia, atrial fibrillation, and heart failure. COVID-19 triggers systemic coagulopathy and inflammatory responses, leading to CAC, which increases the risk of ischemic strokes. Serial histological analyses of 100 autopsies from COVID-19-positive patients revealed widespread microthrombi and microinfarcts in the neocortex of 58 brains, with microthrombi often linked to small, patchy infarctions. Immunohistochemical staining also demonstrated robust angiotensin-converting enzyme 2 (ACE2) receptor expression in intraparenchymal blood vessels.[39] These findings align with neurological symptoms such as headaches, altered senses, cognitive defects, and increased mortality. These symptoms are attributed to neurocoagulation, resulting from excessive thrombin generation and disrupted BBB function. Post-mortem studies also suggest brain endothelial injury in COVID-19 patients, likely caused by host defense mechanisms rather than direct viral presence.

In a current finding, Douaud *et al*.[40] examined 785 patients from the UK Biobank and analyzed magnetic resonance imaging data collected over 3.2 years from individuals who had contracted COVID-19. Consistent with the frequent reports of smell and taste impairment in COVID-19, the study found greater structural abnormalities and indicators of tissue damage in regions associated with the primary olfactory cortex, including the anterior cingulate cortex, orbitofrontal cortex, insula, amygdala, ventral striatum, hippocampus, and parahippocampal gyrus. Notably, the authors observed a marked reduction in the total brain volume, even in patients who had experienced only mild COVID-19 symptoms, suggesting a potential increase in cognitive decline.[40] Most brain imaging studies on COVID-19 have focused on acute cases, examining individual reports or small case series using computed tomography, positron emission tomography, or magnetic resonance imaging scans. These studies have revealed a range of brain abnormalities, including white matter changes, reduced cerebral blood flow, and signs of ischemic events, especially in the cerebrum.[17,18,41] However, it is still unclear whether these abnormalities predated SARS-CoV-2 infection. It is possible that these brain changes indicate pre-existing vulnerabilities that make certain individuals more susceptible to the effects of COVID-19 or more likely to experience severe symptoms, rather than being a direct result of the disease itself.

Systematic analyses of various studies and case reports suggest that SARS-CoV-2 not only alters brain architecture but also causes cognitive impairment in individuals with mild or acute COVID-19 infection.[42] Previous reports have demonstrated traces of SARS-CoV-2 in the human brain; however, COVID-19 can induce physiological changes in the brain even without direct viral presence.[17,19] While most reports recognize the significant impact of SARS-CoV-2 on brain architecture, there remains a limited understanding of how these structural changes interact with the aging process. Therefore, we highlight a key hypothesis related to the mechanisms underlying the neurological consequences caused by long COVID (Table 1).

4. Neuroinvasion: The beginning of viral persistence

Brain cells are extremely selective and allow only specific candidates to enter. Neuroinvasion is a crucial step that ensures the persistence of the virus in the brain.[43,44] Neurovirulent viruses, including members of the *Coronaviridae* family, employ multiple routes for neuroinvasion[45] (Figure 2). Two crucial pathways for CNS penetration are the hematogenous route and the neuronal route.[46] In addition, the olfactory route plays a critical role in CNS penetration.[36] Pathways such as the nasal epithelium route, lymphatic tissue, and CSF infection may also play significant roles.[47]

The first established theory of SARS-CoV-2 invasion into the brain involves the host ACE2 receptor, which is present at the surface of alveolar epithelial cells, brain cells, and cells in the gastrointestinal (GI) tract.[48,49] Study has demonstrated that among respiratory viruses, SARS-CoV-2 exhibits the highest affinity for the ACE2 receptor.[50] This unique feature of SARS-CoV-2 enhances neuroinvasion and viral persistence in the brain. Host proteases such as transmembrane serine protease 2, cathepsin L, and furin are involved in the cleavage of the spike protein.[51] In addition, the neuronal co-receptor neuropilin-1 can facilitate viral internalization.[52]

Following primary infection, SARS-CoV-2 can disrupt the epithelial barrier and invade the bloodstream, where it may persist before reaching the CNS.[53] Neuroinvasion through the bloodstream has also been described for other coronaviruses, such as Middle East respiratory syndrome coronavirus (MERS-CoV), which enters the bloodstream and subsequently infects endothelial cells.[47] Paniz-Mondolfi *et al.*[54] observed SARS-CoV-2-like particles in endothelial cells, pericytes in brain capillaries, and astrocyte processes, supporting the hypothesis of hematogenous endothelial neuroinvasion of SARS-CoV-2. The virus can infect the endothelial cells at the blood–CSF barrier and subsequently spread into the CNS.[48,54] In addition, circumventricular cerebral organs and the choroid plexus, which are not protected by the BBB, could serve as entry points for viral penetration.[55] In acute infections, cytokine release or a cytokine storm can alter BBB permeability, particularly by disrupting tight junctions, allowing virus particles to enter the brain.[56] Recent findings have shown evidence of BBB disruption during acute COVID-19 infection and in long COVID patients with cognitive impairment, often referred

Table 1. Hypothetical mechanisms underlying long COVID-associated neurological sequelae

Proposed consequences of COVID-19 infection	Potential contribution to long COVID-associated neurological symptoms	References
Persistent virus or antigen reservoir	Ongoing immune or inflammatory activation	17,29
Damage in CNS and other associated regions	Reduced brain volume, structural abnormalities, and tissue damage in areas such as the anterior cingulate cortex, orbitofrontal cortex, insula, amygdala, ventral striatum, hippocampus, and parahippocampal gyrus, which are connected to the primary olfactory cortex	17,29
Changes in inflammatory response, cytokine levels, and T-cell profile	Downstream effects from neuroinflammation to neuronal damage	11,17
Vascular endothelial activation or dysfunction	Impact on platelet activation, clotting, microclots, and gas exchange	17
Mitochondrial dysfunction and proteostasis	Overproduction of ROS and RNS, deregulation of UPR	9
Autoimmunity	Potential role of autoimmune autoantibodies or T cells causing endothelial activation, postural orthostatic tachycardic syndrome, myocarditis, and neuroinflammation	17
Microbial dysbiosis	Altered metabolomic programming affecting immune response	9

Abbreviations: COVID-19: Coronavirus disease-2019; CNS: Central nervous system; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; UPR: Unfolded protein response.

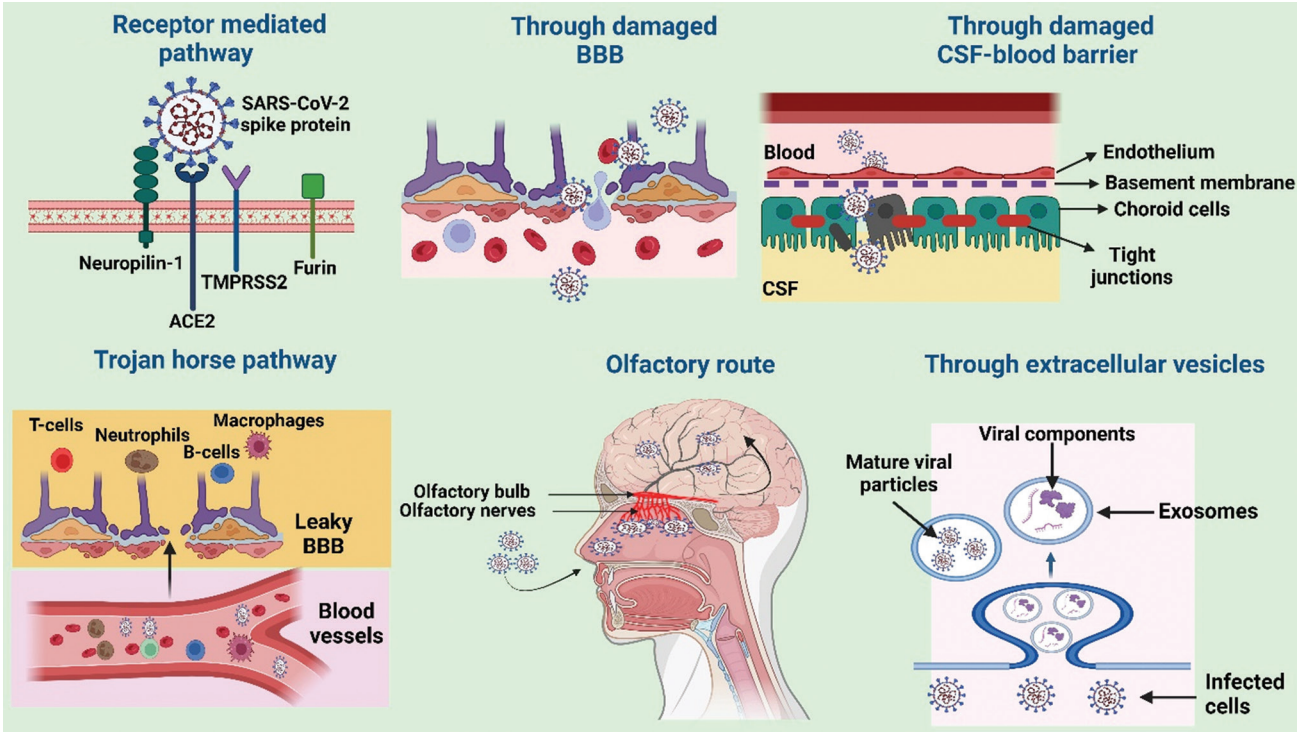

Figure 2. Mechanisms of neuroinvasion. SARS-CoV-2 can enter the brain through multiple routes: (A) Receptor-mediated endocytosis involving ACE2 and proteases such as TMPRSS2 and furin, with neuropilin-1 acting as a neuronal co-receptor. (B) SARS-CoV-2 infection alters the permeability of the BBB, thus permitting viral entry through the membrane. (C) Disruption of the tight junctions that protect the CSF-blood barrier leads to viral invasion of choroid cells. (D) Infected immune cells (T-lymphocytes, neutrophils, B-cells, and macrophages) infiltrate the brain through the leaky BBB (Trojan horse pathway). (E) The virus may invade the olfactory nerve, persisting in the olfactory bulb before invading the CNS. (F) Mature virions or viral components may be transported in exosomes from infected lungs to the brain. Image created with BioRender.com.
Abbreviations: ACE2: Angiotensin-converting enzyme 2; BBB: Blood–brain barrier; CSF: Cerebrospinal fluid; TMPRSS2: Transmembrane protease serine 2.

to as "brain fog." Using dynamic contrast-enhanced magnetic resonance imaging, Greene *et al.*[57] demonstrated BBB disruption in these patients. Transcriptomic analysis of peripheral blood mononuclear cells revealed dysregulation of the coagulation system and suppression of the adaptive immune response in patients with brain fog. These cells exhibited increased adhesion to human brain endothelial cells *in vitro*. In addition, serum from long COVID patients induced the expression of inflammatory markers in brain endothelial cells. These findings suggest that long COVID-associated brain fog is linked to sustained systemic inflammation and persistent localized BBB dysfunction. This disruption, unique to patients with brain fog, observed up to a year after infection, was evident across multiple neuroanatomical regions, including the temporal lobes and frontal cortex.

Another intriguing hypothesis currently under investigation is peripheral immune cell transmigration, or the "Trojan horse" mechanism, wherein infected leukocytes – particularly monocytes and macrophages – serve as viral reservoirs and facilitate entry into the brain.[47,48,58] The human coronavirus 229E binds to human glycoprotein receptor 13 on dendritic cells, and it is speculated that SAR-CoV-2 may interact with this receptor.[47,59] Increasing evidence suggests that SARS-CoV-2 can initially infect peripheral nerve endings and enter the CNS through synapse-associated pathways, a process known as trans-synaptic transmission, which is well documented for hepatitis E virus 67.[47,60] Recent studies have also shown that virus particles can be passively released, diffused, and transported into cell cultures through axonal transport through axoplasmic flow.[47]

Overall, transmission through infected leukocytes and neuronal pathways plays a prominent role in viral persistence; however, additional evidence is needed to substantiate these mechanisms.[61] A recent report supports the olfactory pathway as a route for neuroinvasion, as studies in animal models have shown that the inhalation of coronaviruses leads to cerebral infection.[62] For instance, infection of mice with mouse hepatitis virus (MHV) through the olfactory pathway resulted in the infection of both the brain and muscle, with viral RNA detected in these tissues.[63] The olfactory route of infection is well documented in the human coronavirus-OC43 model,

where viral antigen was detected in the olfactory bulb 3 days after intranasal inoculation in mice. The virus then spreads to the cortex, mesolimbic cortex, hippocampus, amygdala, and finally to the brainstem and spinal cord within 7 days. Ablation of the olfactory bulb after nasal infection with MHV blocked further spread, supporting the theory of spread through the olfactory tract.[63]

An alternative route of transmission is through the vagus nerve and the GI tract, which may play a central role in the retrograde penetration of SARS-CoV-2 into the CNS.[64] Another important mechanism is the extracellular vesicular transport of SARS-CoV-2 or key components of its proteome from the site of primary infection to the CNS. Neuronally enriched extracellular vesicles, including exosomes from individuals with PASC, are enriched with markers of neurodegeneration, such as amyloid, low-molecular-weight neurofilament subunit protein, total tau, phosphorylated tau, and neurogranin. This enrichment suggests that these vesicles may play a critical role in the amplification of AD pathology in patients following COVID infection.[65,66] In addition, membrane-bound exosomes originating from the lungs, which contain transcription factors linked to neuronal gene regulation in Alzheimer's and Parkinson's diseases, have been documented to be transported into the brain through the trans-neuronal pathway.[67] Cumulatively, SARS-CoV-2 utilizes multiple mechanisms to disable and evade the host immune response, which ensures its persistence over an extended period (Table 2).

5. The role of SARS-CoV-2 proteins in viral persistence in the brain by manipulating immunoregulatory pathways

Numerous clinical studies on long COVID have shown that viral particles are present in the brain and affect brain architecture.[5,17,40] However, it remains crucial to understand how SARS-CoV-2 manipulates host immunoregulatory mechanisms, contributing to persistent neuroinflammation (Figure 3). The most important strategy of SARS-CoV-2, shared by other coronaviruses, is replicating within double-membrane vesicles. This strategy prevents the activation of retinoic acid-inducible gene 1 (RIG-I)-like receptors, which recognize viral double-stranded RNA intermediates.[68,69]

SARS-CoV-2 is not only adept at evading detection but also at disguising itself. Chen *et al.*[70] showed that the non-structural protein (Nsp) 14 of SARS-CoV-2 possesses guanine N7 methyltransferase activity, which allows it to mimic the cap structure on viral RNA. In addition, the Nsp16 protein of SARS-CoV-2 modifies this cap-like structure through its 2'O-methyltransferase activity, enabling the virus to evade recognition by melanoma differentiation-associated protein-5 (MDA5).[71]

Table 2. Mechanisms of neuroinvasion by SARS-CoV-2

Mode of invasion	Mechanism of invasion	References
Receptor-mediated pathway	The s-protein of SARS-CoV-2 binds to the ACE2 receptor and co-receptor neuropilin-1; host proteases such as TMPRSS2, cathepsin L, and furin help in the cleavage of the s-protein	44,46,47,50,51
Hematogenous pathway	SARS-CoV-2 disrupts the alveolar epithelial barrier following primary infection and reaches the CNS through bloodstream	45,46,48
Through blood-CSF barrier	The infection spreads from the blood–CSF barrier into the CNS	46,54
Through BBB barrier	Heightened interferon response and cytokine storm may alter the architecture of BBB, thus making it permeable to the virus	46,52,55
Trojan horse pathway	The infected leukocytes, monocytes, and macrophages may infiltrate into the CNS through BBB	46,56
Trans neuronal pathway	SARS-CoV-2 may initially infect the peripheral nerve endings and then enter the CNS through a synapse-connected route	46,58
The olfactory pathway	The viral particles reach the olfactory bulb via the nasal-epithelial pathway and then spread into the CNS	46,60
Through GI tract	The spike-protein of SARS-CoV-2 binds to an ACE2 receptor present on the epithelial cells lining the gut, and through retrograde axonal transport, it reaches the CNS	46,62
Extracellular vesicular transmission	Extracellular vesicles released from alveolar epithelial cells may contain viral genome or key components of viral proteome that reach to CNS by invading BBB or through trans-neuronal pathway	46,63,65

Abbreviations: ACE2: Angiotensin-converting enzyme 2; BBB: Blood–brain barrier; CNS: Central nervous system; CSF: Cerebrospinal fluid; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2; TMPRSS2: Transmembrane protease serine 2.

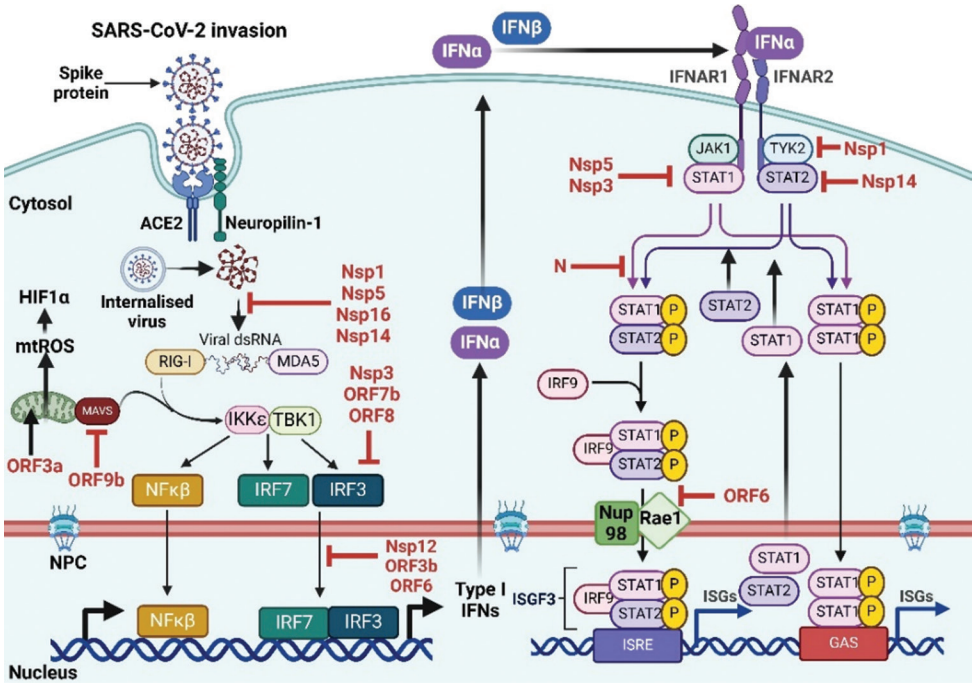

Figure 3. SARS-CoV-2 proteins orchestrate viral persistence in the brain by manipulating immunoregulatory pathways. The spike protein of SARS-CoV-2 interacts with cell surface receptors. Internalized viruses escape innate immune responses using Nsps. These Nsps impact host transcription factors such as NF-κβ, IRF7, and IRF3, which are critical for type I interferon response. Nsps also deregulate the JAK-STAT pathway and inhibit STAT3 translocation of STATs to the nucleus, preventing the activation of interferon stimulatory genes. Moreover, Nsps alter mitochondrial MAVS pathways, compromising host immunity for a longer period of time. Image created with BioRender.com.

Abbreviations: ACE2: Angiotensin-converting enzyme 2; GAS: GMP-AMP synthase; HIF1α: Hypoxia-inducible factor 1-alpha; IFN: Interferons; IFNAR1: Interferon alpha receptor; IFNAR2: Interferon beta receptor; IKKε: Inhibitor of nuclear factor κB kinase; IRF: Interferon regulatory factor; ISGF: Interferon stimulated gene factor; ISRE: Interferon stimulated response element; JAK1: Janus kinase 1; MAVS: Mitochondrial antiviral-signaling protein; MDA5: Melanoma differentiation-associated protein 5; mtROS: Mitochondrial Reactive Oxygen Species; N: Nucleocapsid; NF-κβ: Nuclear factor kappa-light-chain-enhancer of activated B cells; Nsp: Non-structural protein; NPC: Nucleopore complex; Nup98: Nucleoporin 98; ORF: Open reading frame; RIG-I: Retinoic acid-inducible gene I; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2; STAT: Signal transducer and activator of transcription; TBK1: TANK binding kinase; TYK2: Tyrosine kinase 2.

SARS-CoV-2 with mutated Nsp16, which is crucial for altering immunity, exhibits reduced virulence dependent on MDA5.[72] This camouflage strategy could play a pivotal role in allowing the virus to persist longer in brain cells. A study by Siu *et al.*[73] further revealed that the membrane protein of SARS-CoV-2 binds directly to innate sensors and sequesters them in the membrane-associated cytoplasm. Moreover, the membrane protein of SARS-CoV-2 inhibits type I and type III interferon (IFN) production by targeting RIG-I/MDA5 signaling.[74] Consequently, the Nsp3 protein of SARS-CoV-2 inhibits the phosphorylation and nuclear translocation of IFN regulatory factor (IRF) 3, impairing the induction of IFN-I and IFN-III responses. This mechanism, observed in SARS-CoV through open reading frame (ORF) 3a and in MERS-CoV through ORF4a, ORF4b, and ORF5,[66] has not yet been fully clarified in the SARS-CoV-2 model. However, the sequence identity between SARS-CoV and SARS-CoV-2 suggests that this mechanism could plausibly dampen the IFN signaling pathway, ensuring viral persistence in brain cells.[75] In addition, a recent study by Kehrer *et al.*[76] suggests that ORF6 of SARS-CoV-2 plays a crucial role by interfering with the nuclear import of transcription factors, such as IRF and signal transducer and activator of transcription. Specifically, ORF6 binds to nuclear pore complex proteins and ribonucleic acid export 1, thereby blocking the nuclear transport of mRNA in K18 human ACE2 transgenic mice and Syrian golden hamsters. Analogous to this observation, the ORF6 polymorphism observed in the Omicron subvariants, due to the D61L mutation, significantly disrupts ORF6 protein function at the nucleopore complex, impairing innate immune evasion and potentially affecting viral fitness.[76]

There is increasing evidence that viral proteins interfere with the host immune mechanism, creating an immunocompromised microenvironment in host tissues. This low-grade chronic inflammation is one of the main features associated with neurological disorders. The current studies do not provide direct evidence of how viral proteins regulate inflammatory responses in

the CNS, but it is worth hypothesizing that even if the viral genome is not present in the brain, viral remnants may create a degenerative environment that could be associated with long-term COVID symptoms. Although the functions of SARS-CoV-2 proteins are well described in the literature, there is a lack of insights into how individual SARS-CoV-2 proteins modulate the immune response in neurons, glial cells, and astrocytes (Table 3). An emerging theory is that acute inflammation in alveolar epithelial cells may contribute to neurological consequences, suggesting communication between organs. However, the available evidence is insufficient to confirm this phenomenon, and further investigation is needed to identify the unique viral signaling pathways that contribute to these outcomes.

6. SARS-CoV-2 persistence in the context of the aging microenvironment in the brain

Viral infections are increasingly being recognized as important contributors to the development of neurological disorders, with growing evidence suggesting they can mimic the aging microenvironment in the brain.[77] This phenomenon involves several key processes that overlap with the biological mechanisms of aging, including chronic inflammation, oxidative stress, mitochondrial dysfunction, impaired proteostasis, epigenetic alterations, cellular senescence, and gut-brain dysbiosis[8,78-80] (Figure 4). These processes are associated with the most prevalent neurodegenerative diseases, including AD, Parkinson's disease, amyotrophic lateral sclerosis, stroke, and several other neuropathies.[79,81] Recent reports suggest that SARS-CoV-2 infection may exacerbate protein aggregation, particularly amyloid-beta (Aβ) peptides and α-synuclein, which are major pathogenic hallmarks of AD and Parkinson's disease.[82] Furthermore, it has been suggested that the apolipoprotein E gene (*APOE4*), a significant risk factor for AD, could also serve as a biomarker for severe COVID-19.[83] Specifically, the type 4 allele of the gene (*APOE ε4*) is a major susceptibility factor for both AD and COVID-19.[84] Research has shown that the *APOE* genotype influences susceptibility to or resistance against pathogens in various infectious diseases. The protein products of the *APOE* cluster genes may even function as receptors for SARS-CoV-2, as they have been identified as receptors for several viruses, including herpesvirus and hepatitis C virus.[85] This attribute has been observed in both acute and mild cases of COVID-19. Moreover, the persistence of COVID-19 in the brain coincides with the features of pathological aging, potentially contributing to long-term neurological sequelae. Overall, SARS-CoV-2 infection not only mimics the aging microenvironment in the brain but also exacerbates the pathological features associated with neurodegenerative diseases. By triggering key events such as chronic inflammation, oxidative stress, impaired proteostasis, and other aging-like processes, the virus can accelerate the onset and progression of diseases such as Alzheimer's and Parkinson's diseases.[82,86] Understanding these mechanisms underscores the importance of addressing the long-term consequences in COVID-19 patients and reinforces the need for targeted therapeutic strategies to mitigate these effects.

6.1. Chronic inflammation and COVID-19: Enduring fire in the brain

The pathophysiology of PASC, particularly its association with neurological sequelae after mild or moderate SARS-CoV-2 infection, is still largely unexplored. One of the simplest explanations for accelerated age-related neurodegeneration in PASC is chronic neuroinflammation. The viral persistence of the virus can trigger prolonged inflammatory responses in the brain.[87] Interaction with specific receptors activates brain cells – including neurons, oligodendrocytes, and microglia – which amplifies immune signaling and leads to the release of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6.[88] COVID-19 brains have shown degeneration and extensive inflammation, even in individuals without neurological symptoms, and overlap has been observed between AD marker genes and genes upregulated during COVID-19 infection. Inflammatory biomarkers such as TNF, IL-6, IL-1, complement proteins, and galectin-3 have been proposed as common prognostic biomarkers for both SARS-CoV-2 infection and AD.[89] In addition, viral infection can activate the brain's resident T lymphocytes, further increasing neuroinflammation and neurodegeneration, thereby accelerating brain aging.[87] In individuals with long COVID, T-cell alterations have been found, including an increased population of exhausted T cells, decreased numbers of $CD4^+$ and $CD8^+$ effector memory cells, and increased expression of programmed death-1 on central memory cells, persisting for at least 13 months.[90] Type I and type II IFNs also persist for at least 8-month post-infection.[87] Infected individuals with long COVID also exhibit increased non-classical monocytes, activated B cells, double-negative B-cells, and elevated levels of IL-4 and IL-6, while the number of conventional immune cells decreases.[91] In conjunction with these T-cell changes, patients with PASC show a dysregulated pro-inflammatory cytokine profile in their blood, with increased levels of IL-1β, IL-6, and TNF-α-cytokines known to increase in the aging brain.[87,88] A contemporary study by Ng *et al.*[92] has demonstrated that IFN-β has a profound effect on viral persistence. While IFN-α controls early viral spread, blocking of IFN-β improves

Table 3. Postulated function of SARS-CoV-2 proteins in post-acute consequences of COVID-19

SARS-CoV-2 proteins	Function in SARS-CoV-2 replication	Plausible mechanism contributing to neurodegeneration	References
Nsp1	Promotes host mRNA degradation; downregulates host gene expression; binds to the small subunit of ribosome and blocks host translation and suppresses the innate immune response; suppresses apoptosis	May promote viral replication and persistence; translation block of host protein led to unfolded protein accumulation and neurodegeneration	137,139
Nsp2	Interacts with the proteins GIGYF2, EIF4E2 and ZNF598, which act as translation inhibitors	Increased viral protein load may cause impaired UPR response and promote misfolded protein accumulation	70,137,139
Nsp3	Facilitates mRNA transcription and translation while suppressing host protein synthesis; interacts with nucleocapsid protein; PLPro/deubiquitinase domain; cleaves polyprotein pp1a and pp1ab	May accelerate ER stress, oxidative stress, a major contributor to age-associated neurodegeneration	
Nsp4	Transmembrane scaffold protein with a prominent role in vesicle formation; induce morphogenic changes in ER membrane; assembly of replicative structures	Nsp4 induces mitochondrial dysfunction and autophagy deregulation, thereby perturbing proteostasis, the primary cause of age-associated neuronal loss.	137,139
Nsp5	Role in RNA replication and double membrane; Nsp5 acts as an inhibitor of the RIG-1- MAVS–IFN pathway by proteolytically cleaving the 10 N-terminal amino acids from RIG-I, thereby inhibiting MAVS activation; Nsp5 can increase MAVS stability through SUMOylation, activating the NF-κB signaling pathway and promoting the expression of inflammatory cytokines; nsp5 reduce avSG formation	Promote viral replication and persistence through vesicular trafficking, thus inducing neuroinflammation and inflammaging	137,139
Nsp6	Together with Nsp3 and Nsp4, it connects the double membrane vesicles to form the replication complex and interacts with ATP6AP1, disrupting lysosome acidification and consequently impairing autolysosome formation, thereby activating the inflammasome response	Promotes mitochondrial dysfunction and impaired proteostasis, which may trigger the formation and accumulation of oligo-peptides, neurofibril tangles	137,139
Nsp7	Forms complex with Nsp8; act as a primer-independent RNA polymerase	Promote viral replication and persistence through vesicular trafficking, thus inducing neuroinflammation and inflammaging	137,139
Nsp8	In association with Nsp7, it has primase activity	RNA processing enhances liquid phase transition and promotes mitochondrial dysfunction, thus generating oxidative stress in the brain cells	137,139
Nsp9	ssRNA binding protein phosphatase; acts as a host virulence factor; together with Nsp8, binds to signal recognition particle (SRP) and suppresses membrane protein trafficking in the host cells	Impaired host protein trafficking may impair proteostasis	137,139
Nsp10	Activates Nsp14 and Nsp16, thus forming a ternary complex		137,139
Nsp11	Required for replication; possible role in ribosomal frameshift	May promote viral persistence and replication in the brain	137,139
Nsp12	RNA-dependent RNA polymerase; coding sequencing contains the ribosomal frameshift	May promote viral persistence and replication in the brain	137,139
Nsp13	Involved in the initial steps of RNA capping at the 5'-terminus of viral RNA; prevents Tank binding kinase (TBK1) phosphorylation, therefore, blocking IFN-I response	Promote versicular trafficking of the viral genome in a host; helps in viral disguising, thus ensuring viral persistence	137,139

(Cont'd...)

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

SARS-CoV-2 proteins	Function in SARS-CoV-2 replication	Plausible mechanism contributing to neurodegeneration	References
Nsp14	Shows exoribonuclease activity; involved RNA capping	N7 methyltransferase activity mimics the cap structure on the true viral RNA, thus making it unrecognized by the host immune system for a long period of time	70,137,139
Nsp15	Endoribonuclease activity, cleaving RNA substrate at the 3' of uridines, evading detection by the innate immune response	Escaping host detection ensures viral persistence for a longer time in brain cells	71,137,139
Nsp16	Responsible for methylating the 5'-end of viral mRNAs, generating cap1 structures that shield viral mRNA from MDA5 recognition	Ensure viral persistence in the brain and compromised immune response; promote viral replication and neurodegeneration	70,137,139
Spike	Virus-host cell membrane fusion protein binds to ACE2 and co-receptors	Highest affinity towards ACE2 receptors on the cell surface, thus promoting viral invasion and persistence in the brain; Nsp1 binds to the cleaved S1 domain of S protein and amplifies the process of internalization, inducing lipid modification, which may impact BBB permeability	137,139
Nucleocapsid	Viral genome packaging binds to chemokines with the ability to block chemotaxis of immune effector cells	May promote chronic neuroinflammation by blocking the chemotaxis of immune effector cells	137,139
Envelope	Viroporin functions as a ligand for TLR2, promotes cytokine production, and has an impact on spike protein processing and maturation by promoting its retention in the ER-Golgi intermediate compartment	May promote chronic neuroinflammation and ER-stress	137,139
Membrane	Inhibit IFN-antiviral response; interacts with RIG-I/ MDA-5-MAV	May promote chronic neuroinflammation	73,74,137,139
Orf3a	Viroporin is involved in virus replication; activates NLRP3 inflammasome complex and IL-1β release; promotes phagophore nucleation by favoring PI3KC3-C1 formation; activates UPR by activating ATF6; promotes the activation of NF-κB, TLR3 or TLR4, promotes the production of HIF-1α through the generation of ROS in the mitochondria and subsequent mitochondrial disruption; enhances the production of pro-inflammatory cytokines IFN-β, IL-6, and IL-1β.	Activation of NLRP3 inflammasome may result in microglial activation and pyroptotic cell death or sustained pro-inflammatory response associated with age-associated neurodegeneration.	137,139
Orf3b	IFN antagonist	Promote chronic neuroinflammation	75,137,139
Orf3c	ORF3c localizes to mitochondria and interacts with both MAVS and PGAM5 (mt serine/threonine protein phosphatase); ORF3c expression leads to a reduction in IFNB transcripts and IFN-β protein levels	Mitochondrial dysfunction may lead to ROS production and impaired glycolysis, leading to neuronal death or deregulation	75,137,139
Orf3d	Suppress immune activation by an unknown mechanism		137,139
Orf6	Binds to NPC proteins (Nup98-Rae1) to inhibit STAT1 and IRF3 cytosolic-nuclear translocation thus blocking IFN signaling;	Generate immune compromised microenvironment; induce chronic neuroinflammation	75,76,137,139
Orf7a	Interacts with human $CD14^+$ monocytes, leading to the limitation of antigen presentation; promotes the up-regulation of certain chemokines such as CCL11, CCL17, CCL19, CCL21, CCL22, CCL25, CCL26, CCL27	Altered cytokine and chemokine profiles may induce CNS as well as PNS dysfunction, including cognitive and motor impairments	137,139

(Cont'd...)

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

SARS-CoV-2 proteins	Function in SARS-CoV-2 replication	Plausible mechanism contributing to neurodegeneration	References
	and CXCL9; promotes cytokine production such as IL−1α, IL−1β, IL−6, IL−8, IL−10, TNF−α, and IFNβ; inhibits the signaling IFN-I by blocking the phosphorylation of STAT2		
Orf7b	Transmembrane protein promotes apoptosis via TNFR1, TNF-α, and caspase 8	Increased neuronal damage	137,139
Orf8	Downregulates MHC-I; interacts with several ER proteins; IFN-1 response antagonism; evasion of host innate immune response	Generating ER-stress-induced ROS production and sustained neuroinflammation	137,139
Orf9a	single-stranded RNA-binding protein, which displays an oligosaccharide/oligonucleotide binding fold	May promotes viral persistence and replication in the brain	137,139
Orf9b	Suppression of innate immunity by targeting MAVS signalosome	Mitochondrial dysfunction which may cause ROS leakage and subsequent events leading to neurodegeneration	98,137,139
Orf9c	Membrane-associated protein that suppresses antiviral response by interference with IFN signaling and antigen presentation	No prominent role; may promote low-grade inflammation in the brain	137,139
Orf10	Suppress IFN signaling, inhibits antigen processing and presentation, complement signaling, IL-6 signaling; degradation of MAVS	No prominent role; may promote low-grade inflammation in the brain	137,139

Abbreviations: ACE2: Angiotensin-converting enzyme 2; avSG: Antiviral stress granule; ATF6: Activating transcription factor 6; ATP6AP1: ATPase H+ transporting accessory protein 1; CCL: C-C motif chemokine ligand; CD: Cluster of differentiation; CNS: Central nervous system; CXCL9: C-X-C motif chemokine ligand 9; EIF4E2: Eukaryotic translation initiation factor 4E family member 2; GAS: GMP-AMP synthase; GIGYF2: GRB10 interacting GYF protein 2; HIF1α: Hypoxia-inducible factor 1-alpha; IFN: Interferons; IFNAR1: Interferon alpha receptor; IFNAR2: Interferon beta receptor; IKKε: Inhibitor of nuclear factor κB kinase; IL-1β: Interleukin-1beta; IRF: Interferon regulatory factor; ISGF: Interferon stimulated gene factor; ISRE: Interferon stimulated response element; JAK1: Janus kinase 1; MAVS: Mitochondrial antiviral-signaling protein; MDA5: Melanoma differentiation-associated protein 5; MHC-I: Major Histocompatibility-1; mtROS: Mitochondrial reactive oxygen species; N: Nucleocapsid; NF-κβ: Nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3: Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; NPC: Nucleopore -complex; Nsp: Non-structural protein; Nup98: Nucleoporin 98; ORF: Open reading frame; PGAM5: Phosphoglycerate mutase 5; PI3KC3-C1: Phosphatidylinositol (PI) 3-kinase complex I; PNS: Peripheral nervous system; RAE1: Ribonucleic acid export 1; RIG-I: Retinoic acid inducible gene I; ROS: Reactive oxygen species; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2; SRP: Signal recognition particle; STAT: Signal transducer and activator of transcription; TBK1: TANK binding kinase; TLR: Toll-like receptors; TNF-α: Tumor necrosis factor alpha; TNFR1: Tumor necrosis factor receptor 1; TYK2: Tyrosine kinase 2; ZNF598: Zinc finger protein 598.

T-cell responses and accelerates the clearance of persistent viruses in a lymphocytic choriomeningitis virus-infected mouse model. These observations suggest that mild or acute SARS-CoV-2 infection alters the T-cell profile and increases the number of circulating immune cells, potentially leading to immune cell infiltration into the brain through the BBB. Emerging evidence suggests that repeated mRNA vaccination may be linked to a proinflammatory response and an increase in immunoglobulin G4 (IgG4) levels. Rather than providing protection, this rise in IgG4 could indicate immune tolerance to the spike protein, potentially allowing unopposed SARS-CoV-2 infection and replication by weakening natural antiviral defences.[93] In addition, elevated IgG4 synthesis from repeated vaccination with high antigen doses might contribute to the development of conditions such as cerebral venous sinus thrombosis, Guillain–Barré syndrome, and stroke.[94] A recent study by Mavrikaki *et al.*[78] revealed molecular signatures of aging in post-COVID brains. Transcriptomic analysis of the human frontal cortex, a region central to cognitive function, revealed downregulation of genes related to synaptic function and cognition and upregulation of genes involved in the immune pathways. Specifically, genes encoding S100 calcium-binding protein A9, myosin light chain 12A, and rho-related BTB domain-containing protein 3 were upregulated, while those encoding calmodulin 3, inositol polyphosphate 4-phosphatase type I A, glutamate ionotropic receptor AMPA type subunit 1, and glutamate [NMDA] receptor subunit 3A were downregulated, reflecting patterns commonly observed in the aging brain.[78] Cumulatively, the available data suggest a link between neuroinflammation induced by viral persistence and the aging process. However, it remains intriguing to explore how individual viral components modulate host immune mechanisms in favor of viral propagation.

