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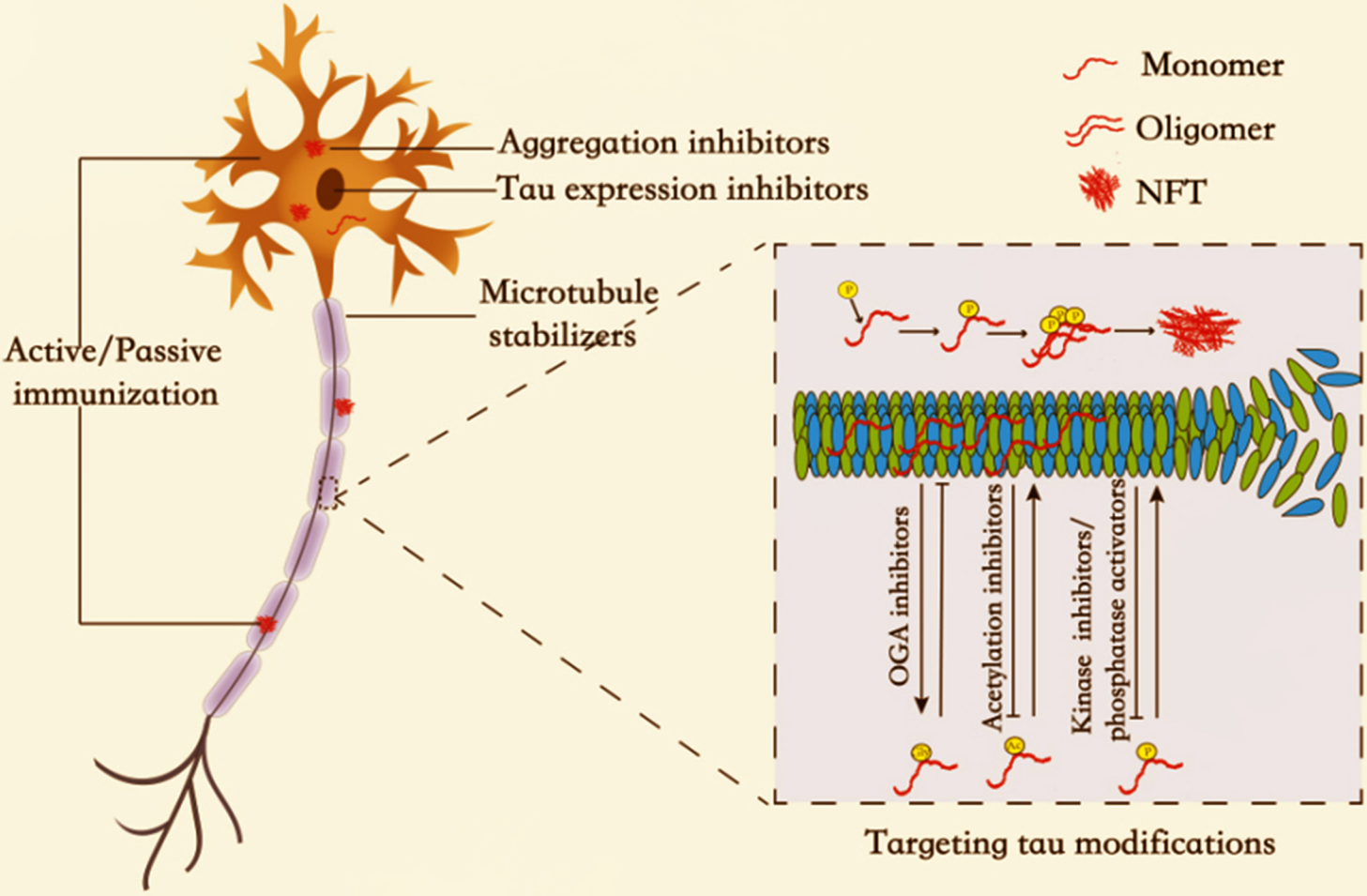
AGEING AND NEURODEGENERATIVE DISEASES



Tau-targeting therapy in Alzheimer's disease: critical advances and future opportunities

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High-quality original articles, reviews, case reports, commentaries are welcomed from ageing and neurodegenerative diseases, particularly on the basic mechanisms of ageing and their roles in the onset and progression of neurodegenerative diseases. The journal aims to report innovative research advances on the following topics:

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2. The associations between neurodegenerative diseases and the biological bases of ageing with a focus on: genomic instability, epigenetic alterations, telomere attrition, protein degradation system failure, mitochondrial dysfunction, cellular senescence, nutrient sensing deregulation, stem cell exhaustion, intercellular communication impairment, etc.;

3. Translational research into prevention and treatment of age-related neurodegenerative diseases;

4. Mechanistic bases for epidemiological observations in aging-related neurodegenerative diseases.

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AND is a peer-reviewed and open access multidisciplinary journal that publishes high-quality original articles, reviews, case reports, commentaries, letters to editor, etc. Ageing is a major risk factor for neurodegeneration, and the prevalence of ageing-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, etc. continues to rise with the increased ageing population. Unfortunately, there are no effective treatments available for the age-related neurodegenerative diseases. Thus, to develop successful interventions, it is important to investigate the basic mechanisms of ageing and their roles in the onset and progression of neurodegenerative diseases. Therefore, we plan to launch this new journal, which is aimed to report innovative research advances on the following topics:

1. The cellular and molecular mechanisms of ageing and the pathogenesis of neurodegenerative diseases;
2. The associations between neurodegenerative diseases and the biological bases of ageing with a focus on: genomic instability, epigenetic alterations, telomere attrition, protein degradation system failure, mitochondrial dysfunction, cellular senescence, nutrient sensing deregulation, stem cell exhaustion, intercellular communication impairment, etc.;
3. Translational research into prevention and treatment of age-related neurodegenerative diseases;
4. Mechanistic bases for epidemiological observations in aging-related neurodegenerative diseases;
5. Psychology and Geriatric Mental Illness;
6. Neuropharmacology.

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Ageing and Neurodegenerative Diseases

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Ageing and Neurodegenerative Diseases

Original Article

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One-year self-reported neurological sequelae in older COVID-19 survivors

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Abstract

Aim: With the increasing number of patients recovered from COVID-19, the long-term health consequences of this disease have attracted much attention. Neurological complications are commonly seen in the acute phase of COVID-19, especially in older adults. This study aimed to investigate the long-term neurological sequelae in older COVID-19 survivors.

Methods: A total of 1438 COVID-19 survivors were recruited in this study. One year after hospital discharge, information about self-reported symptoms of the central and peripheral nervous system was collected. Comparisons of these neurological symptoms between COVID-19 survivors with severe and nonsevere cases were performed.

Results: A total of 139 (53.46%) COVID-19 survivors with severe cases and 328 (27.84%) survivors with nonsevere cases reported at least one neurological symptom one year after discharge. Most of these neurological symptoms were symptoms of the central nervous system. Specifically, 126 (48.46%) survivors with severe cases and 306 (25.98%) survivors with nonsevere cases reported at least one CNS symptom. The most frequently reported symptoms were memory deficit [234 (16.27%)] and attention deficit [80 (5.56%)]. Disease severity was

associated with increased risks of long-term neurological sequelae of COVID-19.

Conclusion: This study demonstrated that neurological sequelae of COVID-19 are common one year after patient discharge, suggesting that the effects of COVID-19 on the neurological system are prolonged.

Keywords: COVID-19, neurological sequelae, survivors

INTRODUCTION

COVID-19 has infected over 4 billion people worldwide and the number is increasing. With the increasing number of patients who recovered from COVID-19, the long-term health consequences of COVID-19 have attracted much attention[1]. Neurological manifestations of COVID-19 are commonly observed in the acute phase of the disease, including symptoms of the central and/or peripheral nervous system[2]. Neurological sequelae such as headache, dizziness, movement disorders, and attention deficits were observed, and these symptoms are more prevalent in survivors with severe cases[3,4]. We have previously demonstrated that COVID-19 had long-term effects on the cognitive performances of older survivors, especially those who survived severe cases[1,5]. Therefore, it is more urgent to demonstrate the neurological sequelae of COVID-19 in older adults, especially survivors of severe cases. This study aims to investigate the self-reported neurological sequelae of COVID-19 one year after patient discharge.

METHODS

Subjects

This cross-sectional study was conducted one year after patient discharge, which included 1438 COVID-19 survivors aged 60 years or above. These subjects were discharged from three COVID-19 designated hospitals in Wuhan, China, from February 10 to April 10, 2020. Among these subjects, 260 were severe cases and 1178 were nonsevere cases. This study was conducted simultaneously with our previous reports, which aimed to determine the long-term effects of COVID-19 on cognition in older hospital survivors[1,5]. Therefore, the inclusion and exclusion criteria were consistent with these two publications.

The research protocols were approved by the institutional review boards of Daping Hospital, as the medical staff of this hospital worked in the COVID-19-designated Huoshenshan Hospital and Tongji Taikang Hospital, which were dismissed after the pandemic. Since this study was conducted based on telephone interviews, the requirement for written informed consent was waived, but verbal informed consent was obtained from all participants or their legal guardians. The findings of this study were reported following the Strengthening the Reporting of Observational Studies in Epidemiology Checklist (STROBE) for cohort studies.

Clinical examinations

The demographic information, including age and sex, and clinical characteristics, including body mass index (BMI) and coexisting disorders, including hypertension, diabetes, hyperlipidemia, stroke history, coronary heart disease, and chronic obstructive pulmonary disease (COPD), the treatment during hospitalization, such as intensive care unit (ICU) admission, mechanical ventilation, high flow oxygen therapy, length of hospital stay, antiviral therapy, antibacterial therapy, intravenous globulin (IVIg) use, and glucocorticoid use, were collected from the medical records.

The diagnosis of COVID-19 was made based on the World Health Organization interim guidance[6]. The severity of COVID-19 was defined as severe or nonsevere following the American Thoracic Society (ATS) guidelines for community-acquired pneumonia[7]. Accordingly, severe cases with COVID-19 were defined

as confirmed SARS-CoV-2 infection plus one of the following conditions: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air. SARS-CoV-2 infection was confirmed by high-throughput sequencing or real-time reverse-transcriptase polymerase-chain-reaction assays of nasal and pharyngeal swab specimens.

Participants were interviewed by telephone and were asked to report their neurological manifestations one year after hospital discharge. These symptoms were classified into sequelae of the central nervous system, including dizziness, headache, memory deficit, attention deficit, ataxia, and seizure, and those of the peripheral nervous system, including taste problem, smell problem, vision problem, nerve pain, and myalgia [Supplementary Table 1].

Statistical analysis

The demographic and clinical characteristics of participants were presented as medians (IQRs) for continuous variables and absolute values along with percentages for categorical variables. For the comparison of demographic and clinical characteristics among groups, the Kruskal-Wallis test, χ^2 test, Fisher's exact test, or Mann-Whitney *U* test was used where appropriate.

Logistic regression models were used to explore risk factors associated with neurological symptoms one year after discharge, adjusting for age, sex, BMI, and coexisting disorders. Statistical analyses were conducted using SPSS statistical package version 25 (IBM SPSS Statistics for Windows, Armonk, NY, USA) and R software version 3.6.2 (R Foundation for Statistical Computing).

RESULTS

Demographic characteristics of participants

This study included 1178 COVID-19 survivors with nonsevere cases and 260 survivors with severe cases. Severe cases were older than nonsevere cases [median (IQR): 71 (67, 79) *vs.* 68 (66, 73), $P < 0.001$]. Severe cases had lower education levels [median (IQR): 12 (6, 12) *vs.* 12 (9, 12), $P = 0.05$] and higher BMI [median (IQR): 24.38 (22.90, 25.64) *vs.* 23.93 (22.44, 25.33), $P = 0.009$] than nonsevere cases. Severe cases had higher proportion of subjects with hypertension [number (%): 133 (51.15) *vs.* 426 (36.16), $P < 0.001$], diabetes [number (%): 65 (25.00) *vs.* 208 (17.66), $P = 0.01$], stroke history [number (%): 42 (16.15) *vs.* 37 (3.14), $P < 0.001$], coronary heart disease [number (%): 71 (27.31) *vs.* 121 (10.27), $P < 0.001$] and COPD [number (%): 43 (16.38) *vs.* 99 (8.40), $P < 0.001$]. As expected, severe cases had higher proportion of subjects received ICU treatment [number (%): 72 (27.69) *vs.* 0, $P < 0.001$], mechanical ventilation [number (%): 83 (31.92) *vs.* 0, $P < 0.001$], high flow oxygen therapy [number (%): 106 (40.77) *vs.* 184 (15.62), $P < 0.001$], delirium [number (%): 82 (31.54) *vs.* 10 (0.85), $P < 0.001$], and had longer length of hospital stay [median (IQR): 28 (22, 34) *vs.* 19 (14, 23), $P < 0.001$]. Furthermore, severe cases had higher proportion of subjects who received antibacterial therapy [number (%): 143 (55.00) *vs.* 131 (11.12), $P < 0.001$], IVIg treatment [number (%): 143 (55.00) *vs.* 22 (1.87), $P < 0.001$] and glucocorticoid treatment [number (%): 144 (55.38 *vs.* 152 (12.90), $P < 0.001$] than nonsevere cases [Table 1].

Neurological sequelae of COVID-19 survivors

One year after patient discharge, 467 (32.48%) survivors reported at least one neurological symptom. Specifically, 432 (30.04%) survivors reported at least one symptom of the central nervous system, and 45 (3.13%) survivors reported at least one symptom of the peripheral nervous system. The most-reported symptom was memory loss [234 (16.27%)], followed by dizziness [96 (6.68%)], headache [80 (5.56%)], attention deficit [80 (5.56%)], smell loss [16 (1.11%)], and taste loss [15 (1.04%)].

Table 1. Demographic and baseline information of participants

	Total group (*n* = 1438)	Severe cases (*n* = 260)	Nonsevere cases (*n* = 1178)	*P* value
Age - Median (IQR), year	69 (66, 74)	71 (67, 79)	68 (66, 73)	< 0.001[a]
Male - No. (%)	691 (48.05)	133 (51.15)	557 (47.28)	0.27[b]
Education - Median (IQR), year	12 (9, 12)	12 (6, 12)	12 (9, 12)	0.05[a]
BMI - Median (IQR), kg/m^2	23.99 (22.54, 25.38)	24.38 (22.90, 25.64)	23.93 (22.44, 25.33)	0.009[a]
Coexisting disorders - No. (%)				
Hypertension	561 (39.01)	133 (51.15)	426 (36.16)	< 0.001[b]
Diabetes mellitus	274 (19.05)	65 (25.00)	208 (17.66)	0.01[b]
Hyperlipidaemia	142 (9.87)	31 (11.92)	111 (9.42)	0.25[b]
Stroke history	79 (5.49)	42 (16.15)	37 (3.14)	< 0.001[b]
Coronary heart disease	193 (13.42)	71 (27.31)	121 (10.27)	< 0.001[b]
COPD	142 (9.87)	43 (16.38)	99 (8.40)	< 0.001[b]
ICU admission - No. (%)	72 (5.01)	72 (27.69)	0 (0)	< 0.001[b]
Mechanical ventilation, No. (%)	83 (5.77)	83 (31.92)	0 (0)	< 0.001[b]
High flow oxygen therapy, No. (%)	290 (20.17)	106 (40.77)	184 (15.62)	< 0.001[b]
Delirium, No. (%)	92 (6.40)	82 (31.54)	10 (0.85)	< 0.001[b]
Length of hospital stay (IQR), day	20 (15, 25)	28 (22, 34)	19 (14, 23)	< 0.001[a]
Antiviral therapy - No. (%)	1107 (76.98)	209 (80.38)	898 (76.23)	0.17[b]
Lianhua Qingwen	703 (48.89)	136 (52.31)	567 (48.13)	0.24[b]
Arbidol	530 (36.86)	106 (40.77)	424 (35.99)	0.16[b]
Kaletra	125 (8.69)	28 (10.77)	97 (8.23)	0.18[b]
Oseltamivir	52 (3.62)	10 (3.85)	42 (3.57)	0.85[b]
Ribavirin	9 (0.63)	2 (0.77)	7 (0.59)	0.67[b]
Other antiviral drugs	20 (1.39)	3 (1.15)	17 (1.44)	1.00[b]
Antibacterial therapy - No. (%)	274 (19.05)	143 (55.00)	131 (11.12)	< 0.001[b]
IVIg treatment - No. (%)	165 (11.47)	143 (55.00)	22 (1.87)	< 0.001[b]
Glucocorticoid - No. (%)	296 (20.58)	144 (55.38)	152 (12.90)	< 0.001[b]

Other antiviral drugs included chloroquine phosphate, hydroxychloroquine, and ritonavir. [a]Mann-Whitney *U* test. [b]Pearson $\chi 2$ test. IQR: Interquartile range; BMI: body mass index; ICU: intensive care unit; COPD: chronic obstructive pulmonary disease; IVIg: intravenous immunoglobulin.

Severe and nonsevere cases had similar frequencies in dizziness [number (%): 20 (7.69) *vs.* 76 (6.45), P = 0.49], ataxia [number (%): 2 (0.77) *vs.* 1 (0.08), P = 0.09], taste problem [number (%): 5 (1.92) *vs.* 10 (0.85), P = 0.17], nerve pain [number (%): 2 (0.77) *vs.* 1 (0.08), P = 0.09] and myalgia [number (%): 4 (1.54) *vs.* 5 (0.42), P = 0.06]. Severe cases had higher proportion of subjects with headache [number (%): 27 (10.38) *vs.* 53 (4.50), P < 0.001], memory problem [number (%): 72 (27.69) *vs.* 162 (13.75), P < 0.001], attention deficit [number (%): 28 (10.77) *vs.* 52 (4.41), P < 0.001], seizure [number (%): 2 (0.77) *vs.* 0, P = 0.03], smell loss [number (%): 8 (3.08) *vs.* 8 (0.68), P = 0.003] and vision problem [number (%): 2 (0.77) *vs.* 0, P = 0.03] [Table 2].

Associations between disease severity and neurological sequelae of COVID-19

In this study, we investigated the associations between disease severity and neurological sequelae of COVID-19. We found that severe disease was associated with a higher risk of any neurological symptom [OR (95%CI): 2.908 (2.809, 3.011)], any CNS symptom [OR (95%CI): 2.623 (2.533, 2.716)] and PNS symptom [OR (95%CI): 4.486 (4.153, 4.846)]. Furthermore, severe disease was associated with a higher risk of almost all the symptoms, including dizziness [OR (95%CI): 1.259 (1.182, 1.342)], headache [OR (95%CI): 2.198 (2.066, 2.337)], memory loss [OR (95%CI): 2.157 (2.072, 2.246)], attention deficit [OR (95%CI): 1.142

Table 2. Neurological sequelae one year after discharge in COVID-19 survivors

	Total group (*n* = 1438)	Severe cases (*n* = 260)	Nonsevere cases (*n* = 1178)	*P* value
Any - No. (%)	467 (32.48)	139 (53.46)	328 (27.84)	< 0.001
CNS sequelae				
Any - No. (%)	432 (30.04)	126 (48.46)	306 (25.98)	< 0.001
Dizziness - No. (%)	96 (6.68)	20 (7.69)	76 (6.45)	0.49
Headache - No. (%)	80 (5.56)	27 (10.38)	53 (4.50)	< 0.001
Memory deficit - No. (%)	234 (16.27)	72 (27.69)	162 (13.75)	< 0.001
Attention deficit - No. (%)	80 (5.56)	28 (10.77)	52 (4.41)	< 0.001
Ataxia - No. (%)	3 (0.21)	2 (0.77)	1 (0.08)	0.09
Seizure - No. (%)	2 (0.14)	2 (0.77)	0 (0)	0.03
PNS sequelae				
Any - No. (%)	45 (3.13)	21 (8.08)	24 (2.04)	< 0.001
Taste problem - No. (%)	15 (1.04)	5 (1.92)	10 (0.85)	0.17
Smell disorder - No. (%)	16 (1.11)	8 (3.08)	8 (0.68)	0.003
Vision problem - No. (%)	2 (0.14)	2 (0.77)	0 (0)	0.03
Nerve pain - No. (%)	3 (0.21)	2 (0.77)	1 (0.08)	0.09
Myalgia - No. (%)	9 (0.63)	4 (1.54)	5 (0.42)	0.06

All comparisons were conducted using Pearson $\chi 2$ test.

(1.078, 1.210)], taste loss [OR (95%CI): 2.054 (1.780, 2.370)] and smell loss [OR (95%CI): 4.577 (4.042, 5.182)] [Figure 1]. Notably, these associations remained significant after adjusting for age, sex, BMI, and coexisting disorders, including hypertension, diabetes, hyperlipidemia, stroke, coronary heart disease, and COPD, suggesting that severe disease had a significant impact on postinfection neurological sequelae beyond these factors.

DISCUSSION

In this study, we investigated the self-reported neurological symptoms of COVID-19 in a cohort of older survivors one year after discharge. Overall, older COVID-19 survivors, especially those who survived severe cases, had a high burden of neurological sequelae. Symptoms of the central nervous system were more common than those of the peripheral nervous system. The most-reported symptom was memory loss, followed by attention deficit and dizziness.

Neurological symptoms are common in both the acute phase and post-acute phase of COVID-19[8]. The neurological sequelae of COVID-19 include a variety of symptoms, such as smell problems, taste problems, and memory deficit[9,10]. COVID-19 also increases the risk of a panel of neurological diseases, such as stroke[11] and autoimmune diseases[12]. We have recently reported that older COVID-19 survivors had an increased risk of longitudinal cognitive decline and that severe cases had a higher speed of cognitive decline than nonsevere survivors and uninfected subjects[1], which is consistent with the present findings that memory deficit was reported by 16.27% of older COVID-19 survivors. The postinfection memory deficit might be attributed to the fact that older adults are at a higher risk of cognitive impairment and that the postinfection low-grade inflammation or hypoxia status would further exacerbate long-term cognitive impairment.

Other symptoms, such as attention deficit, dizziness, and headache, have also been reported months after COVID-19 infection. It is suggested that attention deficits are very commonly seen in COVID-19

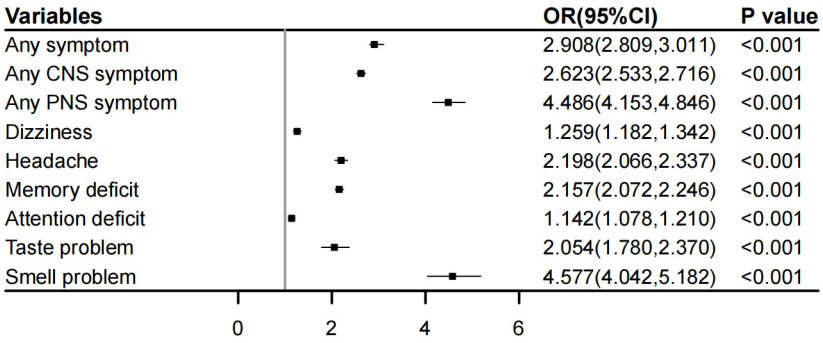

Figure 1. Associations between disease severity and neurological symptoms one year after discharge. Logistic regression models with adjustment for age, sex, body mass index, and coexisting disorders.

survivors[13]. This sequela of COVID-19 would dramatically impact the living quality and work efficiency of survivors. The possible association between COVID-19 and attention deficit might be attributed to the altered neurotransmitter secretion profile after SARS-CoV-2 infection[14]. Headache and dizziness after COVID-19 have also been reported by other studies[9]. However, we found in this study that symptoms of the periphery nervous system were rarely reported in COVID-19 survivors, suggesting that symptoms such as smell and taste problems could be reversible and more attention should be focused on the long-term impact of COVID-19 on the central nervous system. This might also be because our study did not utilize objective measures to determine the smell and taste of participants.

The common mechanism of postinfection neurological symptoms might be multifactual. Evidence regarding the direct invasion of the virus into the brain was limited. The most possible contributions of COVID-19 to postinfection neurological sequelae might be associated with chronic inflammatory[15,16] and long-term hypoxia status after COVID-19[17]. Other pathways by which COVID-19 insults the brain also exist. For example, recent studies have found that postinfection autoimmunity may damage neurons[18]. Neuronal reactive autoantibodies were found in COVID-19 survivors, and these autoantibodies were associated with the neurological symptoms and neuronal damage biomarkers of patients[19], suggesting an autoimmune element of neurological insult of COVID-19. Studies found that the incidence of autoimmune diseases such as Guillain-Barré syndrome[20] and multiple sclerosis[21] is increased after SARS-CoV-2 infection, further supporting this notion. Furthermore, SARS-CoV-2 is suggested to induce a wide variety of transcriptome changes in the brain regions that were associated with cognition and memory[22,23]. Neurodegenerative biomarkers are altered in biofluid of COVID-19 survivors[24]. Besides, COVID-19 is associated with mental health outcomes such as depression and anxiety, which could contribute to increased self-reported symptoms[25].