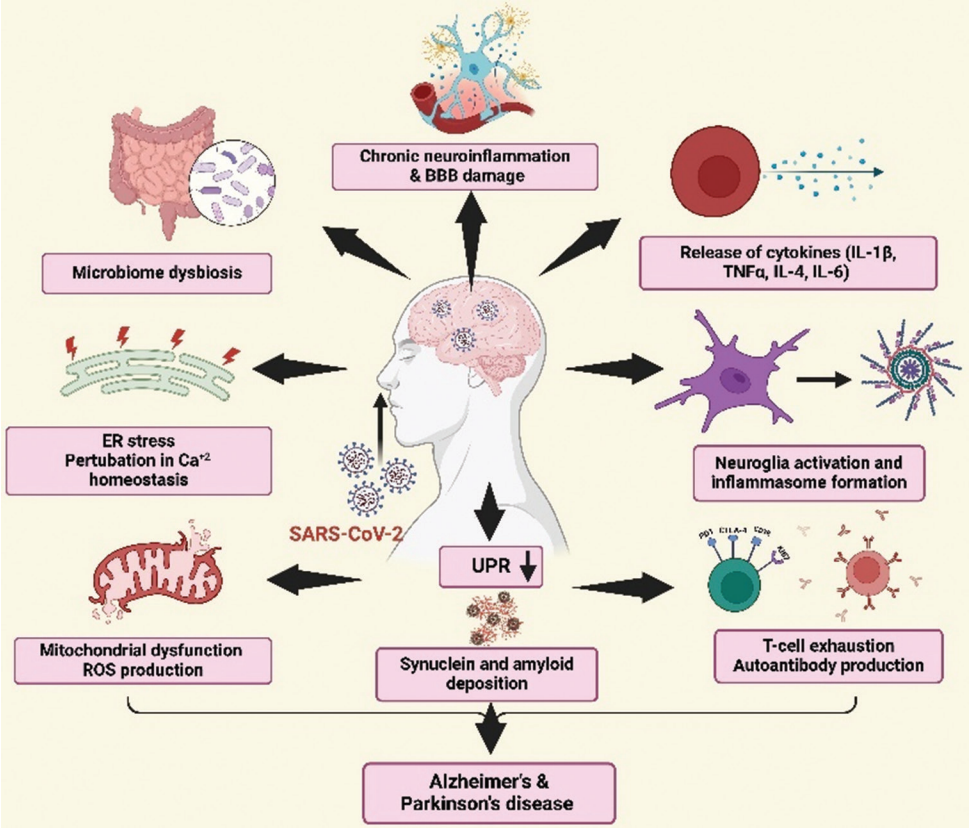

Figure 4. SARS-CoV-2 persistence mimics the aging microenvironment in the brain. Viral persistence in the brain or distant organs induces an aging-like environment in the brain. SARS-CoV-2 infection accelerates the hallmarks of aging, which include sustained neuroinflammation, prolonged cytokine release, microglia activation, T-cell exhaustion, autoimmunity, deregulated UPR followed by plaque formation, increased oxidative stress, ER stress, and microbiome dysbiosis. All these events cumulatively promote neuronal loss and neurodegeneration. Image created with BioRender.com.
Abbreviations: BBB: Blood–brain barrier; CD38: Cluster of differentiation 38; CTLA-4: Cytotoxic T lymphocyte antigen-4; ER: Endoplasmic reticulum; IL: Interleukin; Ki67: Kiel 67; PD1: Programmed cell death protein 1; ROS: Reactive oxygen species; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2; TNF-α: Tumor necrosis factor alpha; UPR: Unfolded protein response.

6.2. COVID-19-induced mitochondrial dysfunction and oxidative stress: Fast-tracking brain aging

Oxidative stress and mitochondrial dysfunction play a central role in the pathogenesis of age-associated neurodegenerative diseases.[95] Prolonged neuroinflammation due to viral infection can increase the formation of reactive oxygen species (ROS), which in turn cause mitochondrial dysfunction and further contribute to neuroinflammation.[96] SARS-CoV-2's Nsps can interact with mitochondrial proteins, evading the mitochondria-mediated innate immune response and establishing infection. A recent report by Duan *et al.*[97] suggests significant changes in mitochondria-related gene expression and metabolic pathways in COVID-19 patients after analyzing RNA sequencing data collected from lung tissue and blood samples. Moreover, the SARS-CoV-2 protein ORF9b can directly alter mitochondrial function to evade host cell immunity.[98] Together with ORF9b, ORF6 impairs mitochondrial antiviral signaling (MAVS) protein function and suppresses the innate immune response.[78] Disruption of mitochondrial dynamics also induces the release of mitochondrial DNA release, which can trigger microglial activation and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome formation.[99] These sequential events lead to persistent neuroinflammation and neuronal damage. In addition, tau aggregation neurodegeneration may be caused by the activation of the NLRP3 inflammasome, triggered during SARS-CoV-2 infection.[27] Another hypothesis suggests that the increased risk of AD in COVID-19 patients could be related to Aβ, which acts as an antimicrobial peptide. It is postulated that the SARS-CoV-2 neuroinvasion could promote Aβ generation as part of the immune response, initiating the Aβ cascade and leading to extracellular Aβ deposition.[100] Furthermore, compared to control patients, patients with AD exhibit higher levels of ACE2 receptor expression. Notably, ACE2 expression is not influenced by age, indicating a potential link between ACE2 expression and AD.[101]

Interestingly, the SARS-CoV-2 protein ORF3a is involved in the activation of hypoxia-inducible factor 1-α (HIF1α) by inducing mitochondrial damage and the production of mitochondrial ROS. HIF1α, a master regulator of glycolysis and other metabolic pathways, subsequently enhances viral replication and the pro-inflammatory response.[102] Ajaz *et al.*[103] investigated functional alterations in mitochondria within living peripheral blood mononuclear cells from patients with COVID-19, revealing changes in the immune system that suggests oxidative stress mediated by mitochondrial dysfunction contributes to inflammaging-like features in long-term COVID situations. In addition, Prasada Kabekkodu *et al.*[104] have suggested that SARS-CoV-2 proteins may localize in the mitochondria, increasing mitochondrial leakage and interfering with Ca^{2+} signaling. Calcium homeostasis plays a crucial role in synaptic transmission, synaptic plasticity, apoptosis, and cell survival, all of which may be linked to cognitive decline and motor dysfunction.[104]

An emerging theory describes how MAVs binding to RIG-I inhibits the interaction of MAVs with hexokinase, thereby impairing glycolysis.[105] Carpenè *et al.*[106] have demonstrated that lactate levels in the blood of COVID-19 patients were significantly higher than those of control subjects. Since cells of the CNS have high energy demands, such disruptions in energy metabolism due to viral infection not only prolong the presence of viral components in the body but also promote slow and persistent neurodegeneration. Furthermore, the induction of pattern recognition receptors and the IFN-1 signaling pathway leads to increased ROS production by xanthine oxidase, nitric oxide synthase, and the mitochondrial respiratory response.[107] SAR-CoV-2 can activate NADPH oxidase 2 through toll-like receptor-7, which inhibits the immune response while increasing ROS levels and supporting viral infection.[108] This overproduction of ROS can lead to the oxidation of phospholipids and macromolecules, altering BBB permeability. In particular, the binding of the viral spike protein to ACE2 leads to excessive production of angiotensin II, which activates NADPH oxidase, further enhancing oxidative stress.[109] The production of excessive cytokines, including IL-1β, IL-2, and IL-6, stimulates ROS, reactive nitrogen species, and the formation of oxygen radicals.[88] According to the "free radical theory" of aging, the accumulation of ROS not only accelerates aging but also impairs protein folding responses, potentially leading to cognitive and motor deficits.[110] Overall, it can be speculated that the persistence of SARS-CoV-2 and its components in the brain induces a vulnerable state that may accelerate the onset of age-related neurodegeneration.

6.3. Proteostasis perturbations in long-term COVID and accelerated brain aging

Proteostasis is a critical factor in the interplay between viral infection and the onset of neurodegenerative diseases. Viruses, including SAR-CoV-2, often manipulate host proteostasis mechanisms to facilitate their own translation.[10] Upon infection with COVID-19 infection, increased inflammatory response, ROS generation, and endoplasmic reticulum (ER) stress due to high viral protein expression lead to elevated levels of heat shock proteins, triggering cellular apoptosis and necrosis. Unfolded protein response (UPR) is primarily driven by three different factors, including protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1α, and activating transcription factor 6 (ATF6), all of which respond to ER-membrane stress. Dysregulation of ER stress-induced UPR plays a critical role in age-related neurodegeneration.[111] Emerging evidence suggests that the spike protein of SARS-CoV-2, or ORF8, is sufficient to activate UPR.[112] Conversely, the N-terminal fragment of NSP3, namely NSP3.1, interacts with ATF6 and suppresses ATF6-mediated UPR pathways. Similarly, MHV triggers the activation of X-box binding protein 1 and the PERK pathway, while hindering UPR-responsive genes such as pro-apoptotic transcription factor C/EBP homologous protein (*CHOP*).[81] In acute COVID-19 patients, host heat shock proteins, such as GRP78, GRP94, binding IgG protein, protein disulfide isomerase, calreticulin, and calnexin, are required for the rapid replication of viral proteins.[113] However, excessive viral protein expression increases the percentage of unfolded and misfolded proteins in the ER lumen, inducing ER stress and UPR. Deregulation of proteostasis pathways due to high viral replication can lead to the formation of oligomers, amorphous aggregates, and amyloid fibers, which are clinical hallmarks of age-associated neurodegenerative diseases.[81] A recent report by Lee *et al.*[81] has described the upregulation of apoptosis-associated genes, such as FAS-associated protein with death domain, Bcl-2-like protein 4 (*BAX*), BH3 interacting death domain, and Caspase 9, as well as autophagy-regulating genes such as WD-repeat β-propeller 45 protein and tectonin beta-propeller repeat containing 2 in human dopaminergic neurons following human preformed fibril treatment. These effects were further amplified with SARS-CoV-2 infection.[81] In contrast, the expression of key autophagy marker proteins, such as microtubule-associated proteins 1A/1B light chain 3B and sequestosome-1 (SQSTM1/P62), was downregulated upon SARS-CoV-2 infection.

Although direct evidence linking proteostasis deregulation to long COVID-related neurological manifestations remains insufficient, it is clear that ER

stress-induced UPR deregulation plays a pivotal role in age-associated neurodegeneration. SARS-CoV-2 proteins, including the spike protein, ORF8, and NSP3.1, significantly impact UPR pathways, potentially exacerbating the formation of protein aggregates characteristic of neurodegenerative diseases such as Parkinson's disease, AD, and amyotrophic lateral sclerosis. These findings underscore the importance of understanding how viral influences on cellular stress responses may accelerate the onset or progression of these debilitating conditions.

6.4. Gut–brain dysbiosis in COVID-19: Implications for neurodegenerations

In the intricate harmony of human health, the gut microbiome plays a crucial role in maintaining brain function. Over the past few decades, it has become evident that viral infections, particularly SARS-CoV-2, have a profound impact on the mammalian gut.[64] Impairment of the gut microbiota can trigger numerous neurological signals that may lead to long-term neurological sequelae in COVID-19 patients.[114] These mechanisms are regulated by inflammatory cytokines, GI hormones, neurotransmitters (e.g., 5-hydroxytryptamine), and short-chain fatty acids secreted by intestinal epithelial cells.[115]

The microbiota–gut–brain axis is a bidirectional communication pathway that influences inflammatory signaling.[116] Gut-brain dysbiosis is often associated with the manifestation of neurological disorders, including AD, Parkinson's disease, and amyotrophic lateral sclerosis.[117] During the course of the COVID-19 infection, viral replication can occur within the GI tract for an extended period. Once the infection resolves, microbiota dysbiosis may develop. This imbalance in gut bacterial populations can lead to long-term symptoms. SARS-CoV-2 enters the intestine through ACE2 receptors located on the brush border of enterocytes in the small intestine, making these cells susceptible to infection.[118] ACE2 also forms a complex with the amino acid transporter B0AT1, which mediates the uptake of tryptophan into intestinal cells.[119] Conversely, mTOR, an essential sensor of intracellular amino acids, regulates gut microbial composition. Thus, the downregulation of the ACE2 receptor due to COVID-19 infection reduces the secretion of antimicrobial peptides, further enhancing peripheral immune deregulation.[120]

Although substantial evidence is still lacking regarding increased susceptibility to chronic neurological diseases, such as Parkinson's disease, following COVID-19 infection, it is speculated that SARS-CoV-2 infection could predispose patients to long-term neurological disorders. Such an effect could occur through ACE2 dysfunction, which may compromise the integrity of the intestinal barrier. This event may lead to elevated levels of circulating lipopolysaccharides and the subsequent formation of α-synuclein deposits in the enteric nerve.[121] Viral persistence in intestinal cells or heightened inflammation may also decrease the abundance of commensal bacteria, such as Ruminococcaceae and Lachnospiraceae, while inducing the colonization of pathobionts, such as Enterobacteriaceae and Desulfovibrionaceae.[122] This phenomenon is associated with increased levels of peripheral inflammatory cytokines in the gut, including IL-6, TNF-α, C-reactive protein, IL-1β, and IL-2, which are correlated with psychiatric and neurodegenerative disorders. Notably, these peripheral cytokines can reach the CNS through the BBB or through the circumventricular organs, contributing to microglial activation.[123,124]

Along with the gut-brain axis, the gut-lung axis also plays a critical role in neurological manifestations.[124] Multi-omics data collected from COVID-19 patients suggest that acute SARS-CoV-2 infection not only impacts species abundance and diversity but also impairs secondary metabolite production, which may directly affect neurotransmitter release and synapse formation.[122] Therefore, treatments with prebiotics, probiotics, and fecal microbial transplantation could open up new therapeutic avenues. However, further evidence showing a direct correlation between long COVID and gut dysbiosis is essential.

7. Advancement in therapeutic approaches for neurological disorders associated with long-term COVID

The emergence of SARS-CoV-2, which triggered COVID-19-associated neurological deficits, stands as one of the greatest medical emergencies of recent times.[17] Substantial investment in life sciences over the past few decades has facilitated a prompt scientific response, including advancements in viral characterization, testing, and sequencing.[17] This rapid progress permitted the development of highly effective vaccines within a short period, offering partial protection. However, contrary to popular belief, available drug treatments have delivered limited benefits so far. Due to the enormity of the pandemic, clinical trials primarily focused on acute symptoms, leaving some potential therapies insufficiently tested. At present, published therapies provide evidence supporting the treatment of early symptoms, but the understanding of COVID-19's impact on complex organs like the brain remains limited. Therapeutics for long-term COVID-associated neurological symptoms are under investigation, including anti-viral therapies, anti-inflammatory therapies, neutralizing antibodies, and anti-coagulant therapies.[125]

Significant efforts are underway to identify biomarkers associated with biological aging, which could serve as

prognostic tools for evaluating the risk of developing specific age-related neurological diseases. One promising strategy involves repurposing existing drugs, which are not only cost-effective but also may partially mitigate long-term impact.[17,125] Drugs such as remdesivir (a polymerase inhibitor initially developed for hepatitis C virus) and molnupiravir (originally developed for Venezuelan equine encephalitis and influenza) have efficiently reduced hospitalization and death, but their long-term efficacy is yet to be evaluated.[126] Other drugs targeting SARS-CoV-2 proteins, such as polymerase, helicase, replication-transcription complex, proofreading mechanisms, and 5'-capping, are under investigation.[125,127] Developing pharmacological agents targeting these pathways could be beneficial in preventing viral persistence in brain cells, although clinical trials should focus on the efficacy of such drugs in crossing the BBB.

An innovative approach involves a soluble recombinant form of the ACE2 receptor, which prevents the viral spike protein from binding to cell surfaces and reduces viral load *in vivo*.[128] Inflammatory mediators, including IL-1, IL-2, 1L-6, 1L-7, 1L-1β, granulocyte-macrophage colony-stimulating factor, and IFNs, play critical roles in chronic neuroinflammation, encephalitis, and increased risk of age-related disease. IL-6 concentrations, in particular, correlate with viral load and are elevated in acute cases. Early studies with tocilizumab, which binds to IL-6 receptors, showed mixed results, suggesting IL-6 could be a prognostic marker for long-term neurological sequelae.[129] Future clinical trials should encompass patients with acute COVID-19, mild COVID-19, and long COVID-19 to compare cytokine profiles and cognitive symptoms. This approach could help identify novel tissue-specific biomarkers that could mitigate the long-term impact of SARS-CoV-2 on the CNS.

Janus kinase inhibitors target key pro-inflammatory cytokines and provide transient protection against excessive cytokine release.[130] Clinical trials targeting other inflammatory mediators are underway; however, the most commonly used monoclonal antibody against TNF – a valuable marker of brain aging – has not yet been decisively evaluated for long COVID patients.[131] Special attention should be given to understanding the complex interaction between the immune system and the coagulation pathway, which could lead to microclot formation in brain blood vessels, increasing the risk of ischemic stroke and other neurological complications.[132]

Previous research into biomarkers suggests that levels of extracellular vesicles, immune markers, and oligopeptides may be indicative of long-term COVID-associated neurodegeneration.[17,66] Discovering biomarkers that indicate immunosenescent phenotypes would be highly beneficial for developing new therapeutics and targets.[18] Past studies reported the presence of Aβ42 and Aβ40, described as abnormal neuro-PASC in the CSF, which indicates impaired amyloid processing in long-term COVID patients.[87,133] Peripheral biomarkers for CNS injury, such as plasma neurofilament light chain and plasma glial fibrillary acidic protein, could serve as prognostic markers for long-term COVID-associated CNS impairment.[134] Other emerging approaches to mitigate the risk of long-term COVID-associated neurological diseases include antioxidant therapy, probiotics, and small-molecule inhibitors. For instance, antioxidants such as resveratrol, ascorbic acid, Q10, fisetin, and quercetin have been shown to improve the redox environment post-COVID.[17] Available treatments with antioxidants and probiotics may help restore intestinal flora, potentially reducing the peripheral inflammatory response and ameliorating chronic neuroinflammation (Table 4).

The SARS-CoV-2 pandemic has exposed significant gaps in our understanding of viral persistence and its effects on brain aging. While traditional research on neuro-aging focused on neurotropic viruses, the concept of "neuro-COVID" has emerged, illustrating how COVID-19 can impair neurological function and cognitive abilities. By combining recent advancements with existing knowledge, researchers have the potential to improve the diagnosis, treatment, and management of COVID-19-associated neurodegeneration, ultimately enhancing the quality of life for affected individuals.

8. Discussion and future directions

The severe outcomes of SARS-CoV-2 infection are likely driven by a pathological hyperinflammatory response, initiating unregulated local tissue damage, systemic cytokine storm, vascular leakage, and thrombosis. These events contribute to both immediate symptoms and neurological deficits.[1,2,135,136] As described earlier, viral invasion of the brain can occur through multiple routes. Viral persistence in the brain is speculated to be caused by (i) early defects in the IFN-1 response, which fails to clear the pathogen or its remnants from the primary site of infection and blood; (ii) failure of the host immune system to recognize the processed viral genome; and (iii) dysregulated immune cell infiltration into the brain.[137] Despite these insights, the exact molecular mechanisms associated with progressive neuronal loss remain unclear, necessitating direct experimental evidence to evaluate the role of viral proteins in neurodegeneration.

Studies on long-term COVID suggest the potential involvement of neurological autoimmune diseases,

Table 4. Advancement of therapeutics in PASC

Therapeutics	Rationale	Plausible role in mitigating PASC	References
Anti-viral therapies			
Anakinra	Anti-IL-1 mAb	Reduce neuroinflammation	17
COVID-19 mRNA booster vaccine	Boost immunity to augment viral clearance	Clear viral remnants and reduce neuroinflammation	17
Infliximab	mAb to clear viral reservoir	Clear viral remnants and reduce neuroinflammation	17
Paclovid	Anti-viral to clear viral reservoir	Clear viral remnants and reduce neuroinflammation	17
Remdesivir	Polymerase inhibitor	Inhibit viral replication; reduce viral load	17,81
Molnupiravir	Polymerase inhibitor	Inhibit viral replication; reduce viral load	17,126,127
SCB-2019	COVID-19 protein vaccine to augment viral clearance	Clear viral remnants and reduce neuroinflammation	17
Siltuximab	Anti-IL-6 mAb	Reduce neuroinflammation	17
Vitamin D	Boost immunity	Promote viral clearance	17
Anti-oxidant therapies			
Fisetin	Entry inhibitor	Reduce viral persistence and neuronal damage	17
Resveratrol	Activate SIRT1 and improve mitochondrial function	Improve energy metabolism, mitochondrial function, and prevent ROS production	17
Quercetin	QR inhibits viral entry, absorption, and penetration	Reduce viral persistence and neuronal damage	17
Modulating gut microbiota			
Fecal microbial transplantation	Reduce dysbiosis	Improve microbiome diversity; reduce PNS inflammation; improve neurotransmitter release	17,117
Probiotics	Reduce dysbiosis	Improve microbiome diversity; reduce PNS inflammation; reduce pathobiont population	17,116,117

Abbreviations: COVID-19: Coronavirus disease-2019; IL: Interleukin; mAb: monoclonal antibody; PASC: Post-acute sequelae of COVID-19; PNS: Peripheral nervous system; ROS: Reactive oxygen species; SIRT1: Silent mating type information regulation 2 homolog.

driven by the excessive production of autoantibodies during the infection.[11] However, this aspect is still under-researched and requires more attention. Most of the available data was collected during the pandemic, a period characterized by severe restrictions, limited social interactions, and reduced access to rehabilitation facilities. Therefore, it is difficult to distinguish between the direct effects of COVID-19 and the effects of pandemic-related stress. Recent advances in neuroscience, glial biology, and neuroimmunology have significantly contributed to our understanding of neurological deficits associated with long-term COVID.[17,18,87,91] Insights gained from other viral models, such as H1N1, human immunodeficiency virus (HIV), and herpes simplex virus, which are known to induce cognitive impairment, aging, and neurodegenerative diseases, are particularly valuable.[138] These studies reveal several common pathways that can accelerate our understanding and the development of therapeutic interventions for long-term COVID-associated neurological sequelae.

Emerging theories propose that long-term COVID-associated oxidative stress, mitochondrial

dysfunction, and hypoxia contribute to neurological deficits, opening new avenues for combating these symptoms.[95,102,107] Oxidative stress and mitochondrial dysfunction are critical factors in neurodegenerative diseases, and their association with long-term COVID suggests that targeting these pathways could alleviate neurological symptoms. In addition, hypoxia, resulting from impaired respiratory function, exacerbates neuronal damage and contributes to cognitive deficits.[128] Addressing these factors through targeted therapies could alleviate the neurological burden of long-term COVID-19.

In the future, fundamental research will drive the development of therapies that can be tested in larger clinical trials. Therapies that have shown promise in treating HIV- and H1N1-related cognitive impairment should be evaluated for their effectiveness against neuro-COVID.[138] One potential strategy involves resetting microglia to their homeostatic, non-reactive states, which could benefit a wide range of neurological disorders. In addition, enhancing neural plasticity mechanisms, even in the presence of ongoing neuroinflammation, could help alleviate cognitive symptoms. Another critical area of research is determining whether inflammation is driven by persistent viral infection or viral remnants in the lungs, intestinal tract, and other reservoirs. Identifying the root cause of distal inflammation could help reduce the neurological burden of long-term COVID-19 by eliminating the primary source of inflammation. This approach necessitates comprehensive studies on viral persistence and the long-term effects of SARS-CoV-2 infection on various organs.

Effective clinical management of long-term COVID-associated neurodegeneration requires a multidisciplinary approach that includes comprehensive assessment and monitoring, targeted therapies, symptomatic management, and preventive measures. Such an approach involves collaboration between neurologists, immunologists, psychiatrists, and primary care physicians to address the complex interplay of viral persistence, immune response, and brain aging.[18] Regular monitoring of patients for neurological symptoms and cognitive decline is essential for early intervention and management. Targeted therapies should focus on reducing neuroinflammation, oxidative stress, and mitochondrial dysfunction while enhancing neural plasticity and cognitive function.[17,87,139] Symptomatic management should address specific symptoms, such as cognitive impairment, depression, and anxiety, through both pharmacological and non-pharmacological interventions. Preventive measures, including vaccinating, promoting overall health, and implementing strategies to reduce the risk of severe COVID-19 infection, are also essential.

9. Conclusion

In conclusion, the neurological aftermath of SARS-CoV-2 infection is a multifaceted issue requiring comprehensive research and clinical strategies. Studies suggest that up to 30-50% of individuals who recover from COVID-19 report persistent neurological symptoms, including cognitive impairment, fatigue, and headache. The risk and severity of neuro-COVID are notably higher in older adults, partly due to age-related brain changes and immune dysregulation. For instance, in individuals aged 65 and above, the risk of developing dementia-like symptoms post-COVID is roughly doubled compared to those without a COVID history. Imaging studies indicate that SARS-CoV-2 can lead to structural changes, such as a reduction in gray matter in the frontal and temporal lobes, which are linked to cognitive functions. One study found that, even in mild cases, patients exhibited brain volume loss comparable to that typically seen in a decade of natural aging. Beyond physical neurological symptoms, COVID-19 survivors have an increased prevalence of mental health issues, including anxiety, depression, and PTSD. In a study, over 30% of long COVID patients experienced moderate to severe depression symptoms, underscoring the need for integrated mental health support. By understanding the underlying mechanisms and developing targeted therapies, we can mitigate the long-term impact of COVID-19 on brain health and improve the quality of life for survivors. Effective management of long-term COVID-associated neurodegeneration hinges on a holistic approach that addresses the intricate relationship between viral persistence, immune response, and brain aging.

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Conflict of interest

The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

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PERSPECTIVE ARTICLE

Shuntogram technique for diagnosing shunt failure in patients with programmable valves: A literature review and a case scenario

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Abstract

Diagnosing shunt patency is crucial in the neurosurgical care of patients with shunted hydrocephalus. The shuntogram is a commonly used method to assess patency, yet its utility in accurately diagnosing shunt failure remains inconsistent. In this article, we examined studies on shuntograms performed in patients with fixed-pressure valves to clarify the techniques used and establish criteria for interpretation. We further reviewed conflicting evidence in the literature regarding the utility of shuntograms and outlined our institution's protocol in the context of programmable valves. Studies included in this review detailed the following elements of shuntogram techniques: patient age, indication for shunt failure assessment, positioning, skin preparation, imaging protocol, contrast agents, patency testing, criteria for negative and positive results, troubleshooting shunt, and sensitivity and specificity of findings. Selection criteria included vague symptoms of shunt malfunction in the presence of non-diagnostic imaging. Patients were typically positioned supine or recumbent, with skin prepared using iodine-based products. Shunts were accessed using a 23 – 25-gauge needle, observing cerebrospinal fluid pressure and aspirate. The contrast agent most frequently used was ^{99m}Tc-diethylene-triamine-pentaacetate, although the exact volume injected was inconsistently reported. Imaging protocols were not standardized, with delayed imaging intervals usually ranging from 9 to 20 min. Failure criteria for shuntograms varied significantly, with limited guidance on follow-up interventions or troubleshooting. Notably, there was no mention of shunt reprogramming during these studies. This review highlights the significant variability in shuntogram techniques and outcomes. We present our institution's protocol and a case scenario demonstrating successful implementation. With proper techniques and protocolization, shuntograms hold the potential as a valuable resource for accurately diagnosing shunt patency.

Keywords: Shuntogram; Ventriculoperitoneal shunt; Technique; Shunt failure; Protocol; Cerebral shunt patency

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1. Introduction

Ventricular shunts for hydrocephalus are known in the literature to have a failure rate exceeding 30% in the 1st year and up to 81% within 12 years.[1-3] While definitions of

"failed" shunts vary among institutions, one minimally invasive method for objectively assessing shunt function is the shuntogram.[4] In patients presenting with symptoms of shunt failure, the decision to perform surgery, regardless of ventricular size, is often informed by shuntogram findings.[5] A shuntogram, most commonly a nuclear medicine procedure, involves aseptic skin preparation over a ventricular shunt valve and the injection of a radioactive tracer into the device's lumen. Scintigraphic images are taken periodically to monitor flow and track tracer spread within the cerebrospinal fluid (CSF) column, both distally and proximally. A diagnosis of shunt obstruction is made when the contrast fails to progress distally beyond the shunt's distal tip, often necessitating surgical intervention.

Despite the longstanding use of the shuntogram, no consensus exists on a standardized, stepwise procedure, which may contribute to the reported misinterpretation rates.[6,7] Furthermore, we propose that an understanding of fluid dynamics and the design of shunts to accommodate variable intraventricular pressures has not been widely accounted for in the current shuntogram techniques.[8-10] As a result, recent studies continue to report inconsistencies in the reliability and predictive value of shuntograms.[5,11]

In this article, we aim to clarify the different techniques documented in studies on shuntogram performance. We will further discuss the conflicting evidence regarding the shuntogram's utility and present our institution's protocol in the context of programmable shunt valves. This discussion will be supplemented by a case scenario that demonstrates how our protocol accurately identified shunt patency after an initial misdiagnosis of shunt obstruction.

2. Literature review: Shuntogram technique and interpretation

The shuntogram remains one of the most common procedures for assessing shunt function in the United States.[4] However, the current body of literature on shuntograms presents a heterogenous and poorly defined set of techniques, which obscures a comprehensive understanding of the procedure's accuracy in diagnosing shunt failure. Despite numerous studies making recommendations for specific procedural steps over the years, no standard protocol has emerged.[11] These protocols vary widely in terms of technique, criteria for assessing shunt patency, and definitions of shunt failure.

Patient selection criteria for shuntograms also vary significantly across institutions, often relying on non-specific symptoms of shunt malfunction, such as headache, dysphoria, nausea, irritability, lethargy, behavioral changes, pyrexia, disequilibrium, or other neurologic changes in cases where imaging findings are inconclusive.[2,5,8,9,11-15] Patients undergoing a shuntogram are often positioned supine or recumbent for variable durations before contrast injection and imaging.[3,9,11,12,16-18] Standard preparation includes shaving the area and performing sterile skin preparation with either iodine-based or alcohol/chlorhexidine-based solutions, followed by sterile draping.[3,8,9,11,12,16-18]

Access to the shunt reservoir or port is typically achieved with a 23 – 25-gauge needle.[5,9,11-13,16,17,19] French and Swanson[9] notably suggested making a small incision on the skin with a scalpel before penetration to reduce the risk of introducing squamous cells or bacteria into the port.[9] In some protocols, opening pressure measurements are taken, and/or CSF is aspirated to observe its characteristics.[13,16,19] The contrast medium is then injected, with ^{99m}Tc-diethylene-triamine-pentaacetate (DTPA) being the most commonly used tracer, followed by ^{111}In-DTPA, iohexol, iopamidol, metrizamide, meglumine diatrizoate, and omnipaque.[2,3,5,7-9,11,12-14,16-23] The exact volume of the injected tracer is seldom specified in the literature, and some protocols include flushing after injection.[24] Imaging may commence immediately to ascertain tracer presence within the shunt lumen, potentially creating an immediate ventriculogram depending on the amount of injectate used. Delayed imaging protocols vary widely, lacking standardization across studies.

Criteria for defining a failed shuntogram are also inconsistent. Typically, a positive (failed) shuntogram is indicated when the contrast remains within the shunt lumen without progressing distally (stagnating in the reservoir or ventricles) or failing to pass the distal tip within an accepted time frame. Time thresholds described include failure to pass the distal tip by 9, 12, 15, or 20 min post-injection, with imaging sometimes continuing for up to an hour. Positional changes during observation are generally unreported. In some series, shunt patency was determined through clinical follow-up, with a patent result indicated if the patient did not return to the emergency department within 30-day post-shuntogram or if they were lost to follow-up.[2,7,12,16,18,19] In addition, factors such as CSF reflux into the ventricles and successful contrast administration influenced interpretations, while intraoperative findings often served as the final determinant of failure or patency.[7]

Shunt flow failure can typically be attributed to discontinuities or obstructions at one of the three mechanical areas: the valve, the ventricular catheter, or the peritoneal catheter. An obstruction at any of these sites may present similarly, with shuntogram imaging helpful in identifying the precise site of obstruction. Shunt failure or occlusion can result from protein deposits or other organic materials, particularly within the ventricular catheter, causing a lack of passive or active reflux of contrast proximal

to the valve. This presentation, however, may also occur in patent shunts. Complete occlusion of the peritoneal catheter results in no distal flow, though tracer may still appear in the ventricular system, spinal canal, and kidneys. Partial occlusions are identified by slow tracer transit or accumulation, while a disconnection often results in tracer widening or accumulating at the discontinuity site. Occlusion involving the valve itself may produce symptoms resembling proximal or distal obstruction, depending on the valve's position relative to the reservoir.[25,26]

2.1. Case presentation

2.1.1. The case

Here, we describe a case of a 73-year-old male diagnosed with normal pressure hydrocephalus who had previously undergone placement of an adjustable right occipital ventriculoperitoneal shunt (Codman Certas, Integra LifeSciences, New Jersey). The patient presented to our neurosurgery clinic with intermittent episodes of cognitive decline and gait/balance issues despite stable imaging. These symptoms prompted an evaluation of shunt patency through a shunt function study. It was unclear from his records if the shunt valve pressure had been adjusted during previous clinic visits. The patient was referred to the Department of Nuclear Medicine for a shuntogram, and coordination of contrast administration was performed by a neurosurgery resident. The patient underwent standard preparation with chlorhexidine and alcohol, and shaving was not performed. Under sterile technique, the right occipital shunt reservoir was accessed with a 25-gauge butterfly needle, and a 400 µL solution containing technetium-99 was injected into the reservoir. Between 0 and 5-min post-injection, counts were observed in the ventricles but were absent in the tubing or abdomen (Figure 1A). The shuntogram findings, which indicated no flow, suggested shunt malfunction. To investigate further, the patient was repositioned from a recumbent to a seated position for 5 min, and the scan was repeated, yet no flow was detected. Examination of the shunt valve revealed that it had inadvertently been set to 5 – a relatively high-pressure setting for this device. To confirm that this high setting was causing the flow failure, and the shunt was adjusted to the lowest possible setting. Upon re-imaging, immediate counts were noted in the distal tubing and abdomen (Figure 1B and C). At the conclusion of the procedure, the shunt setting was re-adjusted to 4, with plans for close follow-up in the outpatient clinic. Following this intervention, the patient experienced improvement without the need for surgery. The radiologist remarked that he would have initially diagnosed the shunt as occluded based on the initial images and acknowledged that he had gained valuable insight from this case.

2.1.2. Shunt protocol

The patient is positioned in a semirecumbent posture, and the site is prepped without shaving, using at least two chlorhexidine prep sticks (ChloraPrep™, Becton, Dickinson in Franklin Lakes, NJ, USA) and some abrasive cleansing of the site. This seated positioning is preferred during preparation to avoid temporarily disturbing the shunt system's fluid dynamics, although patients who are bed-confined may remain lying down. Using a sterile technique, a 25-gauge butterfly needle is inserted to access the shunt reservoir. Correct needle placement is confirmed by withdrawing a small volume of CSF, typically < 0.1 mL. A tuberculin (1 mL) syringe is then used to inject a volume of radiotracer containing technetium-99, usually between 0.1 and 0.2 mL, strictly no more than 0.5 mL. Care must be taken to avoid significant fluid removal or addition during the procedure, as this approach could create a flushing effect on the system, potentially temporarily dislodging obstructive material or generating positive pressure that could cause the tracer to flow into the distal system without actual

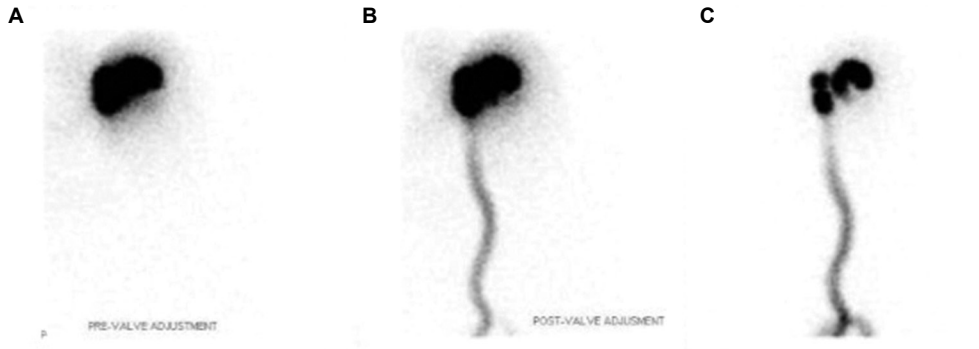

Figure 1. Shuntogram images with 111In-DTPA injected into the reservoir. (A) Pre-valve adjustment: No flow noted in the tubing or abdomen. (B) Post-valve adjustment (Setting 5 to 1): Immediate flow into the distal tubing and abdomen. (C) Delayed image: Continued drainage into the abdomen and emptying of the ventricle

patency. Ideally, the initial images should display only this small tracer volume within the reservoir of the shunt, with no proximal or distal flow, although proximal flow may occur in low-pressure systems. The initial image review also confirms that the injectate is within the shunt lumen and not in subcutaneous tissue. If no distal flow is observed within the first 5 – 10 min, the patient is then placed upright in a chair or allowed to stand for 5 min before re-imaging in the supine position to assess for siphoning-related flow. If the flow is still absent and a programmable valve is present, the valve should be re-read to confirm its setting and reprogrammed to the lowest setting if not already set there. Absence of flow after these steps indicates an obstruction. As a final adjunctive step, the shunt reservoir can be manually pumped to determine whether the obstruction is partial (flow occurs under elevated pressure) or complete (no flow despite pumping). This final step disturbs the steady state of the shunt system and is not used to rule out shunt failure, but it helps determine if the system remains in continuity (Figure 2).

When iodinated contrast is used rather than nuclear medicine agents, images are obtained using static X-rays of the skull and abdomen. We recommend starting with a lateral or oblique view in the sagittal plane aligned with the shunt valve (Figure 3). X-rays are taken at 0 min and then every 5 min, as detailed above. The X-ray tube and plate can be adjusted caudally as needed to keep the distal injectate within view.

3. Results and discussion

Table 1 summarizes the characteristics of the 25 publications discussing shuntogram techniques. These studies exhibit considerable variability in areas such as patient age, clinical indication, positioning, skin preparation, duration, contrast agents, imaging protocols, patency assessment, diagnostic criteria (negative and positive), adjustments to shunt flow following positive failure, and measures of sensitivity and specificity or result/clearance times. Further details are available in Table S1.

In the subsequent discussion, we chronologically examine the evolution of shuntogram techniques from 1981 to 2024.

In 1981, French and Swanson[9] published a study on radionuclide imaging shuntography to evaluate shunt patency. They conducted 78 shuntograms in patients exhibiting clinical features of obstruction. Patients were placed in a supine position, and scalp preparation involved shaving and disinfecting with betadine. A 23-gauge needle was inserted through a small incision in the scalp into the reservoir, and ^{99m}Tc-pertechnetate or ^{111}In-DTPA was injected. Over 100,000 images were obtained using the

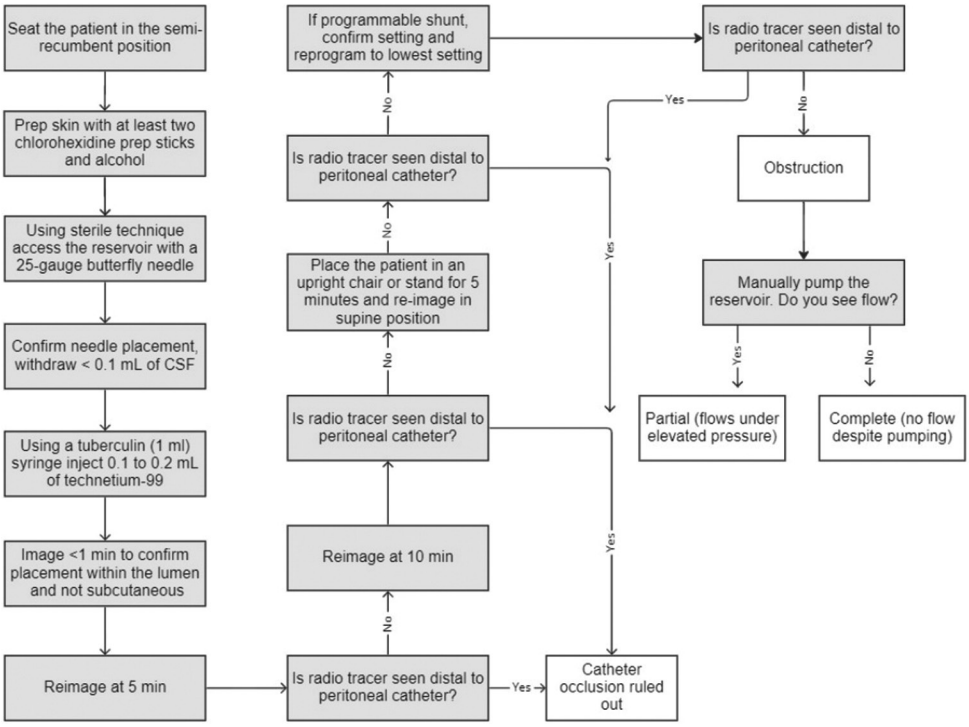

Figure 2. Flowsheet for troubleshooting and diagnosing impaired contrast flow on shuntogram
Abbreviation: CSF: Cerebrospinal.