This study has several limitations. First, the symptoms were self-reported by survivors, and no objective measures were used. Second, this study only included older adults; thus, it is not clear whether younger survivors had similar sequelae. Third, this study did not include a control group with other viral infectious diseases; thus, it could not be determined whether COVID-19 had a greater long-term impact on the neurological system than other infectious diseases. This study is limited by its cross-sectional nature and no longitudinal cohort investigations were involved; therefore, it cannot be known whether these symptoms were reversible. However, this study added novel information about the long-COVID syndrome.

DECLARATIONS

Authors' contributions

Designed this study and drafted the manuscript: Wang LR, Yang Y
Conducted the interviews: Jiang L, Liu XY, Yan XQ
Had critical reading of the manuscript: Liu YH, Wang YJ

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study was conducted in strict accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Chinese People's Liberation Army Specialty Medical Center.

Consent for publication

All participants agree to publication.

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Ageing and Neurodegenerative Diseases

Review

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Tau-targeting therapy in Alzheimer's disease: critical advances and future opportunities

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by two pathological hallmark lesions: extracellular plaques composed of β-amyloid (Aβ) peptide and intracellular neurofibrillary tangles made up of highly phosphorylated tau protein. Over the past two decades, most disease-modifying therapies against AD have been developed mainly on the basis of the amyloid cascade hypothesis with a focus on Aβ. However, these agents yielded only limited benefits against disease progression, which prompts us to revitalize the long-neglected tau hypothesis. Tau protein is a microtubule-associated protein, which can stabilize microtubules, regulate microtubule assembly, and affect the morphology and growth of neuronal axons. Much more importantly, the degree of tau pathology is more closely related to cognitive decline in AD patients than that of Aβ pathology. Therefore, tau-targeting therapy seems to be a promising approach to combat AD. This review describes the research progress of tau-targeting therapy in AD, with an emphasis on immunotherapy. The current challenges and future perspectives in this field are also discussed.

Keywords: Alzheimer's disease, post-translational modifications, tau protein, tau-targeting therapy, immunotherapy

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by progressive memory loss and cognitive impairment, accounting for 50%-75% of dementia cases. In the USA, an estimated 6.5 million people aged 65 and older had clinical AD in 2021, which will increase to 13.8 million by 2060 unless medical breakthroughs are developed to prevent, slow, or cure AD[1]. It is estimated that total medical expenditures for people suffering from AD and other dementias will increase from $321 billion in 2022 to just under $1 trillion by 2050[1]. In addition, patients with AD or other dementias have a higher chance of having more chronic diseases, which will generate more financial burden for treating comorbidities[2]; thus, it has become a major global health problem. The strategies for AD therapy mainly focus on two hallmark lesions: β-amyloid (Aβ), which forms extracellular plaque, and hyperphosphorylated tau, which forms intraneuronal neurofibrillary tangles (NFTs)[3].

It has long been widely accepted that changes in Aβ promote the disease process and drive tau pathology and neurodegeneration in AD[4]. Aβ can promote phosphorylation[5,6] or cleavage[7] of tau by activating related proteases, which in turn promotes tau aggregation and enhances tau-induced neurotoxicity. Beyond this triggering function, it has been commonly assumed that Aβ and tau act independently without specific interaction. However, accumulating evidence suggests there is a bidirectional interplay between Aβ and tau. Increased CSF tau levels were also demonstrated in APP/PS1 transgenic mice overexpressing Aβ, while tau deficiency ameliorated Aβ deposition[8]. The combination of Aβ and tau synergistically promotes the decrease of glucose metabolism, induces brain atrophy in AD brain, and leads to the declined cognitive function[9-11].

Currently, the drugs approved by the Food and Drug Administration for the treatments of AD include acetylcholinesterase inhibitors (donepezil, galantamine, and rivastigmine), NMDA receptor antagonist (memantine), and passive immunotherapy antibody aducanumab. However, these drugs only temporarily delay the disease progression of AD and ameliorate symptoms but cannot prevent or reverse the neuronal loss, brain atrophy, and the consequent progressive cognitive decline[12]. Since the introduction of the amyloid cascade hypothesis, most preventative or therapeutic candidates that were discovered and developed focus on Aβ. Aβ is mainly generated from amyloid precursor protein (APP) through sequential cleavage by β- and γ-secretase[13]. Therapeutic studies targeting Aβ have focused on inhibiting Aβ production by inhibiting β- or γ-secretase[14-16] and blocking Aβ aggregation[17], but these drugs yield only limited benefits and even induce serious adverse effects. Immunotherapy targeting Aβ was once a research hot spot; however, vaccine development has consistently struggled to bypass adverse outcomes such as excessive autoimmunity[18,19]. To date, most Aβ-targeting therapies have failed, which prompted us to re-examine the Aβ cascade hypothesis. Moreover, increasing lines of evidence suggest that tau pathology is more closely related to the cognitive decline in AD than Aβ, which revitalizes the long-neglected tau hypothesis, and more research attention has shifted from Aβ to pathological tau as a feasible target for disease intervention[20-23].

Tau protein is encoded by the microtubule-associated protein tau gene (MAPT) located on chromosome 17q21 and is abundant in neuronal axons. The adult human brain contains six tau isoforms ranging in size from 37 to 46 KD, which result from alternative splicing of exons 2, 3, and 10 of the MAPT gene [Figure 1]. Tau, an intrinsically disordered protein, can stabilize microtubules, regulate microtubule assembly, and affect the axonal morphology and growth of neurons[24]. Under pathological conditions, including AD, progressive supranuclear palsy (PSP), frontotemporal dementia (FTD), and other neurodegenerative diseases, tau's abnormal assembly forms insoluble aggregates, accompanied by a series of neurodegenerative diseases such as synaptic dysfunction and nerve cell death[24,25]. As the course of AD progresses, abnormal

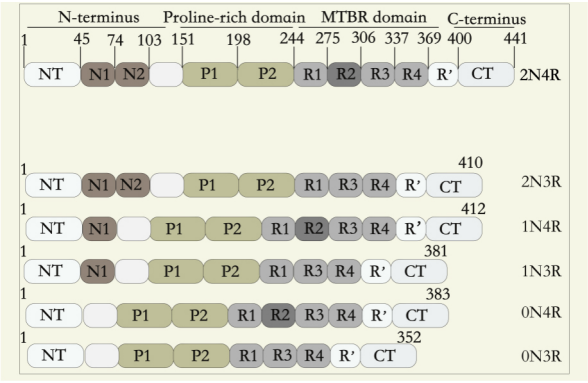

Figure 1. Schematic representation of the protein structures of tau. Six tau isoforms (2N4R, 2N3R, 1N4R, 1N3R, 0N4R, and 0N3R) of 352-441 aa are formed due to alternative splicing of exon 2 (E2), E3, and E10. Tau consists of four regions: N-terminus, proline-rich domain, microtubule-binding domain, and C-terminus. The expression of human tau is developmentally regulated: the 0N3R isoform is expressed only in the fetal brain, and all six isoforms are expressed in the adult brain. In the adult brain, the levels of the 3R and 4R forms are approximately equal, while the 0N, 1N, and 2N tau isoforms account for ~37%, ~54%, and ~9% of total tau, respectively.

tau aggregates to form NFTs existing in a characteristic distribution pattern, allowing the differentiation into six stages, namely Braak grades (transentorhinal stages I-II, clinically silent cases; limbic stage III-IV, incipient AD; and neocortical stages V-VI, fully developed AD)[26]. According to the pathological characteristics of tau, the prion-like aggregation and transmission of tau are proposed: the pathological tau takes itself as a template to induce the conformational changes of normal tau protein, so that it is easier to aggregate and induce more peripheral tau pathological changes, resulting in the spread of tau pathology to wider brain regions[27]. Studies have shown that, compared with the healthy control group, a large amount of tau protein is accumulated in the brain tissue of AD patients[28].

Currently, tau-targeting therapy has become a hot topic in this field, including post-translational modifications (PTMs) of tau, inhibition of tau aggregation, stabilization of microtubules, and tau clearance by immunotherapy [Figure 2 and Table 1].

TARGETING THE POST-TRANSLATIONAL MODIFICATIONS OF TAU

Pathological tau in AD has severe PTMs, and this process begins before the formation of NFTs, even decades before the onset of symptoms[29]. Not all PTMs are pathological, which is determined by the location, type, and amount of modification[30]. Hyperphosphorylation is the most studied PTM of tau and one of the earliest discovered modifications. Early studies found that aggregated tau isolated from AD brains had 3-4 times more overall phosphorylation level than healthy controls (2-3 mol per protein)[31]. Hyperphosphorylation of tau reduces the binding ability of tau to microtubules and reduces the stability of microtubules. The dissociated tau will self-aggregate, form oligomers and NFTs, disrupt the normal structure of cells, block intracellular material exchange, inhibit ubiquitin-proteasome activity, and cause aggregation, finally leading to neurodegeneration[32,33]. Both acetylation and N-glycosylation are also increased in AD. Acetylation inhibits the degradation of tau protein and promotes pathological tau aggregation and propagation[34-36], whereas N-glycosylation stimulates tau polymerization by promoting phosphorylation and conformational changes[37]. Nitration of the Tyr29 site of tau, found only in AD patients, significantly affects tau polymerization and facilitates tau aggregation[38]. The cleavage modification of caspase in AD patient brain also plays a key role. Caspases 2 and 3 cleave the C-terminus at Asp314 and Asp421, respectively, while caspase 6 cleaves the N-terminus[39,40]. In addition to the effect on microtubule binding and aggregation, C-terminal cleavage of tau is also associated with mitochondrial and synaptic damage[41,42]. However, not all PTMs accelerate tau pathology and AD progression. Ubiquitination promotes

Table 1. Research mechanisms and progress of related drugs targeting tau protein therapy

Name	Synonyms	FDA status	Mechanism of action	Company
Targeting tau PTMs				
Tideglusib	NP031112, Nypta®, Zentylor™, GSK-3β inhibitor, NP12	Alzheimer's Disease (discontinued)	GSK-3β inhibitor	Zeltia Group
Lithium		MCI (phase 4), expected to be completed by 2023	GSK-3β inhibitor	
Sodium selenate	VEL015	Alzheimer's Disease (phase 2)	PP2A activators	
LY3372689		Alzheimer's Disease (phase 2)	O-GlcNAcase inhibitor	Eli Lilly & Co.
Salsalate			Acetylation inhibitors	
Aggregation inhibitors				
LMTM	TRx0237, LMT-X, Methylene Blue, TAI	Alzheimer's Disease (phase 3)	Aggregation inhibitors	TauRx Therapeutics Ltd
ACI3024	Tau MorphomerTM	Alzheimer's Disease (inactive)	Aggregation inhibitors	AC Immune SA
Curcumin	Diferuloylmethane, Longvida™	Alzheimer's Disease (phase 2)	Aggregation inhibitors	Verdure Sciences
Tau expression inhibitors				
BIIB080	IONIS-MAPTRx, ISIS 814907	Alzheimer's Disease (phase 1)	Inhibit the translation of tau mRNAs into protein	Biogen, IONIS Pharmaceuticals
Microtubule stabilizers				
Davunetide	NAP, AL-108	MCI (discontinued)	Neuroprotective active agent that stabilizes microtubules in neurons	Allon Therapeutics Inc., Paladin Labs Inc.
Epothilone	BMS-241027	Alzheimer's Disease (discontinued)	Microtubule stabilizers	Bristol-Myers Squibb
Active immunization				
AADvac1	Axon peptide 108 conjugated to KLH	Alzheimer's Disease (phase2)	Targeting tau 294KDNIKHVPG GGS305 epitope	Axon Neuroscience SE
ACI-35		Alzheimer's Disease (phase2)	Targeting tau393VYKSPVVS GDTSPRHL408 epitope with the phosphorylation of Ser396/404 site	AC Immune SA, Janssen
Passive immunization				
RG7345	RO6926496	Alzheimer's Disease (discontinued)	A humanized monoclonal antibody targeting the tau phosphoepitope pS422	
Gosuranemab	BIIB092, BMS-986168, IPN007	Alzheimer's Disease (discontinued)	A humanized IgG4 monoclonal antibody targeting the N-terminal region of tau protein	Biogen, Bristol-Myers Squibb
Semorinemab	RO7105705, MTAU9937A, RG6100	Alzheimer's Disease (phase2)	A monoclonal IgG_4 antibody targeting extracellular tau, can bind the N-terminus of all six Tau isoforms of human tau	AC Immune SA, Genentech, Hoffmann-La Roche
Zagotenemab	LY3303560	Alzheimer's Disease (discontinued)	Recognizes the MC1 epitope, and binds soluble oligomers	Eli Lilly & Co.
JNJ63733657		Mild AD (phase2), expected to be completed in March 2025	A humanized IgG_1 monoclonal antibody targeting MTBR of tau protein with a high affinity for pT217	Janssen
Bepranemab	UCB0107, UCB 0107, Antibody D	Alzheimer's Disease (phase2), will run until November 2025.	A humanized IgG_4 monoclonal antibody targeting tau235-250 near the MTBR of tau	Hoffmann-La Roche, UCB S.A.
PNT001		Alzheimer's Disease (phase 1)	A monoclonal antibody targeting the cis isomer of tau phosphorylated at Thr 231	Pinteon Therapeutics
BIIB076	NI-105, 6C5hulgG$_1$/l	Alzheimer's Disease (phase 1)	A human recombinant IgG_1 monoclonal antibody targeting mid-domain of tau	Biogen, Eisai Co., Ltd., Neurimmune

Tilavonema	C2N8E12, ABBV8E12, HJ9.3	Alzheimer's Disease (discontinued)	A humanized IgG_4 antibody recognizes 25-30aa of tau and targets extracellular tau	AbbVie, C2N Diagnostics, LLC
E2814		Alzheimer's Disease (phase1/2), expected to be completed in April 2024	A humanized IgG_1 monoclonal antibody targeting the second and fourth HVPGG sequences of tau protein MTBR	Eisai Co., Ltd.
Lu AF87908		Alzheimer's Disease (phase 1), expected to be completed in June 2022	A humanized mouse IgG_1 monoclonal antibody targeting tau386-408aa epitope with phosphorylated at Ser396 and Ser404	Lundbeck

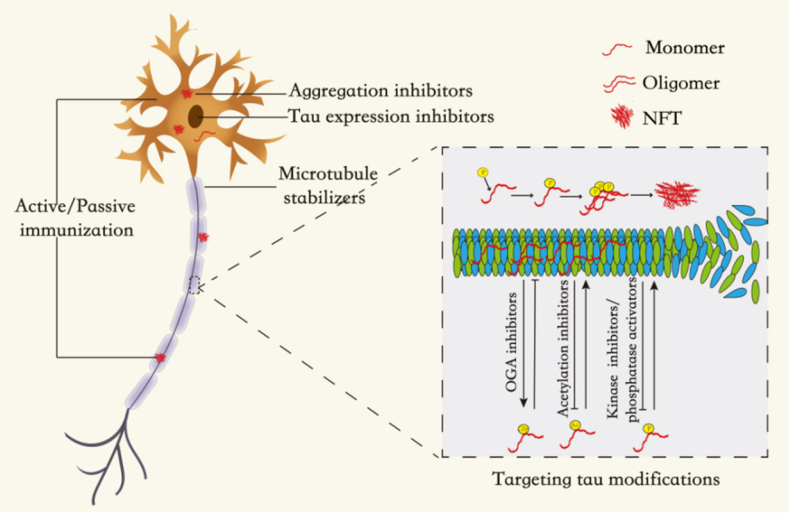

Figure 2. The therapeutic mechanism of targeting tau protein: the therapeutic research of targeting tau protein is mainly through targeting tau PTMs, the inhibition of tau protein aggregation and expression, stabilization of microtubules, and immunotherapy.

the degradation of tau by autophagy and proteasome, inhibits tau aggregation, and maintains microtubule stability[43]. Lysine methylation is part of the normal PTMs of tau in the human brain and can partially serve to protect against pathological tau aggregation[44]. Dityrosine (DiY) cross-links, another tau PTM induced by oxidative stress, was observed on human AD-derived tau oligomers and PHFs[45]. DiY cross-links of tau dGAE (tau297-391 fragment of the full-length tau) can facilitate the formation of non-toxic, soluble tau oligomers, inhibit the formation of β-sheets and further extension of prefibrils[46], and increase the insolubility and stability of tau fibrils in AD[45]. Different from the reported toxic tau oligomers, which are thought to be insoluble and β-sheet rich, tau oligomers formed by DiY cross-links are ThS-negative and random-coil rich[47]. These findings indicate that DiY cross-links seem to be beneficial in the progress of AD. Although tau has different isomers and the full-length tau has five tyrosines, the current research is mainly focused on the Y310 site of dGAE, and further research is needed to explore whether other tau isomers and tyrosine sites can form DiY cross-links and how they function.

Tau phosphorylation inhibitors

Tau protein has multiple phosphorylation sites, and its ability to bind to microtubules depends on the phosphorylation state. Hyperphosphorylated tau protein has less affinity for microtubules and even loses the ability to bind to microtubules, resulting in an unstable cytoskeleton. In addition, hyperphosphorylated tau can accumulate in neurons to form PHFs and eventually lead to neuronal death. Therefore, inhibition of tau hyperphosphorylation is crucial to maintaining normal neuronal physiological functions. The phosphorylation level of tau is mainly determined by the balance of various protein kinases and

phosphatases, and the disruption of this balance will lead to the abnormal phosphorylation of tau observed in AD, especially the hyperactivation of glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5 (CDK5), as well as the inhibition of protein phosphatase-2A (PP2A)[48,49]. Inhibiting the activity of protein kinases and increasing the activity of phosphatases have been considered reasonable strategies for hampering tau pathology and AD therapy.

Protein phosphorylation inhibitors

GSK-3β inhibitors

Tideglusib (NP031112, Nypta®, Zentylor™, NP12) is an irreversible inhibitor of GSK-3β, a widely studied tau kinase, and its inhibition will counteract tau hyperphosphorylation[50]. Preclinical studies have shown that tideglusib can reduce a range of disease outcomes, including tau phosphorylation, Aβ deposition, neuron loss, and gliosis, in mouse entorhinal cortex and hippocampus and reverse spatial memory impairment in transgenic mice[51]. Moreover, the neuroprotective, anti-inflammatory, and neurogenesis-inducing effects of tideglusib have also been confirmed in animal models[52,53]. In a phase 2 trial involving mild to moderate AD patients, tideglusib had an acceptable safety profile for a short-term (26 weeks) treatment except for a transient increase in serum transaminase levels and diarrhea (14%-18% in active, 11% placebo) but failed to slow cognitive dysfunction, with only a small number of patients showing significant reductions of β-secretase in CSF[54,55]. Currently, the study of tideglusib in AD has been discontinued.

Lithium is a well-established drug for the treatment of bipolar disorder, and its role in AD has been studied due to its potential to inhibit GSK-3β[56,57]. On the one hand, after 10 weeks of lithium treatment in patients with mild or mild-to-moderate AD, clinical trials did not show significant improvement in cognitive scores and the therapeutic effects on GSK-3β activity or CSF-based biomarker concentrations[58]. On the other hand, in a 12-month double-blind trial in patients with amnestic mild cognitive impairment (MCI), the level of phosphorylated tau in CSF decreased in the lithium treatment group and cognitive ability was improved compared with the placebo control group. Moreover, the treatment of lithium was safe and well tolerated at a serum concentration range of 0.25-0.5 mmol/L[59,60]. In AD patients, a 15-month study of microdose lithium (300 μg/day) treatment also demonstrated its efficacy in preventing cognitive loss[61]. This is inconsistent with previous studies that failed to find a significant effect of lithium on cognition or AD-related biomarkers, possibly due to the high side effects[62] (renal and neurologic dysfunction, endocrine abnormalities, *etc.*) and withdrawal rate associated with higher lithium levels, shorter follow-up periods, and the included patients with cognitive degradation are related to the later stage. Taken together, the clinical data suggest that long-term microdose lithium therapy is necessary for a beneficial effect, and the use of microdose lithium may be able to resolve the toxicity problems that have pushed the development of the medication[62,63]. Currently, one phase 4 clinical trial of lithium in patients with MCI is underway and is expected to be completed by 2023[64].

AZD1080 is a novel and potent small molecule, orally bioavailable and brain-permeable selective GSK-3 inhibitor, which can inhibit tau phosphorylation in a dose-dependent manner in cells that overexpress tau protein and rescue synaptic plasticity deficits in rodent brain[65]. Meanwhile, AZD1080 has the ability to inhibit the GSK enzyme in humans, and the PK/PD profile demonstrates acceptable safety and tolerability in healthy volunteers after multiple oral ascending doses[65]. Injection of AZD1080 in MPTP mice suppressed the expression of phosphorylated tau and phosphorylated GSK-3β and improved motor functions[66]. Given the overall pharmacological profile, preclinical data, and the completion of additional toxicity studies, AZD1080 may have both disease-modifying and symptomatic potential in the treatment of AD and related tauopathies.

It is worth noting that, despite tau-phosphorylation, GSK-3β is also involved in various signaling pathways and physiological functions. Therefore, the non-specificity of GSK-3β inhibitors might contribute to the potential side effects as therapeutics against AD. Moreover, another important challenge and risk factor to overcome for a GSK-3β inhibitor to be converted into an effective and safe drug for AD treatment is its specific brain distribution.

CDK5 inhibitors

CDK5 is a proline-directed serine/threonine protein kinase activated by interacting with its activators, p35 and p39, or their cleavage products (p25 and p29, respectively), which governs various cellular processes in neurons. The dysregulation of CDK5 is largely due to the formation and accumulation of neurotoxic p25 and p29. The hyperactivation and mislocalization of CDK5 lead to hyperphosphorylation of its substrates[67]. Tau, as an important substrate of CDK5, can be phosphorylated by CDK5, then separates from microtubules and self-aggregates, and finally forms PHFs and insoluble NFTs in neurons[68]. Knockdown of CDK5 in triple transgenic (3xTg) AD mice, as well as inhibition of CDK5 with CDK5 inhibitory peptide *in vitro*, has been shown to reduce tau hyperphosphorylation and NFT formation[69]. Minocycline alleviates AD-like pathology and improves cognitive impairment by inhibiting the CDK5/p25 signaling pathway[70]. In the advanced stages of the disease, CDK5 primes phosphorylation sites on tau for GSK-3β and thereafter synergistically promote the GSK-3β-mediated tau hyperphosphorylation[71]. In contrast to this synergistic manner, one previous study reported that administration of CP681301, a CDK5 inhibitor, enhanced tau phosphorylation in p25-overexpressing transgenic mice. CDK5 can indirectly phosphorylate GSK-3β at S9 and inhibit its activity, suggesting that CDK5 inhibition may enhance tau phosphorylation through the activation of GSK3β[72,73]. Despite contradictory reports about the impacts of CDK5 on tau phosphorylation, another important concern is the tau-specificity of CDK5 inhibitors. Since CDK5 can phosphorylate molecules other than tau, more attention should be paid to developing tau-targeting agents with CDK5 inhibitory activity. In fact, to date, no successful small molecule candidates have been reported to selectively inhibit CDK5 kinase activity.