Table 1. Summary of shuntogram technique characteristics in 25 publications

Parameter	Value
No. of studies (*N*)	25
Series	12 (48)
Case studies	11 (44)
Other	2 (8)
Age	
Adult	7 (28)
Pediatrics	12 (48)
Both	4 (16)
Neither	2 (8)
Indication	
Clinical presentation	13 (52)
Imaging	9 (36)
Positioning	
Supine	6 (24)
Recumbent	2 (8)
Skin prep	
Iodine-based cleanser	5 (20)
Chlorhexidine and alcohol	2 (8)
Asepsis	4 (16)
Time	
<10 min	4 (16)
15 – 45 min	4 (16)
>45 min	5 (20)
Pumped manually	4 (16)
Radio contrast	
Variants of ^{99m}Tc	13 (52)
Metrizamide	2 (8)
Iopamidol	2 (8)
Iohexol	3 (12)
^{111}In-DTPA	3 (12)
Meglumine	1 (4)
Diatrizoate	1 (4)
Unspecified	5 (20)
Needle gauge	
23	3 (12)
25	7 (28)
Injectate amount	
<1 mL	1 (4)
3 ml	1 (4)
Changed position	2 (8)
Sensitivity (37.5 – 92.6%)	3 (12)
Specificity (59.5 – 100%)	3 (12)
False negative (8 – 25%)	5 (20)
False positive (0 – 100%)	4 (16)

Note: Values are expressed as *n* (%) unless otherwise specified. Additional reference citations can be found in Table S1.

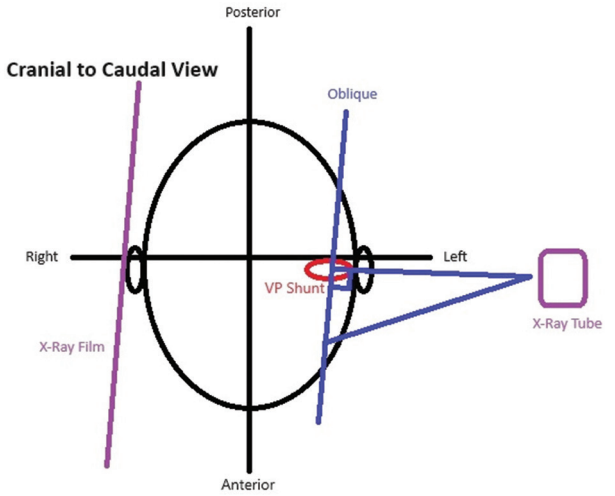

Figure 3. Positioning of the X-ray for oblique view in the sagittal plane for ventriculoperitoneal shunt series

novel scintillation camera (fluoroscopy), capturing images at 10, 20, and 60 min, with additional imaging in an erect position if migration was not observed after pumping. A negative result was verified if, after 6 months, no revision was necessary.

Mirfakhraee *et al.*[12] examined the effect of positioning using a supine position using the head in a lateral orientation. Their timeline for "normal" distal flow was narrow, defined as 3 to 9 min; however, only patients with hydrocephalus on presentation were included in the study. A few years later, Sweeney and Thomas[13] performed a study on 250 shuntograms, enrolling patients with irritability, vomiting, headaches, or pyrexia. Preparation involved shaving the scalp, employing an aseptic technique, and using a 23-gauge butterfly needle for CSF aspiration. Iopamidol was injected, with contrast distal to the valve observed and manual pumping applied as needed to assist distal flow. This approach led to 16 false interpretations, with nine cases requiring shunt revision. These two studies did not specify how contrast progression was monitored, and definitions of shunt failure were inconsistently applied.

By 1991, Benzel *et al.*[16] maintained a supine position for patients 1 h before the procedure, although contrast agents remained variable across institutions.[9,13] They noted that lowering shunt drainage pressure when the flow was initially stalled allowed 18 of 22 shunts to resume flow after surgically placing a lower-pressure valve.[16] Pumping was frequently employed across studies to stimulate flow.[8,16,18] However, Piatt's[27,28] studies in 1992 and 1996 highlighted the limitations of the pumping test, reporting a sensitivity of 50%, specificity of 64%, and a likelihood ratio of 1.39. It was concluded that the pumping test alone was neither

sufficiently sensitive to confirm patency nor specific enough to justify diagnostic intervention.[28]

Vernet *et al.*[18] analyzed 56 radionuclide scans, observing a trend in flow with clearance half-time for the reservoir ranging from 1 – 7.5 min. They concluded that a negative scan was characterized by contrast distal to the tip within 10 min, extending up to 30 min after injection. However, even with these new time guidelines, subsequent studies reported false-negative rates of 14 – 25% and near-zero false positives over the following decade, with a recommended flow detection window of 10 – 15 min.[2,17]

Due to the significant variability in findings, Ouellette *et al.*[7] conducted a retrospective study on 69 patients presenting to the emergency department who underwent ^{99m}Tc-DTPA shuntograms. Three blinded investigators interpreted the results: patients who did not return within 4 weeks were classified as negative, while those with intraoperative confirmation of shunt issues were classified as positive. The study reported a sensitivity of 92.6% and a specificity of 59.5% in patients who had a shuntogram.

Six years later, Thompson *et al.*[3] highlighted the challenges in determining shunt failure through a large-scale study reviewing 259 shuntograms. The inclusion criteria were discordant symptoms with stable ventricle size. Imaging was performed every 15 min over 1 h. A "normal" shuntogram was defined in four different ways based on contrast movement into the distal site. Despite "normal" times ranging from 15 to 45 min, they found low sensitivity and high specificity. Ultimately, they determined that a true negative result occurred when a patient with a normal shuntogram did not return to the emergency department within 30 days. This finding underscored the need for clinical judgment and ongoing observation in interpreting shuntogram studies.

In 2020, Quezada and Gordon[11] conducted 146 radiopharmaceutical flow studies in patients presenting with headache, nausea, vomiting, irritability, and altered neurologic function despite normal computed tomography or magnetic resonance imaging. Patients were placed in a recumbent position and prepared with chlorhexidine and isopropyl alcohol before a 25-gauge butterfly needle was used to inject ^{99m}Tc-DTPA. Fluoroscopy images were taken every 15 min over 1 h. Rapid flow through the distal catheter into the peritoneum or vascular system was considered negative. They reported a true negative rate of 91% and a true positive rate of 70%. Around the same time, a retrospective study of 95 patients found that predicting shunt revision within 30 days had a negative predictive value of 68.3%.[5] Both studies highlight that, despite advancements in procedural techniques and imaging, shuntogram interpretation continues to have limitations in accurately delineating shunt function after 40 years.

In 2024, Nandoliya *et al.*[24] reported on 211 nuclide procedures performed from 2003 to 2022. They injected 0.5 ml or less of nuclide followed by a small saline flush. Patients were encouraged to ambulate, if possible, during the 30-min imaging period. No valve setting adjustments were made. The study reported a sensitivity of 92.3% and a specificity of 96.2%, with no mention of programmable valve adjustments.

These studies do not specifically address the current widespread use of programmable shunt valves. It is possible that limited or absent flow may occur simply because the patient's intracranial pressure is below the valve setting at any specific moment. Therefore, shunt obstruction cannot be accurately diagnosed unless the valve pressure has been sufficiently reduced to allow flow, even if the intracranial pressure is extremely low. In addition, variability in radiocontrast usage persists within the same study, with ^{99m}Tc-DTPA remaining the most commonly administered contrast agent in shuntograms.

Research into biodegradable nanoshells (e.g., hyaluronic acid) encapsulating radiotracers is growing. Ganau *et al.*[29] highlighted the diagnostic potential of nanoshell technology in inflammatory and oncologic pathologies, noting its optimization of contrast media for neuroradiology and nuclear medicine, including applications with ^{99m}Tc-DTPA. Further studies may benefit from this technology, potentially establishing a standardized radiotracer for shuntograms.

4. Conclusion

The burden of shunt-dependent hydrocephalus underscores the need for reliable methods to accurately detect shunt failure. This article presents evidence highlighting the heterogeneous techniques and outcomes associated with shuntograms, alongside an example of successful implementation following our institutional protocol. With proper techniques and protocols, shuntograms have the potential to serve as a valuable tool for diagnosing shunt patency.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data used in this work are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

Advanced Neurology

ORIGINAL RESEARCH ARTICLE

Unpredictable mild stress accelerates the emergence of motor deficits in a rat model of progressive parkinsonism

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Abstract

Parkinson's disease (PD) is a multifactorial condition associated with genetic and environmental factors. In recent years, the role of chronic psychophysiological stress as a predisposing factor for PD is gaining increasing attention. Clinical and experimental evidence indicates that chronic stress exerts adverse effects on the brain. Nevertheless, the potential role of chronic stress in the predisposition to PD remains poorly understood. This study aimed to investigate the effects of exposure to a chronic unpredictable mild stress (UMS) protocol on the onset of parkinsonism in Wistar rats repeatedly treated with a low dose of reserpine. Wistar rats were either exposed to UMS for 1 week or not exposed (control group). Then, the animals were repeatedly treated with a low dose of reserpine or vehicle every other day for 20 days. Behavioral motor evaluations were conducted using catalepsy and open field tests. Moreover, plasma corticosterone (CORT) levels and lipid peroxidation were evaluated. As expected, the UMS protocol increased plasma CORT levels, and reserpine treatment led to a progressive enhancement of cataleptic behavior. Animals exposed to UMS and treated with reserpine exhibited motor alteration earlier during the protocol. No differences were observed in oxidative stress between experimental and control groups, as evaluated through lipid peroxidation assay. Our results showed that chronic mild stress accelerated the onset of motor deficits in reserpine-treated animals.

Keywords: Oxidative stress; Corticosterone; Motor impairment; Parkinson's disease; Neurodegeneration

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1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder associated with aging. It is characterized by the progressive death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and subsequent depletion of dopamine in the striatum.[1] This disruption in dopaminergic transmission affects the thalamocortical pathways involved in the control of voluntary movement,[2,3] resulting in the development of

motor symptoms characteristic of PD. However, no studies have clarified the relationship between this pathological hallmark and various non-motor symptoms of the disease, such as cognitive deficits, autonomic dysfunction, anxiety, sleep impairments, and mood disorders.[4]

Although the precise mechanisms underlying the loss of dopaminergic neurons in the nigrostriatal pathway are not fully understood, PD is recognized as a disease with a multifactorial etiology.[2,5] Among several factors, oxidative stress is believed to be a relevant mechanism in several neurodegenerative disorders.[6] The central nervous system is particularly vulnerable to oxidative stress due to its higher oxygen consumption than other tissues. Furthermore, neurons are less proliferative and have higher levels of nitric oxide than other cells. In this regard, the metabolism of nitric oxide is associated with the generation of reactive oxygen species, which enhances the deleterious effect of this compound.[6,7]

Mapping studies in patients with familial forms of PD using DNA markers have demonstrated a strong association between the early development of motor signs of parkinsonism, such as bradykinesia, resting tremor, muscle rigidity, postural instability, and genetic inheritance.[8] However, only 5 – 10% of the cases are familial.[6,7] The primary genes involved in these mechanisms include those encoding proteins related to the pathophysiology of PD, such as α-synuclein and parkin.[6,8,9] Most patients present with the idiopathic form of the condition, which typically occurs in people aged >60 years.[2,9]

Stress is among the several factors identified as potential predisposing conditions for the development of neurodegenerative processes. Specifically concerning PD, a case report described that a previously healthy 38-year-old woman with no history of neuropsychiatric illnesses presented with resting tremor 1 week after experiencing an acute episode of intense psychological stress, thereby developing early symptoms of PD.[10] Furthermore, a preclinical study showed that the neuroprotective effects of physical exercise in rats with experimentally induced PD were counteracted by exposure to mild stressors.[10] Although several studies have suggested an association between PD and psychophysiological stress,[11-14] most have focused on the relationship between stress and the worsening of cognitive and motor symptoms rather than investigating the possible causal associations.[11,15]

Stress is a physiological response to external and internal disturbances and is useful for the body to adapt to different adverse conditions.[16,17] Stress response can also occur in the presence of adverse psychosocial situations or due to the cognitive perception of unpredictability.[16,18] These processes induce physiological changes that restore the homeostatic balance or prepare the body for possible future imbalances (anticipation). However, excessive stress responses can result in deleterious alterations.[18,19] The response of the body to stress is similar irrespective of whether it is triggered by psychological, environmental, or physiological stimuli.[18] This response involves the release of vasopressin and corticotropin-releasing hormone by the hypothalamus, which induces the release of adrenocorticotropic hormone by the anterior pituitary, ultimately leading to the release of glucocorticoids by the adrenal glands.[20,21] In turn, corticosterone (CORT) stimulates glucocorticoid receptors in the hypothalamus and pituitary gland to regulate the hypothalamic-pituitary-adrenal (HPA) axis through a negative feedback mechanism. This mechanism is essential to limit the body's response to stressors.[17,21] Nevertheless, the stress response is not confined to the HPA axis alone; there is also integration among several brain regions. This integration is facilitated by the activation of the paraventricular nucleus of the hypothalamus.[20,22]

Moreover, the mechanisms of oxidative stress are implicated in the changes caused by psychophysiological stress in the HPA axis.[6,20] For instance, mitochondrial disturbances can cause dysregulation of the enzyme NADPH oxidase, which contributes to the release of free radicals in the central nervous system.[6,23] The generation of oxidative stress is related to increased corticotropin-releasing hormone and adrenocorticotropic hormone levels, resulting in behavioral changes consistent with the reaction to psychological stress.[24] As mentioned earlier, oxidative stress is a key feature in both PD and psychophysiological stress, emphasizing the importance of investigating the possible relationship between psychological stress and the development of this disease.[6]

Overall, the evidence suggests that psychological stress could be a significant predisposing factor for idiopathic PD. In this context, animal models have been valuable tools for investigating several aspects of neurodegenerative diseases, including the risk factors for PD.[2] Nevertheless, classical models that induce parkinsonism using neurotoxins generally promote acute severe motor impairments, making it difficult to evaluate the possible aggravation or acceleration of the process.[2,25,26]

Repeated administration of a low dose of reserpine, which is a blocker of vesicular monoamine transporter 2, has been proposed as a progressive pharmacological model of parkinsonism.[2,27] This protocol induces progressive motor and non-motor signs reminiscent of the disease as well as PD-related alterations in the nigrostriatal pathway. These alterations include increased oxidative stress (reflected by increased membrane lipid peroxidation),

increased levels of inflammatory factors (such as increased astroglia activation and production of inflammatory cytokines), reduced proportion of tyrosine hydroxylase–positive cells in the SNpc, and augmented immunostaining for α-synuclein.[27-30] This progressive protocol has the advantage of gradually promoting motor impairments in rodents, which has been shown to be bidirectionally modified by pharmacological and non-pharmacological interventions.[31-36]

Considering the abovementioned findings, the present study aimed to investigate the effects of unpredictable mild stress (UMS) on the onset and progression of repeated reserpine-induced parkinsonism in Wistar rats. Furthermore, this study examined the effect of UMS on brain oxidative stress (evaluated by membrane lipid peroxidation assay) and plasma CORT levels of rats subjected to the protocol of repeated reserpine administration.

2. Methods

2.1. Animals, general procedures, and experimental design

This study was approved by the Ethics Committee of Universidade Federal de São Paulo (protocol 1365020516), and all procedures were conducted in accordance with the Brazilian legislation for the use of animals in scientific research (Law Number 11,794). A total of 76 6-month-old male Wistar rats were used in the experiments (Experiment I: $n = 36$ and Experiment II: $n = 40$). Groups of 4 – 5 animals were housed in polypropylene plastic cages measuring 33 × 40 × 17 cm and placed under the following conditions: controlled airflow, acoustic isolation, temperature (22°C ± 1°C), and a 12/12-h light/dark cycle (lights on at 7:30 am). All efforts were made to minimize animal pain or discomfort.

In Experiment I, the animals were randomly categorized into four groups; three groups were subjected to the UMS protocol for different durations (1, 2, or 3 weeks), and the remaining group was not subjected to the UMS protocol (control group).

In Experiment II, the rats were randomly assigned to one of four experimental groups, namely, control-vehicle (Ctl-Veh, treated with vehicle only), control-reserpine (Ctl-Res, treated with reserpine only), stress-vehicle (St-Veh, animals subjected to the UMS protocol and treated with vehicle), and stress-reserpine (St-Res, animals subjected to the UMS protocol and treated with reserpine). Rats subjected to the UMS protocol were housed in a different room compared with those in the control groups. The control groups were placed under the following conditions: controlled ventilation and temperature (20°C – 23°C), free access to food and water, and light/dark cycle of 12/12 h (lights on at 6:30 am). The animals were handled for a period of 5 min daily during the 14-day period before the beginning of the experiment to habituate them to the experimenter. All experiments were conducted during the light phase and by researchers blinded to the treatment and stress protocol.

Stressors (as listed in Table 1) were applied 6 days weekly. In Experiment II, the animals were subjected to the UMS protocol for 1 week (days 1 – 7). The duration of exposure to the UMS protocol in Experiment II was determined based on the results of Experiment I. After the UMS protocol, treatment with either vehicle or reserpine (0.1 mg/kg) was performed from days 9 to 29. Catalepsy tests were conducted every 3 days from days 8 to 29, and the open field test was performed twice, on days 19 and 29. On day 31, 48 h after the last injection of vehicle or reserpine, the animals were euthanized via decapitation, without anesthesia, and blood samples were collected for the determination of plasma CORT levels. Moreover, the striatum was dissected and stored for lipid peroxidation assay. Figure 1 provides a summary of the experimental design.

2.2. UMS protocol

The rats that were subjected to the UMS protocol were placed in a separate room from the control groups throughout the study. This procedure was implemented to prevent changes in the behavior of the control rats due to possible alert ultrasonic vocalization from the stressed rats.[37] The stress procedures were performed daily from Sunday to Friday, each lasting 12 h, with no procedures conducted on Saturdays. The stressors used in the protocol are listed in Table 1.

2.3. Drug treatment

Reserpine (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 1% glacial acetic acid and diluted with distilled water to the correct concentration (0.1 mg/mL). The vehicle consisted of the same concentration of acetic acid as in the reserpine solution and water. Either the vehicle or 0.1 mg/kg reserpine was injected subcutaneously every other day, in a total of 10 injections.

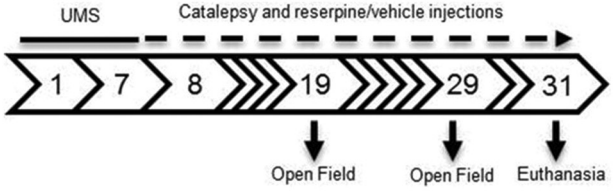

Figure 1. Schematic of the experimental design (Experiment II)
Abbreviation: UMS: Unpredictable mild stress.

Table 1. Unpredictable mild stress protocol

Week	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
1	Moist cage	Small cage	Water in the cage	30°	Total light	No water
2	Water in the cage	30°	Small cage	Total light	No water	Moist cage
3	Small cage	No water	30°	Moist cage	Water in the cage	Total light

The following procedures were used: Small cage: The rat was housed alone in a mouse cage (29×18×12 cm). Water in the cage: Empty cage (without wood shavings) with water up to a level of 2 cm. 30°: Cage inclined at 30°. Total light: Continuous light in the dark period, from 6:30 pm to 6:30 am. No water: Water deprivation at night, from 6:30 pm to 6:30 am. Moist cage: 250 mL of water in the wood shaving layer, starting at 6:30 am. All procedures lasted 12 h.

2.4. Catalepsy test

Catalepsy is defined as the inability to change an imposed position, and this behavior is associated with the decreased motor function observed in patients with PD.[38] Catalepsy behavior was evaluated by placing the animal's forepaws on a horizontal bar positioned 9 cm above the surface of the bench. The duration of catalepsy, defined as the duration for which the animal maintained an immobile posture with both forepaws on the bar, was measured up to a maximum of 180 s. Each animal underwent three trials on each observation day, and the mean duration across these trials was calculated. Cataleptic behavior was measured every 3 days across the protocol, and the results were analyzed considering two observational days in each block of results.

2.5. Open field test

The locomotor activity was evaluated using the open field test conducted in a circular open field arena with a diameter of 84 cm, surrounded by a 32-cm high wooden wall, which was painted black. A digital camera positioned above the arena was used to record the behavioral session, and the camera was connected to a computer placed in a separate room, where the experimenter monitored the behavioral session. The animals were placed alternately in the open field arena and allowed to freely explore the apparatus for 5 min. The distance traveled by each animal was recorded using Anymaze video-tracking software (Stoelting Co., USA). The open field behavior was evaluated on days 19 and 29 of the protocol, which were the midpoint and endpoint of the reserpine protocol (evaluations after 5 and 10 injections of the drug), respectively.

2.6. Oxidative stress: brain lipid peroxidation

The effects of the manipulations on oxidative stress were evaluated through membrane lipid peroxidation assay. At 48 h after the end of reserpine or vehicle treatment, the animals were euthanized by decapitation, the brains were removed, and the striatum was immediately dissected bilaterally. The dissected tissue samples were immediately weighed and frozen at −80°C. At the time of analysis, the tissue samples were homogenized in 0.1 M potassium phosphate buffer and centrifuged for 10 min at 22,673 ×*g* and a refrigerated temperature. A duplicate of each homogenized sample was used to determine the levels of malondialdehyde (MDA, a byproduct of lipid peroxidation, formed from the reaction of this aldehyde with thiobarbituric acid). The MDA levels were determined by quantifying the fluorescent product (excitation at 315 nm and emission at 553 nm) of the reaction with thiobarbituric acid in a plate reader. Results were expressed as nmol of MDA/g of tissue and calculated by comparison with a standard MDA curve. The procedure was performed as described previously.[29]

2.7. CORT levels

CORT levels were measured to evaluate the response to the mild chronic stress protocol, serving as a marker of psychophysiological stress. In Experiment I, animals were euthanized by decapitation, and trunk blood samples were collected for the measurement of CORT levels. In Experiment II, trunk blood samples were collected concurrently with the removal of the brain for lipid peroxidation assay (described in section 2.6). Blood samples were collected in test tubes containing 6% ethylenediaminetetraacetic acid and centrifuged for 20 min at 1209 ×*g* and 4°C. The plasma samples were stored at −20°C until the determination of CORT levels.

For determining the stress hormone levels, 100 µL of a precipitant solution (1 M zinc sulfate) was added to a 100-µL aliquot of plasma, and the mixture was agitated for 30 s. Then, 50 µL of an internal standard solution (10 µg/mL cortisol in 1:1 methanol) was pipetted into this mixture, followed by 1 mL of ether, with ongoing agitation for 1 min. Next, the samples were centrifuged (1086.5 ×*g*, at room temperature) for 5 min, and the resulting supernatant was transferred to another tube for evaporation in compressed nitrogen for 30 min. Finally, the sample was resuspended in 100 µL of 1:1 methanol: water and 0.1% formic acid under agitation for 10 s, after which 20 µL of this sample was injected into a Shimadzu Class VP high-performance liquid chromatography apparatus coupled

to a Waters Quattro Micro Mass Spectrometer (liquid chromatography/tandem mass spectrometry system). Sample separation was performed using a Phenomenex Kinetex column (50 mm × 2.1 mm × 2.7 μm). A six-point (10, 25, 50, 100, 250, 500, and 1000 ng/mL) curve for CORT concentration (Sigma-Aldrich) was used as the standard in the analysis of the samples. The analysis was performed using MassLynx software. The procedure was performed as described previously.[39]

2.8. Data analysis

Data were tested for homogeneity of variance using Levene's test and for normality using the Kolmogorov–Smirnov test. Thus, all data were analyzed using parametric methods. In Experiment I, CORT levels were analyzed using a one-way analysis of variance (ANOVA), considering the duration of UMS (1, 2, or 3 weeks) as the main factor. In Experiment II, catalepsy and open field test data were analyzed using two-way ANOVA with repeated measures, considering the stress protocol (with or without UMS) and treatment (reserpine or vehicle) as the main factors and behavioral observations as repeated measures. The results of catalepsy test were compiled in blocks of two observations. Data on CORT plasma levels and brain lipid peroxidation were analyzed using two-way ANOVA, considering the stress protocol and treatment as factors of analyses. All *post hoc* analyses were conducted using the Tukey *post hoc* test. A significance level of $P < 0.05$ was considered in all tests. The analyses and graph designs were performed using GraphPad Prism 8.

3. Results

3.1. Experiment I

In this experiment, rats were subjected to different durations of the UMS protocol to determine the most suitable duration, as indicated by the highest plasma CORT levels. One-way ANOVA revealed a significant effect of protocol duration (F [3, 32] = 7.463; P = 0.0006). Tukey's *post hoc* test revealed that only 1 week of UMS resulted in higher plasma CORT levels than those in the group that was not exposed to stressors (P = 0.0003; Figure 2). Thus, the duration of 1 week of UMS was selected to investigate the effects of mild stress on reserpine-induced parkinsonism in Experiment II.

3.2. Experiment II

3.2.1. Catalepsy

A repeated measures ANOVA revealed significant effects of blocks (F [3, 144] = 12.10, $P < 0.0001$), treatment (F [1, 144] = 12.45; P = 0.0006), and stress protocol (F [1, 144] = 36.01; $P < 0.0001$). A significant interaction between the blocks and stress protocol (F [3, 144] = 5.737; P = 0.001) was also detected. Tukey's *post hoc* test showed that the catalepsy duration was significantly longer in the Ctl-Res group than in the Ctl-Veh group only in block 4 (P = 0.003). Conversely, the St-Res group showed a longer catalepsy duration than the Ctl-Veh group in blocks 2 (P = 0.008), 3 (P = 0.006), and 4 (P = 0.03). The St-Res group also exhibited increased catalepsy compared with the St-Veh group in blocks 3 (P = 0.006) and 4 (P = 0.04). These data are illustrated in Figure 3. Overall, the results indicated an earlier onset of cataleptic behavior in the group that was subjected to the UMS protocol and received reserpine treatment.

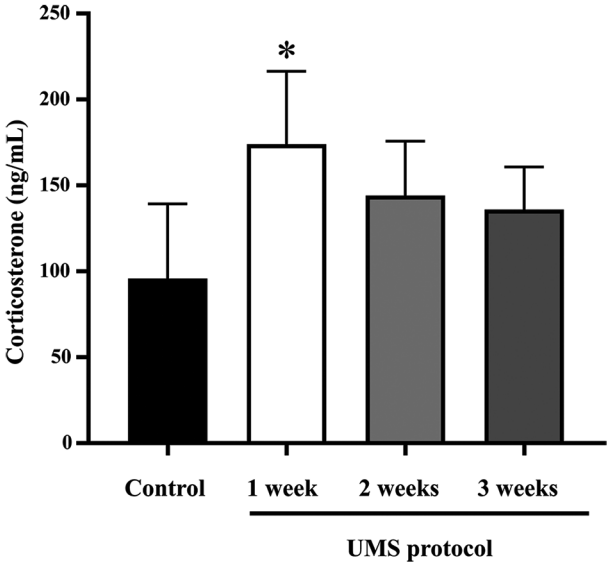

Figure 2. Plasma corticosterone levels of rats exposed to different durations of the unpredictable mild stress (UMS) protocol. Rats (n = 8 – 10 rats/group) were subjected to the UMS protocol for 1, 2, or 3 weeks or remained in their home cages (control) before blood sampling. *P = 0.0003 compared with control (ANOVA with protocol duration as the main factor, followed by Tukey's *post hoc* test). Data were expressed as mean ± SEM

Abbreviations: ANOVA: Analysis of variance; SEM: Standard error of mean.

3.2.2. Open field test

The open field test was conducted on days 19 and 29 of the protocol. A two-way ANOVA with repeated measures revealed significant effects of observation day (F [1, 71] = 57.14; $P < 0.0001$), stress protocol (F[1,71] = 19.53; $P < 0.0001$), and treatment (F[1,71] = 10.45; $P < 0.0001$). Moreover, there was a significant effect of the interaction between the observation day and treatment (F [1, 71] = 9.926; P = 0.0024). Tukey's *post hoc* test revealed that rats in the St-Res group traveled a shorter distance than those in the St-Veh group during the test performed on day 29. Furthermore, all groups (except for

the St-Veh group) showed reduced ambulation on day 29 compared with that on day 19. On day 29, the St-Veh group exhibited increased ambulation compared with the other groups. These data are illustrated in Figure 4. Overall, the results showed that the animals became habituated to the open field from the first to second observations (except for the animals that were subjected to the UMS protocol and received vehicle treatment). Moreover, reserpine treatment reduced locomotor activity only on day 29, irrespective of the stress protocol.

3.2.3. CORT plasma levels

A two-way ANOVA revealed a significant effect of stress on CORT levels (F [1, 36] = 46.01; $P < 0.0001$). However, there were no significant effects of the treatment or of the interaction between the treatment and stress protocol. Tukey's *post hoc* test revealed increased plasma CORT levels in all the St groups compared with that in the respective Ctl groups. These findings are illustrated in Figure 5. Overall, the data showed that the UMS protocol induced increased plasma CORT levels irrespective of treatment.

3.2.4. Lipid peroxidation

A two-way ANOVA revealed no significant effects of treatment or stress on MDA quantification. However, there was a significant interaction between stress and treatment (F [1, 20] = 4.8; $P = 0.04$). Tukey's *post hoc* test revealed no significant differences among the groups. These data are shown in Figure 6. Overall, the results indicated that neither reserpine nor the stress protocol promoted changes in membrane lipid peroxidation.

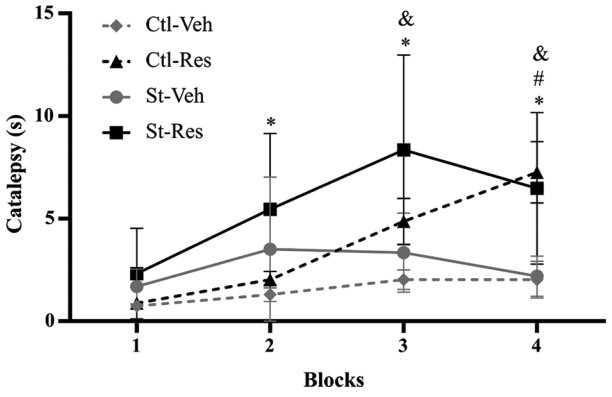

Figure 3. Effects of repeated administration of reserpine preceded by 1 week of unpredictable mild stress (UMS) on catalepsy behavior across the protocol. Data were expressed as mean ± SEM. Groups (n = 10) were subjected (St) or not subjected (Ctl) to the UMS protocol and treated with 10 subcutaneous injections of 0.1 mg/kg reserpine (Res) or vehicle (Veh) on alternate days. $^{*}P < 0.05$ compared with St-Res and Ctl-Veh groups; $^{\&}P < 0.05$ compared with the St-Res and St-Veh groups; $^{\#}P < 0.05$ compared with the Ctl-Res and Ctl-Veh groups (repeated measures ANOVA with stress protocol and treatment as main factors, followed by *post hoc* analysis through Tukey's test)
Abbreviation: SEM: Standard error of mean.

4. Discussion

This study investigated whether a previous exposure to mild stress affects the progression of parkinsonism induced by repeated administration of a low dose of reserpine. First, to determine the appropriate duration of

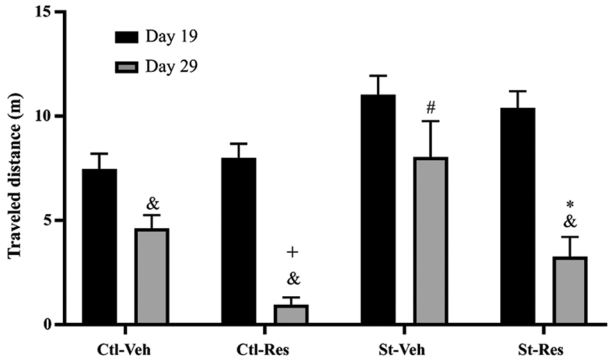

Figure 4. Effects of repeated administration of 0.1 mg/kg reserpine (Res) or vehicle (Veh) on open field behavior. Distance covered in the open field arena by rats on days 19 and 29 of Experiment II. Data were expressed as mean ± SEM. Groups (n = 10) were subjected (St) or not subjected (Ctl) to the UMS protocol and treated with 10 subcutaneous injections of 0.1 mg/kg Res or Veh on alternate days. $^{*}P < 0.05$ compared with the St-Veh group on the same observation day. $^{\#}P < 0.05$ compared with all other groups on the same observation day. $^{\&}P < 0.05$ compared with the same group on day 19. $^{+}P < 0.05$ compared with the Ctl-Veh group on day 19 (repeated measures ANOVA with stress protocol and treatment as main factors, followed by *post hoc* analysis through Tukey's test)
Abbreviations: SEM: Standard error of mean; ANOVA: Analysis of variance; UMS: Unpredictable mild stress.

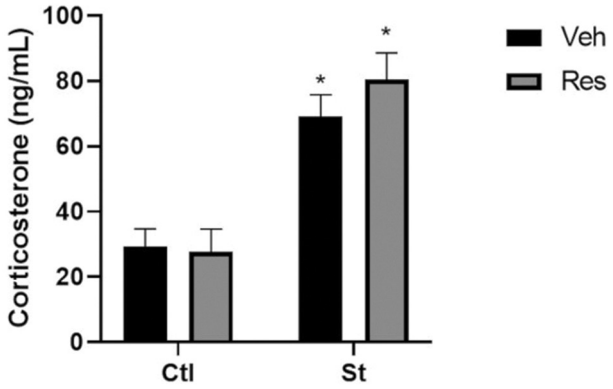

Figure 5. Effects of repeated administration of 0.1 mg/kg reserpine (Res) or vehicle (Veh) on plasma corticosterone levels in rats subjected to chronic unpredictable mild stress for 1 week (St) or those who remained in their home cages (Ctl). Data were expressed as mean ± SEM (n = 10) in all groups. $^{*}P < 0.05$ compared with St and Ctl groups (two-way ANOVA with stress protocol and treatment as main factors)
Abbreviations: ANOVA: Analysis of variance; SEM: Standard error of mean.

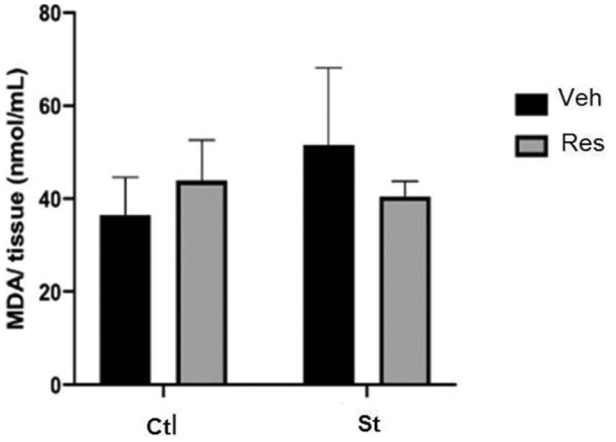

Figure 6. Effects of repeated administration of 0.1 mg/kg reserpine (Res) or vehicle (Veh) on lipid peroxidation (MDA formation) in the striatum of rats subjected to chronic unpredictable mild stress for 1 week (St) or those who remained in their home cages (Ctl). Data were expressed as mean ± SEM (n = 6 for all groups). Statistical analysis did not reveal any significant effects
Abbreviations: SEM: Standard error of mean; MDA: Malondialdehyde.

the UMS protocol, plasma CORT levels were measured after different durations of the protocol, which revealed significantly elevated levels of the stress hormone after the 1st week of the UMS protocol.

When prolonged stress protocols are used, animals tend to habituate to the stressors, and the rate of habituation depends on the type of stressor. Using a protocol with a variety of stressors, creating unpredictability has been shown to delay this adaptation.[18,40] Nevertheless, although unpredictable repeated stress may delay habituation, the delay eventually occurs as animals adapt to different stressors. This may facilitate the activation of the HPA axis after exposure to subsequent stressors.[40]

In Experiment I, we aimed to determine the optimal duration of exposure to unpredictable mild stressors. Results indicated that animals subjected to 1 week of unpredictable stress showed an increase in plasma CORT levels compared with those who were not. A trend toward decreasing CORT levels was observed with prolonged exposure periods, supporting the abovementioned habituation mechanism. Based on these results, we opted for a 1-week duration of exposure to unpredictable stress in Experiment II. Previous studies by our group.[27-30,33,41] have already demonstrated that low doses of reserpine can progressively increase catalepsy duration and reduce locomotor activity in the open field test. Our current findings indicate that exposure to the stress protocol applied before reserpine treatment accelerated the onset of motor impairment. Specifically, rats subjected to the stress protocol and reserpine treatment exhibited increased catalepsy duration starting from block 2, whereas those treated with reserpine without stress exposure showed motor impairment only in block 4.

In the open field test, no significant differences were detected between the groups on the first observation day (day 19 of the protocol), although there was a trend toward increased ambulation in the groups exposed to the UMS protocol. Conversely, rats treated with reserpine exhibited reduced spontaneous ambulation compared with that in rats treated with vehicle in the second evaluation (day 29 of the protocol). Although the decrease in spontaneous motor activity due to reserpine was expected, the effect was subtle throughout the treatment. This is consistent with previous studies indicating that motor activity in the open field is the least affected, or is the last to decline, following repeated reserpine treatment.[27,29,41]

Remarkably, the spontaneous activity evaluated in the open field test reflects a more general measure of motor function compared with that evaluated in the catalepsy test. This outcome is explained primarily by the motivation to explore a new environment, which significantly influences behavior in the open field arena. Interestingly, irrespective of experimental manipulations, ambulation decreased over repeated exposures to the open field arena, probably due to habituation to the apparatus, which reduces the novelty-induced motivation to explore.

Conversely, the stress procedure increased the distance traveled in the open field, an effect that was reversed by reserpine administration. The hyperlocomotion observed in stressed rats is consistent with the literature showing increased motor activity in open field tests and heightened anxiety-like behaviour.[42] The absence of increased locomotor activity in the St-Res group is probably explained by the strong negative impact of reserpine on the spontaneous motor activity. To summarize, Experiment II demonstrated the following: (1) an increase in plasma CORT levels in the stress groups compared with that in the controls, (2) a progressive induction of motor deficit by repeated administration of reserpine, and (3) an accelerated and worsened onset of motor impairment in stressed rats treated with reserpine compared with that in control rats undergoing the same treatment, as indicated by the catalepsy test results.

Investigations using other animal models of parkinsonism have demonstrated that acute or chronic stress exacerbates motor symptoms.[43-46] However, these studies used protocols of predictable stress. In the present study, we used the UMS protocol with various stressors. Specifically, we adapted a model of induction of moderate chronic stress, which is generally used in mood disorder research.[47] This was an interesting approach because

it minimizes the animals' habituation to stressors.[40] In this context, our results corroborate those of other studies that explored the role of stress and CORT levels in PD. For instance, Rudyk *et al.*[48] investigated the role of unpredictable stress in the protocol of PD induced by paraquat. Interestingly, they found an increase in plasma CORT levels. Another study using the 6-OHDA-induced parkinsonism model combined with the restraint stress protocol showed that elevated CORT levels aggravated neuronal loss in the nigrostriatal pathway and motor symptoms in the PD model.[46] Our study extended these findings to the reserpine treatment model, demonstrating an anticipatory effect of stress on the onset of motor symptoms using a progressive approach with repeated application of a low dose.

An explanation for the acceleration of motor symptoms due to unpredictable stress could be the exacerbation of oxidative stress, as evaluated via lipid peroxidation assay. Fernandes *et al.*[29] revealed that a low dose of reserpine (0.1 mg/kg) increased lipid peroxidation 48 h after the 10th injection. It is well-established that oxidative stress can cause motor[6,49,50] and cognitive impairments.[6,51] Differences in the study protocol, such as the fact that euthanasia was performed 72 h instead of 48 h after the last reserpine injection, may explain the disagreement between our findings and those of previous studies.[29] Despite these differences, our study demonstrated that acceleration of motor symptoms did not occur due to increased striatal lipid peroxidation. This is possibly due to the small sample size of the study, which could have masked the results. Furthermore, evaluating oxidative stress using other techniques could provide a more comprehensive analysis. Therefore, further studies are warranted to elucidate the mechanisms underlying the stress-induced acceleration of motor impairment observed in the present study.

An alternative explanation could be the involvement of inflammatory processes in the striatum and substantia nigra in animals subjected to this protocol, as neuroinflammation is known to be a key factor in the progression of motor impairment in PD.[28,45] Repeated low-dose reserpine treatment has been shown to induce neuroinflammation in the nigrostriatal pathway.[28] Furthermore, although Rudyk *et al.*[48] found no differences in the number of Iba1-positive cells in the SNpc of rats subjected to chronic unpredictable stress and paraquat administration, Sugama *et al.*[52] observed an increase in the number of OX42 microglia in the SNpc and locus coeruleus in rats exposed to chronic restraint stress. Altogether, these findings suggest that the neuroinflammation generated during stress increases in an animal model of PD.

Furthermore, a previous study showed that repeated administration of exogenous CORT causes motor impairment, accompanied with structural and functional changes in the motor cortex of rats.[53] Another study demonstrated that exogenous CORT administration and immobilization stress independently cause motor impairments.[54] Hence, the stress-induced aggravation of motor symptoms in PD could be related to increased CORT levels. Nonetheless, this seems unlikely in the present study because reserpine did not significantly promote the increase in CORT levels induced by UMS under our experimental conditions.

5. Conclusion

Chronic UMS anticipates and aggravates motor impairment in the animal model of progressive parkinsonism treated with repeated low-dose reserpine, accompanied with increased plasma CORT levels. Furthermore, these effects on motor impairment were not associated with striatal oxidative stress. Considering these data, additional research is warranted to clarify the mechanisms underlying the stress-induced exacerbation of motor symptoms in PD.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

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Writing – original draft: Debora M. G. Cunha, Laura F. M. Olivatto
Writing – review & editing: Alvaro C. Lima, Marcela Becegato, Regina H. Silva

Ethics approval and consent to participate

The local Ethics Committee (Comissão de Ética no Uso de Animais da Universidade Federal de São Paulo) approved this study (protocol 1365020516), and all procedures were conducted in accordance with the Brazilian law for the use of animals in scientific research.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author on reasonable request.

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ORIGINAL RESEARCH ARTICLE

Mind Marvel platform: Transforming attention deficit hyperactivity disorder challenges into opportunities through interactive gaming

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a neurological disorder marked by a pattern of inattention, hyperactivity, and/or impulsivity. Since the signs and symptoms of ADHD can be observed in the early stages of childhood, a prompt diagnosis, along with effective treatment, is of utmost importance. Once a child is diagnosed, various treatments are available, including behavioral therapy, medication, and cognitive training. Recent research has shown that individuals with ADHD tend to respond well to interactive games and technologies, such as virtual reality (VR) since such activities require timely task completion, attention to detail, and concentration. Thus, the purpose of this study is to leverage on game therapy for ADHD and develop *Mind Marvel*, a unique platform for individuals with ADHD. This platform combines the design of interactive webpages with a VR environment, transforming ADHD challenges into opportunities through such interactive gaming. In this case, the user answers a set of questions and plays a VR game by using a computer mouse and keyboard or a commercial VR controller. This platform has been tested under laboratory conditions, after which preliminary results indicate that the capabilities of the *Mind Marvel* system effectively support individuals with ADHD and raise awareness of their conditions.

Keywords: Attention deficit hyperactivity disorder; Attention deficit disorder; ADHD; Treatment; Therapy; Virtual reality

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1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that is marked by a persistent pattern of inattention, hyperactivity, and/or impulsivity found in children and adults.[1] However, the effects of ADHD vary from person to person. For example, some individuals find it difficult to perform their daily activities, while others can cope with such difficulties. Meanwhile, patients with ADHD tend to have deficits in the higher-level cognitive functions required for mature, goal-directed behaviors and appropriate growth and development.[2] As for the signs and symptoms, they include being fidgety, impulsive, and talkative, with interruptive behaviors, especially in children. Thus, with appropriate diagnoses by primary care providers and clinicians/psychiatrists,

individuals with ADHD can live productive and enjoyable lives.

1.1. ADHD

ADHD is not a newly discovered disorder. In fact, its history dates back approximately two centuries, with clinical reports in Europe noting the unruly behavior of children. Since then, the research on ADHD has been based on two different perspectives: biomedical and historical-behavioral psychology.[3] From the former perspective, ADHD is a neurobehavioral condition in individuals, starting from childhood. According to the National Health Service (NHS), individuals with ADHD exhibit various characteristics such as restlessness, diminished focus, deficient concentration skills, and the propensity to act impulsively. It is not a spreadable disease, but a group of hyperactive symptoms that cause changes in an individual's behavior, emotional intelligence, psychological well-being, and learning capabilities.[4]

The signs and symptoms of ADHD can be observed in the early stages of childhood, becoming more evident by the changing environment (e.g., when the child starts school). Subsequently, this disorder in a child can overlap with normal behavior development. In this regard, diagnostic manuals, such as the DSM-5, ICD-11, and RDoC, prefer the diagnosis of ADHD in individuals under the age of 12, especially if they display multiple inattentive and hyperactive-impulsive signs.[5] Meanwhile, according to *The World Federation of ADHD International Consensus Statement*, 208 evidence-based conclusions indicate that 5.9% of youth and 2.5% of adults are diagnosed with ADHD symptoms.[6] When gender is taken into account, males are more likely to be diagnosed with ADHD, with a male-to-female ratio of up to 4:1, especially among children.[7]

1.2. Treatment methods

When an individual is diagnosed with ADHD, the common procedure is to follow a treatment regime. Table 1 presents the wide array of available ADHD treatment methods. This comprehensive overview of approaches shows how treatments can assist in the recovery (or management) of individuals with ADHD.

In regard to these different approaches, it is worth noting that games can be designed and applied as a treatment method for improving the cognitive abilities of users. For instance, various brain games, puzzles, brain teasers, and quizzes can foster the development of specific brain functions, including memory, problem-solving, and thinking skills. Video games may also aid children in leveraging feelings and emotions such as sadness, anger, and competitiveness among other players. In this context, since the era of COVID-19, there has been a notable increase in the number of online game players. According to a study by Statista, the number of video gamers in 2023 was 2.64 billion, which is expected to increase by 2027.[11]

Table 1. ADHD treatment methods

Treatment method	Approach
Behavioral therapy	• A therapeutic approach for reducing ADHD symptoms. • The initial treatment is performed when the diagnosis is positive for ADHD.[8]
Medication	• Medications can help manage ADHD symptoms but do not rectify them. • Medications act differently in different people. • Proven to have side effects such as sleep deprivation and reduced appetite.[8]
Cognitive training[9,10]	• Helps the individual control ADHD symptoms by altering his/her thoughts and behaviors. • Cognitive training is achieved via talking therapy, training exercises, or cognitive improvement games.
Food diet[9]	• Focuses on a healthy and balanced diet.

Abbreviation: ADHD: Attention deficit hyperactivity disorder.

As a possible treatment approach for ADHD, video games may represent a useful tool. Previous studies have shown that people generally respond well to game instructions and follow them within the designated time frame. Such games can have numerous benefits such as timely task completion, attention to detail, concentration skills, persistence, and the motivation to learn and succeed.[8-11] Based on all these factors, video games can remediate the attitudinal, behavioral, and emotional processes of individuals with impulse-related disorders.[12] With the ongoing technological advancements, doctors and health professionals should search for more sophisticated and cost-effective treatment methods, which include video games. Some of the latest video games that are designed to treat, control, and even cure certain health conditions are presented in Table 2.

1.3. Design and purpose of ADHD games

Thus, the purpose of this study is to leverage on game therapy for ADHD and develop a unique platform for individuals with ADHD. This research was inspired by the study of Craven and Groom, which focused on the following three approaches.[13]

First, there is the *task-focused* approach, which aims to enhance human performance, attention, and perception by challenging the user with a dynamic environment that tests his/her speed and agility in completing the task. In this regard, Oei and Patterson conducted research on the executive functions of gamers and found that action, puzzle, or memory games tend to have improvised

Table 2. Video games for improving certain health conditions.

Health condition/Disorder	Video games	Approach
Cancer	*Re-Mission*	This game helps patients stay positive, educates them about the treatment, and motivates them to fight cancer. Game link: https://hopelab.org/case-study/re-mission/
Asthma	*Asthma Sense*	This game teaches patients about using inhalers, asthma conditions, and treatment procedures. Game link: https://www.mobihealthnews.com/tag/asthmasense-app
HIV	*Play Forward: Elm City Stories*	This game teaches players about the effects of HIV and how to avoid all risky behaviors. Game link: https://schellgames.com/portfolio/playforward-elm-city-stories
Obesity	*Escape from the Diab*	This game teaches children about healthy food habits and proper diets. It also addresses the issue of obesity. Game link: https://artsandculture.google.com/asset/escape-from-diab/7wHm5mz7rff4IA?hl=en
Anxiety or depression	*Mind Light*	This game helps people overcome anxiety and depression. It provides a safe environment based on scientifically proven technology. Game link: https://www.theplayniceinstitute.com/
Stroke	*Rehabilitation Gaming System (RGS)*	This game uses highly innovative VR and new technology to enhance the cognitive functions of stroke survivors. Game link: https://www.aal-europe.eu/projects/rgs/
Diabetes	*Packy and Marlon*	This game provides an educational platform for children to learn about type 1 diabetes. It also teaches patients about self-care behaviors. Game link: https://en.wikipedia.org/wiki/Packy_and_Marlon

Abbreviation: HIV: Human immunodeficiency virus.

cognitive functions.[13] Second, there is the *neuro-feedback* approach, which aims to improve ADHD conditions by focusing on brain-related aspects such as the theta/beta ratio and slow cortical potential amplitude using time-to-time feedback.[13] In other words, it improves the user's cognitive function, which, in turn, reduces ADHD symptoms. Under the umbrella of this approach are games that include gaze control (to track eye movement) and applications (e.g., CogMed) that target working memory through challenging tasks.[13] Finally, there is the *improving the medical condition* approach in which games are used to help therapists/clinicians analyze the health conditions of this disorder. As shown in Table 2, many digital online games can help detect and evaluate the symptoms of various health conditions, including ADHD.

1.4. Cognitive improvements and limitations

As for video games, it is important to note the emergence of virtual reality (VR) technology. Specifically, VR enhances the user's senses by presenting a simulated environment that replicates real-world experiences. Such environments can also trigger cognitive processes, including memory, problem-solving, and concentration skills, making it an optimal method for improving the user's cognitive functions through enhanced engagement, multiple sensory stimulation, training tasks, and rapid response.

Previous studies have indicated that VR is a systematic and controllable approach that utilizes data visualization and immediate feedback to analyze the user's performance.[14] In this regard, Chiu, Hsu, and Ouyang found that VR technology interacts with motor and cognitive skills, reduces stress, depression, anxiety, and fatigue, and improves overall relaxation and coping skills.[15] VR interactions also offer immersive simulations that induce emotions and mitigate potential psychological conditions. For example, VR games, such as *EndeavorRx* (Table 3), can potentially train individuals with ADHD to comprehend their conditions, foster their cognitive skills, and progressively mitigate related symptoms.

Although digital games have numerous benefits for individuals with ADHD, some negative aspects should be noted. First, according to research conducted by The ADHD Center (a leading ADHD assessment clinic in the United Kingdom), individuals with ADHD tend to have more impulsive and curious behaviors, potentially leading to video game addiction.[16] Especially among children, increased impulsivity can reduce the ability to maintain self-control. Second, excessive gaming can lead to hyperfocus in which the individual becomes so engrossed that he/she avoids or blocks out the necessary information. Finally, games are sometimes used to escape uncomfortable real-world situations, potentially impeding personal growth and the development of social skills. Thus, individuals with ADHD should utilize digital media as a treatment method under the guidance and supervision of therapists/clinicians.

Table 3. Relationship between the user's responses and the corresponding results.

The *Mind Marvel* game generates two reports based on the user's performance	**Result 1: The results of the assessment indicate that ADHD symptoms are present in the user.**		**Result 2: The results of the assessment suggest that the user has excellent attention skills and good vision.**
Answers for Question 1 to receive the corresponding report	The user failed to correctly answer the number of balloons popped.	ADHD symptoms • Short attention span • Distraction from the simulations	The user correctly answered the number of balloons popped.
Answers for Question 2 to receive the corresponding report	*Scenario 1* The user correctly answered the number of animals but failed to correctly answer the number of balloons popped.	ADHD symptoms • Impulsivity • Lack of attention • Easily distracted • Shifting focus from the primary task	The user correctly answered the number of balloons popped. However, the number of animals entered was correct or incorrect.
	Scenario 2 The user failed to correctly answer both questions.		
Answers for Question 3 to receive the corresponding report	The user answered "white," instead of "yellow."	ADHD symptoms • Shifting focus from the primary task to the surrounding movements	The user answered "yellow."

1.5. Aims and objectives

Based on the aforementioned considerations, this study develops an ADHD game called *Mind Marvel* to support individuals with ADHD. The objectives of this platform are as follows:

1. To leverage on game therapy to support the cognitive skills of individuals with ADHD.
2. To encapsulate the current findings on ADHD vs. our design.
3. To first compare different applications and then build and optimize the *Mind Marvel* platform.

Overall, the goal is to develop an easily accessible, enjoyable, informative, and supportive application in which the user will be able to understand more about ADHD and be equipped to recognize his/her symptoms.

The remainder of this study is organized as follows. Section 2 focuses on the materials and methods as well as the proposed design and testing of the platform, while Section 3 examines the results. Section 4 includes the discussion, while Section 5 presents the conclusions.

2. Materials and methods

This section delineates the methodologies and technologies employed to create *Mind Marvel*, which is conceived as a dedicated platform for promoting awareness of ADHD and identifying its potential symptoms (if present) in the user. The technological tools used in its development include React, JavaScript, and VR (described in the following section). These technologies and game components were strategically selected to align with the research findings and help facilitate ADHD-related challenges. Through this methodological approach, it is hoped that the *Mind Marvel* platform will have a positive impact on the well-being of individuals with ADHD.

2.1. Tools and technologies

Mind Marvel is a dynamic web-based application created on the React platform, incorporating VR technology. The entire platform is coded in JavaScript, while A-Frame is used in addition to HTML to build an immersive environment for the game. The details regarding the design of the system are as follows:

- *Web Application* – A program that is coded in a remote server and delivered to a browser via the Internet.[12]
- *Virtual Reality* – Digital technologies that build an immersive environment for a better user experience.[17] In this regard, VR was chosen because of its ability to immerse users in a simulated environment in which they can control the virtual surroundings with a keyboard, mouse, and/or other devices. On the *Mind Marvel* webpage, users are introduced to an immersive VR game designed to fully engage their visual/auditory senses and integrate them into the gaming environment.
- *React/React.js* – This open-source JavaScript library, developed by Jordan Walke (Facebook/META®), is the framework for constructing the *Mind Marvel* webpage. Traditional JavaScript applications require manual document object model manipulation to reflect data changes, leading to full-page reloads. However, React introduces single-page applications, which optimize performance by selectively updating portions of the webpage, resulting in a more dynamic user experience.[18]
- *Node/Node.js* – This is a JavaScript library that is mainly used to run JavaScript on the server.[19] Specifically, Node.js is downloaded into the machine,

after which nm is used to install additional packages. In this case, React and Node.js are combined in the platform, for the purpose of building a dynamic, data-driven, extensive web-based application across multiple platforms.

- *A-Frame* – This web-based framework is the most widely used method for developing a VR environment.[20] Specifically, A-Frame (built on HTML) offers straightforward implementation for users. At its core is a robust entity-component framework that is compatible with various VR commercial headsets.
- *Three/Three.js* – This is a JavaScript library for creating and displaying 3D models in applications. In our *Mind Marvel* platform, Three.js is used to assist A-Frame in two ways: (1) Loading 3D models into the scene through GLTFLoader; and (2) keeping track of child-related elements in the scene. In this case, A-Frame is built on top of Three.js.
- *Sketchfab* – This is an online platform that provides 2D and 3D models for free downloads. Incorporating a VR environment with 3D models enhances the user's experience by closely simulating real-world scenes. In our platform, the 3D models include the *Monkey Model*, *White and Yellow Duck*, and the *Lake Model,* which are available at the following links:
  1. *Monkey Model*: https://skfb.ly/VYJ9
  2. *White Duck*: https://skfb.ly/oBRTA
  3. *Yellow Duck*: https://skfb.ly/6ZpIF
  4. *Lake Model*: https://skfb.ly/MYJw

2.2. Rationale and concept

In the initial stage, the blueprint of the *Mind Marvel* platform was designed by using a flowchart diagram. The main features and functionalities are presented in Figure 1.

As shown in this figure, the primary task was to outline the representation and algorithm of the application. Once this was completed, the next step involved identifying the users and their interactions with the application (Figure 2).

In this figure, the concept is that when the user enters the *Mind Marvel* platform, a home page appears with three tabs: (1) A tab for the ADHD *Awareness* page; (2) another tab for the purpose of the project; and (3) a final tab for the ADHD game itself. The overall purpose of the game is to help users recognize potential indicators of ADHD within themselves, while also providing feedback on their performance.

Finally, it is important to note that our idea of developing an immersive game for individuals with ADHD originated from an experiment conducted by National Geographic to explore brain function.[21] In this experiment, the audience (participants) counted the number of jumps made by the girls wearing green tops in a double Dutch game. Despite the straightforward task, the participants were bombarded with distractions such as changing backgrounds, additional players entering the scene, etc. In this case, the objective was to determine whether the brain could maintain focus and complete the designated task. Based on the findings, the majority of participants had difficulty tracking all the alterations. In other words, when overloaded with stimuli, the brain prioritizes one aspect, decreasing attention on other aspects.[22] This experiment provided the groundwork for developing our ADHD-focused game.

2.3. Design

This section documents the mechanisms of the *Mind Marvel* webpage. Specifically, once the nm command initiates the client-side operations in React, the webpage becomes accessible via a browser. As for the website's color

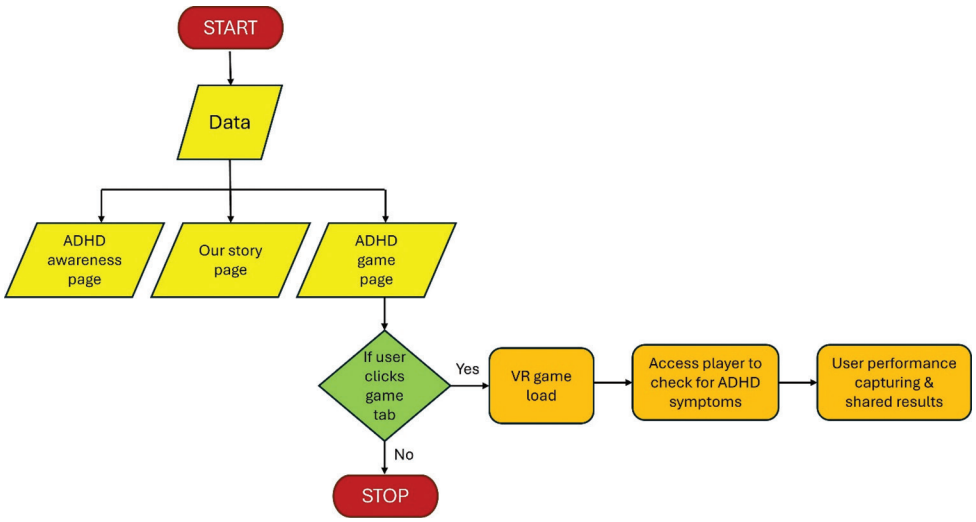

Figure 1. The *Mind Marvel* platform. Image created by authors

scheme, it was deliberately chosen to enhance the aesthetic appeal and the overall quality of the webpage. Regarding the choice of font and colors, it was made to facilitate the ease of reading for users. In this case, the majority of the text on the page is in *Courier New*, *Courier*, and *monospace*, while the background color is *hdl* (348, 40%, 25%).

2.4. The *Mind Marvel* webpage

Figure 3 depicts the home page that users initially encounter on the *Mind Marvel* platform. This page includes three sections: the header, the navigation bar, and the footer. The header includes contact details, such as email and phone number, while the navigation bar features three tabs, each facilitating users to learn more about ADHD. Finally, the footer provides information about the developers.

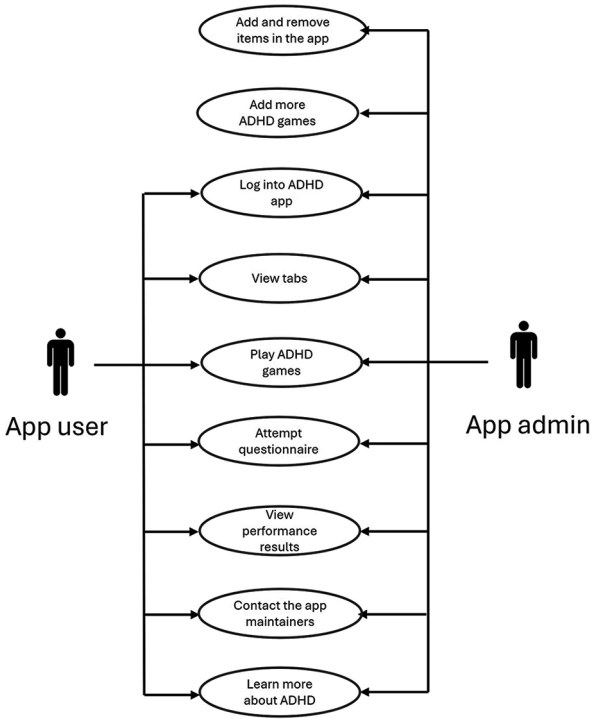

Figure 2. Use a case diagram of the *Mind Marvel* platform. Image created by the authors.
Abbreviation: ADHD: Attention deficit hyperactivity disorder.

The purpose of the *ADHD Awareness* page (Figure 4) is to advocate for ADHD. Despite its well-known acronym, there is still a lack of widespread recognition of ADHD as a legitimate disorder among the public. In fact, many countries, such as the United States, have passed the Disabilities Act to protect individuals living with ADHD.[23] However, awareness is still a pressing need to ensure the implementation of these legal protections. Meanwhile, there is a prevailing tendency in society that expect individuals with ADHD to exert more effort to mitigate their symptoms, which can hinder them from actually receiving treatment, attention, and care in the early stages. As mentioned earlier, there are some individuals with ADHD who are completely unaware of their conditions. Thus, this webpage aims to foster a better understanding and awareness of ADHD in society.

Upon clicking on the *ADHD Awareness* tab, three issues are displayed: (1) *What is ADHD?*; (2) *Signs and Symptoms*; and (3) *Common Facts about ADHD*. This webpage is designed to encourage the user to interact with its components, creating an engaging and enjoyable experience. For example, upon hovering over each box with the cursor, the answer to each question is automatically displayed. To enhance the design of the webpage, random images of animals appear every 5 s. At the end of the session, the user is presented with a question to raise awareness of their attention and concentration levels. Meanwhile, the home icon displayed at the bottom of the page provides seamless navigation back to the home page.

The project's vision and reason for developing this platform are explained to the user on the *Our Story*

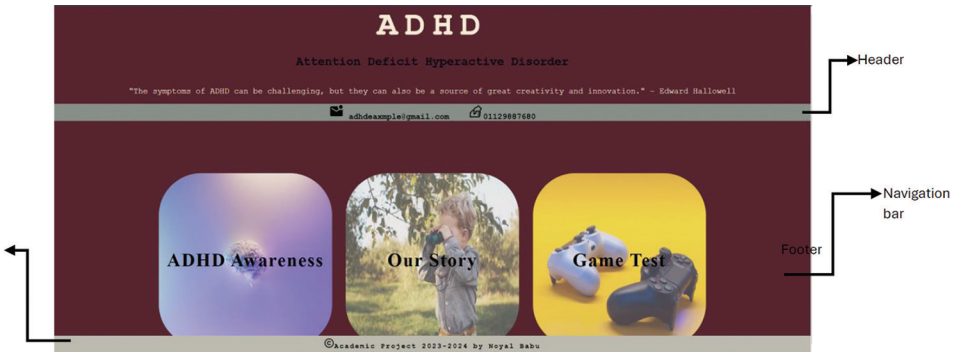

Figure 3. Home page of the *Mind Marvel* platform. Image created by the authors.
Abbreviation: ADHD: Attention deficit hyperactivity disorder.

Figure 4. *ADHD Awareness* page. Image created by the authors.
Abbreviation: ADHD: Attention deficit hyperactivity disorder.

page (Figure 5). It also outlines the forthcoming updates and provides details about the various features and functionalities to enhance the user experience. In this case, the story humanizes the invested effort in the project, fostering a deeper connection with the user. It is hoped that this will attract more potential customers to the application.

Since individuals with ADHD often have difficulty maintaining sustained attention, this webpage integrates minimal messaging and relevant icons. This approach succinctly and expediently communicates our message to the user, acknowledging his/her diverse needs. Again, the home icon at the bottom of the page allows the user to return to the home page.

2.5. Gaming and testing

Upon clicking the *Game Test* tab, the user is directed to the ADHD assessment game, which is an immersive VR game developed to evaluate the user's cognitive skills.

2.5.1. Gaming

Figure 6 presents the *Instruction* page, which outlines the designated tasks and procedures. First, it explains that the user will be teleported into a VR environment that includes sounds, images, and models, and asked to complete a single task in 30 s. When ready, the user clicks the *Test Me!* button to initiate the game. At this point, a lakeside forest simulation appears, which was designed by using 3D models from Sketchfab. In addition, three animals are depicted, with each one randomly appearing to distract the user. Meanwhile, random static balloons (blue) are displayed. During the initial 15-s interval, the user is immersed in a visual depiction of a lakeside forest environment featuring an animated yellow duck and a stationary monkey. At the 15-s mark, a white duck is introduced, followed by the departure of the aforementioned animals.

Previous research has indicated that individuals with ADHD tend to have difficulty differentiating colors. In this case, the retinal mechanism of the dopaminergic neurotransmitter system is responsible for the short wavelength cone sensitivity. According to the Journal of Child Psychology and Psychiatry,[24] some children and adults with ADHD exhibit poorer performance on color perception tests, especially discriminating against the color blue. In this context, a German version of the Stroop-Color-Word Test was conducted on children with and without ADHD. Specifically, in the first scenario, the participants were tasked with reading color words, while in the second scenario, they had to name the colors of the bars printed in these colors. Then, in the third scenario, they were asked to name the colors of the color words printed in incongruous colors. Based on the findings, the children with ADHD exhibited more errors, particularly with blue–yellow stimulation, indicating potential problems in their retinal dopaminergic mechanisms. It also suggests that color perceptions tend to change with ADHD.

As shown in Figure 7, the blue balloons are randomly positioned in the VR environment, after which the user is asked to pop the balloons by clicking on them. In this case, the user is given 30 s to pop as many balloons as possible, while counting the number of popped balloons. However, the following are the challenges that this game presents to the user:

1. The task is to count the number of popped balloons. However, the environment includes numerous obstacles and distractions, such as the animals' random appearance/disappearance and movement in the game. For example, two ducks and a monkey randomly appear in the simulation.
2. The blue balloons. As previously mentioned, certain colors can trigger signs and symptoms of ADHD.

At the end of the game, indications of ADHD are identified

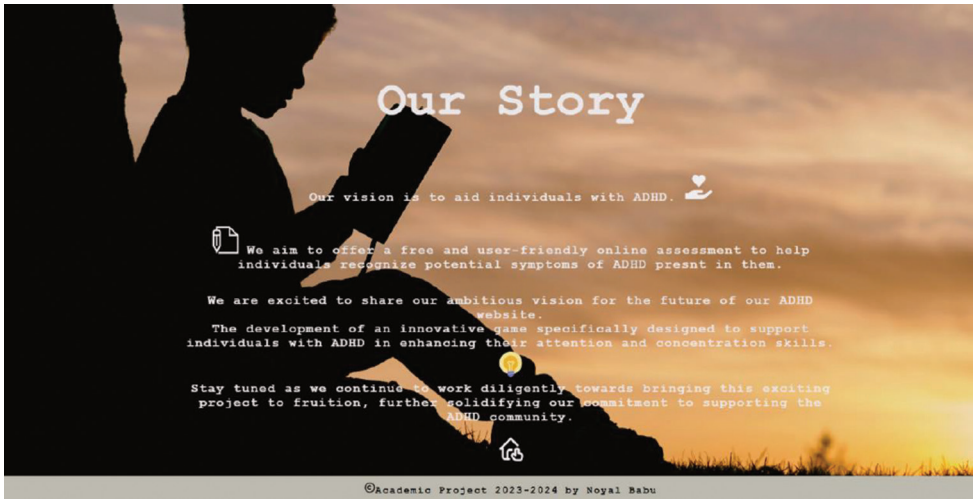

Figure 5. The *Our Story* page describes the main purpose of this project. Image created by the authors.
Abbreviation: ADHD: Attention deficit hyperactivity disorder.

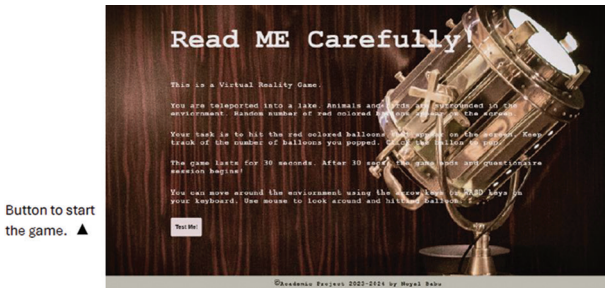

Figure 6. The *Instruction* page. Image created by the authors.

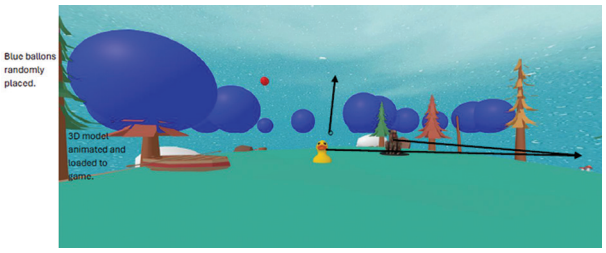

Figure 7. VR game overview. Image created by the authors.
Abbreviation: VR: Virtual reality.

(if presented by the user) through various symptoms such as diminished attention, reduced concentration, and heightened susceptibility to distractions. The user is then directed to the *Questionnaire* page (Figure 8).

As shown in this figure, three questions were designed to assess the user's performance. The first question is regarding the number of balloons that the user popped, which is used to analyze the capacity to focus on the prescribed task. Second, the game tests the user's attention and focus level by asking about the number of animals spotted in the game. Finally, the user is examined to

Figure 8. The *Questionnaire* page. Image created by the authors.

understand how easily he/she gets distracted by small simulations. The answers are then analyzed to explain the user's performance.

Since the game was developed by using React, A-Frame, and Three.js, it can be played on computer devices and on VR devices that support these technologies. On computer devices, the arrow keys or the WASD keys on the keyboard are used to move around the surroundings, while the mouse is used to look around the gaming environment. In this case, when moving the mouse, the user can see a black ring on the screen. In VR and 3D environments created with A-Frame, mouse controls are configured inside the camera elements and a cursor is often included to indicate where the user is pointing or looking. This ensures that the black ring intersects with the object to click, drag, or move it in the scene. It also serves as a visual feedback

mechanism to help the user interact with the objects in the gaming environment.

2.5.2. Testing

The techniques employed to monitor the user's performance in the *Mind Marvel* platform are detailed in Figure 9, which shows the process of gathering data on the number of balloons popped by the user. The platform is coded so that blue balloons are randomly generated in the beginning of the game. In this case, each balloon is assigned a unique identifier comprising a numerical value, followed by the term "balloon-." By utilizing a property from Three.js called "children. Length," the number of child elements within the scene is obtained. This number is then appended as the ID for each generated balloon by using "sceneRef.current.child.length."

Finally, the event listener is attached to each balloon to facilitate the user's interaction through clicks. In this case, upon clicking a balloon, a function is triggered to tally and store the balloon popped by the user in a variable, after which the balloon is simultaneously removed from the scene. Thus, a function is developed to track the number of balloons that the user pops. The accumulated data is then passed on to the *Questionnaire* section to analyze the user's performance.

3. Results

3.1. Analyzing the user's performance

In the *Mind Marvel* platform, the *Questionnaire* section is the component in which the user's performance is evaluated. Here, the user is asked specific questions about the game and the gaming environment. The answers are then analyzed to determine the presence of ADHD. As mentioned earlier, the task is to pop the blue balloons in the scene and keep a count of them. However, the color blue can trigger ADHD symptoms in the user,[2] including difficulty distinguishing the balloons when there are many blue-colored ones. Additionally, the environment is designed to distract the user from completing the task, with the animals randomly appearing and disappearing in the scene.

```
balloon.setAttribute('id', `balloon-${sceneRef.current.children.length}`);
balloon.setAttribute('position', `${Math.random() * 10 - 5} 1 ${-Math.random() * 10}`);
balloon.setAttribute('static-body', true);
balloon.addEventListener('click', () => removeBalloon(balloon));
```

```
//Here, the balloon disappers when clicked
const removeBalloon = (balloon) => {
  setTotalBalloonsHit(prevHit => prevHit + 1);
  sceneRef.current.removeChild(balloon);
  play();
};
```

Figure 9. Code snippet of the balloon hit method. Image created by the authors.

Figure 10. Example of the responses to Question 1 vs. correct and incorrect inputs. Image created by the authors.

The following questions (Figures 10 and 11) focus on the relevance and function of the *Questionnaire*:

Question 1 – How many balloons did you pop?

- *Objective* – This question is regarding the number of balloons the user popped. In this case, it tests the user's ability to focus on the given task and assesses his/her memory skills.
- *Mechanism* – The user is asked to enter the number of balloons popped, after which the given answer is compared to the data from the game. If the user fails to accurately report the number of balloons popped, then it indicates potential challenges in maintaining attention and focus during repetitive activities. In this case, the randomly placed animals might have garnered the attention of the user, while the color of the balloons might have triggered the ADHD symptoms in the user. As stated earlier, individuals with ADHD often demonstrate susceptibility to distraction, particularly in contexts involving detailed simulations.
- *Outcome* – Correct responses from the user represent strong attentional abilities and accurate information reports, whereas incorrect responses indicate the presence of a short attention span.

Question 2 – Count the creatures, animals, and birds, that you have observed in the game.

- *Objective* – This question tests the user's awareness of the simulated environment and his/her ability to complete the given task. It also determines if the user was distracted by the various simulations and sounds.
- *Mechanism* – The VR environment in this game was created with 3D animated models, which offer the user an immersive simulation of real-world scenarios, while simultaneously evaluating his/her ability to focus on completing the designated task. In addition, the use of animals as distractions introduces an element of impulsivity. In this case, individuals with ADHD tend to have difficulty avoiding external interactions when focusing on the main task.

In this game, the tendency of individuals with ADHD to impulsively engage with the animals reflects impulsivity,

Figure 11. Example of the responses to Question 2. Image created by the authors.

which is another common ADHD symptom. In this regard, asking the user to recall the VR game scene assesses his/her awareness of the virtual surroundings. Here, the user is given three options and asked to select the correct answer. It is important to note that Question 2 can only be answered after answering Question 1. Moreover, the answer provided by the user can determine the presence of ADHD, depending on his/her answer in Question 1.

- *Outcome* – If the user correctly answers the number of balloons popped as well as the number of animals in the game, then it indicates that his/her brain successfully focused on the assigned task and remained aware of the surroundings. In addition, the user demonstrates commendable attentional prowess, exhibiting strong focus and an impressive capacity to recall past information. However, if the user correctly answers the number of balloons popped, but cannot recall the number of animals in the game, then it indicates that his/her brain was solely focused on the given task and avoided the urge to notice other elements in the game. Meanwhile, if the user fails to complete the task, but correctly identifies the number of animals, then it indicates impulsivity, a characteristic often associated with ADHD. In other words, the user's focus shifted from the primary task to the distraction. This supports the notion that individuals with ADHD tend to have difficulty avoiding external distractions, thus affecting their ability to maintain focus on specific tasks.

Question 3 – Can you name the color of the first duck that appeared in the game?

- *Objective* – This question determines if the user was distracted by the simulations and small movements in the game, explaining the deviation of the user's focus from the primary task to such distractions.
- *Mechanism* – This game includes two ducks, which appear at different times. For example, at the start of the game, a yellow duck appears, but after 15 s, a white duck appears. This mechanism is designed to distract the user from counting the balloons popped, hindering him/her from recalling such information.
- *Outcome* – If the user correctly names the color of the duck, then it indicates that he/she gazed on the alternative simulations in the scene. If the user fails to name the color of the duck, then it indicates that his/her brain was solely focused on the given task.

3.2. Additional results

In this game, once the user's performance is recorded, the results (including the potential signs and symptoms of ADHD) are provided to him/her. Specifically, by observing gameplay behavior and performance, the user can receive valuable information about his/her attentional control, impulsivity, sensory processing, and executive functioning, all of which are relevant to ADHD diagnosis and management. However, it is important to note that *Mind Marvel* does not officially diagnose ADHD, but mainly functions as a screening tool. In this case, this tool employs a brief questionnaire that only comprises three questions, with a limited range of responses to analyze the user's performance. This game also analyzes these responses and generates one of two possible results. Table 3 illustrates the relationship between the user's responses and the corresponding results.

4. Discussion

This study examined the effectiveness of the *Mind Marvel* platform, as a game-based approach toward neurological disorders (e.g., ADHD) that integrates a webpage design with VR technology. The development of this platform was driven by the need to identify and address potential ADHD symptoms exhibited by the user. The completed application encompasses three primary aspects of an ADHD-based game: task-oriented focus, educational attainment, and the identification of neurological conditions.[13] Set against a backdrop of dynamic surroundings featuring vibrant colors, animated characters, and background audio, this game evaluates the user's attention span, impulsivity, hyperactivity, and concentration, all of which are indicators of potential ADHD.

The *Mind Marvel* platform also offers an interactive educational webpage on ADHD Awareness, with insights into understanding ADHD, its signs and symptoms, and facts about the condition. This webpage is structured in an easy-to-understand format, ensuring that the user can access information about ADHD with a single click. As for

the interactive backgrounds, they are designed to provide a fun and engaging experience, while raising awareness about ADHD. Once the game ends, the user's performance is analyzed to generate an appropriate outcome regarding ADHD symptoms. Hence, this project successfully reflects the perspectives of Craven *et al.*[13]

Following our analysis of ADHD and digital games, two existing games, i.e., *Snappy App* and *EndeavorRx*, were examined, the results of which are presented in Table 4. Based on the findings, a comparison of these games with the *Mind Marvel* platform can be drawn.

According to this table, *Mind Marvel* (like *Snappy App* and *EndeavorRx*) is a sophisticated game developed by conducting meticulous research and employing highly recommended methodologies. This table also illustrates how the game effectively functions as a suitable ADHD intervention that is similar to the other two applications.

Overall, the *Mind Marvel* platform provides the user with an immersive VR experience through its ADHD game. It also tests the user's cognitive skills such as recalling information, decision-making, problem-solving, and concentration skills. With such a dynamic environment, the game is able to expose the presence of ADHD signs, including short attention span, distraction to simulations, carelessness, and short memory (if present in the user). Meanwhile, pleasant backgrounds and soothing soundtracks in the game induce emotions and increase the potential psychological conditions.

There are clearly implications from this research that also involve other types of technologies such as wearable technologies. In this regard, more informative platforms should be designed in which wearable sensors are used to detect (and trigger) data acquisition. For instance, the detection of meltdown events or other considerable episodes (e.g., stress episodes) could activate (or deactivate) a specific set of VR environments or, in the context of this research, a different set of questions.

Table 4. Comparison of *Mind Marvel*'s performance with two ADHD games, i.e., *Snappy App* and *EndeavorRx*.