In addition to the kinases mentioned above, there are many other kinases that play an important role in the phosphorylation of tau. CAMP-dependent protein kinase A (PKA) is a serine/threonine protein kinase, and its activator forskolin can induce hyperphosphorylation of tau at Ser202/Thr205, Ser214, and Ser396[74]. However, PKA phosphorylates tau at Ser214, which reduces the pathological assembly of this protein[75]. The formation of PHFs is inhibited if tau is phosphorylated before GSK-3β, whereas PHFs are promoted if GSK-3β phosphorylates tau before PKA, suggesting that high PKA activity and low GSK-3β activity may inhibit the formation of NFTs[76]. AMP-activated protein kinase (AMPK) is a sensor of cellular stress and maintains energy homeostasis by controlling the activity of several metabolic enzymes. Studies have demonstrated that AMPK is dysregulated in the brains of AD patients, where it co-localizes with phosphorylated tau in pre-tangled and tangled neurons[77]. AMPK activation in mouse primary neurons increased tau phosphorylation at multiple sites, while AMPK inhibition rapidly reduced tau phosphorylation[77]. Phosphorylated AMPK accumulates in neuropil threads and dystrophic neurites around amyloid plaques and appears in over 90% of neurons with pre-tangles and tangles. *In vitro* experiments showed that AMPK could directly phosphorylate tau at Thr231 and Ser396/404, decreasing the microtubule binding affinity of tau[78]. However, one study also found that AMPK reduced tau phosphorylation, improved brain function, and inhibited GSK-3β activity in an AD-like model[79]. The microtubule-affinity regulating kinase 2 (MARK 2) is able to phosphorylate serine residues of tau protein's KXGS motif[80]. Death-associated protein kinase (DAPK) activates MARK1 and MARK2, inhibits microtubule assembly, and destabilizes microtubules[81]. Furthermore, DAPK enhances tau toxicity and MARK-induced tau phosphorylation, and mice lacking

DAPK show a reduction of tau phosphorylation in their brain[81].

Phosphatase activator

The phosphatases are mainly composed of PP2A, PP5, PP1, and PP2B, with proportions of 71%, 11%, 10%, and 7% of the total tau dephosphorylation activity, respectively, in the human brain[82]. Significant decreases in total phosphatase activity, PP2A activity, and PP5 activity toward tau were observed, but an increase in PP2B activity was also detected in AD brain[82]. The abnormal hyperphosphorylation of tau may be partly caused by the downregulation of PP2A activity, and its activity can be reduced by 50% in AD brain, which is consistent with the level of tau phosphorylation, whereas the activity of PP1 and PP5 is much less downregulated[82,83]. *In vitro*, co-incubation of tau aggregates with PP2A restored the binding of tau to microtubules to a level similar to the control group (about 80%)[84]. Therefore, PP2A is the major tau phosphatase, and its activators show promising potential for AD treatment.

Sodium selenate (VEL015): a preclinical study found that chronic treatment with low-dose VEL015 can reduce tau phosphorylation in cellular and mouse models of AD and prevent memory and motor deficits, NFTs formation, and neurodegeneration in tau transgenic mice[85]. In a 24-week, phase 2a, double-blind, randomized controlled trial in patients with mild to moderate AD, VEL015 was safe and well-tolerated[86]. However, no alterations of other AD biomarkers (CSF tau and Aβ levels) and cognitive performance were observed except for the reduction of neurodegeneration assessed by diffusion MRI[86]. In an open-label extension study, chronic VEL015 treatment (up to 23 months) in AD patients was also safe and well-tolerated. Moreover, the cognitive measures showed a slowed disease progression, but this was unable to be confirmed due to the uncontrolled trial design. Further investigations of VEL015 as a treatment for AD are warranted[84,87].

At present, few compounds have been proved to be effective in a tau transgenic mouse model, and fewer compounds have entered clinical trials. In addition to the general challenges of drug discovery and development for this type of disease (crossing the blood-brain barrier (BBB), target engagement, pharmacokinetics, long-term administration, *etc.*), developing kinase inhibitors with specificity for kinases (single-target *vs.* multi-target), formation of multiple substrates (off-target and potential side effects), or inhibition intensity (most tau kinases have many physiological functions) are also important issues to be addressed.

Targeting tau glycosylation

O-GlcNAcylation is a form of PTMs present in the nucleus and cytoplasm, in which a single N-acetylglucosamine (GlcNAc) is linked to the hydroxyl group of serine/threonine of proteins through O-glycosidic bonds. O-GlcNAc modification has important physiological significance, such as participating in signal transduction, gene expression, and cell cycle and proteasomal degradation. O-GlcNAc modification reduces the tendency of tau protein to form toxic aggregates[88,89]. O-GlcNAcase (OGA) catalyzes the removal of O-GlcNAc, whereas OGA inhibitors promote the O-GlcNAc of tau, prevent its aggregation, and stabilize tau in a soluble, nonpathogenic form. In different mutant human tau-expressing transgenic mice, the OGA inhibitor thiamet-G can increase O-GlcNAc-modification of tau and reduce the formation of NFTs and neuronal loss[90-92]. Impaired glucose uptake/metabolism can lead to AD and results in abnormal tau phosphorylation and NFTs formation through GSK-3β hyperactivation and reduction of O-GlcNAc[93]. Therefore, increasing the O-GlcNAc modification level has the potential to reduce tau phosphorylation, which helps to improve and slow down the progression of AD[94].

OGA inhibitor LY3372689, by increasing the level of O-GlcNAc modification, indirectly reduces the phosphorylation level of tau protein, prevents tau aggregation, and stabilizes tau in a soluble, nonpathogenic form, ultimately slowing the progression of AD[95]. In a phase 1 clinical trial, the drug was well-tolerated without serious adverse events or withdrawal due to adverse events. In September 2021, a phase 2 trial began to evaluate LY3372689 in 330 patients with early symptomatic AD who will receive a low or high dose of LY3372689 or a placebo for a period of 76-124 weeks. The trial is expected to be completed in June 2024.

Targeting tau acetylation

Salsalate is a small molecule nonsteroidal anti-inflammatory drug that inhibits acetylation of tau at Lys174 by the acetyltransferase p300. Acetylated tau is a blood biomarker of neurodegeneration induced by traumatic brain injury in mice and humans. Injury-induced neuronal tau acetylation leads to axonal damage and pathological tau mislocalization, while elevated NAD+ enhances the activity of the deacetylase Sirtl and blocks injury-induced tau acetylation, neurodegeneration, and neurobehavioral impairment[96]. Salsalate treatment for 2-3 months can prevent hippocampal atrophy, reduce tau pathology, and restore spatial memory deficits in PS19 mice[97]. In a phase 1 clinical trial of progressive supranuclear palsy (PSP), salsalate was safe and well tolerated, but it had no detectable effect on disease progression[98].

Targeting tau truncation

Enzyme-mediated (calpain, caspase, *etc.*) truncation of tau at the N-terminal (binding neuroplasmic membrane components) and C-terminal (binding axonal MT) is a contributing factor to AD pathology. Regardless of the protease, once tau is truncated, it is reasonable to presume an irreversible loss of its normal function. These fragments can promote tau aggregation, propagation between neurons through synapses, and expand nerve fiber degeneration to postsynaptic neurons, which has potential cytotoxicity[99,100]. Targeting the clearance of these fragments can significantly improve disease progression. Current therapeutic strategies for tau truncation are focused on targeting/blocking tau cleavage toxicity as well as clearance, including inhibitors of protease substrate interactions and antibody-mediated immune clearance. HJ8.5, a monoclonal antibody recognizing the N-terminal region of tau, is able to effectively *in vitro* block its seeding capacity and reduce pathological tau aggregates accumulation, microglial activation, and brain atrophy in six-month-old P301S tau transgenic mice, thereby improving their motor/sensory-motor and cognitive deficits[101,102]. 12A12, which selectively binds the pathologically relevant neurotoxic tau26-230 fragment of tau rather than reacting with its physiological form, is able to ameliorate the cognitive decline and memory impairment in Tg2576 and 3xTg mice[103]. Small molecule inhibitors targeting caspase-2 markedly blocked tau cleavage at the Asp314 site, prevented excessive accumulation of toxic tau fragments in dendritic spines, and restored excitatory neurotransmission in primary rat hippocampal neurons expressing the P301S tau variant[104]. At present, studies targeting tau fragment-mediated actions have only been performed *in vitro*, and further studies are needed to confirm whether it can be a potential therapeutic target for tau lesions.

Unlike other neurodegenerative diseases, tauopathies do not have potent genetic mutations of tau, and the pathological transformation from a highly soluble protein to an insoluble aggregate may be caused by extensive PTMs. PTMs are constantly and dynamically changing as the disease progresses. Compared with single PTM, combined PTMs seem to be more related to tau lesions, and they jointly affect the physicochemical properties of tau.

PREVENTING TAU AGGREGATION

The spread and accumulation of tau aggregates are related to neuronal loss and clinical symptoms in AD. The strategy of anti-protein aggregation therapy is to prevent the soluble tau protein from forming

polymers and NFTs, so as to reduce the toxicity of tau protein and delay their dissemination. Therefore, targeting tau aggregation is a reasonable approach for AD treatment[105,106]. At the same time, the potential advantage of small molecule tau protein aggregation inhibitors that can target intracellular tau protein makes it the focus of research.

LMTM [Methylene Blue (MB), TRx0237, LMT-X, TAI]: MB showed an inhibitory effect on tau aggregation by blocking the tau-tau binding interaction through the repeat domain *in vitro*[107]. MB inhibits tau aggregation and rescues memory deficits in a mouse model of tauopathy[108]. Phase 2 clinical trials have demonstrated its cognitive benefits in patients with mild to moderate AD, but absorption of the highest dose is limited and tolerated poorly in the presence of starvation[109]. Moreover, MB lacked efficacy in a recent Phase 3 clinical trial[110]. Therefore, to overcome these limitations, methylmethionine (LMTM) - a reduced derivative of MB - was developed, which is more stable with better absorption, bioavailability, and tolerability[111]. In a phase 3 trial involving mild to moderate AD, LMTM failed to slow cognitive or functional decline, but after re-analysis of the data, the brain atrophy rate of patients with LMTM as monotherapy was significantly lower than that of mild AD. LMTM as monotherapy may have potential benefits, but due to factors such as pharmacokinetics, no difference was found between the clinical effects of high and low doses[110,112]. To confirm this hypothesis, a phase 3 trial of lower doses (8 and 16 mg daily) of LMTM is currently underway. The study will be completed in December 2022.

ACI-3024 (Tau MorphomerTM) is a small molecular inhibitor of tau aggregation that disrupts the β-sheet structure[113]. The mechanism is to prevent tau toxicity by reducing intracellular misfolding of tau protein and to slow tau protein spreading by preventing tangle formation or promoting tangle breakdown before tau protein tangles are secreted. Nanomolar concentrations of ACI-3024 alone cause a dose-dependent reduction in intracellular pathological tau and prevent the activation of microglia and neuronal death caused by PHFs. One-month treatment with ACI-3024 reduced aggregated and insoluble tau in the brains of tau lesion rTg4510 mice while decreased the activation of microglia and the total tau of CSF in proportion to the plasma drug concentration[114]. Oral treatment of ACI-3024 is being studied in neurodegenerative diseases and the phase 1 trial has been completed, but no preclinical information on ACI-3024 has been officially released.

Curcumin (diferuloylmethane, Longvida™) is the main ingredient of Indian turmeric spice, which is extracted from the rhizome of turmeric and has an excellent safety profile and a wide range of pharmacological activities with potential neuroprotective benefits, including anti-amyloid properties, inhibition of Aβ and tau aggregation, promotion of neurogenesis, and antioxidant and anti-inflammatory effects[115]. These effects suggest that curcumin may be useful in the treatment against tau-related neurodegeneration. However, curcumin has low bioavailability and can be rapidly degraded *in vivo*. Clinical trials in AD have shown no therapeutic effect of curcumin[116]. These results have led to the development of nanocarriers and analogs, and carrier devices such as liposomes and polymeric nanoparticles have shown promising potential[117]. J-147, an oral curcumin derivative, has been demonstrated to easily cross BBB and enhance memory in aged AD mice[118]. In addition, purpurin[119], ginseng[120], and many other natural compounds are also being investigated, which will offer great potential for the treatment of AD.

In addition to inhibiting tau aggregation, there are still some inhibitors reported to act as dual inhibitors of Aβ and tau. Curcumin can effectively disaggregate Aβ as well as preventing fibril and oligomer formation at low concentration[121]. At the same time, some small molecules (e.g., thiophene and ortho catechol) that have not yet entered clinical research are also playing the same role[122,123]. Many single-target drugs for Aβ and tau have failed in different stages of drug development. Therefore, multi-functional drug molecules targeting

multiple targets are necessary for the treatment of AD.

However, choosing the appropriate inhibitor of pathological protein aggregation is a big challenge. It may be detrimental if the inhibitors dissociate large aggregates into smaller (toxic) oligomers or inhibit the inability of toxic oligomers to continue to form fewer toxic fibrils.

REDUCING TAU EXPRESSION

BIIB080 (IONIS-MAPTRx, ISIS 814907): tau-targeted antisense oligonucleotide (ASO) therapy has been shown to inhibit the translation of tau mRNAs and ameliorate toxin-induced seizures, neuronal loss, and neurofibrosis in adult tau-transgenic mouse models[124]. Infusion of BIIB080 into the CSF of cynomolgus monkeys reduced tau mRNA across different brain regions, and CSF tau levels following ASO exposure were correlated with hippocampal tau levels[124,125]. The results of one phase 1 clinical trial show that BIIB080 could reduce the total tau and pTau-181 protein levels in CSF of mild AD patients in a dose-dependent manner, with good safety and tolerability. Phase 2 trials will be conducted in mid-2022. Although BIIB080 can non-selectively reduce the levels of both normal and pathological tau protein, there are still some safety concerns. Studies showed that tau knockdown can impair the repulsive response of the growth cone[126], delay neuronal maturation[127], accelerate neuronal branching[128], and hyperpolarize neuronal membrane potential[129]. In addition, the reduction of tau may decrease mitochondrial mobility, increase the number of abnormal mitochondria[130], and lead to a loss of its nucleic acid safeguarding functions and increase in DNA and RNA oxidative damage[131]. However, only mild phenotypic changes (such as muscle weakness and hyperactivity) and delayed neuronal differentiation were shown in the tau knockout mouse model[127,132]. The reason may be that the changes caused by tau deletion during neuronal development may be compensated by the increased expression of other microtubule-associated proteins. Hence, tau pathologies are primarily caused by gain-of-function abnormalities caused by tau misregulation. We need to consider its impact on normal tau physiological function when targeting tau protein reduction.

PROMOTING MICROTUBULE STABILIZATION

Microtubules are key cytoskeletal elements in living cells, which are essential for axonal transport, synaptic transmission, and maintenance of neuronal morphology. Tau protein normally stabilizes microtubules, but hyperphosphorylation causes loss of its function. Microtubule dysfunction and subsequent axonal transport disorders in neurons are believed to play a prominent role in the neurodegeneration of AD[133,134].

Davunetide (NAP, AL-108) is derived from a growth factor called activity-dependent neurotrophic protein, which has highly effective neuroprotective activity. It is a peptide that can modulate the pool of microtubules in neurons and glial cells, although its exact mechanism of action is unclear[135,136]. Davunetide is related to the memory and cognitive ability of mice. In the 3xTg mouse model of AD, davunetide reduces the amyloid accumulation and tau hyperphosphorylation and improves the behavioral performance in the Morris water maze[137,138]. Davunetide entered the clinical trial after a successful preclinical study showing positive results[136,139]. The phase 2 trial of intranasal administration of davunetide in prodromal AD subjects showed good safety, but there was no significant improvement in cognitive score[140]. At present, the experiment has been terminated.

Epothilone D (BMS-241027) is a small molecule microtubule stabilizer isolated from myxobacterium *Sorangium cellosum* for the treatment of AD. Epothilone D can cross the BBB and reverse behavioral and cognitive deficits, clear tau pathology, and curb neuron loss in P301S tau transgenic mice[141,142]. A phase 1 trial of weekly infusion of epothilone D (0.003, 0.01, and 0.03 mg/kg) in patients with mild AD for nine weeks was conducted to evaluate the tolerability and pharmacology of BMS-241027. The clinical trial also

monitored adverse effects and measured whether drugs changed the N-terminal fragment concentration of tau in CSF and cognitive performance. However, the study was terminated without data release.

TARGETING TAU INTERCELLULAR TRAFFICKING

It has been found that the intercellular transmission of tau also plays an important role in the development of AD. Therefore, research targeting tau transmission mechanisms seems to offer opportunities for AD treatment and drug development.

In AD, pathological neurofibrillary tau aggregates display an accumulative pattern that begins in the entorhinal cortex and travels through connected pathways to cortical areas, with cognitive impairment manifesting itself when tau inclusions reach the hippocampus. The presence of tau in the CSF of AD patients has long been thought to be simply the result of the passive release of degenerated and dead neurons. Previous studies found that injection of mutant P301S tau-expressing mice's brain extracts into the brains of transgenic wild-type tau-expressing mice induced the assembly of wild-type human tau into filaments and the spread of pathology from the injection site to adjacent brain regions[143]. In addition, young PS19 tau transgenic mice injected with pathological tau promote tau lesions in a time- and dose-dependent manner[144]. Growing evidence suggests that, in most neurodegenerative diseases, misfolded pathological tau proteins act as seeds (oligomers and protofibrils), which then recruit and convert normal monomeric tau proteins within the cytoplasm of recipient neurons into misfolded pathological tau proteins. The misfolded and pathological tau proteins then travel along anatomically related neural pathways in the brain to adjacent normal cells or synaptically interconnected cells, spreading pathology from affected cells to healthy cells and to previously unaffected brain regions, promoting disease progression[145-148]. Current research speculates that the main mechanisms of cell-to-cell transmission of misfolded pathological proteins mainly include the following cellular processes: (1) Donor neurons can release tau seeds by exocytosis or vesicles such as exosomes that fuse with and deliver their contents to recipient neurons. Extracellular tau seeds can be internally taken up by recipient neurons through endocytosis; (2) In addition to the intercellular transfer of tau via the extracellular space, direct intercellular mechanisms via tunneling nanotubes allow for the transfer of cytoplasmic tau directly from the cytoplasm of one cell to another. Tau, which is simultaneously recruited and aggregated on the cellular leaflets of plasma membrane (PM), interacts directly with specific lipids in cholesterol/sphingomyelin/PI(4,5)P2-rich membrane microdomains, and then penetrates through the PM and releases from the PM facilitated by cell surface heparan sulfate proteoglycans (HSPGs).

Currently, identifying the mechanisms of tau propagation has led to the development of monoclonal antibodies or other compounds that specifically bind to, sequester, or disassemble tau conformations relevant to pathological propagation and may delay disease progression in tauopathies. In addition, inhibition of the process by which tau interacts with PM lipids or cell surface HSPGs may reduce the extracellular tau internalization. However, there are many unresolved issues and challenges regarding the mechanisms of tau propagation. Tau has a variety of pathological conformations, but it is unclear whether specific secretory pathways are preferred by certain types of tau aggregates. Moreover, the receptor proteins involved in the interactions between tau and cell membrane are largely unknown. The mechanism of how normal tau proteins are transformed into misfolded tau aggregates by using pathological tau seeds as templates is also unclear and requires urgent and extensive research to confirm.

PROMOTING TAU CLEARANCE

Autophagic clearance of tau aggregation

The ubiquitin-proteasome system and the autophagy-lysosome pathway (ALP) are the two main modes of tau degradation, while abnormally phosphorylated tau and PHF tau are mainly degraded by ALP[43]. In many neurodegenerative diseases, autophagy dysfunction disrupts the efficient clearance of misfolded proteins and cytoplasmic oligomers. A complete autophagic process consists of three consecutive steps, namely induction, autophagosome formation, autophagosome-lysosome fusion, and degradation[149]. The regulation of autophagy involves complex signaling pathways that can be divided into two main aspects: an mTOR-dependent approach and an mTOR-independent approach; however, both regulatory pathways have been found to be aberrant in AD[150].

Inhibition of MTORC1 and autophagic activity directly correlate with tau clearance; activation of autophagy with specific 12/15-lipoxygenase inhibitors or drugs such as rapamycin significantly inhibits tau aggregation and reduces insoluble and phosphorylated tau protein levels in the brain of AD mice, reducing tau lesions and cognitive dysfunction in neuronal cells[151]. Autophagy-based mTOR inhibitors (OSI-027, AZD2014, and AZD8055) potently downregulate tau phosphorylation, prevent the formation of insoluble tau, and promote autophagy-mediated tau clearance, thereby reducing tau-mediated neuronal stress vulnerability[152].

Selenium-methionine activates autophagy via the AMPK-mTOR pathway and then promotes tau clearance from neurons to improve cognitive performance in AD model mice[153]. Nuclear factor E2-related factor2 promotes autophagy and autolysosomal clearance through directly regulating the expression of Bcl-2 associated athanogene 3, autophagic bridging proteins NBR1 and NDP52, and autophagy protein p62, so as to play important regulatory roles in autophagy-mediated degradation of phosphorylated tau protein[154].

miR-132/212 targets tau mRNA to regulate tau expression, whereas deletion of miR-132/212 induces tau aggregation in mice expressing endogenous or human mutant tau. Moreover, treatment of AD mice with miR-132 mimics partially restores memory function and tau metabolism. miR-132/212 levels are correlated with insoluble tau and cognitive impairment in humans[155]. Blocking cholesterol acyltransferase expression with inhibitors revealed enhanced autophagy and induced autophagosome formation in AD mouse models, accompanied by a decrease in phosphorylated tau[156].

Therefore, all these research findings support that reducing the accumulation of intracellular aggregate proteins through autophagic upregulation could be a promising therapeutic strategy for most neurodegenerative diseases. However, low efficacy and severe side effects due to the lack of selectivity towards diseased cells and potential autophagic cell death have limited the further development of autophagic modulators. A recent tauopathy-homing nanocomponent with autophagy-activating capacity appears to address part of the problem by binding to hyperphosphorylated and/or aggregated tau and selectively aggregating in cells with undergoing tauopathy, further specifically promoting the clearance of pathogenic tau by stimulating autophagic flux and thus rescuing neuronal viability and cognitive function in AD rats[157]. Although activation of autophagy is a promising therapeutic approach in neurodegenerative diseases with impaired lysosomal clearance, overactivation of autophagy may contribute to disease progression. There is a dual role for autophagy in neurodegenerative diseases: in the early stages of AD, autophagy is increased, helping the removal of abnormally folded proteins and preventing further development of AD; however, in the advanced stages of the disease, the autophagic system is abnormal and the clearance of autophagosomes does not keep up with the formation of autophagosomes, leading to the formation and aggregation of neurotoxic Aβ and tau protein oligomers[150,158]. Although various autophagy enhancers have been identified to slow the progression of AD and improve cognitive performance, the

beneficial effects in AD patients are still limited. Deep thoughts and a comprehensive understanding of the role of autophagy in the pathogenesis of AD are still required to provide new theoretical and even therapeutic targets for clinical trials of anti-AD drugs.

Active immunotherapy against tau pathology

Earlier tau-targeting therapies primarily focused on inhibiting tau phosphorylation and aggregation or stabilizing microtubules. However, these approaches have mostly failed due to toxicity and/or lack of efficacy. Most of the current therapies targeting tau are moving to immunotherapy. Both active and passive immunization are designed to form antibodies targeting the pathological conformation of tau without responding to non-pathological tau. Promoting the clearance of abnormal tau is expected to reduce neuronal loss and ameliorate clinical symptoms[159,160]. The first vaccine for tau active immunotherapy was designed against recombinant tau protein, which was inoculated into C57BL/6 wild-type mice. Although it can induce tau antibodies, it also induces the histopathological characteristics of AD and tau lesions, manifested by NFTs formation, axonal damage, and gliosis. In addition, mononuclear infiltrates without demyelination in the central nervous system (CNS), accompanied by neurological deficits, were observed (such as lameness and limb paralysis). This is the first study showing that immunization against tau may induce tau-like features, and, to circumvent these effects, active immunization is performed using tau fragments or phosphorylated tau fragments[161].

AADvac1 (Axon peptide 108 conjugated to KLH) is the first clinically tested active vaccine against pathologically modified forms of tau protein with an epitope of 294KDNIKHVPGGGS305, the structural determinants essential for pathological tau-tau interactions. Good safety and immunogenicity have been demonstrated in transgenic AD rat models. The produced antibodies are capable of distinguishing between pathological and physiological tau, thus ensuring the specificity against pathological tau protein and significantly delaying tau pathological lesions and deterioration of cognitive behavior[162]. AADvac1 entered phase 1 clinical trials after successful preclinical toxicology and safety studies. The data from the phase 1 trial demonstrate that AADvac1 was safe and well-tolerated, with the majority of participants producing increasing antibody titers after repeat injections[163]. The 18-month open-label extension study showed decreased antibody titers within six months after the last injection, but the intensive dose restored IgG level without treatment-related serious adverse events. Patients with higher IgG responses tended to reduce hippocampal shrinkage and performed better in some cognitive tests[164,165]. In a 24-month phase 2 safety trial, 185 patients with mild to moderate AD were recruited, and its safety and immunogenicity were validated, with more than 95% of immunized participants developing specific tau antibodies and the concentration in CSF averaged 0.3% of serum. Furthermore, some neurodegenerative markers (such as Nfl, pTau217, pTau181, and total tau) were also significantly altered in CSF compared with placebo[166].