Features offered by the game	Importance of the features	Games that offer this feature	
ADHD Awareness page	• Aids the user in understanding the signs and symptoms of ADHD. • Despite the prevalence of ADHD, a remarkable proportion of individuals are unaware of their condition.	*Snappy App* *EndeavorRx* *Mind Marvel*	✓ ✗ ✓
Our Story page	• Describes the purpose of the application. • Aids the user in understanding how the application serves as a medium in the context of ADHD.	*Snappy App* *EndeavorRx* *Mind Marvel*	✓ ✓ ✓
Instruction page	• Explains the functioning of the application.	*Snappy App* *EndeavorRx* *Mind Marvel*	✗ ✓ ✓
Accessibility	• The game must be available to all people via all devices. *Snappy App* and *EndeavorRx* are mobile applications, while *Mind Marvel* is a web-based application that can be accessed from any device via a browser.	*Snappy App* *EndeavorRx* *Mind Marvel*	✗ ✗ ✓
Analysis and report	• The game analyzes the user's performance, after which it generates an appropriate report. • The user is informed about his/her cognitive skills, based on the resulting assessment.	*Snappy App* *EndeavorRx* *Mind Marvel*	✓ ✓ ✓
Providing medical support to individuals with ADHD	• If the application identifies the presence of or signs of ADHD in the user, then medical/clinical support is necessary. • Further steps are presented to the user.	*Snappy App* *EndeavorRx* *Mind Marvel*	✗ ✓ ✗

5. Conclusion

In this study, we presented the *Mind Marvel* platform, a system that combines the design of interactive webpages and VR technology. After conducting comprehensive research into ADHD, a deeper understanding of the signs and symptoms, treatment modalities, diagnostic procedures, and underlying causative factors was obtained. According to previous research, identifying the early signs of impulsiveness and hyperactivity in children can help control this neurological disorder and channel such energy into building a better life for them.[25] Although genetics or environmental factors play a considerable role in ADHD, they cannot prevent the occurrence of it in individuals. Thus, improving cognitive skills, along with medical interventions and treatment, can help individuals with ADHD in recovery.[26]

Undertaking a study on this neurological disorder opened the path for exploring the importance of digital video games as a treatment or diagnosis tool since such games can improve the user's cognitive functions, focus, multitasking ability, stress management, and thinking skills. In this regard, the *Mind Marvel* platform provides a VR game with a dynamic environment and challenging tasks to assess the presence of ADHD symptoms in the user. The application also assesses his/her performance and provides helpful feedback. As for the importance of VR technology, previous research has highlighted the following benefits:

- VR technology can train cognitive skills.[27,28]
- VR games, such as *EndeavourRx*, can be used as a prescribed treatment method since they can help identify the underlying symptoms of ADHD in individuals.
- VR environments and simulations can provide a soothing effect on users by reducing stress, depression, fatigue, and anxiety.

Although this study showed that this VR game can help identify the symptoms of ADHD, there are potential enhancements for future updates of this application. First, to effectively address ADHD, collaboration with clinicians, psychologists, and medical teams in the design and construction process is imperative, along with the choice of proper technologies.[29-31] While extensive research assisted the development process of our ADHD game, the advice of clinicians and medical teams would have enhanced the overall function of the application. Second, automated reporting of the user's performance to clinicians, with guidance after ADHD detection, should be integrated. Third, using video games as a treatment method can have a negative impact on the user. For instance, since individuals with ADHD tend to be impulsive and hyperactive, there is a potential of developing an addiction to these games. Thus, future iterations of the proposed system should investigate different methods and technological solutions to alleviate the adverse effects of digital ADHD games on users.[29,32-34] Finally, it would be interesting to integrate the *Mind Marvel* platform with another set of solutions from the engineering and computer science fields. For example, wearable sensors can provide important physiological information about the performance and physiological status of the user when performing tasks in this platform.[32,35,36] Moreover, an analysis of this sensorial information (combined with machine learning techniques) can support the detection of stress conditions associated with the designated tasks.[34] In this case, this integrated approach would benefit from clinical validation based on high-quality measurements.[37]

In sum, the *Mind Marvel* platform demonstrated its potential as an effective intervention for identifying ADHD signs and symptoms in users. Furthermore, it underscored the importance of raising awareness about ADHD among the public by providing a dynamic and engaging platform for disseminating ADHD-related information to all users.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Noyal Babu, Neil Buckley
Formal analysis: Noyal Babu
Investigation: Noyal Babu
Methodology: Noyal Babu, Neil Buckley
Writing – original draft: Noyal Babu, Emanuele Lindo Secco
Writing – review & editing: Emanuele Lindo Secco

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The entire development process of the *Mind Marvel* web

application is recorded, and versions are tracked inside the GitHub repository. Visit the link to the repository: https://github.com/Noyalbabu/Dissertation_Implementation.

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ORIGINAL RESEARCH ARTICLE

Drosophila Sirtuin 1 plays a neuroprotective role in altering Alzheimer's disease-related pathologies in flies

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Abstract

Sirtuin, a Class III histone deacetylase enzyme dependent on nicotinamide adenine dinucleotide, plays a pivotal role in aging and age-related diseases. Numerous studies have highlighted the involvement of sirtuins in Alzheimer's and other neurodegenerative diseases; however, their molecular mechanisms and possible interactions with Alzheimer's disease (AD)-associated genes remain unclear. In this study, using a *Drosophila melanogaster* model of AD, we investigated the potential genetic interactions between sirtuin and AD-associated genes, including amyloid-beta 42, Appl, and Tau. Our findings show that the overexpression or downregulation of *Drosophila* Sirtuin 1 alters AD-related pathologies such as the rough eye phenotype, behavioral impairments, and excessive cell death observed in AD model flies. In addition, the observed rescue of AD pathologies appears to be associated with sirtuin overexpression, which modulates c-Jun N-terminal kinase and Notch signaling pathways in flies. These findings show that Sirtuin1 plays a neuroprotective role in AD.

Keywords: Sirtuin 1; Alzheimer's diseases; *Drosophila melanogaster*; Alzheimer's disease-related pathologies; Amyloid protein; Histone deacetylase enzymes

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1. Introduction

Sirtuins, a conserved group within the Class III histone deacetylase family, are dependent on nicotinamide adenine dinucleotide. They remove acetyl groups from lysine residues on both histone and non-histone proteins through hydrolysis and are generally known as "lysine deacetylases".[1-3] In humans, seven (*Sirt1 – 7*) and five (Sirt1 [dsir2, Sirt2 [dsirt2, Sirt4 [dsirt4, Sirt6 [dsirt6, and Sirt7 [dsirt7) sirtuin genes have been identified in humans and *Drosophila melanogaster*, respectively.[4,5] Of the five sirtuin genes, *Sirt1* (*dsir2*) is one of the most conserved genes in *D. melanogaster*, with its overexpression shown to prolong the lifespan of fruit flies.[6,7] Research on *Drosophila* has highlighted *dsir2* as a crucial gene involved in the anti-aging process.[8-11] One study demonstrated that *dsir2* and *dfoxo* are required to maintain the expression of the autophagy-related gene atg18 during aging.[12] Earlier studies have also indicated that *dsir2*, *foxo*, and *4E-BP* play essential roles in regulating the lifespan of *Drosophila*.[13] SIRT1, a nuclear protein, is instrumental in lifespan extension, cell cycle regulation, and histone deacetylation.[5,14,15]

Sirt1 is a well-studied gene in the context of aging and age-related neurodegenerative diseases (NDDs).[3,16] Evidence suggests that it plays a protective role against NDDs, cancer, cardiovascular conditions, and other age-related issues.[3,16] Alzheimer's disease (AD) is a leading cause of dementia and cognitive decline in the elderly,[17-19] characterized by two main neuropathological hallmarks: extracellular senile plaques primarily composed of amyloid-beta (Aβ) peptide, formed by the amyloidogenic cleavage of the APP protein by β and γ-secretase and intracellular neurofibrillary tangles (NFT) associated with tau, a microtubule-binding protein.[20-24]

D. melanogaster, commonly known as the "fruit fly," has been an ideal and powerful invertebrate model organism for genetic research for over 120 years. In the *Drosophila* model of AD, neuronal death results in a rough eye phenotype, impairments in learning, memory, climbing, and phototaxis.[25-28] In addition, studies have shown that the upregulation of AD-associated genes ($A\beta_{42}$, *Tau*, and *Appl*) in *Drosophila* induces cell death (apoptosis) through increased cellular stress.[29-31]

Although *Sirt1* has been widely studied in the context of aging, the molecular details of its interactions with AD-related genes (Tau, Aβ42, and Appl) remain unclear. In this study, we investigated the potential genetic interactions between *Sirt1* and the abovementioned AD-associated genes in *Drosophila*.

2. Materials and methods

2.1. *Drosophila* stocks and genetics

The W^{1118} strain of *D. melanogaster* is used as a wild-type control in experimental studies. Gal4 stocks: Pan-retinal *GMR-GAL4* (Chr. II) drives gene expression in all cells posterior to the morphogenetic furrow (MF) in the developing eye and later becomes active throughout most of the pupal eye.[27,32] Pan-neuronal *elav-Gal4* (Chr. X) drives gene expression in the sensory neurons of the fly brain under the control of *elav*. Both *GMR-GAL4* and *elav-Gal4* flies were used as control groups in the experiment.

In this study, AD-associated genes were expressed in the fly eye using the *GMR-GAL4* driver, which induced retinal degeneration and resulted in a rough eye phenotype. AD model flies were crossed with *elav-Gal4* to express AD-associated genes in sensory neurons, resulting in degenerative phenotypes, including pathological morphologies and behavioral changes associated with AD.

Sirt1 overexpressing/downregulating transgenic fly stocks: *UAS-Sirt1* (Chr. II) [BL#44216] overexpresses the *Sirt1* gene, while *UAS-Sirt1*^{RNAi} (Chr. III) [BL#31636] is an RNA interference (RNAi) line for *Sirt1* downregulation. *Drosophila* c-Jun N-terminal kinase (JNK) *Basket* (*Bsk*) downregulating transgenic fly stocks: *UAS-Bsk*^{RNAi} (Chr. III) [BL#31323] is an RNAi line for *Drosophila* JNK (*Bsk*) gene downregulation. *Notch* downregulating transgenic fly stocks: *UAS-Notch*^{RNAi} (Chr. III) is an RNAi line for *Notch* gene downregulation. Transgenic fly stocks overexpressing or downregulating AD-related gene: *UAS-A*β_{42}*(Human)/CyO* (Chr. II) expresses the human $A\beta_{42}$ gene under the UAS system; *UAS-Tau*$_{WT}$ (Chr. II) [BL# 51362] expresses wild-type *Tau* under UAS control. UAS-*Appl*^{RNAi} (Chr. III) [BL# 28043] is an RNAi line for *Appl* downregulation. Recombined fly stocks include the following: w;*GMR-GAL4-UAS-A*β_{42}*(Human)/CyO;+/+*, a stock with *GMR-GAL4* and *UAS-A*β_{42}*(Human)/CyO*, w;*GMR-GAL4-UAS-Tau*$_{WT}$*/CyO;+/+*, a stock with *GMR-GAL4*; and *UAS-Tau*$_{WT}$*/CyO*, and *GMR-GAL4/+;UAS-Appl*^{RNAi}*/+* (Chr. III) [BL#28043], an RNAi line for *Appl* downregulation.

UAS-ArcAβ_{42} Stocks: UAS-ArcAβ42 (Chr. III) and *UAS-ArcA*β_{42} (Chr. II) express the human Aβ42 fragment of APP with the familial Alzheimer's Arctic mutation under UAS control. *UAS-ArcA*β_{42} (Chr. III) and *UAS-ArcA*β_{42} (Chr. II) were provided by Damin Crowther, Cambridge University, UK.

We used the *UAS-ArcA*β_{42} fly strain for climbing assays, cell death assays, real-time reverse transcription polymerase chain reaction (RT-PCR) analysis, and immunostaining because other AD transgenic flies, such as *UAS-A*β_{42}*(Human)/CyO*, *UAS-Tau*$_{WT,}$ and *UAS-Sirt1*, are present on the second chromosome, making it unfeasible to perform crosses between each AD-related gene and *UAS-Sirt1* with *elav-Gal4*.

All flies were cultured in a biological oxygen demand (BOD) incubator at 22°C ± 1°C on standard *Drosophila* food media, which included agar powder (regular grade for bacteriology, SRL#19661), maize powder, sugar, yeast powder (High sugar eagle instant dry yeast), nepagin (an antifungal agent, methyl-p-hydroxybenzoate sodium salt 99% extrapure, Loba Chemie#04661), and propionic acid (antibacterial, SRL#12931).

2.2. Microscopy imaging

Light microscopy was employed to examine the external eye morphology of adult flies. For this experiment, 10-day-old flies of the selected genotypes were examined under a light microscope. Eye images were captured at 51.2× magnification under a Carl Zeiss Stemi™ DV4 stereo binocular microscope. Measurements were recorded in micrometers using TS Viwe7 (version 7.1.3.7) software. For light microscopic imaging, 40 flies of the desired genotype were used.

2.3. Nail polish imprint of adult eyes

The external eye morphology of adult Drosophila was observed following the methodology described previously.[33] For this procedure, flies from the selected groups were anesthetized, placed on a clean glass slide, and decapitated. The heads were coated with small drops of clear nail polish to create an impression of the eye surface, which was then allowed to dry at 24°C. After drying, the nail polish layer was carefully removed from the eyes using fine dissecting needles, forming an exact replica of the eye's surface, mimicking the goblet-shaped appearance of the adult eye. The nail polish layer was placed on a glass slide with the imprinted side facing upward. To flatten the layer, a coverslip was gently placed over it, and mild pressure was applied using a needle. The preparations were observed using differential interference contrast microscopy for imaging.

2.4. Climbing assay

This assay was conducted as described earlier.[34] For this assay, flies aged 10, 20, and 30 days were placed in a vertical glass tube (30 cm long × 1.5 cm wide) and allowed to acclimatize for 2 min. Flies were then gently tapped to the bottom of the tube, and the number of flies crossing the 8 cm mark within 10 s^{-1} was counted. Twenty flies of each genotype were placed in the tube, and the experiment was conducted 5 times. In total, 100 flies per genotype were used for the climbing assay. The results were expressed as the percentage (%) of flies climbing 8 cm in 10 s^{-1} under standard lighting conditions.

2.5. Phototaxis assay

This assay was also conducted as described previously.[34] For this test, 10-day-old flies were placed in a Y-maze glass tube (with one light arm and one dark arm) and allowed to acclimatize for 2 min. Flies were then gently tapped and allowed to move through the Y-maze for 20 s. The number of flies moving toward the light and dark paths was recorded. Twenty flies of the desired genotype were placed in the Y-maze, and the experiment was repeated 5 times. For the phototaxis assay, 100 flies per genotype were used. The assay was performed under standard lighting conditions (~500 Lux), and results were presented as a light preference index.

$$\text{Light preference index} = \frac{\text{Number of flies traveling toward the light path} - \text{Number of flies traveling toward the dark path}}{\text{Total number of flies}}$$

2.6. Body weight analysis

Body weight analysis was conducted as described in a previous study.[34] For the present study, flies aged 10, 20, and 30 days of the desired genotype were used. The body weights of 20 adult flies were collectively measured using a Sartorius weighing balance (Germany) in milligrams (mg). The experiment was repeated 5 times, using 100 flies from each genotype.

2.7. Survival (lifespan) assay

The survival assay was performed as described previously.[35] Recently eclosed adult flies were collected, with a total of 100 flies (20 flies per vial) used for each genotype. Every alternate day flies from each vial were transferred to fresh food media, and the number of dead flies was recorded daily for up to 50 days. Throughout the assay, flies were kept in a temperature-controlled BOD incubator. Median survival was calculated using the Kaplan–Meier method,[36] and a survival curve was plotted. Statistical analysis was conducted using GraphPad Prism 5.0 software. Significant differences between genotypes and major variations in median survival were evaluated using the Mantel–Cox log-rank test.[37]

2.8. Acridine orange (AO) staining

AO staining was performed as described previously,[35] with minor modifications. Larval eye imaginal discs and larval brains were dissected in 1× phosphate-buffered saline (PBS) and then incubated for 2 min in a 1 μg/mL AO solution (Cat# 877529, Invitrogen, USA) prepared in 1× PBS. The tissues were washed, mounted in 1× PBS, and immediately examined using a laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany). More than 20 larval eye imaginal discs and larval brains were collected from each genotype. Quantification of AO-positive cells was conducted using ImageJ 5.0 software (NIH, USA).

2.9. Quantitative RT-PCR (qRT-PCR)

qRT-PCR was conducted as described previously,[38] with slight modifications. Briefly, 30-day-old fly heads were isolated for mRNA extraction using TRIzol reagent (Cat# 15596026, Invitrogen, USA). cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Cat#AB-1452/B, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Amplification of cDNA was carried out using gene-specific primers. A 20 μl reaction mixture was prepared, containing PowerUP™ SYBR™ Green Master Mix (Cat. #A25742, Applied Biosystems, Thermo Fisher Scientific, USA), primers and cDNA.

qRT-PCR was performed using the Step One Plus system (Applied Biosystems, USA). Relative quantification was conducted using the "Delta-Delta Ct" (ΔΔct) method, normalized with the endogenous gene *RP49*. Data are presented as mean ± standard error of the mean (SEM). Relative mRNA levels were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, using GraphPad Prism 5.0 software. Table 1 provides details of the primers used for qRT-PCR.

2.10. Immunostaining of larval brain

The immunostaining of the larval brain was performed by selecting third-instar larvae of the desired genotype. Larval brains were dissected in 1X PBS and then fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). The dissected brain tissues were washed 3 times in 1% PBST (1X PBS, 1% Triton X-100) for 15 min each and then blocked in a 4% bovine serum albumin solution in 1X PBS for 2 h at RT. The tissues were subsequently incubated overnight at 4°C in a blocking solution containing primary antibodies: rabbit-anti-P-JNK (1:100, Cat #V7931, Promega), mouse-anti-*Drosophila* Notch intracellular domain (NICD, 1:100, Cat#C17.9C6, DSHB), mouse-anti-*Drosophila* Delta extracellular domain (1:50, Cat#C594.9B, DSHB), and rabbit-anti *β*-Amyloid (1-43) (1:50, Cat#E8C2D, Cell Signaling Technology, USA). Following primary antibody incubation, brain tissues were washed 3 times in 0.1% PBST for 15 min each, then blocked with blocking solution at RT for 1 h, and subsequently incubated with secondary antibodies: AF-488 Goat-anti-Rabbit IgG (1:150, Cat# A11008, Invitrogen), AF-488 Goat-anti-Mouse IgG (1:150, Cat# A11001, Invitrogen), and Anti-Mouse Cy3 IgG (1:150, Cat# C2181, Sigma) for 2 h at RT. After secondary antibody incubation, the brain tissues were washed 3 times in 0.1% PBST for 15 min each and mounted in DABCO (Sigma-Aldrich, USA), an antifade medium. The samples were analyzed using a fluorescence microscope, and a total of 20 third-instar larval brains were examined for each genotype.

2.11. Statistical analysis

All data are presented as the mean ± SEM, with the number of biological replicates indicated as "n." For all experiments, the significance between genotypes was assessed using one-way ANOVA with Tukey's test in GraphPad Prism 5.0. Images were created with Adobe Photoshop 7.0®. Histograms were analyzed in GraphPad Prism 5.0, with significance indicated as follows: ns, non-significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

3. Results

3.1. Overexpression of *Sirt1* modulates the AD-related pathologies in *Drosophila*

AD model flies in *Drosophila* showed various AD-related pathologies in flies, including rough eyes, reduced or loss of survival, and impaired locomotor and phototaxis behaviors.[27] To investigate the effect of *Sirt1* overexpression and its genetic interaction with AD-associated genes ($A\beta_{42}$, *Tau*, and *Appl*) in fruit flies, we conducted genetic crosses between fly lines overexpressing and downregulating *Sirt1* with AD model flies in *Drosophila* and examined any phenotypic alterations. The phenotypic changes indicated possible genetic interactions between *Sirt1* and AD-associated genes. In the present study, we used *UAS-*$A\beta_{2}$*(Human)*, *UAS-*Tau_{WT}, and *UAS-*$Appl^{RNAi}$ AD model flies as well as *UAS-Sirt1* to overexpress and *UAS-*$Sirt1^{RNAi}$ to downregulate *Sirtuin1* in *Drosophila*. The UAS

Table 1. The primers used for quantitative real-time reverse transcription-quantitative polymerase chain reaction

Gene	Forward primer sequence	Reverse primer sequence
Sirt1	5′ TTTGCCCGCGAGATATATCC 3′	5′ GCCCTTGGTCTCCAGCATT 3′
$A\beta_{42}$	5′ CGAGCGATTGCTGTTGGA 3′	5′ TCCCGACCGCTTCTGTTC 3′
Tau	5′CAATAGCAACACCACTTCGGATAG 3′	5′ CGTATCTGCTGTTTGGAAACTGA 3′
Appl	5′ CCCAGATTGCCGTTCTCTGT 3′	5′ TGTGGGACCCGGTTGTCTTCT 3′
Grim	5′ TGGATGCTGGGATCTTTTGG 3′	5′ CGCTGGCTCGAACTGTAGCT 3′
Reaper	5′ CGGGAGTCACAGTGGAGATTC 3′	5′ GGTCTTCGGATGACATGAAGTG 3′
Hid	5′ GAGTGCCCCGCAAATCTTC 3′	5′ CCGTGCGGAAAGAACACAT 3′
DIAP1	5′ TTGGTTTGGCTGGGCTTATT 3′	5′ GGCTTGGAGTGCCATCGA 3′
JNK	5′ ATCAGCTCCATGACCAGGTAGAC 3′	5′ACTTGGATCACGACAGAATGTCC 3′
Notch	5′ CGATGCGTTGCCAAAATG 3′	5′ CAAAGGACACTTGCACGAGATG 3′
Delta	5′GCTTCACGAATCCCATCCA 3′	5′ TCGACGATCAGCGAGAAGGT 3′
RP49	5′ GCAAGCCCAAGGGTATCGA 3′	5′ ACCGATGTTGGGCATCAGA 3′

flies were driven by pan-retinal GAL4 "*GMR-GAL4*" and pan-neuronal GAL4 "*elav-GAL4*." *Sirt1* overexpression in control flies (*GMR-GAL4/UAS-Sirt1;+/+*) did not show observable changes in the eye compared to that in control *GMR-GAL4/+;+/+* flies (Figure 1A and A'), whereas slight degeneration in ommatidia and abnormal bristle arrangement was observed in the *Sirt1* downregulation flies driven by *GMR-GAL4* (*GMR-GAL4/+;UAS-Sirt1^{RNAi}/+*) (Figure 1E and E'). Further, we found that the rough eye phenotype, abnormal bristles, and ommatidial arrangement characteristic of AD model flies were significantly improved by *Sirt1* overexpression (*GMR-GAL4-UAS-Aβ$_{42}$(Human)/UAS-Sirt1;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+*, and *GMR-GAL4/UAS-Sirt1;UAS-Appl^{RNAi}/+*) (Figure 1B, B'-C, C'-D and D'), whereas *Sirt1* downregulation in an AD genetic background (*GMR-GAL4-UAS-Aβ$_{42}$(Human)/+;UAS-Sirt1^{RNAi}/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Sirt1^{RNAi}/+* and *GMR-GAL4/+;UAS-ApplRNAi/UAS-Sirt1^{RNAi}*) slightly increased these AD-related pathologies (Figure 1F, F'-G, G'-H, and H').

Further, we investigated the effect of *Sirt1* on learning and memory, as impairments in these areas directly impact behavioral activities in AD model flies.[39-41] We analyzed the effect of *Sirt1* overexpression on phototaxis activity in 10-day-old AD model flies using a phototaxis assay (Figure 1I).

As shown in Figure 1I, *GMR-GAL4-UAS-Aβ$_{42}$(Human)/+;+/+* flies had a light preference index of 7.56, which significantly improved to 15.61 with *Sirt1* overexpression in *GMR-GAL4-UAS-Aβ$_{42}$(Human)/UAS-Sirt1;+/+* flies. Similarly, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies had a light preference index of 9.19, which increased to 14.59 with *Sirt1* overexpression in *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+* flies. In *GMR-GAL4/+;UAS-Appl^{RNAi}/+* flies, the light preference index was 8.80, which significantly increased to 15.3 with *Sirt1* overexpression in *GMR-GAL4/UAS-Sirt1;UAS-Appl^{RNAi}/+* flies.

We also examined the impact of *Sirt1* on the climbing activity of arctic *Aβ$_{42}$* mutants in flies aged 10, 20, and 30 days. Our results showed that *Sirt1* overexpression in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* flies significantly improved climbing activity to 77.4%, 71.6%, and 66%, respectively, compared to same-aged *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* AD model flies, which exhibited lower climbing activity at 52.2%, 46.6%, and 35.4%, respectively (Figure 1J).

We further investigated the effect of *Sirt1* on body weight in *elav-GAL4*-driven AD model flies. The body weights of 10-, 20-, and 30-day-old *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* flies were 15.24, 9.8, and 6.48 mg, respectively, compared to *elav-Gal4/+;+/+;+/+* flies, which had body weights of 17.04, 15.46, and 14.56 mg, respectively (Figure 1K). *Sirt1* overexpression in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* flies significantly increased body weights to 15.24, 14, and 11.82 mg, respectively, compared to *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* flies (Figure 1K).

3.2. Overexpressing *Sirt1* modulates the lifespan of AD model flies

We examined the impact of *Sirt1* overexpression on the lifespan (survival) of AD model flies. Our results showed a significant decrease in the median survival (30 days) of AD model flies (*elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+*) compared to the median survival of 60 days for *elav-Gal4/+;+/+;+/+* flies and 56 days for *w^{1118}* flies (Figure 2). Furthermore, we observed that AD model flies with *Sirt1* overexpression (*elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*) exhibited a significantly increased median survival of up to 48 days compared to control AD model flies (Figure 2). In contrast, AD model flies with *Sirt1* downregulation (*elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*) showed a reduced median survival of 34 days.

Table 2 shows the median survival (days) for all genotypes.

3.3. Overexpression of *Sirt1* decreases the ectopic expression of *Aβ$_{42}$* in third-instar larval brain of Drosophila.

As demonstrated in Figure 3, *elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+* (AD model flies) exhibited increased expression of *Aβ$_{42}$* in the third instar larval brain (Figure 3B and E) compared to the experimental control flies (*elav-Gal4/+;+/+;+/+*) (Figure 3A and E). Furthermore, overexpression of *Sirt1* in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* flies significantly decreased Aβ$_{42}$ expression compared to that in AD model flies (*elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+*) (Figure 3C and E). In contrast, *Sirt1* downregulation in *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* flies significantly increased Aβ$_{42}$ expression compared to AD model flies (*elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+*) (Figure 3D and E). These results indicate that *Sirt1* overexpression has a

Table 2. The median survival (days) of control and experimental group flies

Genotype (data comparison)	Median survival (days)
w^{1118}	56
elav-Gal4/+;+/+;+/+	60
elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+	30
elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+	48
elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+	34

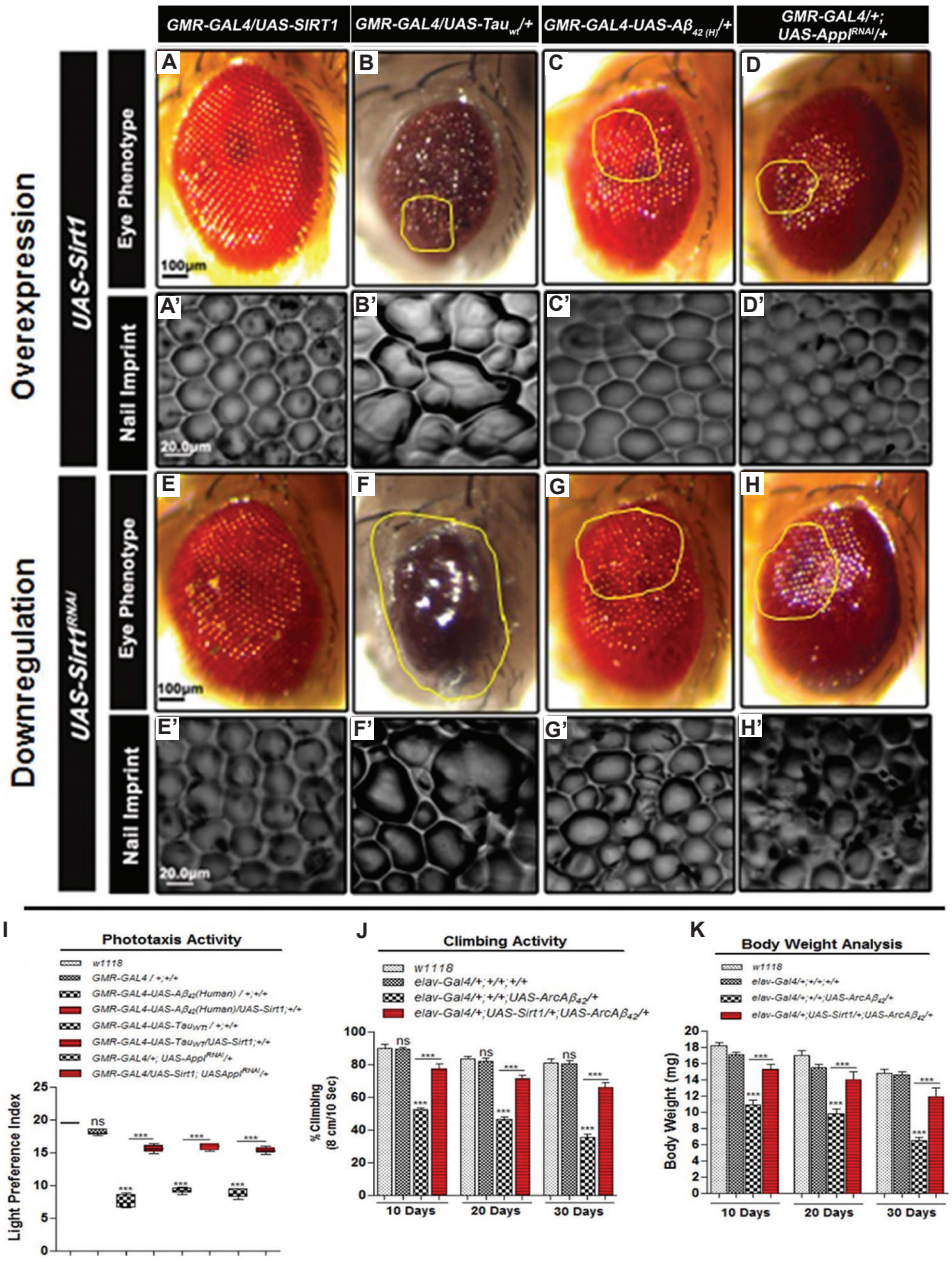

Figure 1. *Sirt1* regulates Alzheimer's disease-related pathologies in *Drosophila*. (A-D) Light microscope images; (A'-D') Images of nail imprints from the eyes of 10-day-old Drosophila with *Sirt1* overexpression using *GMR-GAL4/UAS-Sirt1* (A-A'), *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1* (B-B'), *GMR-GAL4-UAS-Aβ*$_{42}$*(Human)/UAS-Sirt1* (C-C'), and *GMR-GAL4/UAS-Sirt1;UAS-Appl*^{RNAi}/+ (D-D'). Overexpression of *Sirt1* is correlated with AD model flies in Drosophila. (E-H) Light microscope images (E'-H') Images of nail imprints from the eyes of 10-day-old adult flies with *Sirt1* downregulation using *GMR-GAL4/+;UAS-Sirt1*^{RNAi}/+ (E-E'), *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Sirt1*^{RNAi}/+ (F-F'), and *GMR-GAL4-UAS-Aβ*$_{42}$ *(human)/+;UAS-Sirt1*^{RNAi}/+ (G-G'), *GMR-GAL4/+;UAS-Appl*^{RNAi}*/UAS-Sirt1*^{RNAi} (H-H') flies (n = 40). Scale bar indicates 100 μm (A-H) and 20 μm (A'-H'). Yellow-highlighted areas represent the degenerated parts of the eyes, n = 40. (I) The box-and-whisker plot displays phototaxis activity (expressed as light preference index) of 10-day-old adult flies: *w*^{1118}, *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Aβ*$_{42}$*(Human)/+;+/+*, *GMR-GAL4-UAS-Aβ*$_{42}$ *(Human)/UAS-Sirt1;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;+/+*, *GMR-GAL4/+;UAS-Appl*^{RNAi}/+, and *GMR-GAL4/UAS-Sirt1;UAS-Appl*^{RNAi}/+, n = 100. (J) Histogram indicating climbing activity (expressed as % climbing in 8 cm 10 s^{-1}) of 10-, 20, and 30-day-old flies: *w*^{1118}, *elav-Gal4/elav-Gal4;+/+;+/+*, *elav-Gal4/+;+/+;UAS-ArcAβ*$_{42}$*/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ*$_{42}$*/+*. n = 100. (K) Histogram showing body weight analysis of 10-, 20-, and 30-day-old flies: *w*^{1118}, *elav-Gal4/+;+/+;+/+*, *elav-GAL4/+;+/+;UAS-ArcAβ*$_{42}$*/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ*$_{42}$*/+*. n = 100. Error bars represent mean ± standard error of the mean. Statistical significance was calculated using one-way ANOVA with Tukey's test in GraphPad Prism 5.0. Results are indicated as ns: non-significant, *P < 0.05, **P < 0.01, ***P < 0.0001.

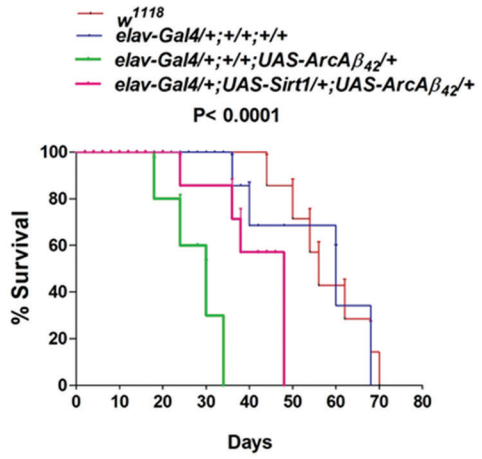

Figure 2. Lifespan (survival) analysis of Alzheimer's disease model flies in *Sirt1* overexpressing genetic background. Survival curves are shown for the following groups: w^{1118} (red line), *elav-Gal4/+;+/+;+/+* (blue line), *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* (green line), *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* (pink line), *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* (black line), n = 100.

neuroprotective effect by lowering Aβ_{42} expression in the larval brain of flies.

To further validate our observations, we conducted RT-qPCR analysis using head samples from 10-day-old adult flies from both control and experimental groups to examine the expression (mRNA) levels of AD-associated genes (Aβ_{42}, Tau, and Appl). In *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* AD model flies, the expression levels of *Aβ$_{42}$*, *Tau*, and *Appl* were significantly increased by 8.5-, 1.7-, and 1.42-fold, respectively, compared to control flies (1.0) (Figure 3F). We also observed that *Sirt1* overexpression in AD model flies (*elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+)* reduced the expression levels of AD-associated genes to 0.81-, 0.73-, and 0.26-fold, respectively, compared to *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* AD model flies (Figure 3F). Conversely, *Sirt1* downregulation in *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* significantly increased the expression levels of AD-associated genes to 1.77-, 1.2-, and 0.36-fold, respectively (Figure 3F), further supporting our observations.

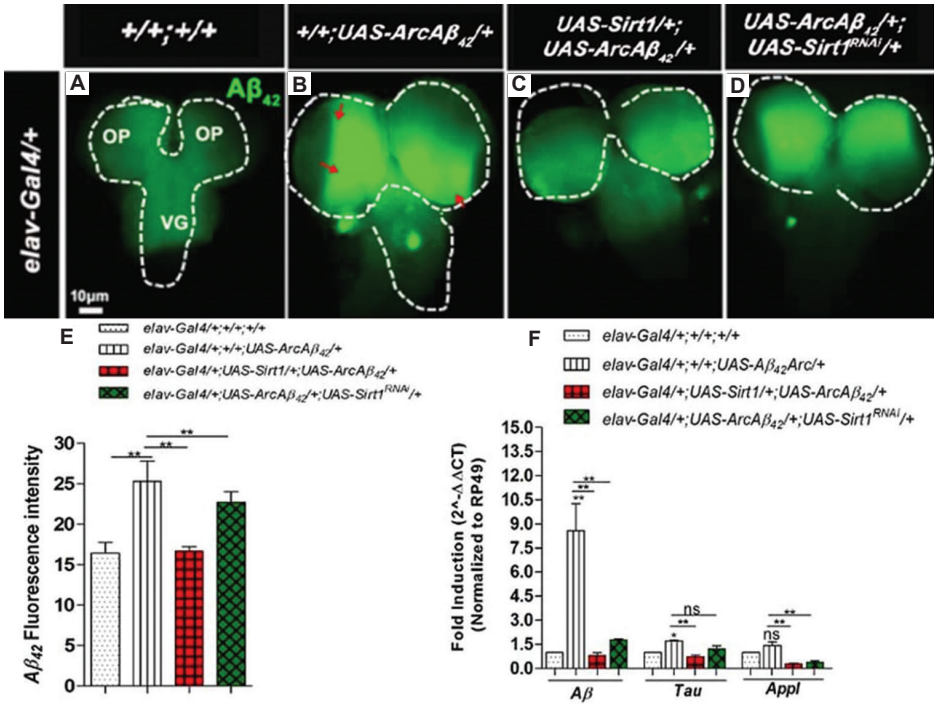

Figure 3. Anti-Aβ_{42} expression in the third instar larval brain and quantitative RT-qPCR analysis of *Aβ$_{42}$*, *Tau*, and *Appl* expression. (A-D) Third instar larval brains from *elav-Gal4/+;+/+;+/+* (A), *elav-GAL4/+;+/+*, *UAS-ArcAβ$_{42}$/+* (B), *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* (C), and *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* (D), showing anti-Aβ_{42} expression. Red arrows highlight increased expression of Aβ_{42} compared to the control (A). Scale bars denote a distance of 10 μm (A-D). A total of 20 larval brains were used for each genotype. The histogram illustrates the average fluorescence intensity of Aβ_{42} in the larval brains of each genotype (as shown above). Fluorescence intensity was measured using ImageJ software, NIH, USA. Error bars indicate mean ± SEM (E). Data significance was determined by one-way ANOVA with Tukey's test in GraphPad Prism 5.0 software, with significance levels set at $^{*}P < 0.05$, $^{***}P < 0.0001$. (F) Histogram illustrating relative expression of *Aβ$_{42}$*, *Tau*, and *Appl*, as quantified by RT-qPCR in 10-day-old adult fly heads: *elav-Gal4/+;+/+;+/+*, *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*. *RP49* was used as an endogenous control, and error bars indicate mean ± SEM. Statistical significance was calculated using one-way ANOVA with Tukey's test in GraphPad Prism 5.0 software and is indicated as ns: non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

Abbreviations: RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; SEM: standard error of the mean; ANOVA: Analysis of variance.