ACI-35 is a liposome-based vaccine targeting the pSer396/404 epitope of tau, which activates the immune system and produces antibodies against the pathological conformers of phosphorylated tau protein. ACI-35 has been shown to be safe for long-term injection and can improve clinical status and indices of tau lesions in the brain of P301L mice, without evidence of neuroinflammation or other adverse neurological effects[167]. However, due to its weak immunogenicity, ACI-35.030, the second-generation vaccine of ACI-35, was developed. The redesigned vaccine induced a stronger immune response in rhesus monkeys, and enhanced injection could improve the antibody titer. In July 2019, ACI-35.030 initiated a phase 1b/2a trial in patients with early AD, and the results show that ACI-35.030 generated positive safety, tolerability, and immunogenicity. All participants in the first two dose groups developed anti-tau IgG and IgM responses preferentially targeting phosphorylated tau, with high IgG titers; these results support plans to further advance this vaccine into phase 2/3, where enrollment at the highest dose is currently underway and is expected to be completed in 2023.

Active immunization targeting various forms of tau protein has been proved to reduce tau pathology, and the lasting immune response makes it a promising choice, but the potential autoimmune response is a major concern. Early preclinical reports observed that this problem may be caused by the use of very strong adjuvants not approved for use in humans[161,168].

Passive immunotherapy against tau pathology

Since the effectiveness of isotope immunotherapy was demonstrated in the JNPL3 tau transgenic mouse[169], researchers reported that exogenous monoclonal anti-tau antibodies can effectively reduce tau lesions in the brain, improve cognitive ability, and delay the development of the disease in animal models[101,170]. Compared with active immunization, passive immunization has the ability to reduce the probability of adverse immune response and target specific epitopes, reduce the chance of targeting non-pathological tau, and change the treatment method according to the stage or type of tau lesions to find which tau epitopes are more common; specific IgG isoforms can be selected and changed to improve efficacy. Once diagnostic technology has advanced enough, treatments can be tailored to the individual based on the stage of the disease.

RG7345 (RO6926496) is a monoclonal antibody specifically targeting the tau phosphoepitope pS422, which is predominantly distributed on dendrites. Phosphorylation of tau at this site is thought to be a pathological form associated with the relocation of tau away from microtubules and toward the soma-dendritic region of neurons[171]. RG7345 enters cells through endocytosis, removes phosphorylated tau protein, and effectively improves the pathological condition of tau[172]. Studies have shown that active vaccination targeting the pS422 tau epitope can induce similar exogenous antibody effects, reduce the level of insoluble pS422 tau, and improve the performance of Thy-Tau22 transgenic mice in the Y maze[173]. Unfortunately, the phase 1 clinical trial of RG7345 lasted less than one year in January 2015, possibly because of poor pharmacokinetics, although no safety or efficacy questions were raised during the trial.

Gosuranemab (BIIB092, BMS-986168, IPN007) is a humanized IgG_4 monoclonal antibody targeting extracellular, N-terminal tau fragments (eTau), which are mainly derived from pluripotent stem cells of familial AD (FAD) patients[68,174,175]. Exogenous addition of eTau causes neuronal hyperactivity, which in turn increases Aβ production and neuronal hyperactivity[176]. In mouse models, neutralization of eTau with antibodies reduces Aβ production, effectively improving tau pathological states and patients' cognitive and behavioral performance[177,178]. In a clinical study in healthy volunteers, infusion caused a dose-dependent increase in blood and CSF of gosuranemab, and a 67%-97% decrease in CSF of unbound eTau after four weeks, without serious or severe adverse events[179]. However, in the gosuranemab phase 2 trial TANGO on patients with mild cognitive impairment caused by AD or mild AD, there was no beneficial effect compared with placebo. On 16 June 2021, TANGO and the development of gosuranemab were terminated.

Semorinemab (RO7105705, MTAU9937A, RG6100) is a monoclonal anti-tau IgG_4 antibody targeting the N-terminal region of tau protein, mainly targeting extracellular tau. Semorinemab can bind the N-terminal of all six human tau protein isomers, both tau monomeric and oligomeric, regardless of the phosphorylation state. By binding with tau protein, it can delay their spread between neuron cells. In addition, the purpose of this antibody is to explore antibodies with reducing effector function in an effort to limit the activation of microglia leading to the inflammatory response[180]. Phase 1 clinical trials did not produce serious adverse effects, but also did not produce good results. The phase 2 trial TAURIEL showed that plasma semorinemab was increased in a dose-dependent manner, with a half-life of 32 days, and approximately 0.3% of the antibody entered the CNS. Both CSF total tau and phosphorylated tau decreased, but the total tau value in plasma increased with increasing dose of semorinemab. The antibody did not alter the downstream CSF

markers of neurodegeneration and inflammation (such as NFL, neurogranin, S100B, IL-6, and sTREM2) but increased the inflammatory marker YKL-40. YKL-40 protein increases with the development of AD and is associated with brain atrophy and other deleterious effects[181]. Another Phase 2 clinical trial, LAURIET, is underway.

Zagotenemab (LY3303560) is a humanized anti-tau antibody targeting the conformational epitope MC1 (amino acids residues 312-322) of tau, which is an early pathological conformation of tau[182,183]. Zagotenemab selectively binds and neutralizes soluble tau aggregates with high affinity rather than monomers, recognizing a conformational epitope in the N-terminal region of tau protein. In tau transgenic mice, treatment with MC1 reduces phosphorylated tau levels and neurofibrillary pathology, which is mediated by microglia-dependent or neuron-dependent tau/antibody clearance[184,185]. In October 2021, an investor disclosed that a phase 2 trial of zagotenemab in subjects with prodromal to mild AD did not meet its primary endpoint, and the development of the antibody was terminated.

JNJ-63733657 is a humanized IgG_1 antibody targeting the microtubule-binding domain (MTBR) of tau protein, which has a high affinity for tau phosphorylated at Thr217 (pT217)[186]. The antibody can more effectively interfere with the transmission of pathogenic and aggregated tau between cells than the antibody against the N-terminal of tau. JNJ-63733657 has been reported to eliminate the "seed" of pathogenic tau in cells and interfere with the dissemination of pathologically aggregated tau proteins in mouse models. A phase 1 clinical trial validated JNJ-63733657 to be safe and tolerable in 72 healthy volunteers. The serum pharmacokinetics was linear with dose and 0.2% ended up in CSF[187]. Single or multiple administrations resulted in a dose-dependent reduction of free p217 tau in CSF[188]. In January 2021, a phase 2 study of 420 patients with early AD symptoms and tau positive PET scan began and is expected to continue until 2025.

Bepranemab (UCB0107, UCB 0107, Antibody D) is a humanized monoclonal IgG_4 antibody targeting 235-250 amino acids near the MTBR of tau, which may be more effective for preventing the transmission of pathogenic tau in cell than an antibody targeting N-terminal tau. It is also true that the antibody showed greater efficacy than other antibodies in preventing the seeding and aggregation of pathological tau in tests on cells. Bepranemab can prevent pathologic tau seeds in transgenic mice injected with AD brain extracts and in mice injected with K18 P301L tau fibrils[189,190]. In the clinical trials, no adverse events or safety issues, drug-resistant antibodies, *etc.*, were reported. While serum and CSF concentrations increased in a dose-dependent manner, the CSF/serum ratio remained unchanged at different doses. The experiment is in phase 2 for patients with MCI or mild AD dementia and will run until November 2025.

PNT001 is a monoclonal antibody targeting the cis isomer of phosphorylated tau at threonine 231 (cis-pT231). Cis-pT231-tau is a neurotoxic conformation that has been shown to be one of the main drivers of neurodegenerative diseases, including traumatic brain injury, chronic traumatic encephalopathy, vascular dementia, and AD. Cis-pT231-tau is resistant to dephosphorylation and degradation, promotes tau aggregation, and accelerates neurodegeneration[191,192]. In the human brain, the level of cis-pT231 in CSF correlates with the severity of brain injury[193]. In a mouse model of vascular dementia, PNT001 reduced neurodegeneration and ameliorated cognitive impairment[194]. PNT001 blocks the diffusion of toxic tau protein by accurately targeting and neutralizing tau protein carrying cis-pT231, thereby protecting the normal functioning of the brain and treating neurodegenerative diseases. Phase 1 clinical trial results show that the antibody produced dose-related blood and CSF concentrations that remained constant over 28 days, and it was well tolerated. A study is currently underway on acute traumatic brain injury and is expected to be completed by January 2023.

BIIB076 (NI-105, 6C5 huIgG$_1$/l) is a human recombinant monoclonal IgG$_1$ antibody targeting the mid-domain of tau, which blocks tau aggregation *in vitro* and tau transmission between neurons[195]. It can recognize monomers, fibrosis, and tau isolated from the brains of healthy subjects and patients with AD. In preclinical studies, following intravenous injection in cynomolgus monkeys, BIIB076 exhibited dose-dependent increases in serum exposures. Total and free tau that were unbound to BIIB076 in CSF decreased significantly, while there were no adverse BIIB076-related toxicology changes or pathology-related findings[196]. These data establish a positive safety profile for BIIB076 for inclusion in a phase 1 trial, which was completed in March 2020. The results show that, as the concentration increased, some side effects occurred, but the safety profile is acceptable. BIIB076 engaged its target, halving the concentration of unbound mid-region-bearing tau in the CSF one week after infusion and the reduction persisted up to three weeks.

Tilavonemab (C2N8E12 /ABBV8E12/ HJ9.3) is a humanized IgG$_4$ antibody targeting 25-30 aa residues at the N-terminal of tau protein, which mainly recognizes aggregated and extracellular pathogenic tau. This antibody cannot enter neuronal cells, and thus only functions outside the cells[101,197,198]. In preclinical studies, the antibody can block tau seeding caused by exogenous tau aggregates and prevent the transneuronal spread of pathological tau[197,198]. The levels of aggregated and hyperphosphorylated tau and brain atrophy can be reduced by injecting antibodies into P301S tau-transgenic mice, thus improving their cognitive performance[101,102]. Although the study found that tilavonemab did not provide more benefits than placebo, phase 2 clinical trials were conducted based on the acceptable safety and tolerability of single-dose tilavonemab to assess the efficacy and safety of multi-dose in early AD or PSP patients[199]. However, the analysis of phase 2 of PSP trial data showed that free tau in CSF was decreased and total tau in plasma increased in the treatment group, but the antibody did not show efficacy[200]. In July 2021, it was announced that the development of tilavonemab was discontinued, and it will be removed from its pipeline.

E2814 is a humanized monoclonal IgG$_1$ antibody targeting the HVPGG sequence that recognizes the second (aa 299-303) and fourth (aa 362-366) repeats of MTBR in tau, which recognizes the 4R and 3R tau isoforms. It is capable of binding extracellular tau, inhibiting the cell-to-cell spread of pathogenic species, and facilitating microglial clearance[201]. In mice injected with K18 P301L tau fibrils, a model of tau transmission, E2814 modestly reduced the propagation of aggregated tau. Some data suggest that tau antibodies of the mid-domain are more effective at interfering with the spread of pathogenic, aggregated tau than N-terminally targeted anti-tau antibodies currently in clinical trials. A phase 1 trial testing the safety and tolerability of a single intravenous infusion in healthy adults showed no significant drug-related clinical changes or dose-limiting events, but there was a dose-related increase in antibody-tau association, which persisted for at least a month. In 2021, a multiple-ascending-dose trial was conducted to detect E2814's safety, pharmacokinetics, and induction of anti-E2814 antibodies. The trial will run until November 2022. Meanwhile, E2814 was selected for evaluation in patients with pathogenic APP and presenilin mutations in the DIAN-TU prophylaxis trial, which is expected to be completed in April 2024.

Lu AF87908 is a humanized mouse IgG$_1$ monoclonal antibody produced by immunization of mice with the tau386-408 epitope and phosphorylating at Ser396 and Ser404. This antibody preferentially binds aggregates of hyperphosphorylated tau and reduces the ability of brain-derived tau to seed aggregates in cultured neurons and rTg4510 tau transgenic mice[202], and it can mediate the uptake and lysosomal degradation of tau aggregates in microglia via the antibody's Fcγ receptor[203]. A phase 1 study is currently underway to test its safety, tolerability, and pharmacokinetics in healthy individuals and AD patients. The study is expected to be completed by July 2022.

CURRENT CHALLENGES AND FUTURE OPPORTUNITIES IN TAU-TARGETING IMMUNOTHERAPY

Challenges in tau immunotherapy

Currently, research on a targeted tau vaccine is still in its infancy. Many unknown fields, such as the amino acid sequence of tau protein suitable for synthetic vaccine, methods to determine vaccine effectiveness, and the dose and mode of vaccination, require further exploration. In the meantime, the following issues must be considered in immunotherapy research targeting tau protein.

BBB crossing

The reason for the failure of most studies is the inability of drugs to cross the BBB. Increasing the concentration of therapeutic drugs in the brain can not only improve the efficacy of antibodies, so as to improve the success rate of clinical trials, but also reduce the production and treatment costs and the number of treatments required. Therefore, it is necessary to strengthen the delivery of antibodies and other biological therapeutic drugs used to treat central nervous system diseases.

Nanotechnology has recently emerged as a promising route for cross-BBB drug delivery to the CNS. The continuous and accurate drug targeting ability of nanoparticles (NPs) can make up for the defects of insufficient drug concentration in the CSF. Ideal NPs should have appropriate physiochemical properties, good biocompatibility, and effective production magnification[204]. NPs are small, nanoscale carrier structures that can be designed or modified to encapsulate or attach to molecules, peptides, proteins, antibodies, and nucleic acids[205]. There is currently a variety of NPs being explored for CNS drug delivery, including gold, hydrogels, liposomes, *etc.*[206]. The use of different nanocarriers is currently one of the most widely used state-of-the-art technologies to overcome the shortcomings of existing drugs and provide new therapeutic approaches, particularly in diseases where access to affected organs is difficult. Currently, some NPs targeting tau have been developed. Ghalandari *et al.* prepared folic acid functionalized gold NPs (FA-AuNPs) and gold-shelled Fe_3O_4 NPs (AuFeNPs), which showed a binding affinity for tubulin and tau[207]. Protein-capped metal (PC-Fe_3O_4 and PC-CdS) NPs present a novel function, which act as a powerful tau aggregation inhibitor in AD[208]. Cur-loaded T807/RPCNP NPs not only effectively penetrate the BBB but also selectively combine with p-tau with high affinity, thereby inhibiting the important pathways in tau-related AD pathogenesis and alleviating the memory impairment of AD mouse models[204]. These NPs have become a promising treatment strategy for AD through their high bioavailability in the brain. However, due to technical and cost limitations, as well as exhibiting long-term toxicity, only a few nanoparticles targeting diseases of the CNS are currently in clinical trials[209,210]. Another delivery system, the exosome, has also become a hot topic of current research, as its lipid bilayer reduces renal clearance, encapsulates and protects drugs that will be degraded, and allows the recipient cells to recognize and absorb the homing properties of vesicles, and its low immunogenicity makes it a promising drug delivery system[211]. Studies have shown that exosomes loaded with drugs can cross the BBB when injected intravenously in mice[211,212]. Advances in these technologies for medical drug development, drug delivery, and novel diagnostic biomarkers may serve as promising option for the treatment and diagnosis of AD as well as other neurodegenerative diseases.

Cell membrane crossing

Tau is mainly concentrated in neuronal cells, and how antibodies cross through membranes has become a major challenge hindering antibody development and application. Previous studies have shown that many antibodies can actually function intra/extracellularly[213,214] and are detectable in neurons[213,215,216], although some antibodies are not taken up into neurons[213,217].

Tau antibodies can cross the BBB and enter neurons, which are then mediated by the FcR or endocytosis[218,219]. Intracellularly, antibodies can bind to tau aggregates within the endosomal-lysosomal

system and promote their breakdown, thereby increasing the pathway for lysosomal enzymes to degrade the aggregates[216], isolating tau assemblies in the cytoplasm and preventing their release from neurons, or promoting proteasomal degradation through the binding of the E3 ubiquitin-protein ligase TRIM21[220].

Of course, there is also a small amount of tau outside the cell. Extracellular tau is therefore crucial in the spread of tau, and extracellular antibodies can change the process of the disease by preventing the transmission of pathological tau. Extracellular antibodies act mainly by isolating tau aggregates, interfering with their assembly, and promoting phagocytosis of microglia, thus blocking the spread of tau pathology among neurons[184,197] [Figure 3].

Vaccine biosafety

Studies have shown that some of the phosphatase sites targeted by immunotherapy are presented in the brains of healthy people. This has raised concerns about the safety of vaccines targeting specific phosphorylation sites. Concurrently, these treatments have been studied by selectively targeting only one or two phosphate sites out of the 45 phosphate sites identified in AD brains. However, it is not known whether all phosphorylation epitopes of pathological tau coexist on the same tau molecule, or whether different pathological tau subspecies display a variety of different phosphorylation sites.

At the same time, compared to Aβ, tau is a much larger antigen, so it is very important to select the correct immunogenic epitopes. Previously, targeting the N-terminus of the tau protein was a hot research topic because of its ability to generate high-affinity antibodies. However, thus far, antibodies that bind to this region appear to have had little success in phase 2 trials. Therefore, antibodies against tau mid-region, especially MTBR, are an important target in recent studies. Several antibodies currently targeting this epitope suggest that mid-region antibodies are more effective at interfering with the spread of pathogenic, aggregated tau than anti-tau antibodies targeting the N-terminus. The reason is that the region can drive tau aggregation, and the antibody binding to this site can prevent the spread of misfolded forms of tau[221].

Future directions

Currently, clinical trials of tau-based drugs aimed at gain-of-toxic-tau function (e.g., dysregulation of PTMs and tau aggregation) or loss-of-function (microtubules instability) have been increasingly conducted in tauopathy patients. In addition, as the understanding of tau proteins increases, more and more studies targeting tau are being conducted.

Biomolecular condensation of intrinsically disordered proteins/regions (IDPs/IDRs) via liquid-liquid phase separation (LLPS) has gained widespread interest as the rapidly unfolding role of phase-separated condensates in a variety of cellular functions and human diseases[222]. LLPS of tau is essential to regulate some cellular physiological functions; however, abnormal phase separation can lead to the transition of soluble tau to fibrotic tau, and the generation of phase transition droplets provides local high protein concentration and positive charge density for the generation of fibrillar amyloid structures, favoring the recruitment of polyanions (ribonucleic acid, heparin, *etc.*) involved in the formation of neurofibrillary tangles[223]. The rapid development of the LLPS mechanism has undoubtedly changed our understanding of various biological activities and disease conditions. The basic research on LLPS and human diseases will continue to be improved and translated into clinical practice.

apoE4 is the strongest genetic risk factor for late-onset AD, which is found in some neurons with NFTs, and the specific interaction of apoE with tau may regulate tau metabolism in neurons and alter the rate of PHF and NFT formation[224]. Removal of apoE4 substantially reduces tau-regulated neurodegeneration and

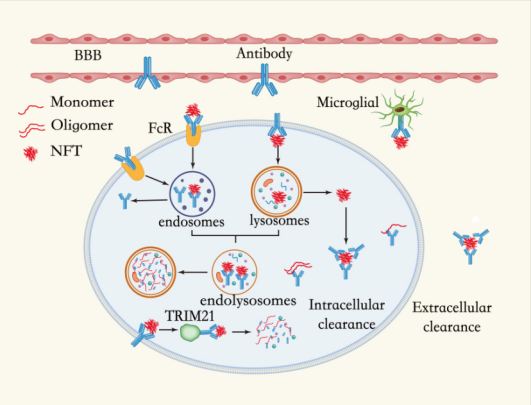

Figure 3. Mechanism of action of tau protein clearance. (1) Intracellular clearance: antibodies can cross the BBB and enter the neuron, and then enter the cell either mediated by FcR or through endocytosis. Intracellularly, antibodies can bind to tau aggregates within the endosomal-lysosomal system and promote their breakdown, thereby increasing the pathway for lysosomal enzymes to degrade the aggregates while isolating tau assemblies in the cytoplasm and preventing their release from neurons or promoting proteasomal degradation through the binding of the E3 ubiquitin-protein ligase TRIM21. (2) Extracellular clearance: extracellular antibodies act to block the spread of tau pathology between neurons by isolating tau aggregates, interfering with their assembly, and promoting phagocytosis by microglia.

decreases p-tau lesions, tau-induced synaptic loss, and phagocytosis of synaptic elements by microglia[225]. In the absence of microglia, the pathological progression of p-tau is prevented and the pathogenesis of p-tau is also largely driven by microglia-mediated inflammation[226]. Overexpression of the apoE metabolic receptor, low-density lipoprotein receptor, in P301S tauopathy mice significantly reduces brain apoE and improves tau pathology and neurodegeneration[227]. These results suggest that an in-depth understanding of the mechanisms of action of tau protein will provide novel therapeutic approaches for AD.

In brief, targeting tau is a promising treatment strategy. However, various challenges, including BBB restricting the entry of drugs and reducing the effective drug concentration, the lack of selectivity and specificity of target proteins leading to insufficient or ineffective treatment effect, or the difference of phenotype between animal models and humans resulting in the failure of clinical transformation of drugs, as well as the selection of disease treatment stages, have limited the development of drugs. With the improvement of diagnostic methods, the intervention time was transferred to MCI stage or early prodromal stage of AD, and treatment should be carried out before symptoms appear. The development of early diagnosis methods allows for diagnosis and treatment early for diseases. Active immunotherapy can prevent the disease in the early stage but may lack the corresponding specificity and produce autoimmune side effects. Once the disease progresses and a large number of pathological proteins appear, a single treatment cannot be enough to alleviate the disease progress. The combination of passive immunotherapy and various treatment methods such as inhibiting its aggregation, transmission, protein expression, *etc.* will improve the effect of treatment to a certain extent. Although many candidates targeting tau have been discovered and developed at present, few are really useful in the clinic. Further continuing basic research on the underlying causes and mechanisms of dysfunction in AD is critical to identifying new targets for intervention.

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Authors' contributions

Research and drafting of the article: Guo Y, Tan J
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Ageing and Neurodegenerative Diseases

Review

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Modeling neurodegenerative diseases using non-human primates: advances and challenges

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Abstract

Neurodegenerative diseases (NDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), are pathologically characterized by progressive loss of selective populations of neurons in the affected brain regions and clinically manifested by cognitive, motor, and psychological dysfunctions. Since aging is the major risk factor for NDs and the elderly population is expected to expand considerably in the coming decades, the prevalence of NDs will significantly increase, leading to a greater medical burden to society and affected families. Despite extensive research on NDs, no effective therapy is available for NDs, largely due to a lack of complete understanding of the pathogenesis of NDs. Although research on small animal and rodent models has provided tremendous knowledge of molecular mechanisms of disease pathogenesis, few translational successes have been reported in clinical trials. In particular, most genetically modified rodent models are unable to recapitulate striking and overt neurodegeneration seen in the patient brains. Non-human primates (NHPs) are the most relevant laboratory animals to humans, and recent studies using NHP neurodegeneration models have uncovered important pathological features of NDs. Here, we review the unique features of NHPs for modeling NDs and new insights into AD, PD, and ALS gained from animal models, highlight the contribution of gene editing techniques to establishing NHP models, and discuss the challenges of investigating NHP models.

Keywords: Neurodegeneration, non-human primate, gene editing

INTRODUCTION

Neurodegenerative diseases (NDs) represent a group of devastating neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). NDs are characterized by progressive loss of specific populations of neurons in the affected brain regions. NDs always manifest clinical symptoms in old age. As the life span and global population increase, the number of ND patients is expected to rapidly rise in the coming decades[1-4].