3.4. Improved AD-related pathologies were linked with reduced cell death in AD flies

Excessive cell death is a major factor in AD and plays an important role in the onset of AD-related pathologies.[42,43] AD model flies exhibit rough eye phenotypes and motor dysfunction, which are indicators of neuronal cell death (Figure 1 A-H).[25-27,35] As discussed above, *Sirt1* overexpression reduces, while *Sirt1* downregulation

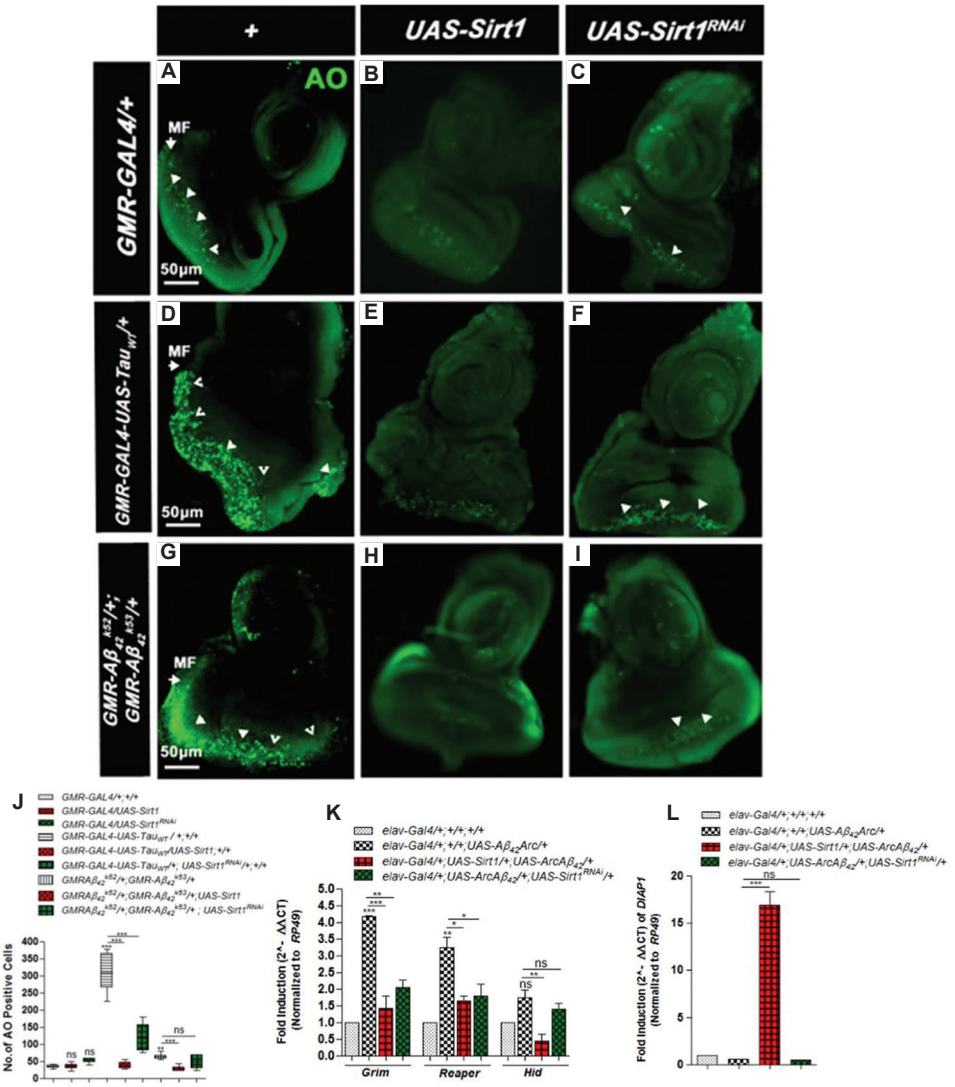

Figure 4. AO staining of third instar larval eye discs in *Sirt1* overexpressing/downregulating AD model flies, along with quantitative RT-qPCR analysis of the *Grim*, *Reaper*, *Hid*, and *DIAP1* in Drosophila. (A-I) Confocal microscopy images of the eye imaginal disc from third instar larvae, stained with AO in *GMR-GAL4/+* (A), *GMR-GAL4/UAS-Sirt1;+/+* (B), *GMR-GAL4/+;UAS-Sirt1^{RNAi}/+* (C), *GMR-GAL4-UAS-Tau$_{WT}$/+;++* (D), *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+* (E), *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Sirt1^{RNAi}/+* (F), *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/+* (G), *GMR-Aβ$_{42}^{k52}$/UAS-Sirt1;GMR-Aβ$_{42}^{k53}$/+* (H), *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/UAS-Sirt1^{RNAi}* (I). AO-positive cells (dead cells) located posterior to the morphogenetic furrow are marked by white arrowheads. Scale bars indicate 50 µm (A-I). A total of 20 larval eye imaginal discs were used for the study. (J) Box-and-whisker plot illustrating the average number of AO-positive third instar larval eye imaginal discs for each genotype (n = 20). Quantification of AO-positive cells was analyzed using ImageJ software, NIH, USA. (K) Histogram illustrating the expression levels of apoptotic genes *Grim*, *Reaper*, and *Hid* in the adult heads of 0-day-old flies of *elav-Gal4/+;+/+;+/+*, *elav-Gal4/+;+/+*, *UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*. *RP49* was used as an endogenous control for normalization. (L) Histogram illustrating the relative expression of *DIAP1* in the heads of 10-day-old adult flies: *elav-Gal4/+;+/+;+/+*, *elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*. *RP49* was used as an endogenous control for normalization. Error bars indicate mean ± standard error of the mean significance was calculated using one-way ANOVA with Tukey's test in GraphPad Prism 5.0 and is indicated as ns: non-significant, ***P < 0.0001.

Abbreviations: RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; SEM: standard error of the mean; ANOVA: Analysis of variance; AO: Acridine Orange.

exacerbates, the rough eye phenotype in correlation with AD model flies (Figure 1 A-H). To investigate whether the rough eye phenotype in AD model flies is associated with excessive cell death in the eyes, we conducted AO staining in the respective larval eye imaginal discs (Figure 4 A-I) of control (*GMR-GAL4/+*), AD model flies (*GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* and *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/+*), and AD model flies with *Sirt1* overexpression (*GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+* and *GMR-Aβ$_{42}^{k52}$/UAS-Sirt1;GMR-Aβ$_{42}^{k53}$/+*) or *Sirt1* downregulation (*GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Sirt1^{RNAi}/+* and *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/UAS-Sirt1^{RNAi}*) backgrounds.

We observed excessive cell death (AO-positive cells) posterior to the MF in the third instar larval eyes of AD model flies (Figure 4D and G) compared to age-matched experimental control flies (*GMR-GAL4/+* and *GMR-GAL4/UAS-Sirt1;+/+*), which showed fewer AO-positive cells associated with developmental apoptosis (Figure 4A and B). Further, AO staining in AD model flies with *Sirt1* overexpression (*GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+* and *GMR-Aβ$_{42}^{k52}$/UAS-Sirt1;GMR-Aβ$_{42}^{k53}$/+)* showed a significant reduction in AO-positive cells posterior to the MF (Figure 4E and H). In contrast, downregulation of *Sirt1* in *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Sirt1^{RNAi}/+* and *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/UAS-Sirt1^{RNAi}* did not show any significant changes in cell death compared to their respective AD model flies (Figure 4F and I). Overexpression or downregulation of *Sirt1* in the *GMR-GAL4* (experimental control) background (*GMR-GAL4/UAS-Sirt1;+/+* and *GMR-GAL4/+;UAS-Sirt1^{RNAi}/+*) showed no visible changes in cell death (Figure 4B and C) compared to the undriven control *GMR-GAL4/+* (Figure 4A). These results indicate that *Sirt1* overexpression exerts a protective effect against apoptosis in *Drosophila*.

In flies, apoptosis is primarily regulated by three proapoptotic genes: Grim, Reaper, and Hid[44,45]. These genes are transcriptionally regulated in response to death-inducing signals and link signaling pathways to the cell death mechanism[45,46].

To further confirm the AO staining results, we analyzed the mRNA expression levels of the proapoptotic genes Grim, Reaper, and Hid through qRT-PCR in the heads of 10-day-old adult flies from both control and experimental groups.

The expression levels of *Grim*, *Reaper*, and *Hid* in *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* flies (Figure 4K) were significantly increased by 4.16-, 3.24-, and 1.73-fold, respectively, compared to control flies (1.0). Overexpression of *Sirt1* in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* flies significantly reduced the expression levels of *Grim*, *Reaper*, and *Hid* by 1.42-, 1.65-, and 0.44-fold, respectively, whereas *Sirt1* downregulation in *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* flies significantly increased the expression of these apoptotic genes by up to 2.0-, 1.79-, and 1.39-fold, respectively, compared to control flies (Figure 4K). These findings support our earlier observations that *Sirt1* overexpression plays an antiapoptotic role and indicate that *Sirt1* is a crucial modulator of AD-related pathologies in *Drosophila*.

To further investigate whether the increased expression of apoptotic genes (*Grim, Reaper*, and *Hid*) was linked to changes in the expression of *Drosophila* inhibitor of apoptosis (*DIAP1*), we analyzed *DIAP1* expression levels through qRT-PCR in the heads of 10-day-old adult flies from both control and experimental groups. Figure 4L shows that *DIAP1* expression was significantly reduced to 0.55 in *elav-Gal4/+;+/+;UAS-ArcAβ$_{42}$/+* AD model flies compared to control flies (1.0). *Sirt1* overexpression in the AD genetic background increased DIAP1 expression up to 16.09 in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+* flies compared to AD model flies, whereas *Sirt1* downregulation significantly reduced *DIAP1* expression to 0.44 in *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* flies compared to AD model flies (Figure 4L). Therefore, it was confirmed that the rescue observed in the eye phenotype was linked to reduced cell death due to *Sirt1* overexpression.

3.5. *Sirt1* plays a neuroprotective effect by altering JNK signaling pathways

JNK is a key component of the mitogen-activated protein kinase (MAPK) pathway[47-49] and has been extensively studied in the context of cell death in *Drosophila*. In *Drosophila*, the sole JNK is *Bsk*.[48] Several earlier studies have indicated that AD correlates with increased phosphorylated JNK expression, which also colocalizes with Aβ.[50-52] In addition, Aβ peptides can induce JNK signaling activation.[53-55] As shown above, AD model flies exhibited increased cell death in eye cells, and JNK signaling is strongly correlated with cell death signaling pathways. Therefore, to analyze the potential genetic interaction between Drosophila JNK (*Bsk*), *Drosophila Sirtuin1*, and AD-associated genes (Tau$_{WT}$) in flies, we conducted a genetic interaction study. In this study, we observed the rough eye phenotype, abnormal bristles, and ommatidial disarrangement in *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies. These pathologies were exacerbated when *Bsk* was downregulated using *UAS-Bsk^{RNAi}* (single/double copy) in *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B* and *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-sk^{RNAi}*, (Figure 5B and C). We further noted significant improvements in these pathologies in *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies when *Sirt1* was overexpressed and *Bsk* was downregulated (single/double copy) in the AD model flies (i.e., *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/*

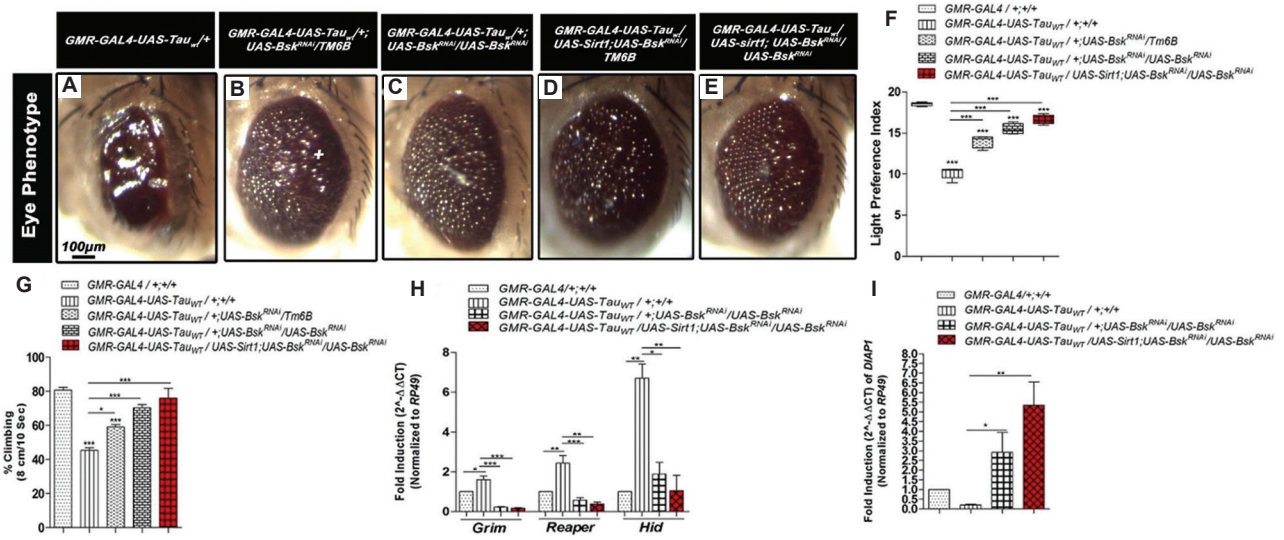

Figure 5. Downregulation of Drosophila *Bsk* and overexpression of *Sirt1* modulate Alzheimer's disease-related pathologies, with RT-qPCR analysis of the *Grim*, *Reaper*, *Hid*, and *DIAP1* in *Drosophila.* (A-E) Light microscope images from 10-day-old AD model flies (*GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*) (A); with *Bsk* downregulation (single copy) (B); *Bsk* downregulation (double copy) (C); *Sirt1* overexpression along with *Bsk* downregulation (single copy) (D); and *Bsk* downregulation (double copy) (E). Scale bar indicates 100 µm. The yellow-highlighted area represents the severely degenerated parts of the eyes. (F) Box-and-whisker plot displaying the phototaxis activity (expressed as light preference index) of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}* flies (n = 100). (G) Histogram indicating climbing activity (expressed as % climbing in 8 cm 10 s^{-1}) of 10-day-old flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}* flies (n = 100). (H) Histogram illustrating the expression level of *Grim*, *Reaper*, and *Hid*, as determined by RT-qPCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*. *RP49* was used as an endogenous control for normalization. (I) Histogram indicating the *DIAP1* gene expression level, as examined by RT-qPCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}, GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*. *RP49* was used as an endogenous control for normalization.
Abbreviation: RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

TM6B, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1*, *and UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*,) (Figure 5D and E).

Moreover, we observed the combined effect of *Bsk* and *Sirt1* on learning and memory impairment in AD model flies. We found that the light preference index in 10-day-old *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies was significantly reduced to 10.13, whereas *GMR-GAL4/+;+/+* flies had a light preference index of 18.50 (Figure 5F). The light preference index in *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies (10.13) increased to 13.92 and 15.48 when *Bsk* was downregulated (homozygous/heterozygous conditions) in *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B* and *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*, respectively. Furthermore, the light preference index in *GMR-GAL4-UAS-Tau$_{WT}$/+* flies (10.13) significantly improved to 16.72 in a genetic background of *Sirt1* overexpression and *Bsk* downregulation (i.e., *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}*). In addition, 10-day-old *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+* flies exhibited 45.4% climbing activity compared to 80.8% in age-matched *GMR-GAL4/+;+/+* control flies (Figure 5G). The climbing activity was significantly restored when *Bsk* was downregulated in both heterozygous/homozygous conditions. Specifically, 10-day-old flies with the genotypes *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B* and *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}* showed 59% and 70.4% climbing activity, respectively. Furthermore, reduced climbing activity was significantly restored when *Sirt1* was overexpressed alongside *Bsk* downregulation in an AD genetic background: 10-day-old *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;UAS-Bsk^{RNAi}/UAS-Bsk^{RNAi}* flies showed 76% climbing activity compared to 59% in *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Bsk^{RNAi}/TM6B* flies (Figure 5G).

As discussed above, proapoptotic genes (Grim, Reaper, and Hid) are activated in AD model flies. To examine the impact of *Sirt1* overexpression alongside *Bsk* downregulation in an AD genetic background, we performed qRT-PCR analysis of *Grim*, *Reaper*, and *Hid* in the heads of 10-day-old adult flies from both control and experimental groups. The expression levels of *Grim*, *Reaper*, and *Hid* were significantly increased by 1.61-, 2.43-, and 6.71-fold, respectively, in *GMR-GAL4-UAS-*

Tau_{WT}/+;+/+ AD model flies compared to control flies (1.0) (Figure 5H). In addition, downregulation of *Bsk* (homozygous) in *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-Bsk^{RNAi}*/*UAS-Bsk^{RNAi}* flies significantly reduced *Grim*, *Reaper*, and *Hid* expression to 0.22-, 0.58-, and 1.89-fold, respectively. In the AD genetic background, Sirt1 overexpression along with Bsk downregulation further decreased the expression levels of *Grim*, *Reaper*, and *Hid* to 0.17-, 0.39-, and 0.78-fold, respectively, in *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Sirt1*;*UAS-Bsk^{RNAi}*/*UAS-Bsk^{RNAi}* flies.

To further verify these results, we examined *DIAP1* expression by conducting qRT-PCR on the heads of 10-day-old adult flies from both control and experimental groups. As shown in Figure 5I, *DIAP1* expression was significantly decreased to 0.19 in AD model flies *(GMR-GAL4-UAS-Tau_{WT}*/+;+/+*)* compared to the experimental control group (1.0). Downregulation of *Bsk* in an AD genetic background increased DIAP1 expression up to 2.9-fold in *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-Bsk^{RNAi}*/*UAS-Bsk^{RNAi}* flies (Figure 5I). Moreover, we observed that Sirt1 overexpression alongside *Bsk* downregulation in an AD genetic background significantly increased *DIAP1* expression to 5.3-fold in *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Sirt1*;*UAS-Bsk^{RNAi}*/*UAS-Bsk^{RNAi}* flies (Figure 5I). This finding supports our observations.

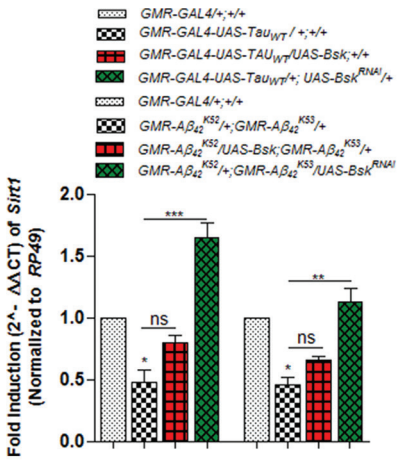

Figure 6. qRT-PCR analysis showing expression of *Sirt1* in Bsk overexpression/downregulation in Alzheimer's disease model flies. The histogram above illustrates the expression level of *Sirt1* quantified by RT-qPCR real-time PCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau_{WT}*/+;+/+, *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Bsk*;+/+, *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-Bsk^{RNAi}*/+, *GMR-GAL4/+;+/+*, *GMR-$Aβ_{42}^{k52}$*/+;*GMR-$Aβ_{42}^{k53}$*/+, *GMR-$Aβ_{42}^{k52}$*/*UAS-Bsk*;*GMR-$Aβ_{42}^{k53}$*/+, and *GMR-$Aβ_{42}^{k52}$*/+;*GMR-$Aβ_{42}^{k53}$*/*UAS-Bsk^{RNAi}* flies. *RP49* (an endogenous control) was used for normalization. Error bars indicate mean ± standard error of the mean. Significance was calculated by one-way analysis of variance with Tukey's test in GraphPad Prism 5.0 and is indicated as ns: non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ Abbreviation: qRT-PCR: Quantitative reverse transcription- polymerase chain reaction

3.6. Overexpression/downregulation of *Bsk* modulates *Sirt1* expression in AD model flies

To further validate the above observations and investigate the association between *Sirt1* and JNK, we analyzed the expression levels of *Sirt1* in the heads of 10-day-old AD model flies with *Bsk* overexpression and downregulation from both control and experimental groups using qRT-PCR. The expression level of *Sirt1* was significantly decreased to 0.47-fold in *GMR-GAL4-UAS-Tau_{WT}*/+;+/+ and 0.45-fold in *GMR-$Aβ_{42}^{k52}$*/+;*GMR-$Aβ_{42}^{k53}$*/+ flies compared to control flies (1.0) (Figure 6). Our study suggests that overexpression of *Bsk* in an AD genetic background increases *Sirt1* expression levels to 0.8-fold in *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Bsk*;+/+ and 0.65-fold in *GMR-$Aβ_{42}^{k52}$*/*UAS-Bsk*;*GMR-$Aβ_{42}^{k53}$*/+ flies compared to AD model flies (Figure 6). In contrast, *Bsk* downregulation significantly increased *Sirt1* expression levels to 1.6-fold in *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-Bsk^{RNAi}*/+ and 1.13-fold in *GMR-$Aβ_{42}^{k52}$*/+;*GMR-$Aβ_{42}^{k53}$*/*UAS-Bsk^{RNAi}* flies compared to AD model flies (Figure 5).

3.7. *Sirt1* regulates the Notch signaling in *Drosophila*

We further explored the potential genetic interaction between *Notch*, *Sirt1*, and AD-associated genes (Tau_{WT}) in *Drosophila*. The rough eye phenotype, abnormal bristles, and ommatidial disarrangement observed in *GMR-GAL4-UAS-Tau_{WT}*/+;+/+ AD model flies (Figure 7A) improved when *Notch* was downregulated using *UAS-$Notch^{RNAi}$* (single or double copy) in *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-$Notch^{RNAi}$*/*TM6B* and *GMR-GAL4-UAS-Tau_{WT}*/+;*UAS-$Notch^{RNAi}$*/*UAS-$Notch^{RNAi}$*, respectively (Figure 7B and C). Furthermore, these pathologies significantly improved when *Sirt1* was overexpressed and *Notch* was downregulated (single or double copy) in AD genetic backgrounds (i.e., *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Sirt1*;*UAS-$Notch^{RNAi}$*/*TM6B* and *GMR-GAL4-UAS-Tau_{WT}*/*UAS-Sirt1*;*UAS-$Notch^{RNAi}$*/*UAS-$Notch^{RNAi}$*, respectively) (Figure 7D and E).

Notch has been reported as a substrate of presenilin/γ-secretase and plays an essential role in memory and learning processes.[56,57] Thus, to examine the effect of *Notch* signaling along with Sirt1 overexpression on behavioral changes in AD model flies, we conducted behavioral assays (phototaxis and climbing activity) in 10-day-old AD model flies with *Sirt1* overexpression alongside *Notch* downregulation. We found that the light preference index in 10-day-old AD model flies (*GMR-GAL4-UAS-Tau_{WT}*/+;+/+) significantly decreased to 10.13 compared to *GMR-GAL4/+;+/+*control flies with a light preference index of 18.50 (Figure 7F). The light preference index of *GMR-GAL4-UAS-Tau_{WT}*/+;+/+ flies (10.13) increased to 13.33 and 14.85 when Notch was downregulated (homozygous/heterozygous condition)

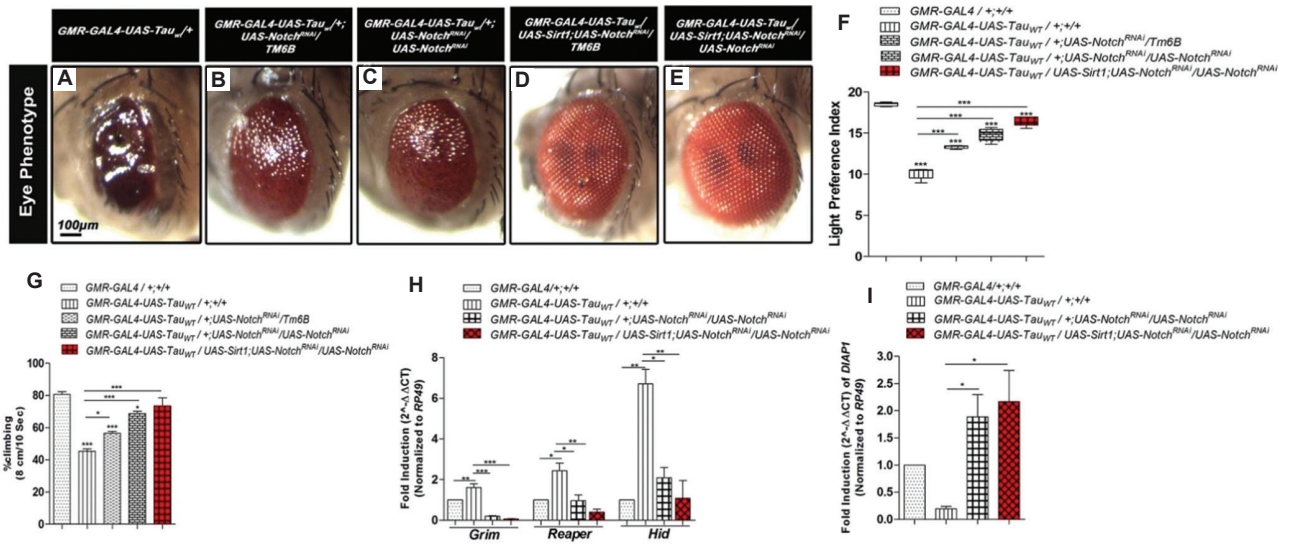

Figure 7. Downregulation of *Notch* and overexpression of *Sirt1* modulate Alzheimer's disease-related pathologies and RT-PCR analysis of *Grim*, *Reaper*, *Hid*, and *DIAP1* in *Drosophila*. (A-E) Light microscopy images of 10-day-old AD model flies: *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+* (A); with *Notch* downregulation (single copy) (B); *Notch* downregulation (double copy) (C); *Sirt1* overexpression with *Notch* downregulation (single copy) (D); and *Notch* downregulation (double copy) (E). Scale bar indicates 100 µm (A-E). The yellow-highlighted area represents degenerated areas in the fly eye. (F) Box-and-whisker plot displaying phototaxis activity (expressed as light preference index) of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/TM6B*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} (n = 100). (G) Histogram indicating climbing activity (expressed as % climbing in 8 cm 10 s^{-1}) of 10-day-old flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/TM6B*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} (n = 100). (H) Histogram illustrating the expression levels of proapoptotic genes (*Grim*, *Reaper*, and *Hid*) as determined by RT-qPCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}. *RP49* was used as an endogenous control for normalization. (I) Histogram indicating the *DIAP1* gene expression level as determined by RT-qPCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}. *RP49* was used as an endogenous control for normalization.
Abbreviations: RT-PCR: Reverse transcription polymerase chain reaction; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/TM6B* and *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, respectively. Furthermore, the light preference index (10.13) in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+* flies significantly increased to 16.55 when *Sirt1* was overexpressed along with *Notch* downregulation in *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} in AD model flies.

In the climbing activity assay, the climbing activity of 10-day-old AD model flies (*GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*) significantly decreased to 45.4% compared to age-matched *GMR-GAL4/+;+/+* control) flies with a climbing activity of 80.8% (Figure 7G). The climbing activity increased significantly when *Notch* was downregulated using *UAS-Notch*^{RNAi} in heterozygous/homozygous conditions (i.e., 10-day-old *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/TM6B* and *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies showed 56.6% and 68.8% climbing activity, respectively, compared to the 45.4% climbing activity observed in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*). In addition, the reduced climbing activity was significantly improved when *Sirt1* was overexpressed along with *Notch* downregulation in the AD genetic background: 10-day-old *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies showed 73.6% climbing activity compared to the 45% shown by *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+* (Figure 7G).

Furthermore, we investigated the effects of *Sirt1* overexpression and *Notch* downregulation on the expression of proapoptotic genes (Grim, Reaper, and Hid). For this purpose, we performed qRT-PCR analysis of these genes in the heads of 10-day-old adult flies from both control and experimental groups. The expression levels of *Grim*, *Reaper*, and *Hid* were significantly increased by 1.61-, 2.43-, and 6.71-fold, respectively, in AD model flies (*GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*) compared to control flies (1.0) (Figure 7H). However, the expression levels of *Grim*, *Reaper*, and *Hid* were significantly reduced to 0.18-, 0.96-, and 2.1-fold, respectively, when *Notch* was downregulated (homozygous) in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies. Furthermore, when *Sirt1* was overexpressed alongside

Notch downregulation in the AD genetic background, the expression levels of *Grim*, *Reaper*, and *Hid* were decreased to 0.05-, 0.4-, and 0.75-fold, respectively, in *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies.

This was further confirmed by assessing the expression level of *DIAP1* via qRT-PCR in the heads of 10-day-old adult flies from both control and experimental groups. *DIAP1* expression was significantly decreased to 0.19 in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+* flies compared to control flies (1.0) (Figure 7I). *Notch* downregulation in the AD genetic background increased *DIAP1* expression to 1.88-fold in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies (Figure 7I). Moreover, we found that *Sirt1* overexpression combined with *Notch* downregulation in the AD genetic background significantly increased *DIAP1* expression up to 2.16-fold in *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies (Figure 7I).

3.8. Downregulation of *Notch* along with *Sirt1* overexpression alters the expression of Delta (Notch ligand) in AD model flies

In AD, the Notch extracellular domain cleaves APP and Tau, contributing to the formation of A*β* plaques and NFT, respectively.[58] Thus, we analyzed the expression level of Delta, the ligand of Notch, in 10-day-old fly heads of AD model flies from both control and experimental groups by conducting qRT-PCR analysis. As illustrated in Figure 8, *Delta* expression was significantly increased up to 16.29 fold in AD model flies (*GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*) compared to control group flies (1.0). In contrast, Notch downregulation in the AD genetic background reduced *Delta* expression to 7.88 fold in *GMR-GAL4-UAS-Tau*$_{WT}$*/+; UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies (Figure 8). In addition, co-expression of *Sirt1* (overexpression) and *Notch* downregulation in the AD genetic background significantly reduced *Delta* expression to 5.63 fold in *GMR-GAL4-UAS-Tau*$_{WT/}$*UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi} flies (Figure 8).

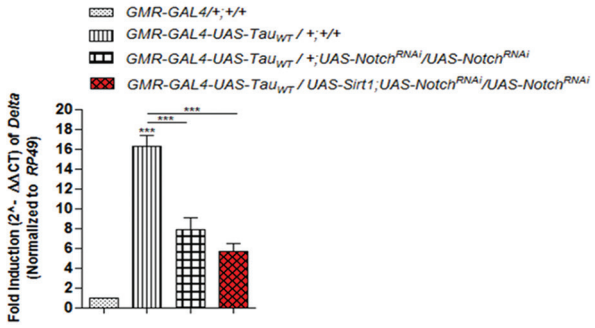

Figure 8. *Delta* gene expression in control, AD model flies, and AD model flies with *Sirt1* overexpression and *Notch* downregulation. The histogram above illustrates *Delta* expression levels as determined by RT-PCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*, *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}, and *GMR-GAL4-UAS-Tau*$_{WT}$*/UAS-Sirt1;UAS-Notch*^{RNAi}*/UAS-Notch*^{RNAi}. *RP49* was used as an endogenous control for normalization. Error bars indicate mean ± standard error of the mean. Significance was calculated by one-way analysis of variance with Tukey's test in GraphPad Prism 5.0, with $^{***}P < 0.0001$.

Abbreviations: AD: Alzheimer's disease; RT-PCR: Reverse transcription polymerase chain reaction.

3.9. Overexpression/downregulation of *Sirt1* regulates *JNK* and *Notch* expression levels in AD model flies

Our findings suggest a genetic interaction between *Sirt1*, *JNK*, *Notch*, and AD-associated genes in *Drosophila*. To further confirm these observations, we assessed *JNK* and *Notch* expression levels by conducting qRT-PCR on the heads of 10-day-old adult flies from both control and experimental groups. Compared to control flies (1.0), the expression levels of *JNK* and *Notch* in AD model flies (*GMR-GAL4-UAS-Tau*$_{WT}$*/+;+/+*) were significantly increased to 2.25- and 2.0-fold, respectively, and to 3.2- and 2.62, respectively, in *GMR-A*β_{42}^{k52}*/+;GMR-A*β_{42}^{k53}*/+* flies (Figure 9A and B). Compared to AD model flies, *Sirt1* overexpression in the AD genetic background significantly reduced *JNK* and *Notch* expression levels to 0.43- and 0.47-fold, respectively. Conversely, *Sirt1* downregulation in the AD genetic background increased *JNK* and *Notch* expression levels to 2.15- and 1.55-fold, respectively, in *GMR-GAL4-UAS-Tau*$_{WT}$*/+;UAS-Sirt1*^{RNAi}*/+* flies and to 2.76- and 1.65-fold, respectively, in *GMR-A*β_{42}^{k52}*/+;GMR-A*β_{42}^{k53}*/UAS-Sirt1*^{RNAi} flies. We also analyzed *JNK* and *Notch* expression levels in the *elav-Gal4* (a ubiquitous GAL4) genetic background. As shown in Figure 9C, *JNK*, and *Notch* expression levels were significantly increased to 1.48- and 2.84-fold, respectively, in *elav-Gal4/+;+/+;UAS-ArcA*β_{42}*/+* AD model flies compared to control flies (1.0). *Sirt1* overexpression significantly reduced *JNK* and *Notch* expression levels to 0.55- and 0.23-fold, respectively, in *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcA*β_{42}*/+* flies compared to AD model flies. The downregulation of *Sirt1* in the AD genetic background increased *JNK* and *Notch* expression to 1.20- and 0.81-fold, respectively, in *elav-Gal4/+;UAS-ArcA*β_{42}*/+;UAS-Sirt1*^{RNAi}*/+* flies compared to AD model flies.

To further validate these observations, we performed staining for anti-active-JNK (phosphorylated JNK), anti-Delta, and anti-NICD in the brains of third instar larvae overexpressing or downregulating *Sirt1* in AD model flies (Figure 10). As shown in Figure 10 B, F, J we observed significantly increased expression of active JNK, Delta, and NICD in the third instar larval brains of AD model flies (*elav-GAL4/+;+/+*, *UAS-ArcA*β_{42}*/+*) compared to

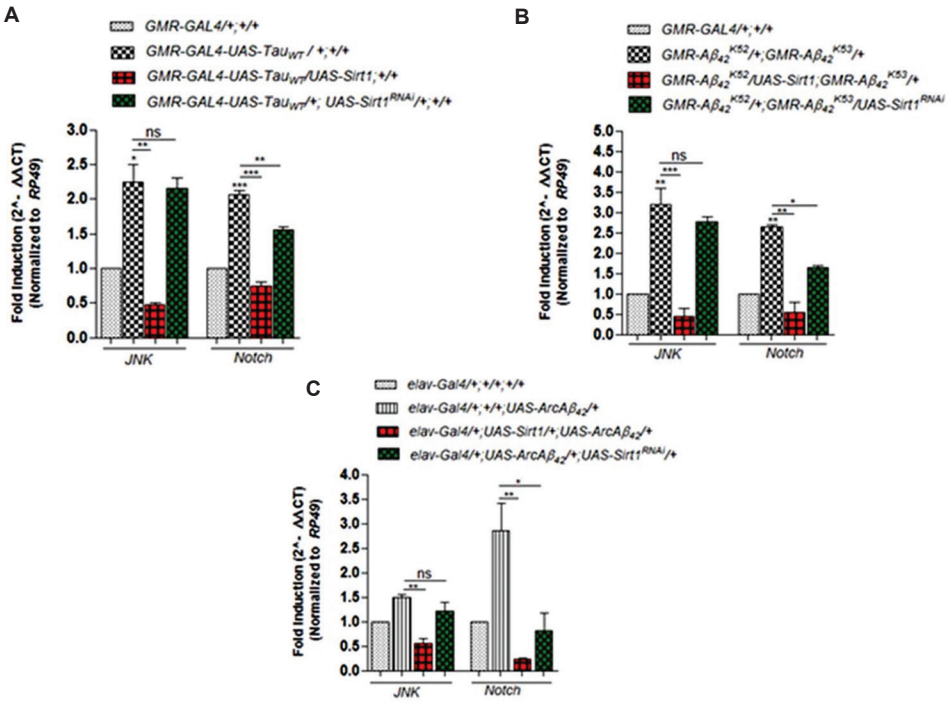

Figure 9. *JNK* and *Notch* expression levels in control and *Sirt1* overexpressing/downregulating AD model flies. The histogram above illustrates *JNK* and *Notch* gene expression levels quantified via RT-qPCR in the heads of 10-day-old adult flies: *GMR-GAL4/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/+;+/+*, *GMR-GAL4-UAS-Tau$_{WT}$/UAS-Sirt1;+/+*, and *GMR-GAL4-UAS-Tau$_{WT}$/+;UAS-Sirt1^{RNAi}/+* (A); *GMR-GAL4/+;+/+*, *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/+*, *GMR-Aβ$_{42}^{k52}$/UAS-Sirt1;GMR-Aβ$_{42}^{k53}$/+*, and *GMR-Aβ$_{42}^{k52}$/+;GMR-Aβ$_{42}^{k53}$/UAS-Sirt1^{RNAi}* (B); *elav-Gal4/+;+/+;+/+*, *elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*, and *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+* (C). *RP49* was used as an endogenous control for normalization. Error bars indicate mean ± standard error of the mean. Significance was calculated by one-way analysis of variance with Tukey's test in GraphPad Prism 5.0, indicated as ns: non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.
Abbreviation: RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

control (*elav-Gal4/+;+/+;+/+*) flies (Figure 10A, E, I). *Sirt1* overexpression in AD model flies (*elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+)* significantly decreased JNK, Delta, and NICD fluorescence intensities (Figure 10C, G, and K). Conversely, *Sirt1* downregulation in AD model flies (*elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*) significantly increased JNK, Delta, and NICD fluorescence intensities (Figure 10D, H, and L). This result clearly indicates that *Sirt1* genetically interacts with JNK, Delta, and Notch signaling in *Drosophila*.

4. Discussion

AD is a neurological disorder affecting millions worldwide.[59] In recent decades, biochemical and pharmacological research has delved into its complexities, providing significant insights into its molecular mechanisms. This enhanced understanding has paved the way for the discovery and development of innovative treatments to address this challenging and debilitating condition.[60]

As discussed above, AD is among the most devastating aging-associated NDDs and *Sirt1* has demonstrated neuroprotective effects. Thus, exploring the connection between AD-associated genes (Aβ$_{42}$, Tau, Appl) and *Sirt1* could be valuable in identifying/designing potential therapeutic targets for the disease. *Sirt1* is an extensively studied gene in the context of aging and age-related diseases.[8-11] In the current study, we utilized various transgenic *Drosophila* fly lines, including *Aβ$_{42}$*, *Tau*, and *Appl*, which mimic AD-related pathologies, such as the rough eye phenotype (Figure 1), behavioral deficits (phototaxis and climbing) (Figure 1I-J), decreased body weight (Figure 1K), and reduced survival (Figure 2) due to ectopic expression of *Aβ$_{42}$*, *Tau*, and *Appl* in *Drosophila* (Figure 3). We further, observed that AD-related pathologies, as described above, were significantly improved with the overexpression of *Sirt1*, whereas *Sirt1* downregulation exacerbated these pathologies (Figures 1, 2, and 3A-E). In addition, our study showed that *Sirt1* overexpression decreased the expression of AD-related genes (Aβ$_{42}$, Tau, and Appl) in AD model flies, whereas *Sirt1* downregulation exerted the opposite effect, increasing the expression of these genes (Figure 3F). Thus, *Sirt1* overexpression reduces *Aβ$_{42}$*-, *Tau*-, and *Appl*-induced toxicity in AD flies and improves AD-related pathologies.

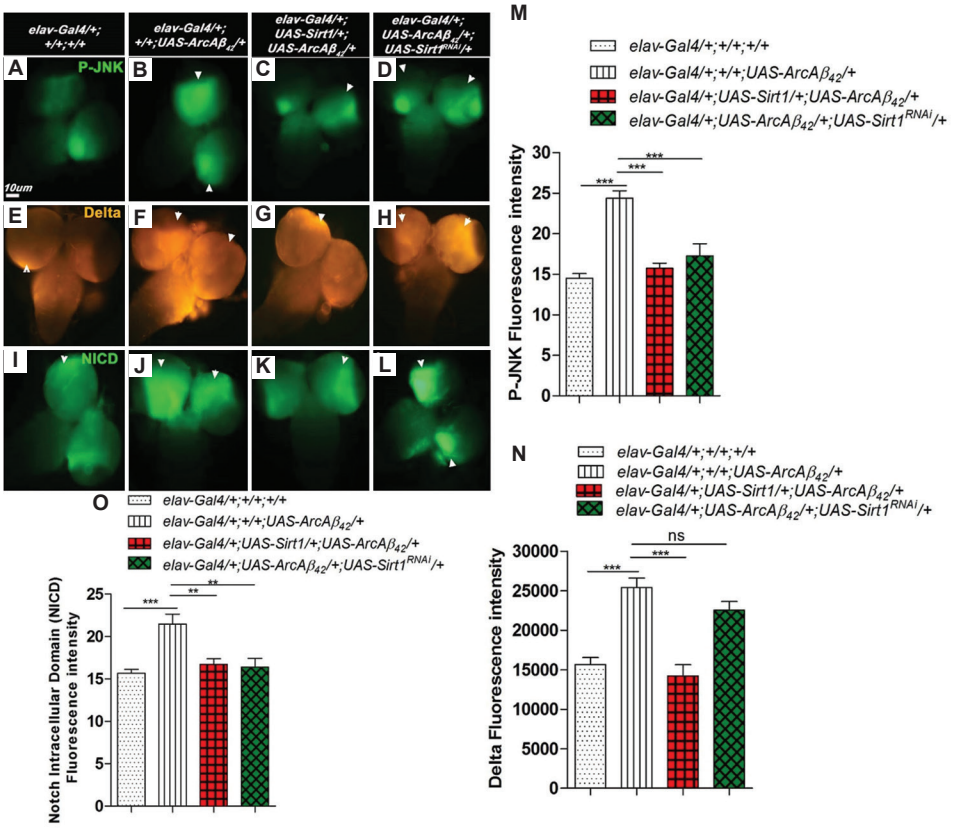

Figure 10. Fluorescence microscopy images and fluorescence intensity measurements showing anti-P-JNK, anti-Delta, and anti-NICD staining in the third instar larval brain. (A-D) JNK expression, (E-H) Delta expression, and (I-L) NICD expression in the third instar larval brains of *elav-Gal4/+;+/+;+/+*, *elav-GAL4/+;+/+;UAS-ArcAβ$_{42}$/+*, *elav-Gal4/+;UAS-Sirt1/+;UAS-ArcAβ$_{42}$/+*, and *elav-Gal4/+;UAS-ArcAβ$_{42}$/+;UAS-Sirt1^{RNAi}/+*. White arrowheads indicate upregulated p-JNK, Delta, and NICD staining compared to *elav-Gal4/+;+/+;+/+* (experimental control) flies (A, E, I). Scale bars indicate 10 μm (A-L). (M-O) Histogram indicating average fluorescence intensity of P-JNK, Delta, and NICD in third instar larval brains of each genotype. P-JNK, Delta, and NICD fluorescence intensities were analyzed using ImageJ software, NIH, USA. Error bars indicate mean ± standard error of the mean. Significance was calculated using one-way analysis of variance with Tukey's test in GraphPad Prism 5.0 and is indicated as ns: non-significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$. A total of 20 larval brains were observed for each genotype.
Abbreviation: NICD: Notch intracellular domain.