At present, no therapy can reverse, halt, or slow NDs, although a lot of progress has been made in the past decades. One of the important reasons for the failure to effectively treat NDs is that the pathogenesis of NDs is still an enigma. Although most (> 90%) NDs are sporadic, small populations of familial NDs are found to carry genetic mutations. Identification of these genetic defects enabled the generation of gene-modified animals, which provide important animal models for us to investigate the pathogenesis of NDs[5,6]. Of these animal models, rodents are widely used and serve as essential animal models for investigating almost all types of NDs, largely because of their small size, fast breeding, and short generation time, as well as the available embryonic stem cells (ESC) that allow for genetically modifying endogenous rodent genes efficiently. As a result, several ND rodent models have been created, and investigation of these animal models has generated a wealth of information regarding the pathological changes and mechanisms of NDs. Although most rodent models can recapitulate the important pathological hallmarks of NDs, overt neuronal loss is often missed[7], making it difficult to use rodent models to rigorously evaluate therapeutic effects on neurodegeneration. In line with this scenario, most therapeutic trials based on findings from rodent NDs models have failed clinically[8]. NHPs are closer to humans than other species of animals, especially in their brain structure and function, as well as the aging process[9,10]. Recently, several NHP ND models have been generated and provided new insights into ND pathogenesis. Here, we review the strengths of NHPs for ND investigation, highlight new pathogenic insights offered by NHP models of NDs with a focus on AD, PD, and ALS, and discuss challenges and perspectives for future studies.

UNIQUE ADVANTAGES OF NHPS FOR ND INVESTIGATION

The brain is the most complex and delicate organ of mammals, responsible for information reception, procession, decision making, and organism behavior. During evolutionary progress, primates developed a sophisticated central never system (CNS), leading to the most intelligent human being[11]. Laboratory NHPs primarily consist of cynomolgus (*Macaca fascicularis*), rhesus (*Macaca mulatta*), and marmoset (*Callithrix jacchus*). The first two are old world monkeys belonging to the macaque genus, descended from the same ancestor 1.8 million years ago[12], and share the same ancestors with humans around 30 million years ago, while the mouse was divergent from humans 70 million years ago[13]. Macaque monkeys are more proximal to humans phylogenetically and more widely used in biomedical research due to their unparalleled high level of similarity with humans in the following important aspects.

First, there is a tremendous similarity in brain structure between macaque monkeys and humans. Human and monkey brains are full of gyrus and sulci at the outer surface, whereas rodent brains lack the important feature of gyrification[14-16]. While there are several differences in brain anatomy between rodents and humans, these differences do not exist in monkeys when compared with humans. For example, the striatum, which is particularly affected in Huntington's disease, is divided into caudate and putamen in both monkey and human brains but lacks these two distinct parts in rodent brains[17-20]. The similar brain anatomy and structure in humans and non-human primates are likely due to the similar timelines for brain

development. For example, the complete formation of CNS in humans and macaque monkeys before birth requires about 280 and 160 days, respectively, which is significantly different from the rapid formation of the mouse brain in 18 days [Table 1]. In addition, the postnatal primate (macaques and human) brain takes several years to reach maturation, while the rodent brain needs less than half a year[21]. Adult neurogenesis is kept in the subventricular zone and subgranular zone of the hippocampus in mice throughout life, while it is controversial in primates[22-24].

Second, the composition and morphology of cell types in the human brain are more similar to those in the monkey brain than in small animal brains[25-27]. Astrocytes and microglia collaborate to sustain brain environment homeostasis, and they also play important roles in aging and neurodegeneration[28-30]. The soma size and morphology of glial cells, as well as their ratio to neurons in mice, differ significantly from those in the primate[31,32]. For example, astrocytes in the non-human primate brains develop more abundant processes in the same manner as human astrocytes than those in the mouse brain[33-36]. Astrocytes are a core element of the blood-brain barrier (BBB), which works at the interface of capillary blood vessels and cerebral parenchyma, stringently regulating the materials exchange[37,38]. BBB breakdown is a hallmark event in several degenerative diseases. Positron emission computed tomography (PET) radiotracers[39,40] and recent adeno-associated virus (AAV)-mediated transgene[41,42] studies have revealed the special role of the NHP BBB in expressing and distributing transgenes. Glial cells are essential for the survival of neurons by both providing neurotrophic, nutritional, and structural support and removing toxic insults[43,44]. It has been well documented that glial dysfunction plays a critical role in NDs[45-47]. Similarities in glial cells between non-human primates and humans are obviously an advantage for NHP models of NDs.

Non-human primates also closely resemble many aspects of humans in genomic regulation, aging process, metabolism, and physiology. The monkey genome holds more variations among individual alleles, offering a more faithful genomic context to interrogate molecular pathogenesis. For example, genome-wide association studies have established that the first genetic risk factor for AD is the APOE ε4 allele, which is present in primates but absent in rodents[48,49].

Non-human primates are particularly useful for examining behavioral abnormalities that also occur in NDs. It is well known that depressive behavior and cognitive impairment are the common features of patients with NDs. These phenotypes, which are hardly assessed in small animal models, can be evaluated in monkeys using well-established behavioral assessments[50,51].

NHP models of AD

AD is the most common neurodegenerative disease, the sixth leading cause of death, and 7%-8% of people over age 65 have AD[52,53]. The typical clinical symptoms of AD consist of memory loss, cognitive dysfunction, and mental as well as behavioral abnormalities[54]. Extracellular senile amyloid plaques and intracellular neurofibrillary tangles (NFTs) are two pathological hallmarks of AD. In addition, cerebral amyloid angiopathy, demyelination, neuroinflammation, brain atrophy, and synaptic ion dyshomeostasis are frequently detected in association with AD pathology[55]. More than 90% of AD patients are sporadic with amnesic manifestation in the mid-60s or later, while fewer than 10% of AD cases are familial form with early-onset symptoms caused by genetic mutations in the amyloid precursor protein (APP), presenilin 1, and presenilin 2 genes[56,57]. Based on these genetic findings, numerous transgenic mouse models expressing familial mutations driven by various promoters have been created[58]. However, although prominent Aβ deposition can be seen, obvious Tau accumulation, the other pathological hallmark, is hardly seen in these mouse models[59-61]. Further, these Aβ transgenic mouse models do not develop overt and robust neurodegeneration as seen in patients with AD[62,63]. Because brain imaging data indicate that symptom

Table 1. Comparison of CNS features across species

Species	Human	Macaque	Mouse
Life span (years)	70-90	30-40	~2
New striatum	Yes	Yes	No
Gestation (days)	280	155	18
Behavior reservoir	large	Moderate	Small
BBB permeability	Strict	Moderate	low
Gyrification	Yes	Yes	No
Circadian	Diurnal	Diurnal	Nocturnal
Cortex thickness (mm)	4-5	2-3	1
Inter cortex communication	High	Moderate	low
Naturally developed AD, PD pathology	Yes	Yes	No

CNS: Central never system; BBB: blood-brain barrier; AD: Alzheimer's disease; PD: Parkinson's disease.

deterioration of AD correlates with Tau aggregation more tightly than Aβ deposition[64], several mouse models expressing human Tau were established[65-67]. Some *Tau* transgenic mice show no obvious neuron loss, even after being crossed with Aβ mouse lines[68]. Many attempts have also been made to recapitulate AD pathogenesis in the minipig[69,70], but no typical pathology was detected in a three-year longitudinal study[71]. Thus, these findings underscore the urgent need to establish a better AD model[72,73].

Recent investigations have found that primates can naturally develop amyloid plaques and NFTs in old age[74-76]. Importantly, the Tau pathology initiates and is spread in the same manner as that in AD patients, strongly suggesting that monkeys possess unparalleled physical context for the occurrence of late-onset sporadic AD[75,77]. Since toxic chemicals in the environment may contribute to AD pathogenesis, Yang *et al.* performed methanol administration to induce an AD monkey model[78,79]. Several investigations attempted to make AD models by intracerebral or lateral ventricle administration of synthetic or patients' Aβ oligomers (AβO), which results in early pathological events, including reduced spines, increased inflammation, and synaptic dysfunction, but with no amyloid plaques or NFTs[80,81]. Since young animals were used in these studies, aging may be an important contributor to the appearance of typical AD pathology. A recent study achieved a remarkable and widespread distribution of massive Aβ aggregate across the entire brain via single focal delivery of synthetic AβOs into cerebral parenchyma, which triggered neuroinflammation and slight Tau phosphorylation, although no brain atrophy or cognition decline was detected[82]. Since Tau hyperphosphorylation and aggregation are considered more cardinal factors for dementia severity[54,83,84], injection of AAV expressing mutant human Tau into the monkey entorhinal cortex, a region in which early AD pathology initiates, was tried to create an AD model. Interestingly, exogenous Tau is spread into various brain regions, highly reminiscent of the AD patient brain, causing disease markers related to AD to rise in blood and CSF[85]. However, no functional examination was presented in studies of this AD monkey model. Because aging is the major risk for AD, more longitudinal studies of the above AD monkey models may reveal the relationship between behavioral phenotypes and pathological changes.

NHP models of PD

As the second most common neurodegenerative disease, Parkinson's disease (PD) affects more than 6.1 million people worldwide[86]. PD is clinically recognizable for dyskinesia, such as resting tremor, rigidity, poor balance, and bradykinesia, and it also frequently presents with constipation, hyposmia, and cognitive, psychiatric, and sleep problems[87]. Pathologically, PD is caused by progressive loss of substantia nigra (SN) dopaminergic neurons, which results from the intracellular accumulation of α-Synuclein, namely Lewy

bodies or Lewy neurites[88]. Similar to AD, most PD cases are sporadic, but roughly 10% of cases are familial, caused by mutations in the genes encoding α-Synuclein, PINK1, Parkin, LRRK2, and DJ-1[89]. Accordingly, many genetically modified mouse models were developed to carry the PD gene mutations, but none of them can recapitulate the dopamine neuron degeneration in PD[90].

PD has long been considered as a human-specific disease; however, recent studies revealed that aged NHPs display prominent synucleinopathy[91], and a monkey naturally showing PD symptoms was identified[92], underlining the great potential of NHPs for PD research. Due to technical difficulties in generating transgenic NHP PD models, most NHP models for PD were generated using chemical toxins. 6-hydroxydopamine (6-OHDA) (a hydroxylated analog of dopamine) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can selectively target SN dopaminergic neurons[93,94], inducing typical behavioral manifestations despite the absence of Lewy pathology. Since the 1980s, the MTPT-induced monkey model has been prevalent for drug and therapy tests[94]. However, phenotypes of this toxin-induced model are unstable and do not exactly mirror slow disease progression. Recently developed protocols attempt to address this issue by stereotaxic injection of MPTP into multiple SN sites[95]. Interestingly, in a monkey model with repeated MPTP administrations, alpha synucleinopathy was detected[96], which opens up a new avenue for researchers to establish a better PD animal model.

Since α-Synuclein is the core component of Lewy bodies, many attempts have been made to generate NHP models by overexpressing human mutant α-Synuclein in the monkey SN[97]. Highly efficient expression of A53T α-Synuclein in SN can be achieved by stereotaxic brain injection, giving rise to pathological hallmark Lewy neurites that can be detected four months post-surgery and loss of over 50% dopaminergic neurons[97]. However, no motor dysfunction and α-Synuclein spreading are displayed[97]. A longitudinal study of disease progression with this model in the future may bring novel insights. An α-Synuclein transgenic PD monkey model carrying a clinical mutation was generated by expressing mutant transgenes in fertilized embryos with lentivirus, and these monkeys showed impaired finger coordination and cognitive dysfunction at three years of age[98]. Because PD is an age-dependent disease, Yang *et al.* performed intracerebral injection of mutant α-Synuclein into the SN in monkeys at different ages and found that aging greatly promotes the accumulation of α-Synuclein and alpha synucleinopathy[99].

As described above, all genetically modified mouse models of PD are unable to develop degeneration of dopamine neurons, a key pathological hallmark[90,100]. However, recent studies on establishing PINK1-targeted monkeys via CRISPR/Cas9 indicate that severe neuronal loss occurs in the monkey brains when PINK1 is lost[101,102]. PINK1 mutations cause autosomal recessive PD with early-onset manifestation. Because of the loss of function mechanism for PD with PINK1 mutations, several groups tried to create PD monkey models by CRISPR/Cas9-mediated PINK1 knockout in fertilized monkey embryos[101,102]. It seems that a large DNA fragment deletion in the PINK1 gene mediated by CRISPR/Cas9 can lead to a severe loss of neurons in the monkey brain[101,103]. However, most PINK1 mutations in humans are point mutations that may partially affect PINK1's function to cause age-dependent neurodegeneration. Thus, CRISPR/Cas9-mediated deletion of PINK1 may completely eliminate the function of PINK1, allowing identification of its critical role in neuronal survival in the primate brain[104]. Indeed, Chen *et al.* used a single strand cutting enzyme D10A nickases to target monkey PINK1 but did not find PD symptoms in the generated monkey model[102]. Conversely, focal disruption of the PINK1 and DJ-1 genes in the adult monkey brain was able to mimic some PD pathology[105], suggesting that both types of genetic mutations and aging could be important for developing PD phenotypes in monkeys.

NHP models of ALS

ALS is a rare and fatal neurodegenerative disease caused by progressive loss of motor neurons in the brain and spinal cord, leading to muscular atrophy and movement disorder[106]. The prevalence of ALS is around 6 cases per 100,000, with a preference for elderly Caucasians[107]. Similar to AD and PD, most ALS patients are sporadic, and about 5%-10% of cases are caused by gene mutations. Mutations in the genes encoding TAR DNA-binding protein 43 (TDP-43), superoxide dismutase 1 (SOD1), fused in sarcoma (FUS), and C9ORF72 can result in ALS[108,109]. TDP-43 is a nuclear protein that is involved in a variety of cellular functions, including gene transcription, RNA processing, and protein homeostasis[110,111]. In addition to ALS, TDP-43 mutation is also associated with frontotemporal lobar degeneration (FTLD), as well as other neurological disorders and pathological conditions. Of the pathological changes in these diseases, cytoplastic TDP-43 accumulation is the common hallmark[112,113]. Normally, TDP-43 is located in the nuclei but redistributed in the cytoplasm under pathological conditions. Mislocation of TDP-43 in the cytoplasm could lead to loss of function in the nuclei and toxic function in the cytoplasm[114].

Several transgenic mouse models have been established to investigate ALS pathogenesis[115-117], but most fail to recapitulate the cytoplasmic mislocation of TDP-43[114,118]. This phenomenon thus encourages the establishment of new ALS animal models using large animals. A TDP-43 transgenic pig model created by expressing mutant TDP-43 in fertilized eggs displayed the cytoplasmic distribution of TDP-43[115]. In addition, a macaque monkey model, which was generated by stereotaxic delivery of a viral vector expressing mutant TDP-43 into the cortex and substantia nigra, showed the cytoplasmic distribution of mutant TDP-43, which is in contrast to the nuclear distribution of the same transgenic mutant TDP-43 in the mouse brain[119]. This finding is consistent with the previous report that exogenous mutant TDP-43 is distributed in the neuronal cytoplasm in the monkey spinal cord[120]. Thus, TDP-43-mediated neuronal pathology is apparently dependent on species-specific factors. In support of this, primate-specific caspase-4 is found to be responsible for the cleavage of mutant TDP-43 and its cytoplasmic accumulation[119]. Comparing mouse and monkey models of ALS also highlights the importance of using non-human primates to investigate NDs.

NHP models of HD

HD is an autosomal dominant neurodegenerative disease with full penetration, as well as a rare inherited monogenic disease with varied prevalence across the world. The Asian population has the lowest incidence, while Western countries have a much higher prevalence. HD is pathogenically caused by CAG repeat expansion (> 36 CAGs) in exon 1 of the HD gene, which is translated to the polyglutamine (polyQ) repeat in the protein huntingtin (HTT)[121,122]. The polyQ expansion renders HTT to misfold and aggregate in the patient's brain, resulting in the preferential loss of the medium spiny neurons in the striatum and extensive neurodegeneration in various brain regions as the disease progresses[122]. Clinically, HD is characterized by involuntary movement, called chorea. This symptom usually appears in middle life, and disease onset, progression, and severity correlate with polyQ number. Currently, there is no effective therapy available for HD[122].

Dozens of HD rodent models carrying various lengths of CAG repeats have been generated. However, none of them can mimic overt progressive striatal neuron death[123,124]. The transgenic HD monkey model is the first gene-modified monkey disease model[125], created by lentivirus injection into fertilized embryos. Lentivirus mediates transgene insertion into the host genome at random sites with uncontrollable copy numbers[126] such that the transgenic HD monkey model generated by this protocol displayed overt phenotypic heterogeneity. Among the first transgenic HD monkeys, animals with 84 CAG repeats showed severe phenotype at the early postnatal stage, while mice carrying the same length of CAG repeats merely displayed subtle symptoms[123,125]. However, lentivirus-mediated transgene expression is germline

transmissible with a stable expression level[127,128]. Importantly, the offspring of the founder animal displayed CAG repeat instability, a critical feature of HD[129]. In subsequent longitudinal investigations, researchers further revealed that this model could resemble progressive striatal and hippocampal morphometric changes as well as motor and cognition impairment, similar to clinical observation[130,131].

Recently, several transgenic swine HD models were also generated[132-134]; these models also showed more obvious neurodegeneration and motor disorders than that of mice with the same length of CAG repeats. Through CRISPR/Cas9-mediated knock-in (KI) on pig fibroblast cells and somatic cell nuclear transfer technique, a HD KI pig model was successfully created[135]. This model expresses 150 CAG repeats under the endogenous HTT promoter, leading to selective neurodegeneration as well as movement disorders, effectively mimicking typical HD pathology and clinic features. Importantly, similar to the transgenic HD monkey, the HD KI pig is also inheritable[135], making it possible to generate a large number of HD pigs in the future based on the potent reproductive capability of swine. Although the HD KI pig seems to be an ideal model, the macaque monkey model is more suitable for investigating emotional and psychiatric activity. Thus, combining HD pig and monkey models will bring us deeper insight into HD pathogenesis as well as advances in HD therapy.

CHALLENGES AND SOLUTIONS

With the development of gene editing technology, especially CRISPR/Cas9, several NHP models of NDs have been established and offer new insights into pathogenesis. For example, aging has been confirmed to be critical for age-dependent neurodegeneration, as expressing the disease proteins (Amyloid-β, Tau, and α-Synuclein) in the brains of old monkeys can faithfully recapitulate neuropathology[82,85,99]. Investigations on NHP models of NDs also revealed that species-dependent factors are critically involved in important pathological events. Depletion of PINK1 can lead to severe neuronal loss in the monkey brain but not in the mouse brain, which is largely due to abundant expression of PINK1 in the primate brain and undetectable level of PINK1 in the rodent brain[101,103]. The lack of cytoplasmic distribution of mutant TDP-43 in most mouse models is perhaps due to the absence of caspase-4, a primate-specific enzyme that can cleave TDP-43 to cause truncated TDP-43 to move from the nucleus to the cytoplasm[119]. With more in-depth studies and the establishment of additional non-human primate models of NDs, greater advances are expected, which will bring new insights into disease pathogenesis.

Despite the great progress that has been made, NHP models of NDs have not been used at a large scale. One key hurdle is that most models are hard to scale up for widespread application. Due to relatively low reproductive capability, a longer time for sexual maturity, and the absence of germline integrating ESC, it is challenging to generate NHP models by following rodent protocols to knock-in or knock-out the endogenous genes in one-cell stage embryos[7]. Although cloning of the macaque monkey[136], especially by somatic cell nuclear transfer[137,138], has been demonstrated to be feasible, the efficiency is suboptimal and remains to be improved. Since many NDs are caused by point mutation, newly emerging base editing and prime editing tools[139,140] will facilitate the generation of better animal models that can precisely mimic human genetic mutations. Recent research indicates that appropriate small molecules are able to direct human ESC to the early stage blastomere state[141], suggesting the opportunity to implement a germline integration strategy in non-human primates [Figure 1].

Aging is the biggest risk factor for ND incidence. When investigating animal models generated from germline genetic manipulations, the animals are likely to show phenotypes and neuropathology when they become old. In this regard, any treatments that can promote the aging process should presumably facilitate the development of disease phenotypes. Considering the high cost for maintaining non-human primates

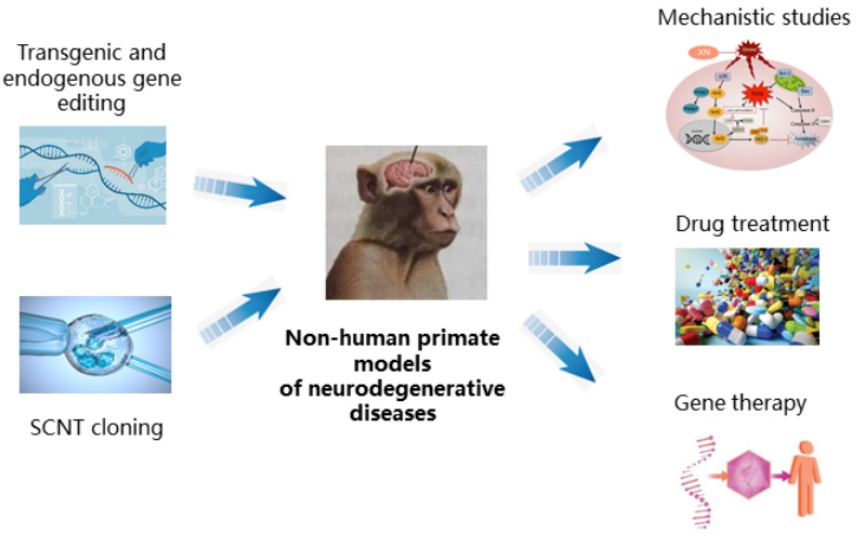

Figure 1. With the optimization of transgene and somatic cell nuclear transfer technique in non-human primate, more and better neurodegenerative disease monkey models will be generated in the future, which will in turn lead to promotion of molecular pathogenesis studies as well as treatment strategies such as drug development and gene therapy.

and their longer life span, it would be more appropriate to use monkeys at old ages to investigate NDs. Because NDs selectively affect distinct brain regions or specific types of neurons, it is feasible to selectively introduce genetic mutations in these brain regions or specific types of neurons by stereotaxic injection of viral vectors that can efficiently transduce neuronal cells. Recent studies using such a strategy have demonstrated its feasibility and generated several monkey models of NDs to offer novel findings that could not be obtained from small animal models[99,104,142]. Investigation of the neuropathology and mechanisms underlying selective neurodegeneration in non-human models should provide highly valuable information regarding pathogenesis for sporadic and familiar NDs, as both types of NDs share the same pathological and behavioral phenotypes. Uncovering the primate-specific factors that contribute to selective neurodegeneration is particularly important for the generation of humanized rodent models that can be widely used to investigate NDs and develop their therapies.

DECLARATIONS

Authors' contributions

Conceived the idea of this review article: Li XJ, Guo XY
Drafted the manuscript: Li B, He DJ, Guo XY
Revised the manuscript: Li XJ
Designed pictures: Guo XY
Completed the pictures: Li XJ

Available of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Ageing and Neurodegenerative Diseases

Review

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Genetically engineered pig models of neurological diseases

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Abstract

Genetically modified animal models are commonly used for *in vivo* studies of human diseases. Mice are the most common animal models used in biomedical research, which have provided important insights into disease pathogenesis and are widely used to find treatments for diseases. However, due to the differences in the anatomical structure and physiological function between human and mouse brains, most genetically modified mouse models cannot fully recapitulate the overt and selective neuronal loss seen in age-dependent neurodegeneration diseases. While non-human primates (NHP) are closer to humans and have been used to model human disease, these models are difficult to be utilized at a large scale due to various limitations including their high costs, prolonged breeding time, community concerns for use of NHP, and high ethical standards. As an important animal resource in agriculture, pigs are also used as animal models in biomedical research. The central nervous system of pigs is highly similar to that of humans, making pig models suitable for investigating neurological diseases. The relatively short breeding period, large litter size, and established somatic cell transfer technology are advantages over NHP for using pigs to model human diseases. The recent development of gene editing tools allows

one to more efficiently generate pig models that can precisely mimic genetic mutations in neurological diseases. In this review, we summarize recent advances in the use of pigs for modeling human neurological diseases, including new approaches for generating genetically modified pig models.