Moreover, we observed excessive cell death in AD model flies, which was significantly decreased when *Sirt1* was overexpressed (Figure 4E, H, and J) but increased when *Sirt1* was downregulated (Figure 4F, I, and J). The ectopic cell death observed in AD model flies was caused by increased expression of *Drosophila* apoptotic genes such as *Grim*, *Reaper*, and *Hid* (Figure 4K) and decreased/altered expression of *diap1* in AD model flies (Figure 4L). This was further supported by qRT-PCR analysis showing decreased expression of apoptotic genes (Figure 4K) and increased *diap1* expression (Figure 4L) when *Sirt1* was overexpressed in the AD model flies' genetic background. These results further suggest that *Sirt1* possesses antiapoptotic properties (Figure 4E, H, J).

Since JNK and Notch signaling are well-studied in the context of cellular stress, cell death, and early developmental processes in *Drosophila*, we observed the status of JNK and Notch signaling in AD model flies. We noted a slight improvement in the rough eye phenotype, abnormal bristles, and ommatidial arrangement in AD model flies when *Drosophila* JNK (*Bsk*) was downregulated. The pathological features of AD were further improved by overexpressing *Sirt1* along with downregulating *Bsk* in the AD model flies' genetic background(Figure 5C, D, and E). In addition, we observed an improvement in behavioral deficits (phototaxis and climbing) when *Bsk* was downregulated. This improvement was further enhanced when *Sirt1* was overexpressed along with *Bsk* downregulation in AD model flies (Figure 5F and G).

The improvement in AD pathologies was associated with reduced cell death due to a decrease in apoptotic gene expression (Figure 5H) and the activation of inhibitors of apoptotic proteins (IAPs) in AD model flies (Figure 5I). This reduction was attributed to the decreased expression

of proapoptotic genes (Figure 5H), and the increased anti-apoptotic activity of *Drosophila IAP1* (*diap1*) (Figure 5I) when *Bsk* was downregulated (in both heterozygous in both and homozygous conditions) and with *Sirt1* overexpression alongside *Bsk* downregulation in AD model flies. This finding supports prior studies indicating that $A\beta_{42}$ accumulation activates JNK-induced cell death in *Drosophila*.[61]

We also observed a significant increase in *Sirt1* expression levels when *Bsk* was downregulated in the AD model flies genetic background (Figure 6). Furthermore, we found that AD-related pathologies improved when *Notch* was downregulated along with *Sirt1* overexpression in AD model flies genetic background (Figure 7C-G). To further confirm these findings, we analyzed Delta protein expression in AD model flies. We observed that Notch downregulation along with *Sirt1* overexpression led to decreased *Delta* expression in AD model flies (Figure 8). We further observed that *Sirt1* overexpression decreased, while *Sirt1* downregulation increased, the expression of *JNK* and *Notch* signaling in *Drosophila* (Figures 9 and 10). Thus, our study indicates that Sirt1 possesses neuroprotective role by regulating the JNK and Notch signaling in *Drosophila*.

Between 2010 and 2023, several therapeutic strategies targeting AD have been attempted, including the identification of early biomarkers, anti-amyloid immunotherapy, Aβ aggregation inhibitors, BACE inhibitors, tau aggregation inhibitors, Selective Aβ42 lowering agents, α-secretase enhancers, anti-tau immunotherapy, and anti-inflammatory agents.[60] The current study is a small step toward identifying therapeutic targets for AD using *Drosophila* as a model organism.

5. Conclusion

Our study demonstrated that overexpression of *Sirt1* in *Drosophila* affects AD-related pathologies by improving the rough eye phenotype, correcting behavioral defects, increasing the phototaxis response, and reducing apoptosis in the *Drosophila* model of AD. Furthermore, these improvements were associated with reduced JNK/Notch activity in the *Sirt1* overexpression genetic background, which reduced neurodegeneration in AD model flies. The present study also showed that *Sirt1* genetically interacts with AD-associated genes (Appl, $A\beta_{42}$, and Tau) in *Drosophila* and could be a potential therapeutic intervention for NDDs. Thus, based on our observations, we concluded the neuroprotective potential associated with *Sirt1* in *Drosophila*.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Investigation: All authors
Methodology: Vidhi Bhatt
Writing – original draft: All authors
Writing – review & editing: All authors

Ethics approval and consent to participate

The study involves use of invertebrate model organism *Drosophila melanogaster* and was approved by Institutional Biosafety Committee (IBSC) Meeting, Agenda Item No 1.1.1, dated January 18, 2021.

Consent for publication

Not applicable.

Availability of data

The data supporting the present study's findings are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Non-invasive electroencephalography-based technique for rapid diagnostics of absence epilepsy in rats

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Abstract

Electroencephalography (EEG) is a crucial tool for diagnosing absence epilepsy, a type of generalized epilepsy characterized by brief lapses of consciousness. Here, we used a Wistar Albino Glaxo from Rijswijk (WAG/Rij) rat genetic model of absence epilepsy, in which spike-wave discharges (SWDs) manifested spontaneously and were linked to absence-like behavior. Conventionally, invasive electrocorticography (ECoG) with surgically implanted chronic electrodes has been used to confirm the absence epilepsy by the presence of SWDs in rats. However, this restricts the utilization of the same rat subject in multiple experiments. Therefore, there is a need for non-invasive EEG-based diagnostic tools in rats. This study introduces a novel, non-invasive EEG-based technique designed specifically for the rapid diagnosis of absence epilepsy. This approach is based on the sedative effect of xylazine and its unique capacity to induce SWDs. This approach was evaluated in a well-accepted genetic WAG/Rij rat model of absence epilepsy, including adult subjects of both sexes. Non-invasive EEG recording lasted 6 – 9 min. During the 6-min post-injection period, xylazine-induced SWDs closely resembled spontaneous SWDs in terms of the spike-wave morphology and frequency. The proposed non-invasive EEG-based technique is rapid, safe, inexpensive, and yields consistent results. Importantly, it can be repeated throughout a rat's lifespan to assess the age-related progression of absence epilepsy.

Keywords: Xylazine; WAG/Rij rats; Spike-wave discharges; Phenotypic variability; Physiobelt

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1. Introduction

Absence epilepsy is a non-convulsive form of epilepsy. Absence seizures may be noted in several types of idiopathic generalized epilepsies, including childhood absence epilepsy, juvenile absence epilepsy, and juvenile myoclonic epilepsy.[1-3] In these conditions, the seizures are typically characterized by generalized 3 – 6-Hz spike-and-wave complexes on electroencephalography (EEG) recordings. EEG is essential for diagnosing various forms of absence epilepsy. The presence of characteristic spike-and-wave discharges (SWDs) is necessary for the accurate diagnosis of idiopathic generalized epilepsies in EEG recordings, particularly when clinical manifestations are subtle or infrequent.[1,4,5] Spike-wave EEG patterns are characteristic ictal EEG hallmarks. However, the SWD structure exhibits significant variations across diverse epilepsy types (References in Hirsch *et al.*[2]).

- Childhood absence epilepsy is characterized by the occurrence of 3-Hz (range = 2.5 – 4 Hz) generalized SWDs. If no generalized SWDs are elicited following 3 min of hyperventilation in an untreated patient, childhood absence epilepsy can be excluded
- Juvenile absence epilepsy is characterized by the occurrence of regular 3 – 5.5-Hz generalized SWDs. If no SWDs are observed after performing hyperventilation for 3 min in an untreated patient, juvenile absence epilepsy can be ruled out
- Juvenile myoclonic epilepsy is characterized by the occurrence of irregular, generalized 3 – 5.5-Hz SWDs and polyspike-wave discharges.

Animal models are frequently employed in basic and preclinical studies of absence epilepsy because of ethical concerns associated with research engaging human participants. Absence epilepsy can be studied in various animal models, including electrical stimulation models, pharmacological treatment models, and genetic models (References in Jafarian *et al.*[6]). The following pharmacological compounds can induce typical absence seizures in rodents and cats: penicillin at high doses (250,000 – 6,000,000 U/kg), pentylenetetrazole (PTZ) at low doses (20 – 30 mg/kg intraperitoneally), and gamma-hydroxybutyrate at a dose of 200 mg/kg intravenously.[6,7] Pharmacological animal models of absence epilepsy can be classified into two categories: acute and chronic.

- In acute models, a pharmacological agent is administered to induce absence seizures for a brief period. These models are valuable for examining the immediate effects of drugs on absence seizures and for discovering novel treatments
- In chronic models, absence seizures are induced by administering a pharmacological agent over a long period. These models are beneficial for studying the long-term effects of drugs on absence seizures and for identifying potential adverse effects.

As idiopathic generalized epilepsies, including childhood absence epilepsy, have a multifactorial genetic cause, genetic animal models are considered more suitable than chemical models. In contrast to externally induced SWDs in chemical or electrical epilepsy models, genetic animal models demonstrate spontaneous SWDs due to their genetic predisposition. Notably, the EEG characteristics of SWDs and the associated behavioral signs in the genetic animal models closely resemble the EEG and clinical manifestations observed in human patients.

SWDs occur spontaneously during absence-like seizures in genetic rat models of absence epilepsy, including the Wistar Albino Glaxo from Rijswijk (WAG/Rij rats)[8-11] and Genetic Absence Epilepsy Rats from Strasburg (GAERS) rats,[12-15] as well as in relatively healthy rat strains, such as Sprague Dawley, Long Evans and Wistar rats.[12,16-20] WAG/Rij and GAERS strains, which are derived from Wistar rats, have been recognized as valid, reliable, and predictive models of human absence epilepsy. Here, we used the WAG/Rij rat genetic model of absence epilepsy,[8-11] in which SWDs appear spontaneously and are associated with absence-like behavior. The WAG/Rij genetic model provides several advantages over chemical models of epilepsy in rodents (The Discussion section for more details). Two technical approaches were employed to execute our study *in vivo*: (1) traditional electrocorticographic (ECoG) examination in free behavior using implanted epidural electrodes and (2) a newly devised non-invasive EEG technique for the rapid diagnosis of absence epilepsy in rats.

Epidural ECoG is a valuable tool for analyzing brain activity and is recommended for preclinical investigation in genetic rat models.[11] In patients with refractory epilepsy, ECoG serves as the primary standard for precisely identifying the exact seizure onset zones that require surgical removal.[21] ECoG generates exceptionally high-quality and more stable signals with exceedingly high-precision spatial resolution than the non-invasive EEG method. Although minimally invasive, the ECoG method offers numerous advantages. First, it generates high-resolution ECoG signals, capturing complex neural activity across the brain's surface. Second, it facilitates prolonged recordings, enabling the monitoring of brain activity over extended periods. Finally, ECoG is compatible with neuroimaging techniques, such as functional magnetic resonance imaging and magnetoencephalography, thereby enhancing the results' interpretive power.

Considering that absence-like seizures can manifest in relatively healthy rat strains.[12,16-20] and the correlation of absence epilepsy in rats with neurobehavioral comorbidities,[22-24] there is a need for non-invasive EEG-based diagnostic tools in rats. EEG-based techniques can provide a rapid and reliable diagnosis of epilepsy while safeguarding the rats. In this study, we used the principle of pharmacological induction of SWDs, as previously described by our research group,[25,26] to diagnose the absence epilepsy in rats. In particular, the systemic administration of low doses of alpha2-adrenoreceptor agonists (such as xylazine, dexmedetomidine, and medetomidine) is known to induce recurrent, long-lasting SWDs in WAG/Rij rats. However, it did not elicit *de novo* SWDs in non-epileptic subjects.[25] The intraperitoneal injection of low-dose dexmedetomidine (dose range, 0.0035 – 0.0307 mg/kg) in symptomatic WAG/Rij rats induced an absence-like behavioral state and elevated SWDs shortly after injection (with durations ranging from 68 s to 6.6 min).[25] In 1990, Buzsáki *et al.* revealed

that among alpha-2-agonists, xylazine (i.p., 2 mg/kg) was the most effective agent to induce high-voltage spike-and-wave spindles (HVS) in Fischer 344 rats.[27] Similar to WAG/Rij rats, HVS is associated with absence seizures and spontaneously occurring SWDs.[10,28] The WAG/Rij rat strain is widely recognized as a valid and reliable genetic model for investigating absence epilepsy.[8-10,29-31] WAG/Rij rats are particularly valuable in preclinical investigations due to their remarkable similarities to human patients with absence epilepsy.[23,30,32] In the present study, we used xylazine for its sedative effect and its strong ability to elicit SWDs in WAG/Rij rats. A prominent absence seizure-inducing effect of xylazine provided the basis for developing a novel, non-invasive EEG technique for rapidly diagnosing absence epilepsy in genetically predisposed rats. The non-invasive EEG technique was effectively validated in a large cohort of WAG/Rij rats aged 5 – 15 months (n = 65, including 32 females and 33 males).

2. Methods

2.1. Animals

This study included adult WAG/Rij rats of both sexes (body weight 300 – 500 g). The rats were bred and reared at the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences (RAS) in Moscow, Russia. The rats were housed in a vivarium and maintained under standard conditions with a 12/12-h light-dark cycle and unrestricted access to food and water. All experiments were conducted in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes. All phases of this study were approved by the Ethics Committee of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of RAS. Specifically, the protocols for ECoG examination in rats (protocol #4, October 26, 2021, and protocol #4, December 13, 2022) and additions for non-invasive EEG examination in rats (protocol #4, May 29, 2024) were approved.

The first group of 16 rats (9 females and 7 males) was subjected to invasive ECoG examination in free behavior during baseline and after i.p. xylazine injection. Among them, three rats (two females and one male) were subjected to non-invasive EEG examination before ECoG examination. The second group including 32 female and 33 male rats was subjected to non-invasive EEG examination under the influence of xylazine.

2.2. Pharmacological provocation of SWDs

SWDs were induced through intraperitoneal administration of xylazine hydrochloride (Xyla, 20 mg/mL xylazine hydrochloride, Interchemie Werken De Adelaar, the Netherlands) in dosages of 2 – 8 mg/kg.

2.3. ECoG

The rats were permanently implanted with epidural screw electrodes to perform long-term recordings during periods of free behavior.

2.3.1. Electrode setup and implantation procedure

The ECoG electrodes were constructed using stainless steel screws with a shaft length of 2.0 mm, a head diameter of 2.0 mm, and a shaft diameter of 0.8 mm. Each electrode was equipped with four screws that were affixed to wires and 4-pin connectors. The surgery was conducted under isoflurane anesthesia using the RWD Stereotaxic Anesthesia Setup (RWD Life Sciences, China). The rat's head was secured in the stereotaxic apparatus (Standard Stereotaxic Instrument, RWD, Life Sciences, China). After shaving the rat's head, the skin and soft tissues were excised from the cranium (Figure 1A). To implant the electrodes, four holes were drilled into the cranium: two holes over the right and left frontal cortices (AP +2 mm and L ± 2.5 mm) for the active frontal electrodes, one hole over the occipital cortex (AP −6 mm and L 4 mm) for the active occipital electrode, and one hole over the right cerebellum for the reference electrode (Figure 1B). The entire assembly was permanently attached to the skull utilizing a methyl methacrylate monomer (Figure 1C). Following the surgical procedure, the rats were administered metamizole (produced by FSSCI Microgen, Russia) intramuscularly (25 mg/kg) to alleviate pain. To prevent damage to the electrode connectors, the rats were individually confined in cages and allowed to recuperate for at least ten days. The pain, suffering, and distress experienced by the rats during the experiment were minimized, in keeping with the experimental protocols approved by the Animal Ethics Committee of our institution.

2.3.2. ECoG recording in free behavior

The rats were placed in Plexiglas cages (25 × 60 × 60 cm), and the ECoG was recorded during free behavior. The four-pin connectors on the rats' heads were connected to an amplifier through a swivel contact. The ECoG signals were transmitted to a multichannel amplifier (PowerLab 4/35, LabChart 8.0 software, ADInstruments, Sydney, Australia). The signals were bandpass filtered between 0.5 and 200 Hz, digitized at 400 samples per second per channel, and stored on the hard disk. The rats' behavior was monitored employing a Genius eFace 1325R video camera and recorded using the video capture module in LabChart software.

2.4. EEG examination

The EEG signal was captured from the scalp using a wireless recording system (Physiobelt, Neurobotics, Moscow, Zelenograd, Russia). Two Physiobelt sensors

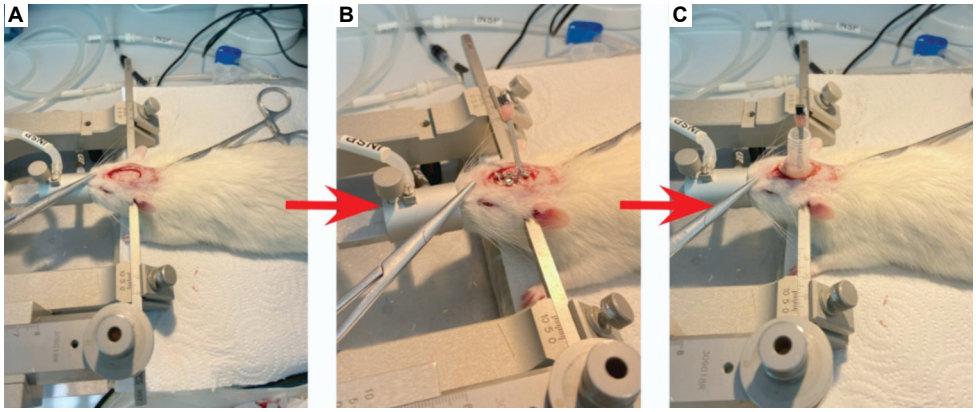

Figure 1. The surgical procedure for implanting epidural electrodes in a Wistar Albino Glaxo from Rijswijk rat under isoflurane anesthesia. (A) The skin and soft tissues were meticulously excised from the cranium. (B) Screw electrodes were precisely inserted at specific coordinates on the cranium. (C) A plastic carrier tubing was inserted, and the entire assembly was permanently secured to the cranium using a methyl methacrylate monomer.

were positioned approximately 1 cm apart in a recording cap (Figure 2A). Using the Bluetooth 4.0 transmission protocol, this montage enabled bipolar recording with a sampling rate of 1000 Hz, an amplitude range of ±3 mV, and a bandpass filter that spanned 1 – 45 Hz. The software Physiobelt v 2.8.0 for Windows 10 was used for EEG data acquisition and initial visualization.

Figure 2 illustrates the step-by-step procedure for non-invasive EEG recording.

- Step 1. Shaving the head and preparing for the EEG recording (Figure 2C)
- Step 2. Medication. Intraperitoneal injection of 2% xylazine in low doses (2 – 8 mg/kg) to induce sedation and trigger epileptic spike-wave activity
- Step 3. Attach the electrodes. Two Physiobelt sensors are placed over the frontal and parietal areas of the rat's head (indicated by arrows in Figure 2C and 2D)
- Step 4. EEG signal recording for 5 – 9 min following xylazine injection. This period is characterized by light sedation and by the presence of SWDs in symptomatic subjects. Figure 2B depicts an example of SWDs recorded using the Physiobelt software
- Step 5. Visualization. EEG signal processing using the LabChart v8 software for visual examination.

2.5. Statistical analysis

The time-frequency analysis of the ECoG and EEG signals was conducted using the LabChart v8 software for Windows 10. The software Physiobelt version 2.8.0 for Windows 10 was used for performing the visual inspection of non-invasive EEG.

In the first group of rats (n = 16 rats, ECoG examination), SWDs were visually identified in the data recorded during two-time intervals: (1) During a baseline 4-h interval (from 0:00 to 04:00 a.m.). (2) Six minutes following an intraperitoneal administration of xylazine. The total duration of the SWDs during each time interval was computed. Pearson correlations were employed to investigate the relationships between the total duration of SWDs during the 2-time intervals.

In the second group (n = 65 rats, EEG examination), 6 – 9 min of EEG recordings were visually inspected to detect SWDs.

3. Results

The WAG/Rij rats were subjected to ECoG examination. Figure 3A illustrates ECoG recording with spontaneous SWDs at baseline. The rats exhibited a typical SWD frequency of 8 – 10 Hz,[9,11,29] which consisted of high-voltage negative spikes and low-voltage waves.[33] As depicted in Figure 3A, the duration of SWDs was approximately 6s, which is consistent with the average duration of SWDs reported in age-matched male WAG/Rij rats.[34]

3.1. SWD-promoting effect of xylazine

Systemic administration of xylazine at a low dose (2 – 8 mg/kg) induced continuous SWDs in symptomatic WAG/Rij rats. Xylazine injections triggered a long-lasting train of SWDs (approximately 1 m 22 s after injection as depicted in Figure 3B). Similar to the baseline, xylazine-induced SWDs were characterized by a series of 8 – 10-Hz high-voltage spikes interspersed with low-amplitude waves (Figure 3A).

The video-ECoG hosted at https://encyclopedia.pub/video/video_detail/1305 demonstrates the acute effect of i.p. injection of 2% xylazine in a 16-month-old female WAG/Rij rat.[35] The recording commenced immediately after the i.p. injection of 2% xylazine (2 mg/kg). Approximately 1 minute following the injection, the first

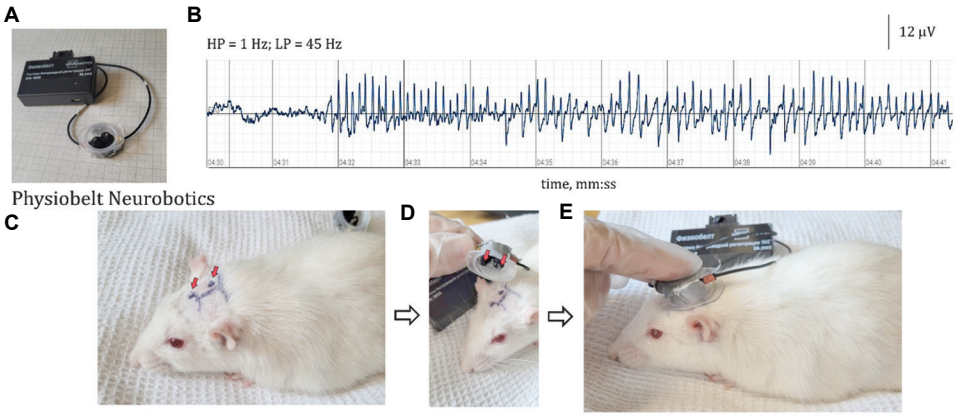

Figure 2. Non-invasive electroencephalography (EEG) recording in a rat utilizing a wireless Physiobelt system. (A) The receiver of the PhysioBelt was connected to a rat's recording cap, which contained two sensors that were positioned 1 cm apart. (B) The EEG signal with SWDs recorded 4 min after xylazine administration (2 mg/kg). The bottom photos illustrate the EEG recording procedure: (C) The rat's head should be shaved; here the projections of cranial sutures are marked on its skin. In the photo, the arrows denote two important points corresponding to the frontal and parietal cortical areas, which are situated approximately 1 cm apart. (D) Two sensors of Physiobelt should be placed over these points. (E) A recording cap should be gently held manually on the rat's head for 5 – 10 min.

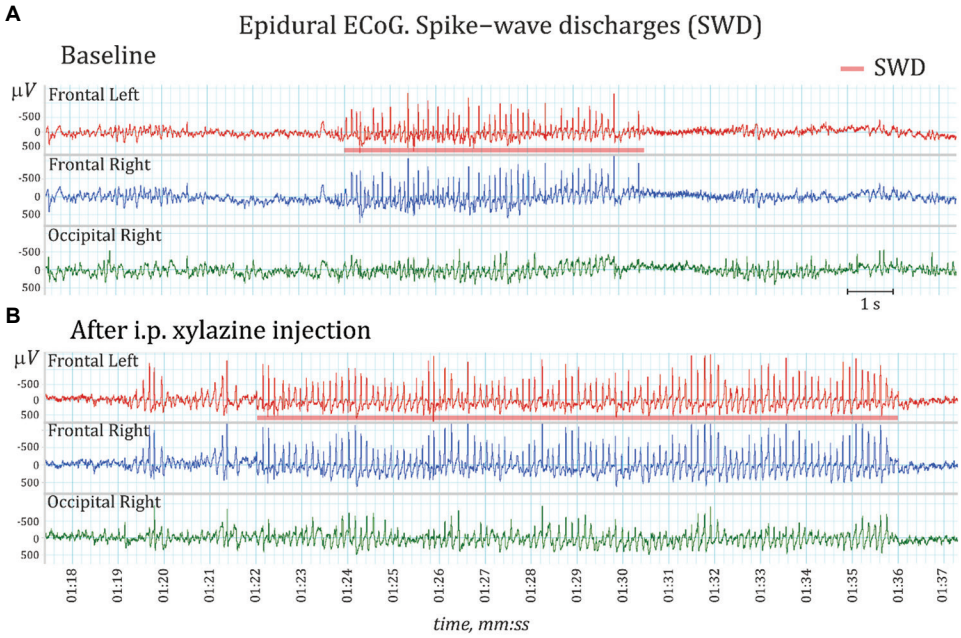

Figure 3. ECoG recordings of bilaterally synchronous SWDs in a symptomatic WAG/Rij rat (male, 14 months old). (A) Spontaneous SWDs at baseline. (B) Xylazine-induced SWDs (dosage 6 mg/kg) in the same subject; the moment of injection was designated as time zero.
Abbreviations: ECoG: Electrocorticography; SWD: Spike-wave discharges; WAG/Rij: Wistar Albino Glaxo from Rijswijk.

absence-like seizure associated with SWDs occurred. The subsequent 5 minutes were characterized by continuous bilaterally synchronous SWDs, which were succeeded by sporadic spike-wave complexes. The most pronounced SWDs were observed within the 1 – 6-second period following xylazine administration. This period of interest was investigated using non-invasive EEG. Through this approach, we have effectively developed a novel technique that combines the sedative properties of xylazine with its remarkable ability to promote absence-like seizures.

3.2. Non-invasive EEG and invasive ECoG recordings of xylazine-induced SWDs

This section describes xylazine-induced SWDs in the same rat subject using a novel non-invasive EEG technique (Figure 4A) and a traditional invasive ECoG technique

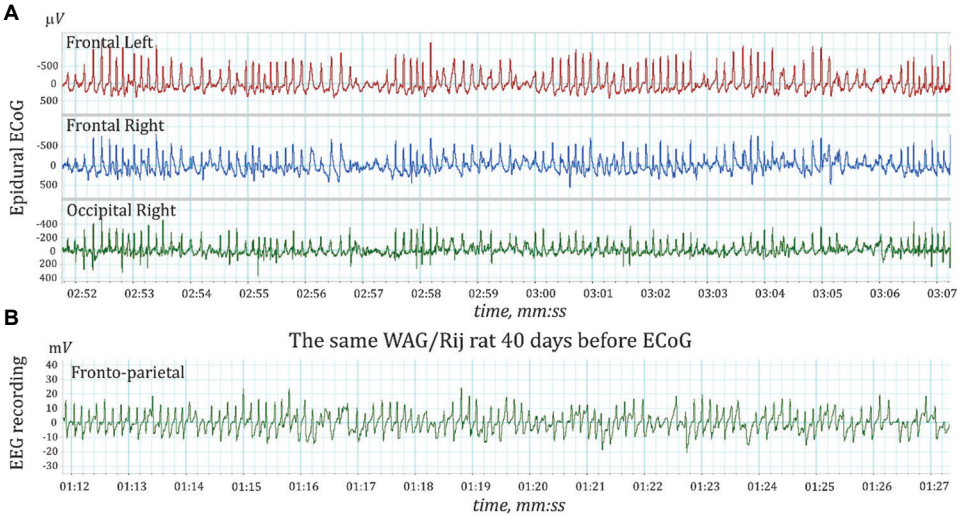

Figure 4. Examples of spike-wave activity induced by xylazine (i.p., 2 mg/kg) in a symptomatic WAG/Rij rat (female, 16 months old). The time of injection was designated as time zero. (A) Continuous SWDs were recorded from symmetrical frontal (left and right) and occipital right cortical locations using epidural ECoG. (B) Continuous SWDs recorded using the EEG technique in the same rat subject 40 days before ECoG.
Abbreviations: ECoG: Electrocorticography; SWD: Spike-wave discharges; WAG/Rij: Wistar Albino Glaxo from Rijswijk.

(Figure 4B). Both recordings were obtained subsequent to xylazine injections at a dose of 2 mg/kg. Note the consistency of the spike-wave activity's waveform in Figure 4A and 4B and the distinctive spike-wave pattern, characterized by remarkably sharp and repetitively occurring spikes.

A statistical analysis was performed to evaluate the SWD-promoting effect of xylazine in a group of 16 rats (9 females and 7 males). All rats were implanted with ECoG electrodes. The mean age of the rats was 14.4 months, with ages ranging from 13 to 16 months. ECoG signals were recorded for approximately 24 h at baseline and after xylazine administration (dose 2 – 8 mg/kg). SWDs were visually identified in ECoG recordings obtained under two conditions: (1) At baseline: spontaneous SWDs were selected during the 4-h interval (from 0:00 to 04:00 a.m.). (2) Immediately after xylazine administration: xylazine-induced SWDs were selected during a 6-min interval following xylazine administration. The spike-wave morphology and frequency of the xylazine-induced SWDs were similar to those of the spontaneous SWDs. Following this initial period, the 8 – 10-Hz SWDs gradually transitioned to 6 Hz SWDs, occasional spike-wave complexes, and eventually into slow-wave activity that characterizes a sedative state.

Figure 5 demonstrates the correlations between the total duration of spontaneous SWDs occurring at baseline (4 h) and the total duration of xylazine-induced SWDs (6-min). A strong Pearson correlation coefficient of 0.72 (P = 0.0016) indicates a significant positive correlation between the total duration of spontaneous SWDs and the

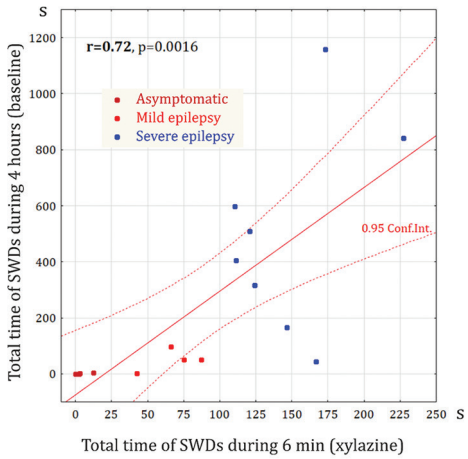

Figure 5. Pearson correlation between the total duration of spontaneous SWDs occurring at baseline (4 h) and the total duration of xylazine-induced SWDs (6 min).
Abbreviation: SWD: Spike-wave discharges.

total duration of xylazine-induced SWDs, indicating a high level of consistency between them. The occurrence of xylazine-induced SWDs was found to be minimal in subjects with no spontaneous SWDs at baseline (Figure 5).

The total duration of SWDs was highly variable among the 16 rat subjects. Four rats (25% from the group, marked with brown dots in Figure 5) did not experience any SWD during the baseline period, and they exhibited minimal spike-wave activity following xylazine administration (<25 s out of a 300-s observation period, or <8%). Therefore, these subjects were considered asymptomatic. The other

four subjects (25% from the group, marked with red dots in Figure 5) exhibited a low number of SWDs at baseline and mild SWDs following xylazine administration (25 – 100s out of the 6-min observation period, or 8 – 33%). The remaining eight rats (50% from the group, marked with blue dots in Figure 5) exhibited SWDs at baseline and severe SWDs after xylazine injections (more than 100 s out of the 6-min observation period or more than 33%).

In conclusion, we observed a remarkably high level of consistency between the total duration of spontaneous SWDs and that of xylazine-induced SWDs. Therefore, the severity of xylazine-induced epileptic manifestations was remarkably similar to that of the baseline condition. The findings indicate that xylazine injections could be a valuable tool for diagnosing absence epilepsy in rats. On invasive ECoG examination of the first group (n = 16), three major epileptic phenotypes were revealed, including asymptomatic (25%), mild epilepsy (25%), and severe epilepsy (50%).

3.3. Non-invasive EEG examination in rats under xylazine

The second group of rats (n = 65) was subjected to non-invasive EEG examination. Each rat was administered xylazine intraperitoneally (dose: 8 mg/kg) to induce sedation and provoke epileptic spike-wave activity. EEG recordings were obtained for more than 6 min after the injection. The rats remained immobile during the EEG recording. We used a portable microamplifier (Physiobelt) to acquire the EEG signals and occasionally encountered signal disturbances due to incidental rat head movements. These movements disrupt the physical contact between the skin and the sensors, causing signal loss or zeroing. High-voltage sine waves were observed a few seconds after the restoration of skin contact before commencing the acquisition of low-voltage electrical signals from the brain (shown by "signal lost/noise" in Figure 6).

Three distinct epileptic phenotypes with varying degrees of severity were identified based on the presence of SWDs during the 6-min post-injection intervals (Figure 6). This classification was exclusively based on the EEG results, excluding any additional behavioral assessments.

(1) Asymptomatic rats did not exhibit 8 – 10-Hz SWDs, despite the presence of abnormalities, including brief 6-Hz SWDs and occasional spike-wave complexes (Figure 6A).
(2) Mild epilepsy. Typical 8 – 10-Hz SWDs with a duration not exceeding 10 s occurring 2 – 8 times/6-min interval (Figure 6B).
(3) Severe epilepsy. Frequent and prolonged 8 – 10-Hz SWDs, some of which could last up to several minutes and were interrupted by brief periods of desynchronization (Figure 6C).

3.4. Xylazine-based tests for diagnosing absence epilepsy

Non-invasive EEG examinations under a xylazine-induced state were performed in the second group of rats (n = 65) between 5 and 15 months of age. Based on the results of EEG-based assessments, the rats were diagnosed with three categories of epileptic conditions: asymptomatic, mild, and severe. Figure 7 demonstrates the diagnostic results grouped by the following age ranges: 5 – 7 months, 7 – 9 months, and older than 9 months.

Twenty-six rats were tested multiple times at varying ages. Among them, six rats (23%) were characterized by an age-related increase in the severity of absence epilepsy. Nine rats (35%) showed no age-related changes in the severity of epilepsy. Eleven rats (42%) were asymptomatic. None (0%) of the rats demonstrated a reduction in the severity of absence epilepsy.

4. Discussion

In this study, we present a novel EEG-based diagnostic method for the rapid diagnosis of absence epilepsy. This method utilizes the sedative effects of xylazine and its distinct ability to induce SWDs in rats with spontaneous SWDs. To validate this method, we implanted WAG/Rij rats (n = 16) with ECoG electrodes, recorded three-channel ECoG, and assessed spontaneous SWDs during baseline and xylazine-induced SWDs. A substantial correlation was observed between the durations of spontaneous SWDs recorded during the 4-h interval at baseline and those of xylazine-induced SWDs measured at 6 min post-injection.

Here, we used the WAG/Rij rat genetic model of absence epilepsy.[8-11] In contrast to chemical or electrical models of epilepsy, WAG/Rij rats exhibit spontaneous SWDs due to genetic predisposition. The PTZ model is one of the most widely used chemical models of epilepsy. It provides a simple and widely applicable method for studying epilepsy mechanisms and screening potential antiepileptic drugs; however, it is not a model of absence epilepsy. PTZ is one of the first proconvulsant drugs used in animal models to induce seizure activity.[36-39] Injections of PTZ primarily induce tonic-clonic seizures rather than absence seizures. PTZ acts as a GABA-A receptor antagonist, suppressing inhibitory synaptic function and leading to increased neuronal excitability.[38] A single high-dose injection of PTZ (above 48 mg/kg) can induce acute, severe seizures. Chemical kindling, which induces repetitive seizures, can result from repetitive low-dose administrations (30 – 35 mg/kg) over time.[36,39] The WAG/Rij rat model is a

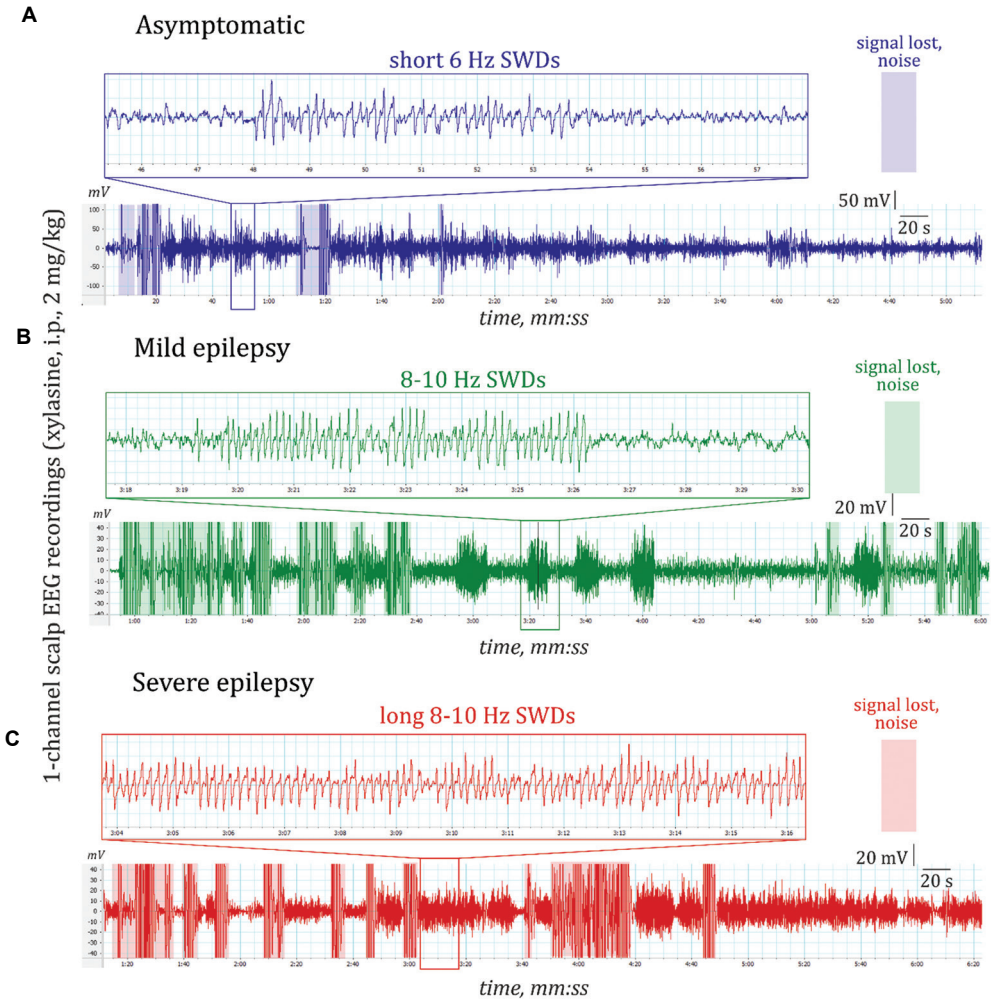

Figure 6. Examples of 6-min noninvasive EEGs recorded immediately after i.p. xylazine injections. The movement artifacts are shaded. (A) Occasional spike-wave complexes and short 6-Hz SWDs manifested in an asymptomatic rat. (B) Typical 8 – 10-Hz SWDs lasting <10 s in a rat with mild epilepsy. (C) Prolonged 8 – 10-Hz SWDs in a rat with severe epilepsy.
Abbreviations: SWD: Spike-wave discharges; EEG: Electroencephalography.

genetic model of absence epilepsy that was introduced by Van Luijtelaar and Coenen in 1986.[29] This model has been comprehensively validated and widely recognized.[10,23,30,31,40] WAG/Rij rats are characterized by spontaneous absence seizures that commence at approximately 3 months of age, with seizure severity increasing with age.[8,32,34,41] The spike-wave seizures typically last for a few seconds (mean: 6 – 8 s) and are characterized by a frequency of 8 – 10 Hz.[8,34,41]

Table 1 summarizes the differences between the genetic WAG/Rij rat model and the PTZ pharmacological models.[6,9,11,23,36,38] These data indicate that the WAG/Rij rat model is more valid and clinically relevant for studying absence epilepsy than the PTZ model.