Keywords: Pig models, neurological diseases, gene editing, genetic modification, genome editing tools, disease models

INTRODUCTION

In vivo experiments using laboratory animals are essential for the verification of important findings from *in vitro* studies. In addition, animal models of human diseases are critical in revealing pathological changes and disease pathogenesis, which provide the theoretical basis for the development of treatments and therapeutic strategies. Small animal models such as mice and rats have been widely used in biomedical research, and animal models generated from mice have greatly advanced our understanding of the pathology and mechanisms of diseases. Small animals can partially mimic the symptoms and pathologic phenotypes of human disease, especially in extremely complex neurodegenerative diseases. That may be due to the considerable differences in development, aging, and fine structures between mouse and human brains. For example, the full development time for mouse brains is 21 days while primates' brains need more than 150 days to reach full maturation[1]. The short lifespan of rodents is another major difference that may cause the different presentation of the neuropathology, since mice can only live for a little over two years, which is much shorter than the human's average of 70 years. Therefore, the rapid development of the brain and the short lifespan of mice may cause neuronal cells to respond less strongly to the production of misfolded toxic proteins than do human neuronal cells. Differences in neural circuits and anatomical and physiological features between rodent and human brains suggest that we should explore other animal models to develop neurodegenerative diseases.

Undoubtedly, non-human primates (NHP) are ideal animal models that can closely mimic human diseases due to the high similarities between NHP and humans in genetics, physiology, development, social behaviors, and cognition. However, it is difficult to create a genetically modified NHP model when compared with small animals due to various factors, including long breeding cycles, lack of effective methods for genetic manipulations, high costs, community concerns, and high ethical standards. As a result, the first transgenic mouse model was generated as early as 1974[2], but the first genetically modified monkey model did not appear until 2001[3].

Considering the shortcomings of small animals and non-human primates in modeling human neurological diseases, pigs have some advantages over other species. Pig models have several unique features that make them a promising alternative animal model[4]. Pigs can produce larger litters and have a shorter maturation and reproduction time with fewer concerns about ethical issues and lower costs than non-human primates[5,6]. In regards to the similarity of pigs to humans, pigs are also highly close to humans in terms of anatomy, physiology, and metabolism[5]. As for the brain, the central nervous system of pigs is very similar to that of humans. For example, both human and pig brains have many sulci and gyri. Anatomically, the dorsal striata of the pig and human brains are both split into two distinct structures of the caudate nucleus and putamen, compared with a single structure in the rodent brain. In addition, the hippocampus in the pig brain more structurally resembles the human hippocampus than that in rodents. The timing of myelin formation in pig brains is also similar to that of humans during brain development[6] [Figure 1]. These similarities make the pig a better animal model for studying neurological diseases. In addition, pigs have the advantages of early sexual maturity (5-8 months), a short reproduction cycle between generations, and a

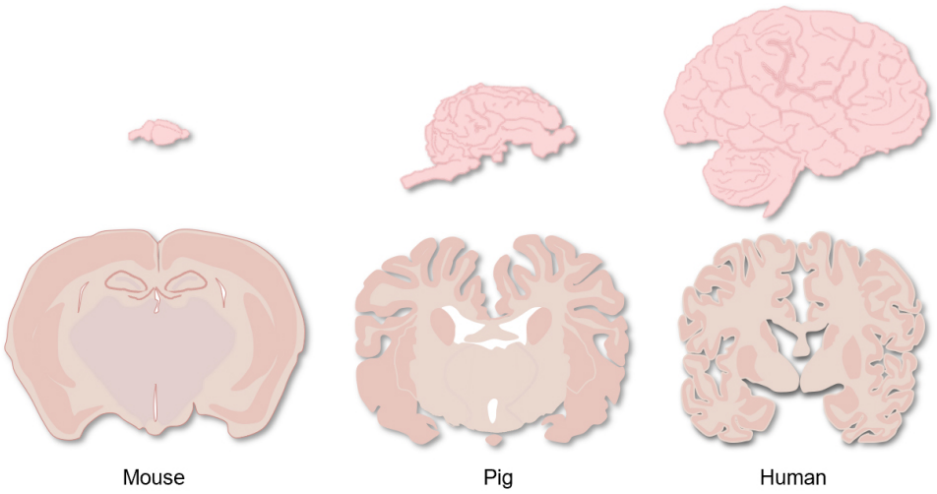

Figure 1. Comparison of brain structures of mouse, pig, and human.

larger litter size (about 10-12 piglets per litter)[7,8]. Moreover, fully established somatic cell nuclear transfer (SCNT) technology combined with recently developed genome editing technology has made it possible to efficiently generate genetically modified pig models[9] [Figure 2]. Here, we briefly discuss how to use related techniques to establish genetically modified pig models and review the established pig models for neurological diseases.

METHODS FOR GENERATING GENETICALLY MODIFIED PIG MODELS

For a long period of time, there have been two main methods to establish genetically modified pig models: embryonic microinjection and SCNT. Microinjection is a traditional method for creating transgenic animals and involves injecting DNA material directly into the pronucleus and transferring the early embryo into the surrogate mother to create a transgenic animal, which introduces transgenes randomly into the genome of the resulting offspring[10]. This method is fairly straightforward, but the efficiency of producing transgenic animals is relatively low, about 10% in mice, 4% in rabbits, and only 2%-3% in pigs[11,12]. Although several strategies have been used to improve the efficiency of embryonic microinjection, including pronuclei or cytoplasmic injection of DNA or mRNA[13,14], there are still many difficulties in using this method to generate genetically modified pig models. For example, due to the high lipid content and low transparency in pig oocytes[15], it is difficult to perform embryonic microinjection. In addition, this method will lead to random integration and poor precision of gene targeting. To improve the accuracy of gene editing, researchers developed a gene targeting strategy using homologous recombination (HR) in embryonic stem (ES) cells, which greatly improves the efficiency of generating gene-targeted animal models[16,17]. The lack of ES cells in pigs hinders the generation of precise genetically modified pig models. To overcome this difficulty, researchers firstly screen and identify the precisely targeted transgenes in cultured pig cells and then use them for SCNT, making it possible to establish gene-targeted pig models. However, the efficiency of HR in modifying pig somatic cells is very low, and the fatality rate is high due to the intrinsic genetic defects[18]. Later, an attempt to improve the efficiency of pig gene targeting was made by the application of several important technologies, including the delivery of gene-targeting vectors using recombinant adeno-associated virus (rAAV)[19,20].

GENOME EDITING TOOLS

Due to low targeting efficiency, for a long time, only a few transgenic pig models had been successfully

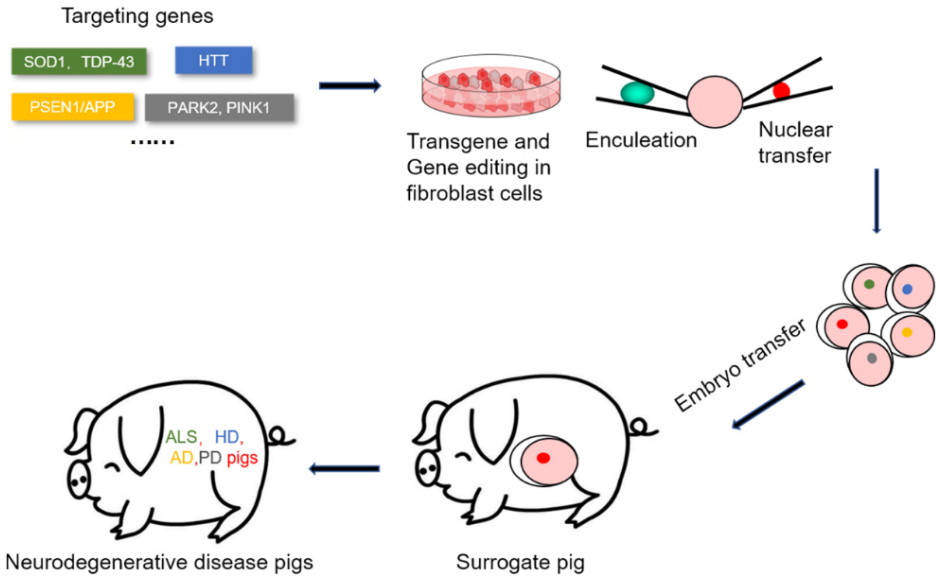

Figure 2. Flow chart of transgenic and gene editing using SCNT to construct neurodegenerative disease pig models. SCNT: Somatic cell nuclear transfer.

established[21-24]. This situation was greatly improved with the development of new precise gene editing tools, which include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas). ZFNs, composed of DNA-binding domains consisting of tandem zinc finger motifs with nuclease domains from the endonuclease FokI, can induce the targeted DNA double-stranded breaks (DSBs) that lead to DNA damage repair mechanisms[25,26]. Although ZFNs have been widely applied in many species, including plants, animals, and mammalian cells in culture[25], they have not been used to create large animal models.

TALENs are an alternative tool for genome engineering[27-29]. They are also fusion proteins of tandem repeats of a TAL effector protein and the FokI nuclease. TALENs induce the targeted DSBs that activate DNA damage response pathways and lead to gene knockout (KO) or knock-in (KI)[30]. As compared with ZFNs, TALENs are easier to design and synthesize, and some animal models of disease have been successfully established using TALENs[31].

Although ZFNs and TALENs have been applied to various species, CRISPR/Cas9 is now the most widely used genome editing tool for generating genetically modified animal models. The CRISPR/Cas9 system confers targeted gene editing by small RNAs that guide the Cas9 nuclease to the target site through base pairing[32]. When the complex is located at the targeting site of the genome, Cas9 cuts both strands at a precise location. Then, the repair mechanism kicks in to rejoin the damaged genomic DNA by non-homologous end joining (NHEJ) or homology-directed repair (HDR), which may result in mutations to inactivate or alter gene function. Based on this damage-repair mechanism, scientists have optimized the CRISPR/Cas9 system to create many genome editing models for small animals, such as mice[33], rats[34], and zebrafishes[35]. Large animal models such as pigs have also benefited from this technology. Here, we focus on genetically modified pig models of neurological diseases.

Pig models of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset progressive neurodegenerative disease caused by the selective death of motor neurons (MNs). With the occurrence of aging, patients with ALS develop

progressive loss of upper and lower MNs, muscle atrophy, and eventually paralysis, and they usually die within 3-5 years after the onset of symptoms[36,37]. Currently, the pathophysiological mechanism of ALS remains to be fully understood. Genetic studies have identified more than 30 gene mutations that are highly associated with the etiology of ALS, including copper/zinc superoxide dismutase 1 (*SOD1*) and TAR DNA-binding protein 43 (TDP-43). Mutations of these genes affect many cellular and molecular processes, leading to increased oxidative stress, mitochondrial dysfunction, excitatory toxicity, neuroinflammation, protein aggregation, and abnormal RNA metabolism. The neuropathology of ALS is characterized by protein aggregation and accumulation of ubiquitinated protein inclusion bodies in the neuronal cytoplasm. In most ALS patients, *SOD1* and *TDP-43* are the main components of these inclusion bodies, suggesting that *SOD1* and *TDP-43* are causative factors for the occurrence and development of ALS. Therefore, several studies have generated pig models that express mutant *SOD1* or *TDP-43* and showed ALS-like phenotypes.

Chieppa *et al.* produced an ALS pig model using SCNT in combination with transfected somatic cells expressing the G93A mutation of human *SOD1*[38]. In 2014, Yang *et al.* used similar techniques to generate transgenic pigs that express the same *SOD1* mutation. The transgenic pigs developed age-dependent neuropathology and movement disorders, which recapitulate the features of the early disease symptoms seen in human ALS[9]. Moreover, transgenic mutant *SOD1* pigs show intranuclear inclusions and an association of *SOD1* with the nuclear protein PCBP1, which were not seen in mouse brains[9]. In addition to *SOD1*, researchers also established transgenic miniature pigs expressing mutant *TDP-43*. They found that transgenic *TDP-43* was also distributed in the cytoplasm of neuronal cells resembling the pathology seen in human ALS brain tissues[39], which was not found in many transgenic *TDP-43* mouse models[40-42]. Therefore, these pig models of ALS have a great value in studying the pathogenesis mediated by cytoplasmic mutant *TDP-43* or intranuclear *SOD1*.

Pig models of Huntington's disease

Huntington's disease (HD) is an autosomal dominant and age-dependent neurological disorder characterized by motor dysfunction, cognitive decline, and psychological disturbance. Pathologically, HD is characterized by selective neurodegeneration, which preferentially occurs in the striatum. Most HD patients develop symptoms in middle age, and the symptoms worsen with age with patients usually dying 10-15 years after symptom onset[43]. HD results from a monogenetic mutation of a CAG repeat expansion in the exon1 of the gene Huntingtin (*HTT*). *HTT* is a multifaceted protein that is expressed ubiquitously and has numerous roles[44]. CAG repeat expansion (> 36 CAGs) in the *HTT* gene is translated to a polyglutamine (polyQ) expansion that causes *HTT* to misfold and aggregate in the brain. HD transgenic mice and HD-KI mice have been widely used, but their brains do not display the selective and striking neuronal loss seen in human HD patients[45].

In 2001, a transgenic pig model for HD was produced by pronuclear microinjection. However, the development of behavioral and neuropathological symptoms of HD in this transgenic pig model remains unclear[46,47]. In 2010, researchers used SCNT to successfully establish a transgenic HD pig model expressing N-terminal mutant *HTT* (1-208 amino acids) with 105Q. This pig model showed apoptosis in the brain and died postnatally. However, mice expressing the same transgene did not produce the brain pathology seen in pigs[48]. Later, another group used lentiviral transduction of pig embryos to establish a transgenic minipig model of HD expressing N-terminal mutant huntingtin (1-548 aa) under the control of human *HTT* promoter. However, this pig model did not develop motor deficits at up to 40 months of age, although mutant *HTT* mRNA and protein fragments were detected in the brain and peripheral tissues[49].

It is apparent that the phenotypes of transgenic HD pig models are dependent on the expression levels of transgenic N-terminal mutant *HTT*. It is important to create a pig model that expresses full-length mutant *HTT* at the endogenous level. With the development of CRISPR/Cas9 technology, precise gene editing of various animal species becomes possible[33], especially for the generation of large animal models[32]. To overcome the shortcomings of the transgenic pig model of HD, Yan *et al*. first used CRISPR/Cas9 to insert a large CAG repeat (150 CAGs) into the pig *HTT* locus in fibroblast cells, and then used SCNT to generate a HD knock-in pig model[50]. The brains of this pig model showed severe and preferential neurodegeneration in the medium spiny neurons in the striatum, an important pathological feature in HD patients. More importantly, the HD pig models displayed dance-like symptoms and breathing difficulties, which were similar to the symptoms in HD patients. Further, the pathogenic and neurologic features of HD pigs can be stably passed to offspring, enabling the establishment of a large animal model of HD for mechanistic study and drug screening.

Pig models of Alzheimer's disease

The incidence rate of Alzheimer's disease (AD) is increasing year by year with aging. Its early neurological symptoms are mainly memory loss and behavioral changes, and, in the late stage, the patients will have cognitive impairment, which severely affects daily life[51]. AD is usually divided into familial AD (FAD) and sporadic AD (SAD) according to different pathologies. Only about 5% of AD cases are FAD and are caused by mutations in β-amyloid precursor protein (APP), presenilin 1 (*PS1*), and/or presenilin 2 (*PS2*). Nearly 95% of patients with AD are classified as SAD, which is caused by a combination of genetic factors and environmental risk factors without documented familial history of AD[52]. The deposition of β-amyloid (Aβ) and hyperphosphorylation of Tau are the major pathological hallmarks, with other pathophysiologic changes including neuroinflammation, oxidative stress, and abnormal lipid metabolism. In addition to Aβ and Tau, apolipoprotein E4 (*APOE4*) and coulomb-receptor expressed on myeloid cells 2 (*TREM2*) are considered to be the risk factors[53]. Various mouse models of AD have been developed to mimic the symptoms of AD. However, due to the complexity of the neuropathology spectrum of AD, none of the available mouse models truly recapitulate the full spectrum of AD neuropathology, which includes Aβ deposition, synapse loss, inflammation, tau hyperphosphorylation, and neurofibrillary tangle formation[54]. To model the characteristics of AD in more human-like species, researchers injected Aβ oligomers into the lateral ventricle of macaques, which diffused into the brain and accumulated in several regions associated with memory and cognitive functions. They found that oligomer injections induced AD-like pathology with neurofibrillary tangle formation in the macaque brain, which was not found in small animal models[55]. Other researchers also used viral delivery of human 4R-tau to generate a tau-based rhesus monkey model of Alzheimer's disease[56]. However, due to the long reproductive cycle of monkeys and immature cloning technology, it was difficult to obtain a large group of monkey models of AD through transgenic methods. Therefore, the establishment of transgenic pig models of AD is needed.

In 2009, Kragh *et al*. tried to develop a pig model of Alzheimer's disease by expressing AD-causing dominant mutation APP^{sw}. The transgene consisted of the cDNA of the neuronal variant of the human *APP* gene with the Swedish mutation. However, no disease phenotype was reported, although it was predicted that accumulation of the Aβ peptide in the brain might develop at the age of 1-2 years[57]. The same group also generated a transgenic miniature pig model expressing a cDNA of the AD-causing gene *PSEN1M146I* driven by an enhanced human UbiC promoter. However, no phenotypic data have been published yet[46,58]. To induce the neuropathology of the increased intraneuronal Aβ plaque formation, this group combined the mutation of *PSEN1* and *APP* together to generate double transgenic Göttingen minipigs that carry one copy of a human PSEN1 cDNA with the Met146Ile (PSEN1M146I) mutation and three copies of a human A βPP695 cDNA with the Lys670Asn/Met671Leu (AβPPsw) double mutations. Their strategy successfully generated a pig model with an intraneuronal accumulation of Aβ42 in the brain between the age of 10 and

18 months, which may represent an early event in the pathogenesis of AD[59]. In 2017, another group used a retroviral multi-cistronic vector to generate an AD transgenic pig carrying three AD-related genes with a total of six well-characterized mutations: *hAPP* (K670N/M671L, I716V, and V717I), *hTau* (P301L), and *hPS1* (M146V and L286P). They confirmed that transgenes were expressed at especially high levels in the brain. The levels of Aβ-40/42, total Tau, and GFAP were high in the brains of these transgenic animals as well. They proposed that more tests are needed in the future to find out if these pigs have age-dependent phenotypes of AD[60].

Pig models of Parkinson's disease

Parkinson's disease (PD), characterized by slowness of movement, limb stiffness, and tremors, is the second most common neurodegenerative disorder in the world. PD patients may also have issues such as cognitive issues, depression, anxiety, olfactory loss, and gastrointestinal disorder. The motor symptoms of PD are caused by the death of dopaminergic neurons in the substantia nigra[61]. Loss of dopamine neurons causes a drop in dopamine levels in the striatum, which leads to disrupted motor control[62]. Many mutations or variants in a number of genes, such as α-synuclein (SNCA), leucine-rich repeat kinase 2 (*LRRK2*), ten-induced kinase 1 (*PINK1*), arkin (*PRKN*), and protein deglycase (*DJ-1*), are found to increase the susceptibility to PD and have been used to create genetically modified animal models of PD[62,63]. However, many mouse models do not recapitulate the selective and progressive neurodegeneration seen in PD[64,65]. Although non-human primate models of PD have been established for investigation[66,67], it is difficult to establish a cohort of PD monkey models. Some teams thus explored the generation of pig models to study the neurological phenotypes of PD.

Yao *et al.* used TALENs combined with SCNT and embryo transfer to generate *DJ-1* KO piglets by disrupting the *PARK7* gene to model the phenotype of PD. Unfortunately, the piglets all died due to cloning defects, although *DJ-1* protein was successfully repressed in all the detected tissues[68]. Another group used CRISPR/Cas9 combined with SCNT to generate *PARK2* and *PINK1* double-gene KO pigs. However, as with mouse PD models, no phenotypic symptoms of PD were observed in the seven-month-old live mutant pigs[69]. In 2016, Wang *et al.* generated a PD pig model using CRISPR/Cas9 system by simultaneously targeting three distinct genomic loci, *Parkin/DJ-1/PINK1*, in Bama miniature pigs. However, the piglets remained healthy with a normal growth rate, and no typical symptoms of Parkinson's disease were observed in the 10-month-old live mutant pigs in this study[70].

BASE EDITING USED IN PIG MODELS

Although the CRISPR/Cas9 system has been widely used to facilitate genome editing, it could induce random insertions or deletions (indels) through error-prone NHEJ rather than the error-free HDR[35]. As a result, indels are obtained much more frequently at targeting sites than single-nucleotide substitutions. However, most human neurological diseases are induced by point mutations, rather than indels[71], which emphasizes the importance of the application of the genome-editing technique of base editing in the establishment of animal models of human neurological disease.

Base editing is a genome-editing technique that generates mutations at single-base resolution[72-74]. All four transition mutations, namely C to T, G to A, A to G, and T to C, can be inserted into the genome with the available CRISPR/Cas base editors (BEs). The cytosine base editor (CBE) can insert a C-G to T-A mutation, while the adenine base editor (ABE) can alter an A-T base pair into a G-C pair. In RNA, conversion of A to inosine (I) is also possible with the RNA base editor (RBE)[75].

The above advanced technologies have already been used to generate many genome editing models, especially in small animals and plants, such as mouse[76,77], rat[78], rabbit[79], sheep[80], rice[81], and wheat[82]. Some groups have also succeeded in applying this tool to large animals[83,84].

As for pigs, Li *et al*. first established pig models created via BE3, which separately targeted the *TWIST2* gene and the *TYR* gene[85]. These pig models were able to reproduce the phenotypes of human diseases, which indicates that base editing systems provide a safer and more efficient approach to generating pig models that can precisely mimic point mutations of human diseases. Another study also indicated that using base editing technology was able to precisely introduce three gene (*GGTA1*, *B4galNT2*, and *CMAH*) base conversions into the pig genome with high efficiency[71]. In summary, there is enormous potential for establishing pig disease models of neurological disease through base editing because of its significant advantages compared with the traditional CRISPR/Cas9 system.

POTENTIAL LIMITATIONS OF USE OF PIG MODELS

Currently, pig models for neurodegenerative diseases provide considerable support for the analysis and treatment of such diseases in humans. In general, pig models have great potential to advance the study of human neurodegenerative diseases, from pathogenesis research to the development of drugs, and even as donors of tissues and organs.

In addition, while pig disease models have greatly accelerated advances in studying genetic diseases and testing drugs and treatments, there are still some problems. First, pigs require more space than rodents in animal facilities and, thus, higher maintenance costs. Second, due to their large size, surgical operations need to be performed by trained personnel, and because its brain is wrapped in a thick skull, the collection of brain tissue requires a high degree of proficiency of the operator, which increases the experimental cost to a certain extent. Third, because of their large size, behavioral tests will be more difficult. However, at present, various behavioral studies of pigs have been gradually improved, for instance, learning and memory study using novel object recognition tests; anxiety and depression measurement using open field[86]; neuropsychological screening for executive function, anxiety, willingness to explore a new environment, and locomotion using the open field test[87]; and motor ability measurement using a 3D kinematic gait analysis system[87].

CONCLUSION

A critical step in studying neurological diseases is to establish suitable animal models. Due to the complexity of neurological diseases, such as AD and PD, as well as the species differences between mice and humans, selective and overt neurodegeneration is not well modeled using mouse models. Pig models have great potential in modeling neurological diseases due to their close resemblance to the human nervous system, and several genetically modified pig models have been established for investigating neurodegenerative diseases [Table 1].

Pigs have very similar brain structure and function to humans. More importantly, pigs have sulci and gyri, and their brain volume is similar to that of humans, offering advantages over small animals for studying important brain diseases. Given their short reproductive cycle (5-6 months of sexual maturity) and multiple litter sizes (average of 7-8 piglets) as well as the availability of techniques for generating specific models of human diseases, pigs also have distinct advantages over non-human primates. Pigs can also be ethically used for translational research. For example, scientists and doctors recently successfully transplanted a pig heart into a patient with end-stage heart disease[88]. This work opened up a new avenue in the study of xenotransplantation.

Table 1. Examples of neurodegenerative disease pigs described in this article

Pig models	Genes	Editing type	References
ALS pig	*SOD1*	TG	[38]
ALS pig	*SOD1*	TG	[9]
ALS pig	*TDP-43*	TG	[39]
HD transgenic pig	*HTT*	TG	[47]
HD transgenic pig	*N-mHTT(105Q)*	TG	[48]
HD transgenic pig	*HTT(1-548)*	TG	[49]
HD KI pig	*HTT*	KI	[50]
AD transgenic pig	*APPsw*	PM	[57]
AD transgenic pig	*PSEN1(M146I)*	TG	[58]
AD transgenic pig	*PSEN1, APP*	PM	[59]
AD transgenic pig	*hAPP, hTau, hPS1*	PM	[60]
PD pig	*PARK7*	KO	[68]
PD pig	*PARK2, PINK1*	M-KO	[69]
PD pig	*Parkin, DJ-1, PINK1*	M-KO	[70]

The table lists genes that have been changed using TG, KO, M-KO, PM, or KI. TG: Transgenic; KO: knockout; M-KO: multiplex knockout; KI: knock-in; PM: point mutation (by HDR); ALS: amyotrophic lateral sclerosis; HD: Huntington's disease; AD: Alzheimer's disease; PD: Parkinson's disease.

The pig models can also be used for preclinical evaluation of stem cell therapy, gene therapy, and drug screening because their body size and metabolism are closer to humans than other species. Their relatively fast breeding and reproduction would provide a sufficient number of animals for evaluation of the therapeutic effects of drugs and other means. Considering the advanced gene editing tools available, we believe that genetically modified pig models will play a more important role in the studies of age-dependent neurological diseases in the future.

DECLARATIONS

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Authors' contributions

Wrote the review paper: Li C, Li J
Revised manuscript: Li S, Yan S
Conceived and designed experiments: Yan S, Lai L
All authors read and approved the final manuscript.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Exploring the causal relationship between dietary macronutrients and neurodegenerative diseases: a bi-directional two-sample Mendelian randomization study

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Abstract

Aim: The associations between dietary macronutrient intake and neurodegenerative diseases (NDDs) have been widely reported; however, the causal effect remains unclear. The current study aimed to estimate the causal relationship between dietary macronutrient intake (i.e., carbohydrate, fat, and protein) and NDDs [e.g., Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS)].

Methods: Mendelian randomization (MR) was applied to evaluate the causal relationship between dietary macronutrient intake and NDDs. We used the single-nucleotide polymorphisms strongly associated ($P < 5 \times 10^{-8}$) with the exposures from the genome-wide association studies as instrumental variables. Inverse-variance

weighted, MR-Egger, weighted median, and the MR pleiotropy residual sum and outlier were used to verify the MR assumptions.

Results: Genetically predicted higher carbohydrate intake was associated with an increased risk of ALS [odds ratio (OR), 2.741, 95% confidence interval (CI): 1.419-5.293, *P* = 0.003). Vulnerability to PD was negatively associated with the relative intake of fat (OR, 0.976, 95%CI: 0.959-0.994, *P* = 0.012) and protein (OR, 0.987, 95%CI: 0.975-1.000, *P* = 0.042). The study also identified the causal influence of AD on dietary carbohydrate intake (OR, 1.022, 95%CI: 1.011-1.034, *P* = 0.001).

Conclusion: We found solid evidence supporting the idea that a higher carbohydrate proportion causally increases ALS risk. Genetically predicted higher AD risk is causally associated with increased dietary carbohydrate intake. Vulnerability to PD may have a causal relationship with a decrease in the dietary intake of protein and fat.

Keywords: Dietary macronutrient intake, neurodegenerative diseases, Mendelian randomization, causality

INTRODUCTION

Neurodegenerative diseases (NDDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), have become one of the disease categories with the largest increase in regard to the global health burden in aging populations[1,2]. Regarding the most common NDDs, approximately 46.8 million people worldwide are estimated to be living with AD, and by 2030, it is estimated that approximately 74.7 million people might suffer from this disease[2]. Furthermore, the global prevalence rates of PD and ALS were 3 per 1000 population and 4.42 per 100,000 population, respectively[3,4]. In the absence of practical therapeutic approaches for NDDs, identifying causal risk factors can lead to progress in the prevention and treatment of these diseases.

In previous studies, metabolic health has been reported to be closely associated with the prevalence of NDDs[5,6]. For example, glucose metabolism dysregulation has been considered a critical regulatory element for the progression of NDDs[7-9]. The primary sources of energy intake are carbohydrates, fat, and protein, and an individual's metabolism of these energy sources is one of the major determinants of the development of NDD risk factors, including diabetes, cardiovascular disease, hypertension, and obesity[10-12]. Appropriate diet composition plays a vital role in reducing these risk factors, thereby reducing the risk of developing NDDs. Hence, further research is needed to clarify the effects of the relative intake of macronutrients (i.e., carbohydrate, fat, and protein) on the risk of NDDs. Unfortunately, high cost and difficulty often hinder the conduct of clinical trials on the effects of macronutrient composition[13]. Additionally, confounding commonly occurs in observational studies, and it is inevitable for macronutrient intake to be influenced by bias[13].

In the absence of high-quality randomized controlled trials, Mendelian randomization (MR) can be considered an alternative approach to assessing the causal relationship between dietary macronutrient intake and NDDs[14]. MR is a novel technique that involves using genetic data to assess and estimate the causal effects of modifiable (nongenetic) risk factors based on observational data[15]. This method depends on the use of genetic variants that are randomly allocated during meiosis, and thus it can decrease susceptibility to measurement errors and largely overcome the limitations of reverse causation and residual confounding[16]. MR analysis has recently been used to explore the causal association between dietary micronutrients (i.e., mineral nutrition) and the risk of NDDs[17]. However, there is no MR study thus far related to dietary macronutrients, including carbohydrate, fat, and protein, or their proportions in one's diet.

The genome-wide association study (GWAS) is used to identify genomic variants that are statistically associated with a specific disease or trait. Single-nucleotide polymorphisms (SNPs), which occur more frequently in people with a certain disease or trait than in people without it, are regarded to be associated with the disease/trait. GWASs may report the association of each SNP with the outcome and provide an estimate of the causal effects on the outcome. In MR analysis, SNPs are considered instrumental variables (IV) to assess causal relationships between exposures (risk factors) and outcomes (diseases). These SNPs are used to calculate the "overall" causal effect of exposure on the risk of diseases[18].

Based on the reviewed literature, we hypothesized that gene-related differences in dietary macronutrient intake would increase susceptibility to NDDs. To test this hypothesis, we conducted a univariable bidirectional two-sample MR study based on publicly available GWAS summary data of dietary macronutrient composition (i.e., carbohydrate, fat, and protein)[19] and NDDs (i.e., AD, PD, and ALS)[20-22].

METHODS

In the present study, we performed a univariable bidirectional two-sample MR analysis to estimate the causal association between three dietary macronutrients (i.e., carbohydrate, fat, and protein) and NDDs (i.e., AD, PD, and ALS). A flowchart of the MR analysis is presented in Figure 1.

Data source

GWAS of dietary macronutrients

We collected summarized GWAS data on dietary macronutrients from the most recently published available studies (release from January 2021). The dietary macronutrient data used in our MR analysis were originally from the Social Science Genetic Association and included 268,922 participants aged 27-71 years. The subjects included in the dataset above were mainly of European ancestry. The data were based on self-report questionnaires containing questions on more than 70 food items in all cohorts. The self-report questionnaires were used to estimate the composition of the three macronutrients, i.e., the proportion of carbohydrate, fat, and protein to the total calories. Summary genetic association estimates were adjusted for educational attainment, the total number of dietary intake measurements, sex and birth year. Full details are provided elsewhere[19].

GWAS of AD, PD and ALS

The analysis used the genetic variants from the International Genomics of Alzheimer's Project (IGAP), including 21,982 AD cases and 41,944 controls[20]; the International Amyotrophic Lateral Sclerosis Genomics Consortium (20,806 ALS cases and 59,804 controls)[22]; and the International Parkinson's Disease Genomics Consortium (33,674 PD cases and 449,056 controls)[21] released on March 2019, March 2018, and December 2019, respectively. For AD GWAS summary data, clinically/neuro-pathologically defined AD might have a more robust/stronger genetic signal. In addition, PD and ALS GWASs were all from the largest Genomics Consortium. The individuals included in the datasets were of European ancestry. Principle covariates, such as age and sex, were adjusted in the association tests in all sources.

Statistical analysis

Selection of instruments

First, we selected the SNPs that reached the genome-wide significance threshold ($P < 5 \times 10^{-8}$) as instrumental variables (IVs) in this analysis[23,24]. Next, we excluded SNPs that were in linkage disequilibrium (r^2 threshold < 0.001 within a 10 Mb window) and extracted the retained SNPs from the outcome datasets[25]. Finally, we harmonized the exposure and outcome SNPs. We also calculated the F statistics to ensure the strength of the exposures, and an F statistic > 10 was considered robust enough against weak

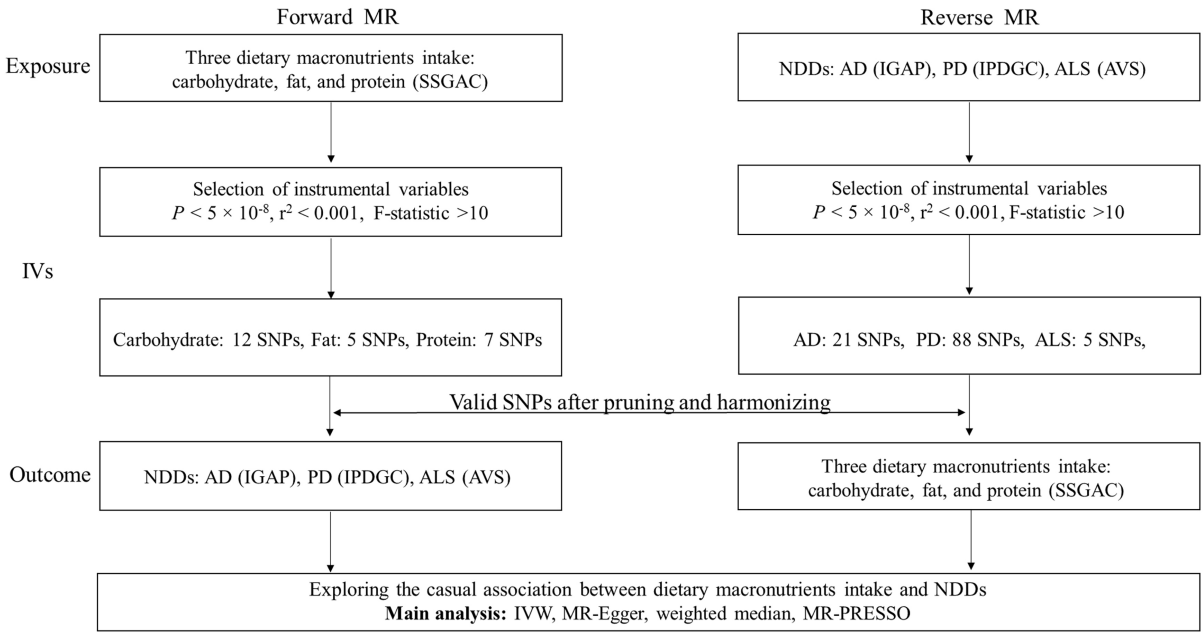

Figure 1. Flowchart of our bidirectional two-sample Mendelian randomization analysis. AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; AVS: ALS Variant Server; IGAP: the International Genomics of Alzheimer's Project; IPDGC: International Parkinson's Disease Genomics Consortium; IVW: inverse-variance weighted; MR-PRESSO: Pleiotropy Residual Sum and Outlier; PD: Parkinson's disease; SNP: single nucleotide polymorphism; SSGAC: Social Science Genetic Association.

instrument bias[26,27]. The R^2 and F statistics of each SNP were calculated according to the formulas $R^2 = 2 \times EAF \times (1\text{-}EAF) \times \beta^2$ and F statistics = $R^2 \times (N\text{-}2)/(1\text{-}R^2)$. Then, we summed them to estimate the overall R^2 and F statistics[28,29] [Table 1].

MR analyses

Data were analyzed between January and April 2022. The inverse-variance weighted (IVW) method was used as the primary analysis approach to assess possible causal effects[30]. Then, we used three alternative methods of two-sample MR [i.e., MR-Egger, weighted median (WM), and the MR pleiotropy residual sum and outlier (MR-PRESSO)] to address variant heterogeneity and pleiotropy effects. The IVW method would be considered the best causal estimation if none of the instruments were found to have substantial heterogeneity or horizontal pleiotropy[31]. The MR-Egger regression and MR-PRESSO global test were used as the main estimation to account for potential pleiotropy[32]. The WM approach was regarded as valid when there was large heterogeneity across all SNPs without horizontal pleiotropy[33]. The MR-PRESSO method provides a correction test by detecting and removing potentially pleiotropic outliers[34]. A predefined decision tree to select methods could yield the best result and was adapted from[35] [Figure 2B]. Cochran's Q statistic evaluated heterogeneity across genetic variants. A Cochran's Q-derived $P < 0.05$ was considered to indicate heterogeneity[36]. The MR-Egger intercept test, as well as the MR-PRESSO global test, was also conducted, and a P value < 0.05 indicated that the IVW results might be invalid due to horizontal pleiotropy[32]. An overview of the MR design is presented in Figure 2.

The MR results were expressed as odds ratios (ORs) with 95% confidence intervals (CIs) interpreted as the estimated effect of a 1-SD change in dietary macronutrients on NDD risk (or the preference of dietary macronutrients per SD increase in log odds of NDDs). All statistical analyses were performed using Rstudio (R version 4.1.1) with the packages "TwoSampleMR" and "MR-PRESSO"[37]. Power calculation was performed using the online power calculator (mRnd)[38] (https://cnsgenomics.com/shiny/mRnd/).

Table 1. The R^2 and F-statistics for the genetic instruments and the power for MR

Exposure		Outcome		nSNP	R^2	F-statistic	Power
Trait	**Sample size**	**Trait**	**Sample size**				
The forward MR analysis							
Carbohydrate intake (%)	268,922	AD	63,926	9	0.0013	359.73	59.2%
Fat intake (%)	268,922	AD	63,926	5	0.0018	478.11	63.9%
Protein intake (%)	268,922	AD	63,926	7	0.0015	411.24	62.7%
Carbohydrate intake (%)	268,922	PD	1,456,300	12	0.0018	484.34	100%
Fat intake (%)	268,922	PD	1,456,300	5	0.0018	478.11	100%
Protein intake (%)	268,922	PD	1,456,300	7	0.0015	411.24	100%
Carbohydrate intake (%)	268,922	ALS	80,610	11	0.0016	441.80	83.2%
Fat intake (%)	268,922	ALS	80,610	5	0.0018	478.11	65.6%
Protein intake (%)	268,922	ALS	80,610	7	0.0015	411.24	51.1%
The reverse MR analysis							
AD	63,926	Carbohydrate intake (%)	268,922	18	0.2424	16071.68	100%
AD	63,926	Fat intake (%)	268,922	17	0.2339	15524.88	100%
AD	63,926	Protein intake (%)	268,922	18	0.2424	16071.68	100%
PD	1,456,300	Carbohydrate intake (%)	268,922	85	0.3085	445039.20	100%
PD	1,456,300	Fat intake (%)	268,922	85	0.3085	445039.20	100%
PD	1,456,300	Protein intake (%)	268,922	85	0.3085	445039.20	100%
ALS	80,610	Carbohydrate intake (%)	268,922	5	0.0323	2578.97	100%
ALS	80,610	Fat intake (%)	268,922	5	0.0323	2578.97	100%
ALS	80,610	Protein intake (%)	268,922	5	0.0323	2578.97	100%

MR: Mendelian randomization; AD: Alzheimer's disease; PD: Parkinson's disease; ALS: amyotrophic lateral sclerosis; nSNPs: number of single-nucleotide polymorphisms; R^2: Variance explained by the SNPs on exposure.

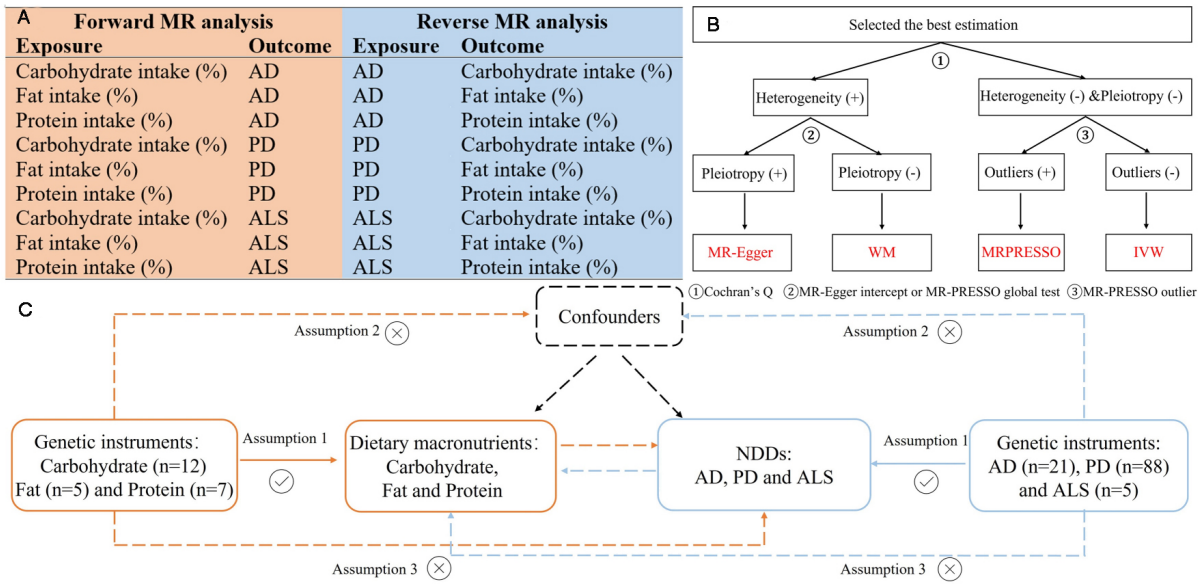

Figure 2. Overview of the design of the present study. (A) We conducted the forward and reverse MR study to explore the bi-directional relationship between dietary macronutrient intake (%) and NDDs. (B) Predefined decision tree for the selection of methods. (C) Sketch of the study design. Assumption 1: the genetic variants are supposed to be strongly associated with the risk of interest; Assumption 2: the genetic variants should not be associated with any confounding factors; and Assumption 3: the genetic variants should affect the risk of the outcome only mediated by the exposures. ALS: Amyotrophic lateral sclerosis; IVW: inverse-variance weighted; WM: weighted median; MR-PRESSO: Pleiotropy Residual Sum and Outlier.

RESULTS

Details of the SNPs used as IVs are presented in Supplementary Tables 1-6. The causal effect estimates of the IVW, MR-Egger, WM, and MR-PRESSO are available in Figures 3 and 4 and in Tables 2 and 3.

The causal effect of carbohydrate intake on NDDs

Considering the best causal estimation, ALS showed evidence of being influenced by carbohydrate intake. The effect estimate of carbohydrate intake on ALS risk was indicated by OR = 2.741 (95%CI: 1.419-5.293, P = 0.003) according to the WM method. As shown in Figure 5, the scatter plot and forest plot visually displayed the relationships between carbohydrate intake and ALS risk. The funnel and leave-one-out plot are also presented in Figure 5. We found that genetic predispositions to carbohydrate intake were not related to AD (IVW: OR = 0.875, 95%CI: 0.409-1.871, P = 0.730) or PD (WM: OR = 0.604, 95%CI: 0.260-1.403, P = 0.241). Cochran's Q test revealed strong pleiotropy for carbohydrate intake on PD and ALS (P_{PD} = 7.33E-07, P_{ALS} = 0.016). The MR Egger intercept test suggested no pleiotropic effects in this analysis, with the results of AD (P = 0.518), PD (P = 0.382) and ALS (P = 0.168). The MR-PRESSO global test also failed to reveal substantial pleiotropy, with P values all greater than 0.05.

The causal effect of fat intake on NDDs

The causal effect estimates in the IVW method were OR = 0.837 (95%CI: 0.446-1.574, P = 0.582), 1.904 (95%CI: 0.883-4.109, P = 0.101), and 0.798 (95%CI: 0.453-1.405, P = 0.435) for AD, PD, and ALS, respectively. No directional pleiotropy or heterogeneity was detected by the MR Egger intercept test and Cochran's Q statistic. The MR-PRESSO global test also showed no evidence that suggested horizontal pleiotropy (P_{AD} = 0.255, P_{PD} = 0.339, and P_{ALS} = 0.289).

The causal effect of protein intake on NDDs

All models consistently suggested that genetically predicted protein intake failed to be associated with the three disorders (AD: OR, 0.774, 95%CI: 0.432-1.384, P = 0.387, IVW; PD: OR, 1.026, 95%CI: 0.422-2.499, P = 0.954, WM; ALS: OR, 0.978, 95%CI: 0.567-1.688, P = 0.937, IVW). Cochran's Q test for protein intake on PD risk revealed substantial heterogeneity across SNPs (P = 0.002). The MR-Egger intercept test and MR-PRESSO global test indicated no remarkable horizontal pleiotropy for protein intake of NDDs.

The causal effect of AD on dietary macronutrient intake

The effect estimates for AD and dietary macronutrients were OR = 1.022 (95%CI: 1.011-1.034, P = 0.001, MR-Egger), OR = 0.990 (95%CI: 0.977-1.003, P = 0.135, WM), and OR = 1.000 (95%CI: 0.992-1.008, P = 0.949, IVW) for dietary intake of carbohydrate, fat, and protein, respectively. There was significant heterogeneity for relative fat intake (P = 0.018). In addition, the MR Egger intercept test detected the presence of horizontal pleiotropy for relative carbohydrate intake (P = 0.017).

The causal effect of PD on dietary macronutrient intake

Genetically predicted higher PD risk was negatively associated with the dietary intake of fat (OR: 0.976, 95%CI: 0.959-0.994, P = 0.012, MR Egger) and protein (OR: 0.987, 95%CI: 0.975-1.000, P = 0.042, WM) but not with carbohydrate intake (OR: 0.999, 95%CI: 0.979-1.020, P = 0.958, MR Egger). Substantial heterogeneity was detected by Cochran's Q test, with the P values of 1.05E-05 for carbohydrate, 0.004 for fat, and 3.08E-04 for protein. In addition, regarding the effect of PD on the dietary intake of fat, the intercept test in MR-Egger found evidence of unbalanced pleiotropy (P = 0.021). The MR-PRESSO global test also indicated strong evidence of directional horizontal pleiotropy for carbohydrate and fat intake. However, the MR-PRESSO results were nonsignificant after correcting for outliers.