Our study demonstrated significant variability in the total duration of SWDs in the WAG/Rij rat model of absence epilepsy. This was inferred from the ECoG results in the first group (n = 16 rats) and corroborated by non-invasive EEG examinations in a larger cohort (n = 65 rats). In both cohorts of rats, we identified three categories of EEG-related manifestations of absence epilepsy in rats: asymptomatic epilepsy, mild epilepsy, and severe epilepsy. In mild epilepsy, each SWD lasted up to 10 s, with 2 – 8 SWDs occurring during the 6 min. In severe epilepsy, frequent and prolonged 8 – 10-Hz SWDs occurred, which lasted up to several minutes. The diversity of absence epilepsy in genetically predisposed patients is well documented. The term "diversity" in this context refers to the wide range of genetic and phenotypic expressions of the disorder. Regarding phenotypic variability, absence epilepsy patients can present with varying degrees of severity and comorbid disorders. This phenotypic variability underscores

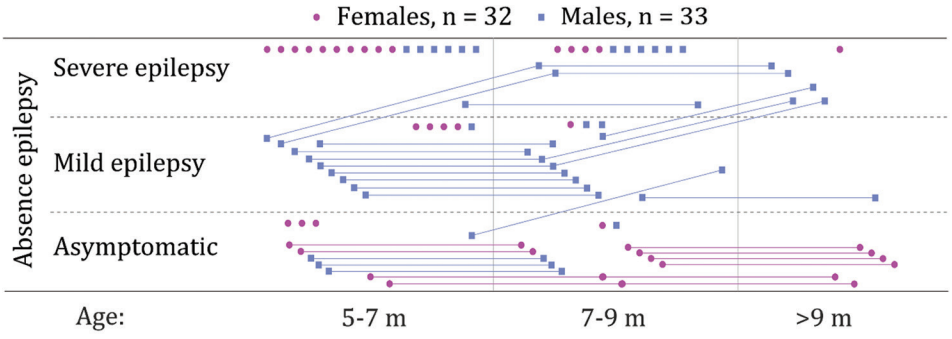

Figure 7. Schematic representation of the results of xylazine-based electroencephalography examinations in the second group of rats aged 5 – 15 months (n = 65). Each dot denotes one subject. Subjects that were evaluated at various ages are indicated by dots that are connected by lines.

Table 1. Comparison between the genetic WAG/Rij rat model and the pharmacological PTZ model of epilepsy[6,9,11,23,36,38]

	WAG/Rij	PTZ
Nature of the models	Genetic model; absence seizures appear spontaneously	Chemical model; seizures are induced by the administration of PTZ
Basic mechanisms	Cortico-thalamo-cortical circuitry and genetic factors	Acute seizure mechanisms
Validity of the models	High face, predictive, and construct validity	Lower validity compared with the genetic models
Durability of the models	Long-term studies, chronic absence epilepsy	Acute studies
Clinical relevance of the models	Similar to human absence epilepsy in terms of clinical presentation and EEG features	Less representative of human absence epilepsy
Mechanisms studied by the models	Spontaneous seizures and their underlying pathophysiology	Acute mechanisms of seizure induction
Model use: drug screening	Can be used for drug screening and may offer more predictive results	Can be used for drug screening

Abbreviations: EEG: Electroencephalogram; PTZ: Pentylenetetrazol; WAG/Rij: Wistar Albino Glaxo from Rijswijk.

the broad spectrum of manifestations associated with absence epilepsy.[42,43] In the present study, we identified three epileptic phenotypes in the WAG/Rij rats. This heterogeneity contradicts the well-accepted homogeneity of seizure severity in WAG/Rij rats. We hypothesize that the phenotypic variability of absence epilepsy in a cohort of genetically prone WAG/Rij rats in the Institute of Higher Nervous Activity and Neurophysiology RAS (Moscow, Russia) is comparable to the phenotypic variability of this disease in human patients.

The phenotypic variability of absence epilepsy in inbred WAG/Rij rats highlights the necessity for developing non-invasive technologies that enable rapid diagnosis of absence epilepsy. It also implies the necessity for a personalized, subject-specific approach to rat models, considering their behavioral features and neurobehavioral comorbidities.[22]

The proposed technique is quick, secure, cost-effective, and yields consistent outcomes. Importantly, it could be conducted repeatedly throughout a rat's lifespan to evaluate the age-related progression of absence epilepsy. In our investigation, 26 rats were subjected to repetitive EEG examinations at ages ranging from 5 to 15 months. Among them, 42% did not exhibit any symptoms of absence epilepsy. The severity of absence epilepsy increased with age in 23% of the rats, remained constant in 35% of them, and never decreased in severity in any of the rats. It is well known that the incidence and duration of SWDs in WAG/Rij rats increases with age.[8,9,11,32,34] Our findings indicate that 23% of the rats exhibited an age-related increase in the severity of absence epilepsy. In our model, the severity of absence epilepsy is empirically measured as a complex characteristic that incorporates both the duration and the number of SWDs that manifested following xylazine administration.

Our findings revealed that during the 6-minute post-injection period, xylazine-induced SWDs closely resembled spontaneous SWDs in terms of spike-wave morphology and frequency. Subsequently, the 8–10-Hz SWDs gradually transitioned into 6-Hz SWDs, occasional spike-wave complexes, and eventually into a slow-wave activity that characterizes a sedative state. Notably, xylazine did not elicit pronounced 8 – 10-Hz SWDs in asymptomatic rats; however, it induced brief 6-Hz SWDs and occasional spike-wave complexes. Alterations in the

waveform of xylazine-induced SWDs during the post-injection period may be linked to the clinical manifestation of absence epilepsy or neurobehavioral comorbidities. The present study did not investigate the waveform patterns and dynamics of intrinsic frequency modulations in xylazine-induced SWDs. In rodents, time-frequency analysis of xylazine-induced spike SWDs could serve as an additional diagnostic and monitoring tool for absence epilepsy and its associated comorbidities.

The technology described in the present study has several limitations. First, this technique can be employed only in anesthetized rats, which need to remain immobile during the EEG recording session. Alpha-2-agonists, in addition to the profound sedation effect, induce SWDs, which is a key feature of the current technique. Second, signal disturbances can occur due to incidental rat head movements. The rats were unable to tolerate the electrode cap, unlike humans, and the examiner had to manually hold the cap on the rat's head (Figure 2C). In this study, we employed a portable microamplifier (Physiobelt) that was suitable for acquiring EEG signals non-invasively in anesthetized rats.

Head movements caused disruptions in the physical contact between the skin and the sensors, resulting in signal loss or zeroing. A few seconds following the restoration of skin contact, high-voltage sine waves were observed before the acquisition of low-voltage electrical signals from the brain.

This newly developed method for rapid, non-invasive diagnosis of absence epilepsy in rats could be advantageous for preclinical studies. This is especially beneficial for ensuring that healthy, non-epileptic subjects are selected as controls and for testing the potential absence seizure-inducing effects of candidate drugs. Future directions for non-invasive EEG recording technology include the following:

- Improvement of techniques for restraining rat subjects to minimize movement and facilitate consistent electrode placement
- Developing electrode caps that ensure close attachment of sensors to the scalp for better signal quality
- Devising a system for the precise positioning of sensors on the rat scalp to optimize EEG recordings.

5. Conclusion

This study presents a novel, non-invasive EEG-based technique for rapidly diagnosing absence epilepsy in genetically predisposed rats. This method utilizes the sedative effects of xylazine and its distinct ability to induce SWDs. Systemic administration of xylazine in low doses (2 – 8 mg/kg) induced continuous 8 – 10-Hz SWDs in symptomatic WAG/Rij rats. Asymptomatic rats exhibited brief 6-Hz SWDs and occasional spike-wave complexes. To validate this technique, we analyzed ECoG results in 16 WAG/Rij rats for the presence of spontaneous SWDs during baseline and xylazine-induced SWDs. The duration of spontaneous SWDs measured in 4-h intervals strongly correlated with the duration of xylazine-induced SWDs measured in the 6-min post-injection interval. This demonstrates the applicability of xylazine for diagnosing absence epilepsy in rats. SWDs induced by xylazine exhibited comparable waveforms in recordings obtained using a novel non-invasive EEG technique and a conventional invasive ECoG technique.

Non-invasive EEG examinations in a larger cohort (n = 65 rats) demonstrated three types of absence epilepsy manifestations in rats: asymptomatic epilepsy, mild epilepsy, and severe epilepsy. The diversity of absence epilepsy in genetically predisposed patients is well documented. We hypothesize that the phenotypic variability of absence epilepsy in a cohort of genetically prone WAG/Rij rats in the Institute of Higher Nervous Activity and Neurophysiology RAS (Moscow, Russia) mimics the phenotypic variability of this disease observed in human patients.

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Conflict of interest

Evgenia Sitnikova is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

Conceptualization: Evgenia Sitnikova
Formal analysis: Evgenia Sitnikova
Investigation: All authors
Methodology: Evgenia Sitnikova
Writing–original draft: Evgenia Sitnikova
Writing–review & editing: Evgenia Sitnikova

Ethics approval and consent to participate

The Ethics Committee of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences (RAS) in Moscow, Russia, approved all phases of

this study. Specifically, the protocols for ECoG examination in rats (Protocol No. 4, October 26, 2021, and Protocol No. 4, December 13, 2022) and additions for non-invasive EEG examination in rats (Protocol No. 4, May 29, 2024) were approved.

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author, Evgenia Sitnikova, on reasonable request. A video-ECoG recording of a symptomatic WAG/Rij rat, in which continuous spike-wave discharges were induced by intraperitoneal injection of xylazine, could be found In the MDPI Encyclopedia URL: https://encyclopedia.pub/video/video_detail/1305 (E. Sitnikova, 2024)

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CASE REPORT

Transforming lives in autism spectrum disorder treatment through acupuncture: A case report

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication, restricted and repetitive behaviors, and narrow interests. Given the high prevalence of ASD and the lack of specific pharmacological treatments, there is a pressing need for alternative therapeutic approaches. Acupuncture has shown promise in improving the clinical symptoms of ASD. This report presents the case of a 6-year-old girl diagnosed with ASD, intellectual developmental disorder, attention deficit hyperactivity disorder, and articulation disorder. She underwent Liu's pediatric neurological rehabilitation acupuncture therapy (PNRAT) for over 2 years. The treatment involved specific scalp acupuncture techniques targeting cognitive and linguistic rehabilitation. After the treatment course, the patient showed significant improvements in cognitive, linguistic, social, and behavioral symptoms, transitioning from limited verbal communication and marked social difficulties to thriving in a mainstream school setting. This case highlights the potential of Liu's PNRAT as an effective intervention for ASD. The observed improvements suggest that acupuncture may offer valuable therapeutic benefits for children with ASD, contributing to the growing interest in its potential as an adjunct therapy. Further research is needed to optimize treatment protocols and explore synergies with conventional therapies.

Keywords: Autism spectrum disorder; Acupuncture; Liu's pediatric neurological rehabilitation acupuncture therapy; Cognitive rehabilitation; Linguistic rehabilitation; Case report

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1. Background

Autism spectrum disorder (ASD) and related conditions significantly impact children's mental health, with ongoing debates about the diagnostic criteria. The release of the diagnostic and statistical manual of mental disorders (DSM-5) by the American

Psychiatric Association marked a pivotal moment in this context, formally introducing ASD and revising diagnostic standards.[1] This revision brought clarity and uniformity to the diagnosis of ASD, helping clinicians and researchers to identify and categorize cases more effectively. However, these changes also sparked discussions and controversies regarding the scope and boundaries of the disorder. The term "spectrum" highlights the broad range of symptoms and severities observed in individuals with ASD, reflecting the complexity and heterogeneity of the condition.[2] This spectrum nature necessitates a nuanced understanding and an individualized approach to diagnosis and treatment.

ASD is characterized by persistent deficits in social communication, interaction, and repetitive behaviors. These deficits vary widely in severity and presentation, making each case unique. From infancy, children with ASD may show minimal or absent social interactions, lack emotional sharing, and exhibit reduced or absent imitation of others' behaviors. Their language is often limited to expressing needs rather than engaging in commentary, sharing feelings, or maintaining conversations. This can significantly hinder their ability to form and maintain relationships. In addition, deficits in nonverbal communicative behaviors may manifest as a lack, reduction, or atypical use of eye contact, gestures, facial expressions, body posture, or tone of voice. These challenges further isolate individuals with ASD, leading to behaviors that can appear inappropriate, passive, or aggressive.[3]

Moreover, many individuals with ASD experience intellectual and/or language impairments, with significant discrepancies between intellectual abilities and adaptive functioning. These impairments can complicate their educational and social experiences. Atypical motor signs, such as odd postures or clumsiness, may also be present, adding another layer of difficulty. Behavioral issues, including self-injury and destructive or provocative behaviors, are not uncommon and can be distressing for both the individuals and their families. Anxiety and depression are prevalent among individuals with ASD, further complicating their social and emotional well-being.[4] Traditional treatments for ASD primarily focus on behavioral interventions, speech therapy, occupational therapy, and pharmacological management. While these treatments can be beneficial, they often face limitations, including variable response rates and the need for long-term intervention to achieve significant improvements. This highlights the necessity for continued research and the development of more effective and comprehensive treatment strategies to better support individuals with ASD and their families.

Traditional Chinese medicine (TCM) does not explicitly reference ASD in its classical literature; however, its conceptual framework may offer insights into the disorder. In TCM, developmental issues such as "language delay" and "weak fetus" are linked to congenital deficiencies and inadequate nourishment of the brain, which can be relevant for understanding ASD.[5] These concepts suggest that imbalances in the body's vital energies might impact cognitive and behavioral development, potentially reflecting aspects of ASD. Acupuncture, a key therapeutic practice in TCM, involves inserting fine needles into specific points on the body to stimulate energy flow and promote healing. This modality has been widely applied to various neurological conditions, with evidence suggesting it may improve clinical symptoms of ASD. Studies indicate that acupuncture can enhance social interaction and communication while reducing repetitive behaviors in individuals with ASD.[6,10-13] However, despite these promising results, the current body of research is limited and varied, highlighting the need for more detailed case reports and rigorous clinical trials. Such studies are essential to validate the efficacy of acupuncture for ASD and to develop optimized treatment protocols. Addressing these research gaps will be crucial for integrating acupuncture into conventional therapeutic strategies and ensuring its effective application in managing ASD symptoms.

In recent decades, pediatric acupuncture, notably Liu's pediatric acupuncture therapy, has gained traction for its efficacy in neurological rehabilitation.[6-8] Liu's pioneering work has led to the development of tailored acupuncture techniques for cognitive rehabilitation, forming a comprehensive approach known as pediatric neurological rehabilitation acupuncture therapy (PNRAT). The characteristics of Liu's PNRAT include painless insertion and enjoyable needle retention, often combined with simultaneous training tasks during the needle retention period. This method aims to integrate traditional acupuncture with modern rehabilitation strategies to enhance treatment outcomes.

This case is unique as it highlights the application of Liu's PNRAT for a 6-year-old girl diagnosed with ASD, intellectual developmental disorder, attention deficit hyperactivity disorder (ADHD), and articulation disorder. The therapy involves using scalp acupuncture techniques specifically targeting cognitive and linguistic rehabilitation areas. The case report demonstrates significant improvements in the patient's cognitive, linguistic, social, and behavioral symptoms after the treatment course. These improvements suggest that acupuncture, particularly Liu's PNRAT, may offer a valuable complementary approach to conventional therapies for ASD. The observed improvements contribute to the growing interest in the potential of acupuncture as an adjunct therapy for ASD symptoms, despite ongoing controversies and the need for more research to optimize treatment protocols.

The significance of this case report lies in its detailed presentation of a clinical scenario where Liu's PNRAT has shown remarkable effectiveness. The case underscores the potential of integrating traditional acupuncture with contemporary medical practices to address complex neurological and developmental disorders like ASD. By documenting the patient's transition from limited verbal communication and social challenges to thriving in a mainstream school setting, this report provides compelling evidence supporting acupuncture as a complementary therapeutic option for ASD. This case report not only highlights the potential benefits of Liu's PNRAT in managing ASD symptoms but also emphasizes the importance of further research. It advocates for the integration of acupuncture into broader therapeutic frameworks to enhance the quality of life for individuals with ASD. The findings presented here pave the way for larger-scale studies and clinical trials to validate the efficacy of acupuncture and refine treatment protocols, ultimately contributing to a more holistic approach to ASD management.

2. Case presentation

A 6-year-old girl was first seen on September 26, 2023, presenting with long-standing difficulties in expressive language and unclear speech. She exhibited limited spontaneous speech, with unclear articulation, and was unable to articulate phrases of more than three words. She can only comprehend simple instructions and demonstrates slightly poor execution ability, along with inadequate comprehension, response, and logical thinking. Her memory and social adaptation are poor, making it difficult for her to learn addition and subtraction within ten, which hinders her ability to attend primary school. She exhibits occasional violent and destructive behaviors and is toilet-trained for urination but requires parental assistance for defecation. She began speaking spontaneously around the age of 2 and started walking independently at 18 months. She has a fair appetite, drinks less water, and complains of occasional abdominal pain (with a history of intussusception). Her bowel movements occur once every 1 – 2 days and are slightly dry. She frequently tosses and turns at night, prefers prone positioning, and occasionally grinds her teeth. On examination, her tongue is pale red with a white, slippery coating, and her pulse is deep and string-like. She was born at full term through vaginal delivery, weighing 2.9 kg and measuring 50 cm, with no history of neonatal jaundice or asphyxia.

The specific assessment instruments (Table 1) used in this case were selected for their established reliability and validity in evaluating various dimensions of ASD and associated conditions. An electroencephalogram (EEG) was conducted to rule out abnormal brain activity, ensuring that no underlying neurological issues were present. The Conners' rating scales and the Conners parent rating scale (CPRS) provided a comprehensive evaluation of behavioral issues, learning disabilities, and symptoms of ADHD, which are often comorbid with ASD. The digit attention test specifically assessed attention deficits, a common problem in ASD. The SNAP-IV stands for Swanson, Nolan, and Pelham-IV offered a detailed assessment of ADHD symptoms and oppositional defiance. The Chinese Wechsler intelligence scale for children (C-WISC) was used to measure cognitive abilities and identify intellectual disabilities. The childhood autism rating scale (CARS) and autism behavior checklist (ABC) were employed to assess the severity of autism symptoms. Finally, the speech assessment evaluated the child's linguistic and cognitive impairments, which is crucial for tailoring the therapeutic approach. Together, these tools provided a holistic view of the child's condition, informing the targeted intervention strategy.

The diagnostic assessments revealed a range of issues in the patient (Table 1 and Figures S1-4). A normal EEG ruled out any significant brain abnormalities. The CPRS and Conners' rating scales indicated mild behavioral problems, attention deficits, and learning disabilities, with additional signs of mild anxiety and depression. The SNAP-IV score confirmed moderate symptoms of ADHD, with a focus on inattention and hyperactivity/impulsivity. The child's performance on the C-WISC was below average, indicating a mild intellectual disability. The CARS and ABC scores fell in the borderline and suspicious ranges, respectively, suggesting a possible diagnosis of ASD. The speech assessment highlighted significant impairments in spontaneous expression, articulation, memory, social adaptation, and learning abilities, leading to an additional diagnosis of articulation disorder. Based on these results, the child's primary diagnoses were ASD, intellectual developmental disorder, ADHD, and articulation disorder. These assessments formed the basis for the tailored acupuncture treatment plan.

2.1. Combined TCM and conventional approaches

The treatment principle for the patient with ASD, in this case, combines the concepts of "clearing the mind," "enhancing intelligence," and "brain health." These principles are rooted in TCM's understanding of ASD as a result of congenital deficiencies and inadequate nourishment of the brain, which affects the heart, spleen, liver, and kidneys. By addressing these imbalances, TCM aims to improve cognitive, emotional, and social functioning.

Before initiating the procedures, the patient's parents were provided with a consent form. The form outlined several important points, emphasizing the relationship

Table 1. Diagnostic assessments

Assessment tool	Score	Interpretation	Diagnosis
Electroencephalogram	Normal range	No abnormal brain activity detected	N/A
Conners parent rating scale	14 points	Positive	Mild behavioral issues
Digit attention test	5 points	Poor	Attention deficit
Conners' rating scales	Behavior problems: 8 (mild), learning disabilities: 6 (moderate), psychosomatic disorders: 0 (normal), impulsiveness: 5 (mild), anxiety and depression: 5 (mild), and attention deficit: 10 (mild)	Mild to moderate symptoms	ADHD, learning disabilities, mild anxiety, and depression
SNAP-IV	Total score: 1.62 (moderate), inattention: 2 (severe), hyperactivity/impulsivity: 1.67 (moderate), ADHD 18-item subtotal: 1.83 (moderate), oppositional defiance: 1.13 (borderline)	Moderate symptoms	ADHD
Chinese Wechsler intelligence scale for children (6 years and above)	58 points	Below average	mild intellectual disability
Childhood autism rating scale	30	Borderline	ASD
Autism behavior checklist	64 points	Suspicious	ASD
Speech assessment	Limited spontaneous expression, unclear articulation, poor memory, social adaptation, and learning difficulties	Significant speech and cognitive impairments	Articulation disorder

Abbreviations: ADHD: Attention deficit hyperactivity disorder; ASD: Autism spectrum disorder.

between treatment efficacy and factors such as the child's age, condition type, and adherence to medical advice during the rehabilitation period. It highlighted the importance of maintaining a light diet, preventing colds, and managing emotional well-being throughout treatment. Parents were advised to promptly report any adverse symptoms, such as fever, dizziness, headache, nausea, or vomiting, to healthcare providers for timely adjustments. The consent form also stressed the need for cooperation among the child, parents, and physician during acupuncture sessions, explaining that normal reactions may include crying or discomfort due to pain or unfamiliar environments. In addition, it included post-treatment care instructions, such as monitoring and managing minor side effects, ensuring the acupuncture site remains dry for 3 h, and avoiding strenuous activities. It also underscored the importance of maintaining a smoke-free, clean hospital environment and protecting hospital property.

The chosen treatment method, Liu's PNRAT, is a specialized approach that incorporates scalp acupuncture techniques. This therapy is based on TCM's meridian theory and the functional localization of the cerebral cortex in modern medicine. By stimulating specific scalp acupoints, it seeks to increase blood flow to the cortex, alleviate ischemia and hypoxia, and promote the development and recovery of various brain functions.

The combination of Liu's PNRAT with conventional cognitive and behavioral interventions was chosen for its potential synergistic effects. Acupuncture addresses underlying imbalances in the body, while conventional approaches focus on skill development and behavior modification. This comprehensive treatment plan aims to optimize the patient's cognitive, linguistic, and social abilities, alleviate associated symptoms, and ultimately facilitate integration into daily life and school settings.

Based on Liu's PNRAT (Figure 1), the following acupoints were selected: *Zhiqi*, emotional zone, language 1/2/3 Area, *Ding Shen*, brain three, and *Jin Jing Yu Ye*.[7-9] The procedure involves routine disinfection using a 1-inch filiform needle inserted into the scalp at an angle of 15° – 30°. The needles are swiftly inserted into *Zhiqi*, emotional zone, language 1/2/3 area, *Ding Shen*, and brain three to a depth of 0.6 – 0.8 inches and left in place for 4 h. Every 1/2 h, the needles are manipulated using twisting, lifting, and thrusting techniques to stimulate cognitive, speech, social, and attention training. In addition, a 3-inch filiform needle is rapidly punctured into the sublingual *Jin Jing Yu Ye* points with 10 punctures per session, performed once daily, and followed by a 15-day break between sessions, totaling 30 sessions per course. During the acupuncture period, the following additional activities were also implemented:

(i) Psychoeducational profile (third edition) assessment: This assessment tool was utilized to regularly evaluate the child's developmental level, including communication, social interaction, motor skills, and adaptive behavior. This ongoing assessment helped monitor treatment progress and adjust the intervention plan as needed.

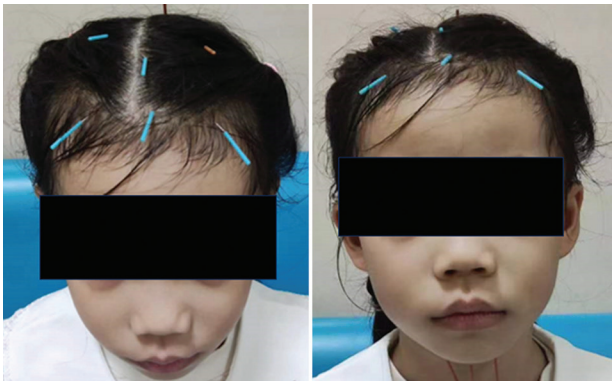

Figure 1. Application of Liu's pediatric neurological rehabilitation acupuncture therapy in the treatment of the patient

(ii) Speech training: This included activities such as pronunciation practice, vocabulary expansion, and language comprehension and expression training. Therapists engaged the child with conversations, guided them in expressing their thoughts and feelings, corrected pronunciation errors, and gradually increased the complexity of language tasks.

(iii) Treatment and education of autistic and related communication-handicapped children: A structured teaching method was employed, including assessment, goal setting, and the development and implementation of teaching and training plans. This approach aimed to create an organized learning environment and daily routine, enhancing the child's self-care abilities and independence.

(iv) Individualized education program: This was developed to set clear learning goals and teaching strategies tailored to the child's specific needs and abilities.

(v) Discrete trial training: This was used to break down complex skills and tasks into smaller, manageable steps, teaching the child incrementally and reinforcing correct responses.

(vi) Natural situational teaching: Teaching occurred in natural settings, leveraging everyday situations to promote the child's learning, and application of skills in real-life contexts.

(vii) Verbal behavior milestones assessment and placement program: This assessment tool was employed to comprehensively evaluate the child's language, social, cognitive, and other abilities, providing guidance for treatment planning.

(viii) Behavior intervention: Behavior analysis and intervention strategies were used to help the child modify undesirable behaviors and develop positive behavior habits.

(ix) Game therapy: Various game activities were used to enhance the child's social skills, emotional regulation, and cooperation abilities. Role-playing games helped the child understand others' perspectives and emotions, while team games fostered cooperation and communication skills.

(x) Applied behavior analysis: This technique was used to observe and analyze the child's behavior, leading to the development of a personalized behavior intervention plan. Positive and negative reinforcement methods helped the child adjust behaviors and cultivate good habits.

(xi) Communication training: The child was taught non-verbal communication methods, such as gestures, facial expressions, and eye contact, alongside improvements in language expression and listening skills.

(xii) Expression training: The child was encouraged to express emotions and thoughts through creative activities such as painting, writing, and crafting, enhancing creativity and self-expression.

(xiii) Cognitive training: This training focused on improving the child's attention, memory, thinking, and problem-solving skills, aiming to enhance cognitive function, learning efficiency, and overall quality of life.

2.2. Follow-up after 1 month of treatment

On October 25, 2023, during the second visit, the child demonstrated improvements in her logical thinking and comprehension abilities. She could gradually understand and follow activity rules, with an enhanced ability to sustain attention. She could complete puzzles in a seated position for 5 – 6 min and actively engage in activities while adhering to rules. Her verbal communication increased, and she started asking questions like "What is this?" and greeting familiar individuals with "Hello" and "Goodbye." Her emotional state improved, with fewer instances of destructive behavior, although occasional aggression remained. In addition, the frequency of abdominal pain decreased, and no teeth grinding at night was reported, consistent with previous observations.

During the third visit on November 25, 2023, the child's response continued to improve. Her logical thinking, comprehension, and memory showed marked improvement. She could fully understand and execute activity rules, actively participate in activities, design games herself, and invite others to join. She initiated conversations more frequently and introduced herself to others by stating her name and age. Her emotional state remained stable, with reduced abdominal pain and less frequent tossing and turning at night compared to before.

By the fourth visit on December 10, 2023, after completing one course of treatment, significant improvements were observed in the child's coordination, language expression,

and social skills. She could express herself independently, demonstrate good logical thinking and comprehension, follow instructions, and exhibit improved memory. In addition, she was able to correctly pick up 20 beads using chopsticks, engage in simple role-playing activities, express emotions, initiate communication, and seek help when needed. Her emotional state was calm, with no signs of aggression or destructive behavior, and she reported no abdominal pain. Bowel movements were regular, and her sleeping pattern had improved slightly. On examination, her tongue appeared pale red with a slight white coating, and her pulse was slightly deep and string-like.

The results of various assessments showed improvements over time, with scores indicating a decrease in symptoms related to ADHD and ASD. The child's overall intellectual functioning also showed improvement, as reflected in her performance on the C-WISC. Subsequent follow-ups revealed that the child's guardians reported a full recovery, with no noticeable differences from typically developing children of the same age, and she had returned to school with successful classroom integration after more than 3 months.

3. Discussion

ASD was first described by Dr. Leo Kanner in 1943, a seminal moment that laid the foundation for our understanding of the condition. However, it was not until the publication of the third edition of the DSM-III in 1980 that ASD was officially recognized as a distinct diagnostic entity, separating it from other developmental disorders and defining its core characteristics. This formal recognition marked a significant shift in how ASD was perceived and treated within the medical community. TCM, with its rich history and unique diagnostic framework, approaches ASD through different conceptual lenses. In TCM, ASD is often categorized under terms such as "Wu Chi" (language delay), "Dai Bing" (stupidity), and "Wu Hui" (lack of wisdom), reflecting a perspective that views the disorder as a manifestation of deeper, underlying deficiencies rather than a standalone condition.[10-12] According to TCM theory, the onset of ASD is believed to occur during critical developmental stages, including the fetal, neonatal, and infantile periods. The etiology and pathogenesis of ASD in TCM are attributed to a combination of congenital deficiencies and postnatal neglect, which result in inadequate nourishment of the brain marrow and insufficient essence. This conceptual framework posits that ASD is primarily a disorder affecting the brain's development and function, with a complex interplay involving the heart, spleen, liver, and kidneys. TCM's approach to ASD emphasizes a holistic view, focusing on restoring balance and enhancing the body's vital energy to address the disorder's multifaceted nature. Recent studies have demonstrated that TCM, particularly through acupuncture, offers significant advantages in treating ASD. Acupuncture, a distinctive therapy within TCM, has shown promising results in improving various aspects of the disorder, such as cognitive function, social interaction, and emotional regulation. This therapeutic approach leverages the body's natural healing processes, aiming to restore balance and promote overall well-being. The efficacy of acupuncture in treating ASD highlights TCM's potential to contribute valuable insights and effective interventions to the broader spectrum of ASD management.[11]

In recent years, substantial research has emerged on the efficacy of acupuncture as an adjunct therapy for ASD. The recent systematic reviews[14,15] assessed studies on acupuncture for ASD and found that while the evidence base is still developing, there is a growing consensus on its potential benefits. The review highlighted that acupuncture, particularly when combined with behavioral therapies, may enhance social communication skills and reduce stereotypical behaviors. Studies included in the review often reported improvements in language development and adaptive functioning among children with ASD, although methodological limitations such as small sample sizes and the absence of standardized treatment protocols were noted. The review concluded that acupuncture holds promise as a complementary therapy for ASD but emphasized the need for well-designed, large-scale, and randomized controlled trials to better understand its efficacy and establish optimal treatment protocols.

Another significant contribution to the understanding of acupuncture's role in neurodevelopmental disorders comes from a recent study by Zhuo *et al*.,[16] which investigated the effects of transcutaneous electrical acupoint stimulation (TEAS) in children with ADHD. This randomized clinical trial found that TEAS significantly improved general symptoms and increased prefrontal cortex blood flow compared to a sham treatment. Although the study focused on ADHD, the findings suggest potential benefits for similar therapies in children with ASD.[17,18] The improvements in cognitive function and neural activity observed with TEAS imply that acupuncture-related therapies could be adapted for ASD, targeting specific neural pathways to enhance cognitive function, social interaction, and behavioral regulation. These promising results highlight the need for further research to explore the long-term efficacy and mechanisms of acupuncture-based interventions in larger samples of children with ASD, potentially offering a valuable complement to existing treatments.[16,19]

Scalp acupuncture, also known as cranial acupuncture, originates from TCM and is based on the theories of

meridians and viscera.[21-24] According to TCM theory, the body's vital activities are maintained by the circulation of *qi* and blood through meridians, with the head being the convergence point of all meridians. The head houses several vital meridians, such as the governing vessel, the conception vessel, and the hand and foot *taiyang* and *taiyin* meridians, forming a complex network. These meridians not only connect the organs and limbs but also regulate physiological functions and maintain internal homeostasis. Scalp acupuncture involves needling specific points on the scalp along meridians and functional regions, such as the 12-head meridians and the four Shen Cong points. By stimulating these points, it can regulate brain function, improve blood circulation, and prevent and treat diseases effectively.[17,18,23,24]

The treatment principles of scalp acupuncture in ASD focus on awakening consciousness, replenishing the brain, and enhancing intelligence.[18,20,23] Liu's PNRAT employs the seven intelligence-enhancing needles to enhance the development of cognitive function areas in the cerebral cortex, process emotional disorders in the emotional area, improve language function development in the language area, and promote the development of advanced cognitive areas and visual nerve functions. In addition, the application of *jin jing* and *yu ye* fluid promotes blood circulation around the tongue, restoring its flexibility.

Recent research has also emphasized the effectiveness of integrative approaches in managing ASD. Studies have shown the advantages of integrating acupuncture with behavioral interventions to enhance social communication skills and diminish stereotypical behaviors in children with ASD.[6] Moreover, acupuncture adjunctive therapy has been linked to notable enhancements in language development and adaptive behavior among individuals with ASD.[13] These results highlight the significance of including complementary therapies such as acupuncture in comprehensive treatment strategies for ASD, aiming to meet the varied requirements of affected individuals.

The integration of TCM and modern cognitive and behavioral interventions offers a holistic approach to managing neurodevelopmental disorders like ASD. Our findings suggest that combining acupuncture with contemporary therapies can create a comprehensive treatment strategy that addresses the cognitive, emotional, and social dimensions of ASD. This integrative approach leverages the strengths of both traditional and modern methodologies, potentially enhancing treatment outcomes by targeting multiple facets of the disorder simultaneously. The substantial improvements observed in cognitive and behavioral symptoms among participants highlight the potential benefits of this combined approach, suggesting that such integrative strategies could be more effective than single-modality treatments.

Despite these promising results, there is a pressing need for further research to fully understand the underlying mechanisms through which acupuncture exerts its effects on ASD. Detailed studies are required to elucidate how acupuncture influences neurological pathways and interacts with modern therapeutic techniques. In addition, refining treatment protocols to better suit individual needs is crucial. Future research should focus on large-scale randomized controlled trials to validate the efficacy of integrated approaches, optimize treatment parameters, and establish standardized practices. By addressing these research gaps, we can develop more targeted and effective treatment strategies for ASD, potentially improving outcomes for a broader range of individuals affected by the disorder.

This study's primary strength lies in its innovative integration of TCM with contemporary therapeutic approaches, offering a multifaceted and comprehensive treatment strategy for ASD. By incorporating Liu's PNRAT alongside conventional cognitive and behavioral interventions, this study provides a holistic treatment framework that addresses the complex and varied needs of children with ASD. The inclusion of acupuncture, with its focus on enhancing cognitive, linguistic, and emotional development, allows for a nuanced exploration of how traditional therapies can complement and potentially enhance modern approaches. This integrative approach not only broadens the scope of treatment options available for ASD but also provides valuable insights into how combining different modalities can achieve more comprehensive and effective outcomes. Furthermore, the study's design includes a variety of therapeutic techniques, such as behavior analysis, speech training, and natural situational teaching, which collectively contribute to a well-rounded assessment of the therapy's impact. This multifaceted approach ensures that the results reflect a more complete picture of the therapy's effectiveness in improving various aspects of the disorder.

Despite its strengths, the study is not without limitations that warrant careful consideration. The relatively small sample size is a significant limitation, potentially affecting the generalizability of the findings and the statistical power to detect subtle therapeutic effects. The lack of randomization and a control group further limits the ability to attribute observed improvements specifically to the acupuncture intervention, as other factors or natural variations in the course of ASD could influence the results. In addition, the absence of long-term follow-up data restricts the understanding of whether the benefits

of the acupuncture therapy are sustained over time or if they diminish after the treatment period ends. These limitations highlight the need for more robust research designs, including randomized controlled trials with larger sample sizes and extended follow-up periods, to validate the efficacy of PNRAT and refine its application in clinical settings. Future studies should aim to address these gaps by employing rigorous methodologies to ensure the reliability and generalizability of the findings, thereby contributing to a more comprehensive understanding of how integrative therapies can optimally benefit individuals with ASD.

4. Conclusion

This case demonstrates that scalp acupuncture therapy has a significant therapeutic effect on the clinical symptoms of children with ASD, enhancing their motor skills, language abilities, and social interactions, thus facilitating positive communication with others. In addition, scalp acupuncture can improve spleen and stomach function, regulate liver *qi*, and provide meaningful support for the rehabilitation of children with comprehensive developmental delays. However, the efficacy of scalp acupuncture therapy may vary from person to person, necessitating personalized treatment based on the specific condition of each child. Future research should further explore the optimal treatment regimens, timing of intervention, and combinations with other therapeutic approaches to enhance treatment outcomes and reduce recurrence rates. It is important to note that the specific effects of scalp acupuncture therapy for neurological disorders may vary due to individual differences and should be administered under the guidance of professional TCM practitioners. Furthermore, scalp acupuncture therapy is typically integrated into comprehensive treatment plans, combined with medication, physical therapy, and other rehabilitation methods to achieve the best therapeutic results.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Zhenhuan Liu, Alan Wang
Formal analysis: Zhenhuan Liu, AlanWang
Investigation: All authors
Methodology: Zhenhuan Liu
Writing – original draft: Yitao Huang, Alan Wang
Writing – review & editing: All authors

Ethics approval and consent to participate

Written consent was obtained from the parents of the child to participate in the study.

Consent for publication

Written consent was obtained from the parents of the child to publish their data and/or images.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

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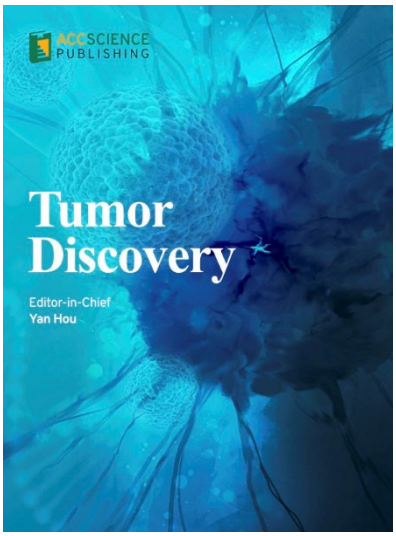

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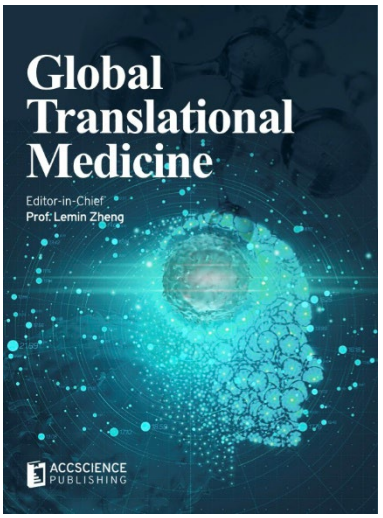

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