Table 2. Forward causal relations of the dietary macronutrient composition with NDDs performed by MR

Exposure	nSNPs	Method	OR (95%CI)	*P*	Q pval	Intercept pval	Global *P*
Carbohydrate intake (%) *vs.* AD	9	**IVW**	**0.875 (0.409, 1.871)**	**0.730**	0.067		
		MR Egger	6.536 (0.019, 2259.250)	0.549		0.518	
		MR-PRESSO	0.958 (0.499, 1.836)	0.899			0.108
		WM	1.029 (0.462, 2.293)	0.944			
		Simple mode	0.989 (0.278, 3.522)	0.987			
		Weighted mode	1.104 (0.328, 3.710)	0.877			
Carbohydrate intake (%) *vs.* PD	12	IVW	0.558 (0.146, 2.134)	0.394	7.33E-07		
		MR Egger	0.009 (1.36E-06, 65.922)	0.326		0.382	
		MR-PRESSO	0.558 (0.146, 2.134)	0.412			0.31
		WM	**0.604 (0.260, 1.403)**	**0.241**			
		Simple mode	0.569 (0.168, 1.931)	0.385			
		Weighted mode	0.588 (0.206, 1.681)	0.343			
Carbohydrate intake (%) *vs.* ALS	11	IVW	2.016 (1.138, 3.570)	0.016	0.016		
		MR Egger	8.835 (0.855, 20.823)	0.094		0.168	
		MR-PRESSO	1.974 (1.170, 3.333)	0.027			0.241
		WM	**2.741 (1.419, 5.293)**	**0.003**			
		Simple mode	3.378 (1.160, 9.842)	0.049			
		Weighted mode	3.344 (1.174, 9.527)	0.047			
Fat intake (%) *vs.* AD	5	**IVW**	**0.837 (0.446, 1.574)**	**0.582**	0.152		
		MR Egger	1.267 (0.393, 4.083)	0.718		0.464	
		MR-PRESSO	0.837 (0.446, 1.574)	0.611			0.255
		WM	1.042 (0.552, 1.966)	0.900			
		Simple mode	1.322 (0.500, 3.495)	0.604			
		Weighted mode	1.245 (0.637, 2.431)	0.557			
Fat intake (%) *vs.* PD	5	**IVW**	**1.904 (0.883, 4.109)**	**0.101**	0.194		
		MR Egger	2.601 (0.561, 12.055)	0.309		0.664	
		MR-PRESSO	1.904 (0.883, 4.109)	0.176			0.339
		WM	2.251 (1.059, 4.785)	0.035			
		Simple mode	2.153 (0.811, 5.718)	0.199			
		Weighted mode	2.190 (1.040, 4.614)	0.108			
Fat intake (%) *vs.* ALS	5	**IVW**	**0.798 (0.453, 1.405)**	**0.435**	0.205		
		MR Egger	1.142 (0.390, 3.344)	0.824		0.489	
		MR-PRESSO	0.798 (0.453, 1.405)	0.478			0.289
		WM	0.913 (0.500, 1.666)	0.766			
		Simple mode	1.042 (0.423, 2.565)	0.933			
		Weighted mode	1.013 (0.511, 2.010)	0.972			
Protein intake (%) *vs.* AD	7	**IVW**	**0.774 (0.432, 1.384)**	**0.387**	0.295		
		MR Egger	0.459 (0.033, 6.303)	0.585		0.704	
		MR-PRESSO	0.774 (0.432, 1.384)	0.420			0.304
		WM	0.594 (0.288, 1.226)	0.159			
		Simple mode	0.627 (0.182, 2.158)	0.487			
		Weighted mode	0.566 (0.213, 1.499)	0.296			
Protein intake (%) *vs.* PD	7	IVW	0.617 (0.174, 2.182)	0.454	0.002		
		MR Egger	5.336 (0.024, 1202.346)	0.571		0.458	
		MR-PRESSO	0.617 (0.174, 2.182)	0.482			0.071
		WM	**1.026 (0.422, 2.499)**	**0.954**			
		Simple mode	1.012 (0.257, 3.984)	0.987			

Protein intake (%) *vs.* ALS	7	Weighted mode	1.247 (0.432, 3.596)	0.697		
		IVW	**0.978 (0.567, 1.688)**	**0.937**	0.303	
		MR Egger	6.186 (0.760, 50.366)	0.149	0.136	
		MR-PRESSO	0.978 (0.567, 1.688)	0.940		0.36
		WM	1.380 (0.717, 2.655)	0.335		
		Simple mode	1.365 (0.480, 3.882)	0.581		
		Weighted mode	1.459 (0.611, 3.487)	0.428		

The selected model is written in bold. NDDs: Neurodegenerative diseases; MR: Mendelian randomization; AD: Alzheimer's disease; PD: Parkinson's disease; ALS: amyotrophic lateral sclerosis; nSNPs: number of single-nucleotide polymorphisms; OR: odds ratio; CI: confidence interval; Q pval: *P*-value of the Cochran Q statistic; IVW: inverse-variance weighted; WM: weighted median; MR-PRESSO: Pleiotropy Residual Sum and Outlier.

The causal effect of ALS on dietary macronutrient intake

Based on the IVW method, we failed to find evidence to support a potential causal relationship between ALS and the relative dietary macronutrient intake, with ORs and 95%CIs of 0.998 (95%CI: 0.970, 1.027), 1.009 (95%CI: 0.987, 1.033), and 1.017 (95%CI: 0.994, 1.041) for carbohydrate, fat, and protein, respectively. For all these estimates, the MR-Egger intercept and MR-PRESSO global test did not indicate the pleiotropic effects of the SNPs in the MR study. Cochran's Q tests also did not provide evidence of heterogeneity.

For SNP conformity, we conducted a leave-one-out analysis and generated forest maps. The forest map indicated stable results [Figure 5C and Supplementary Figures 1-6]. The F statistics for all the SNPs ranged from 28 to 50,488 across the forward and reverse MR analyses, and these values were higher than the conventional threshold of 10, the rule of thumb to distinguish between strong and weak instruments. The statistical power was also calculated, and the results were all higher than 50%. The R^2 and F-statistics for the IVs and the power for MR are shown in Table 1.

DISCUSSION

In this work, we used MR to investigate the causal relationships between dietary macronutrient intake (carbohydrate, protein, and fat) and the most common NDDs (AD, PD, and ALS). It was shown that genetic predisposition to higher carbohydrate intake was related to the increased risk of ALS. Moreover, we found that vulnerability to PD was negatively associated with protein and fat intake. The study also found a potential causal influence of AD on dietary carbohydrate intake.

According to a previous study, high carbohydrate intake may increase the risk of ALS[39]. Regarding the prognosis of the disease, carbohydrate intake was also found to be positively related to the short-term survival of ALS[40]. ALS patients were reported to be affected by glucose metabolism abnormalities, which could be explained by the deficit of the insulin-mitochondrial axis, a glucose-metabolizing pathway[9,41-43]. On the other hand, abnormal glucose tolerance may result from muscle wasting or physical inactivity in ALS patients, leading to a decreased ability to promptly store a large glucose load[44,45]. Therefore, there might exist a vicious cycle between carbohydrate intake and ALS risk that accelerates the progression of the disease. However, two prospective studies recently reported a decreased risk of ALS in persons who were obese and overweight[46,47]. A randomized controlled trial identified the benefits of a high-carbohydrate diet on the progression of ALS via weight gain, supporting a high-carbohydrate diet as a promising nonpharmacologic intervention for ALS[48]. Additionally, studies in a mouse model of ALS have shown that a high-carbohydrate hypercaloric diet increases weight and delays disease progression[49,50]. Therefore, the causal relationship between high carbohydrate intake and the risk of ALS is still unknown. Our study implemented an MR approach with a robustly validated genetic instrument for relative carbohydrate intake and provided robust evidence to support that high carbohydrate intake might cause an increased risk of

Table 3. Reverse causal relations of NDDs with the dietary macronutrient composition performed by MR

Exposure	nSNPs	Method	OR (95%CI)	*P*	Q pval	Intercept pval	Global *P*
AD *vs.* Carbohydrate intake (%)	18	IVW	1.012 (1.004, 1.020)	0.003	0.608		
		MR Egger	**1.022 (1.011, 1.034)**	**0.001**		0.017	
		MR-PRESSO	1.010 (1.002, 1.018)	0.026			0.342
		WM	1.012 (1.001, 1.023)	0.040			
		Simple mode	1.010 (0.991, 1.030)	0.329			
		Weighted mode	1.011 (0.999, 1.024)	0.091			
AD *vs.* Fat intake (%)	17	IVW	0.983 (0.973, 0.994)	0.003	0.018		
		MR Egger	0.974 (0.960, 0.987)	0.002		0.063	
		MR-PRESSO	0.985 (0.975, 0.996)	0.012			0.146
		WM	**0.990 (0.977, 1.003)**	**0.135**			
		Simple mode	0.997 (0.971, 1.023)	0.819			
		Weighted mode	0.993 (0.977, 1.010)	0.435			
AD *vs.* Protein intake (%)	18	**IVW**	**1.000 (0.992, 1.008)**	**0.949**	0.451		
		MR Egger	1.003 (0.992, 1.014)	0.641		0.468	
		MR-PRESSO	1.000 (0.991, 1.008)	0.964			0.223
		WM	1.004 (0.993, 1.016)	0.445			
		Simple mode	1.006 (0.988, 1.024)	0.526			
		Weighted mode	1.004 (0.993, 1.015)	0.528			
PD *vs.* Carbohydrate intake (%)	85	IVW	0.993 (0.984, 1.003)	0.155	1.05E-05		
		MR Egger	**0.999 (0.979, 1.020)**	**0.958**		0.499	
		MR-PRESSO	0.994 (0.985, 1.004)	0.261			< 0.001
		Outlier-corrected	0.997 (0.988, 1.007)	0.607			
		WM	1.001 (0.987, 1.014)	0.936			
		Simple mode	0.992 (0.968, 1.017)	0.546			
		Weighted mode	1.004 (0.987, 1.021)	0.643			
PD *vs.* Fat intake (%)	85	IVW	0.996 (0.987, 1.004)	0.309	0.004		
		MR Egger	**0.976 (0.959, 0.994)**	**0.012**		0.021	
		MR-PRESSO	0.995 (0.986, 1.003)	0.238			0.002
		Outlier-corrected	0.996 (0.988, 1.005)	0.411			
		WM	0.992 (0.980, 1.005)	0.220			
		Simple mode	1.015 (0.979, 1.052)	0.433			
		Weighted mode	0.976 (0.955, 0.997)	0.033			
PD *vs.* Protein intake (%)	85	IVW	0.991 (0.982, 1.000)	0.050	3.08E-04		
		MR Egger	0.997 (0.978, 1.017)	0.779		0.486	
		MR-PRESSO	0.992 (0.982, 1.001)	0.072			0.067
		WM	**0.987 (0.975, 1.000)**	**0.042**			
		Simple mode	0.989 (0.966, 1.015)	0.425			
		Weighted mode	0.987 (0.972, 1.002)	0.091			
ALS *vs.* Carbohydrate intake (%)	5	**IVW**	**0.998 (0.970, 1.027)**	**0.891**	0.199		
		MR Egger	0.994 (0.916, 1.079)	0.903		0.931	
		MR-PRESSO	0.998 (0.970, 1.027)	0.897			0.294
		WM	1.010 (0.981, 1.040)	0.492			
		Simple mode	1.015 (0.970, 1.062)	0.565			
		Weighted mode	1.014 (0.978, 1.050)	0.497			
ALS *vs.* Fat intake (%)	5	**IVW**	**1.009 (0.987, 1.033)**	**0.424**	0.603		
		MR Egger	1.043 (0.985, 1.106)	0.247		0.311	
		MR-PRESSO	1.009 (0.990, 1.029)	0.388			0.636

		WM	1.012 (0.984, 1.040)	0.417			
		Simple mode	1.023 (0.981, 1.066)	0.348			
		Weighted mode	1.016 (0.980, 1.053)	0.444			
ALS *vs.* Protein intake (%)	5	**IVW**	**1.017 (0.994, 1.041)**	**0.149**	0.814		
		MR Egger	1.016 (0.959, 1.077)	0.621		0.982	
		MR-PRESSO	1.017 (1.002, 1.032)	0.083			0.833
		WM	1.019 (0.991, 1.047)	0.184			
		Simple mode	1.027 (0.989, 1.066)	0.243			
		Weighted mode	1.019 (0.985, 1.053)	0.339			

The selected model is written in bold. NDDs: Neurodegenerative diseases; MR: Mendelian randomization; AD: Alzheimer's disease; PD, Parkinson's disease; ALS: amyotrophic lateral sclerosis; nSNPs: number of single-nucleotide polymorphisms; OR: odds ratio; CI: confidence interval; Q pval: P value of the Cochran Q statistic; IVW: inverse-variance weighted; WM: weighted median; MR-PRESSO: Pleiotropy Residual Sum and Outlier.

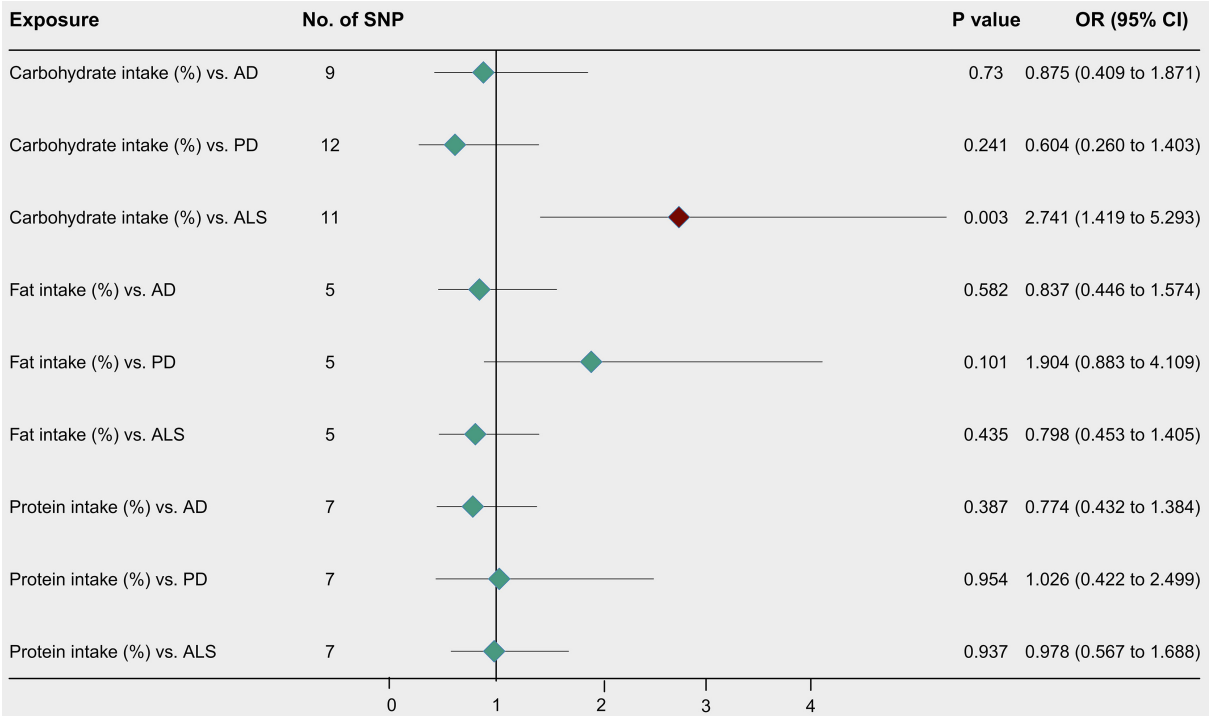

Figure 3. Forward MR analysis estimates of dietary macronutrient intake (%) and NDDs. NDDs: Neurodegenerative diseases; AD: Alzheimer's disease; PD: Parkinson's disease; ALS: amyotrophic lateral sclerosis; CI: confidence interval; OR: odds ratio; SNP: single-nucleotide polymorphism.

ALS. These findings may have important public health implications. Individuals affected by ALS should receive advice about avoiding a high carbohydrate diet, and such a carbohydrate restriction strategy should be included in prevention guidelines for ALS patients regarding macronutrient intake recommendations.

Unexpectedly and interestingly, our primary analysis showed that AD was causally associated with a higher carbohydrate intake, and PD was causally associated with a low intake of protein and fat. However, the underlying mechanisms explaining the associations remain unclear. A previous study reported that AD patients have a greater preference for sweet or sugary food than normal controls and concluded that craving sweet food might be one of the clinical syndromes of AD patients[51]. Decreased serotonin activity could

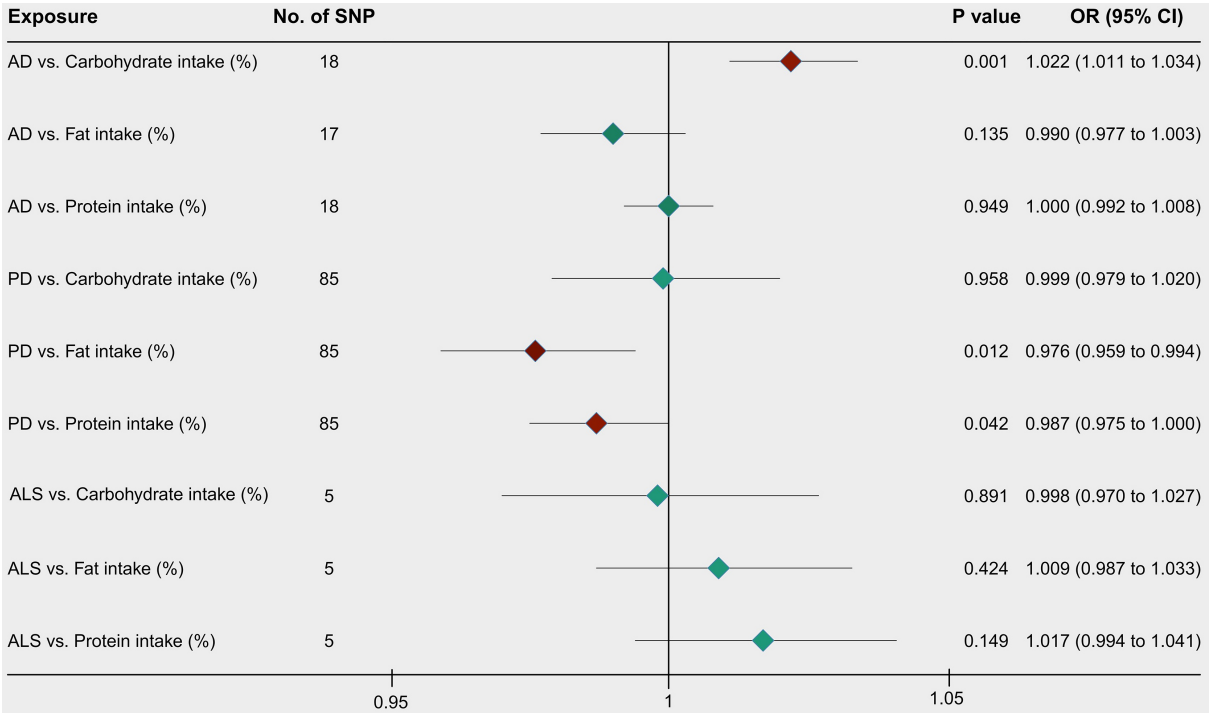

Figure 4. Reverse MR analysis estimates of dietary macronutrient intake (%) and NDDs. NDDs: Neurodegenerative diseases; AD: Alzheimer's disease; PD: Parkinson's disease; ALS: amyotrophic lateral sclerosis; CI: confidence interval; OR: odds ratio; SNP: single-nucleotide polymorphism.

provide a link between AD and carbohydrate preference. Previous studies have found that low brain serotonin levels are linked to impairments in episodic memory and motor speed[52]. Ingestion of carbohydrate-rich food could increase serotonin synthesis via supplementation with tryptophan, the amino acid precursor for serotonin[53]. Therefore, the preference for high-carbohydrate foods would be helpful in alleviating cognitive function decline by increasing serotonin synthesis[54]. A significant body of evidence has pointed to the central role of alpha-synuclein in the pathogenesis of PD. The enteric nervous system, in which alpha-synuclein accumulated, was considered the first vulnerable region of the central nervous system to become affected in PD[55]. The pathologic accumulation of alpha-synuclein is toxic and can interfere with the normal synaptic function of neurons in the gastrointestinal tract, which may contribute to gastrointestinal dysmotility. Food rich in protein and high-fat meats can stress the digestive system due to overwork, thereby aggravating digestive problems[56]. It is reasonable that PD patients will avoid food that is hard to digest in their daily life. Regrettably, there are few clinical trials implying the causal role of PD on protein and fat intake. Thus, further study is needed to elucidate the causal relationship.

However, our MR analyses failed to identify a causal association between dietary macronutrient intake and the risk of AD and PD. These results are inconsistent with those based on prospective studies, which tend to report a significant influence of diet composition on the two disorders[57-60]. Although these studies concluded that dietary factors played a role in the onset and progression of the two diseases, the current evidence is not adequate to support the existence of a causal relationship between them.

The main strength of the MR study is that it is the first MR study to explore the causal relationship between dietary macronutrients and NDDs, which contributes to filling the gaps left by the published observational studies and extends the related research considerably. The present study also has limitations. First, there are

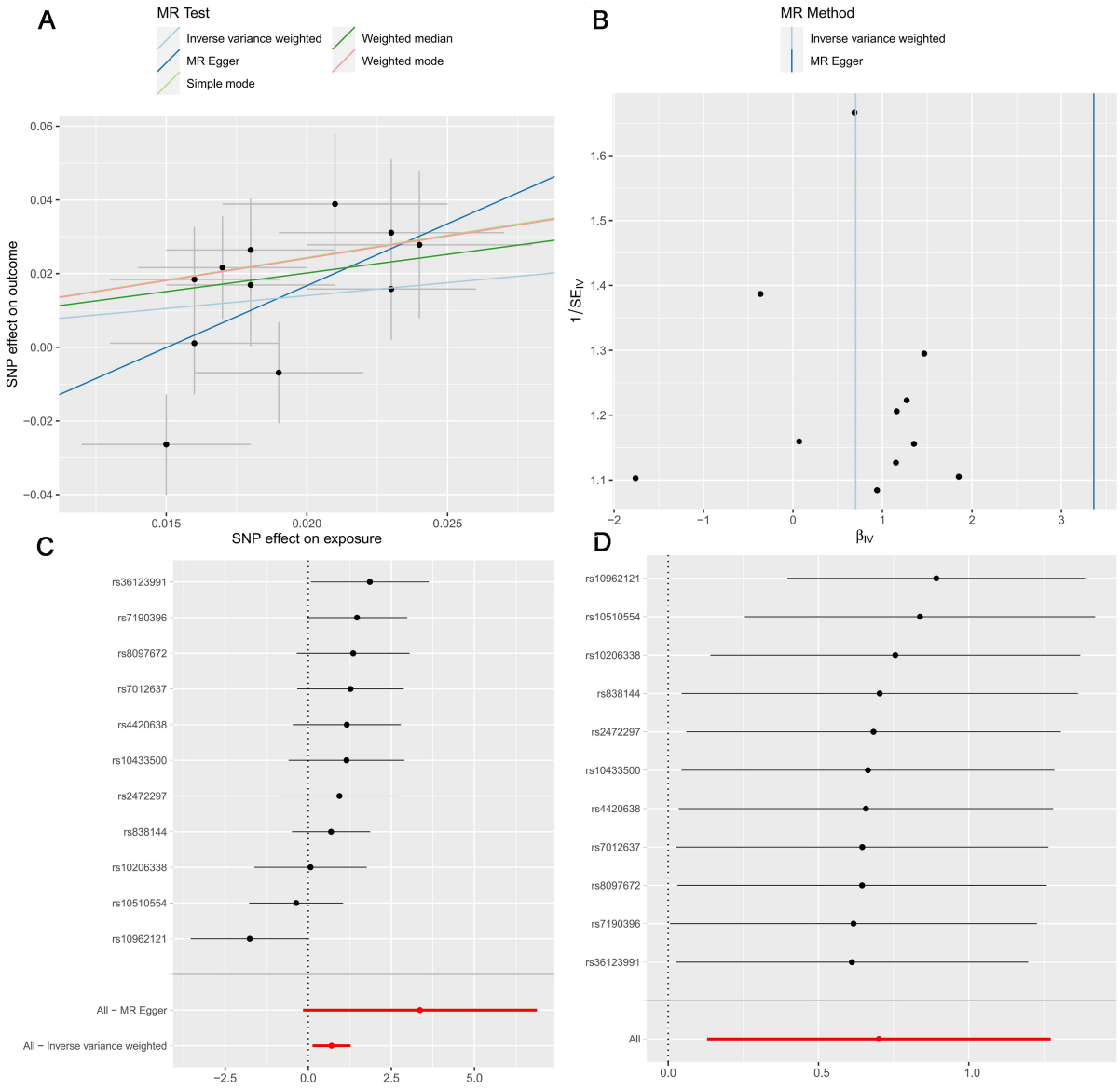

Figure 5. Scatter plot (A), funnel plot (B), forest plot (C) and leave-one-out plot (D) of the causal effect of dietary carbohydrate intake on ALS risk. ALS: Amyotrophic lateral sclerosis; SNP: single-nucleotide polymorphism.

only five available SNPs significantly associated with fat intake and ALS, leading to convergence problems of optimization algorithms within MR methods. To address this issue, a larger population is needed. Second, information regarding the exposure of interest was all from the self-report questionnaires; thus, it is challenging to avoid measurement bias.

In this study, genetically predicted relatively high dietary carbohydrate intake was associated with an increased risk of ALS. In the other direction, genetically predicted higher AD risk is associated with increased dietary carbohydrate intake. We also provided genetic evidence supporting the causal relationship between vulnerability to PD and a decrease in the dietary intake of protein and fat. Future studies are warranted to replicate this finding and elucidate the potential underlying mechanism.

DECLARATIONS

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Authors' contributions

Conceptualization: Tang Y
Data curation: Zheng Y
Formal analysis: Wei T, Wang Z
Methodology: Guo Z, Li X, Hou H
Resources: Tang Y
Software: Guo Z
Writing-original draft: Wei T
Writing-review & editing: Guo Z
All authors read and approved the final manuscript.

Availability of data and materials

All data described in the article are provided within the article.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

Clinical Observation	Clinical observation refers to records of the effects of treatment on hospitalized patients. It details symptoms, diagnosis and treatment of the disease to be reported. The characteristics of clinical reports include new or rare, complex adverse reactions, confusing symptoms or signs, examples of new theories, etc.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether the aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
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Figure caption is placed under the Figure;
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Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background;
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General italic words like *vs*., *et al., etc*., *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

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Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

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Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

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8.1 Initial check

8.1.1 Initial manuscript check

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