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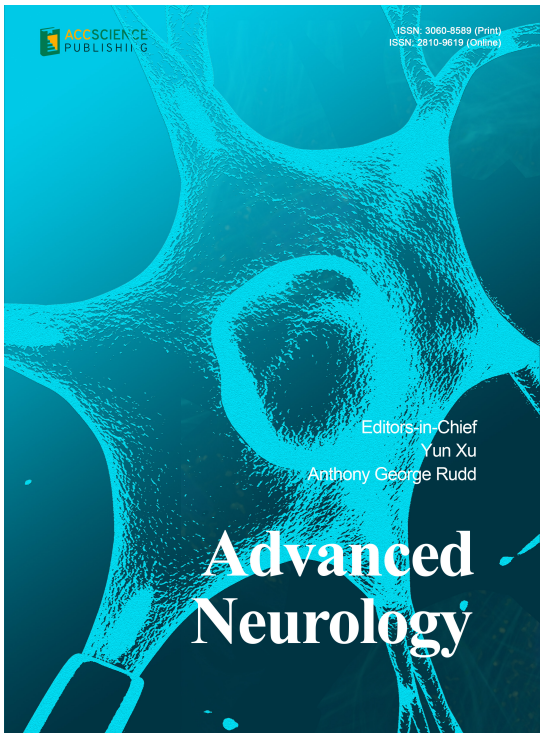
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ADVANCED NEUROLOGY

Editors-in-Chief

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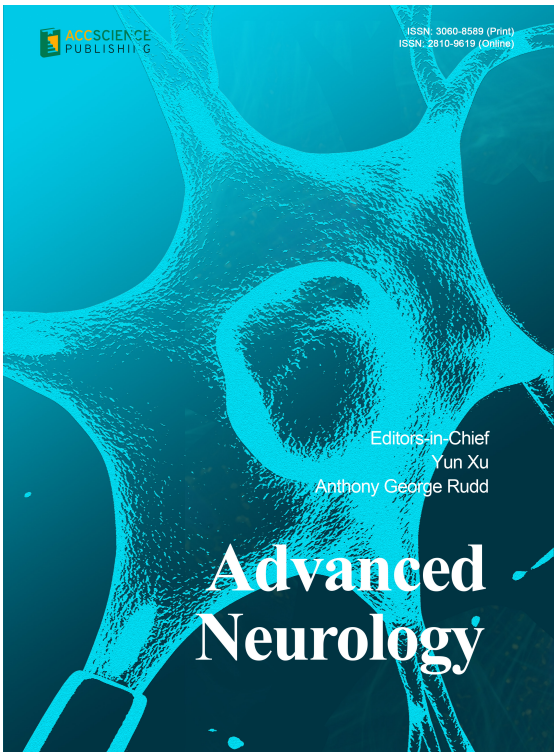
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A close-up graphic image of a neuron

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ORIGINAL RESEARCH ARTICLE

Protective effect of pantothenic acid in kainic acid-induced status eilepticus and associated neurodegeneration in mice

Souravh Bais*, Renu Kumari Rana, Nirmal Dongre, Gaurav Goyanar, Aakash Singh Panwar, Alok Kumar Soni, and Shanti Lal Singune

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Abstract

Pantothenic acid (PA) is a water-soluble vitamin (Vitamin B) that has recently been investigated in various chemical-induced neurotoxicity studies. The present study was designed to explore the biological importance of PA as a neuromodulator by releasing monoamine oxidase (MAO)-A and MAO-B in kainic acid (KA)-induced status epilepticus and the associated neurodegeneration in mice. The mice were intraperitoneally administered with KA at a dose of 10 mg/kg, and the injection solution was maintained at pH 7.2 ± 0.1 before the injection. Subsequently, the mice were observed for various behavioral changes, such as grooming, rearing, hind limb scratching, urination, defecation, jaw movements, salivation, head nodding, incidence of convulsions, and their latency or any mortality, which were recorded during a 4-h period. Further, the animals were euthanized for biochemical and histopathological analysis. The oxidative stress status was determined by measuring levels of glutathione, superoxide dismutase, nitrites, and catalase enzymes. The MAO-A and MAO-B activities, which represent an indicator of brain memory function, and the level of tumor necrosis factor alpha, which is an inflammatory marker in brain tissues, were also measured. The PA pre-treated mice showed a significant increase in retention with latency, as demonstrated in the passive avoidance test, which indicate its protective effect against the KA-induced cognitive deficit. The results showed that the anti-oxidative and anti-inflammatory potential of PA is due to the change in lipid peroxidation, which may prevent mitochondrial damage in neuronal cell, thereby conferring neuroprotection.

Keywords: Epilepsy; Kainic acid; Pantothenic acid; Pharmacological activity; Biochemical analysis; Histopathology

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1. Introduction

Epilepsy is one of the most prominent neurocognitive conditions[1]. Complex partial epilepsy has the poorest prognosis among all patients with temporal lobe epilepsy (TLE), with 60 – 70% of patients experiencing incurable seizures[1,2]. Furthermore, cognitive disorders in human TLE are more frequent[2] but the primary etiology is not clearly known. Administration of kainic acid (KA) as a single infusion has been reported to cause an epileptic state in mice, which results in recurrent seizures and memory impairments[3,4]. KA is a molecular analog of glutamate, an excitatory amino acid neurotransmitter. The

KA-induced epilepsy model has been commonly reported in clinical TLE research[5].

The stimulation of an excitatory amino acid receptor has been reported to cause the production of reactive oxygen species (ROS)[6]. Not only does this pathological loop contribute to the induction of long-lasting seizures, but it may also lead to neuronal damage if not treated[7,8]. Status epilepticus (SE) is an epidemic state where seizures are prolonged and neurological injuries occur in uncontrolled manner[9,10]. Systemic or intracerebroventricular injection of KA induces SE, which causes nerve cell injury and damages to various regions of the limbic system[11]. ROS appears to be involved in the pathway associated with the pathogenesis of SE[12]. Several scientific investigations have demonstrated that antioxidants can facilitate the inhibition of neurotoxicity caused by glutamic agents. The most feasible strategy to determine antioxidant capability would be to scavenge free radicals using non-enzymatic exogenous antioxidants[12].

Treatment offered to SE patients includes drugs that interfere with inflammatory processes, which alter the expression of interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), and IL-6 in in certain brain regions[13]. The results of recent preclinical studies of seizure and primary care indicate the potential role of the inflammatory process in epilepsy etiopathogenesis[14,15]. Pantothenic acid (PA) is a derivative of β-alanine and is available in the dextrorotatory form of calcium salts[16,17]. In humans, deficiency in PA causes different behavioral symptoms, such as depression, personality changes, frequent infection, fatigue, and sleep disturbance, as well as different histological changes in tissues leading to cardiac instability, abdominal pain, and muscle weakness[13,18,19]. Studies have also suggested that PA deficiency causes neurological disturbances[20]. Therefore, the present study was designed to evaluate the effect of PA on KA-induced SE and the associated neurodegeneration in mice.

The anticonvulsant activity of many antioxidant compounds, such as curcumin and vineatrol, has been investigated earlier, and we aimed to examine the anticonvulsant activity of PA using the KA epileptic model. Therefore, the purpose of the present study was to evaluate the effect of PA on epileptic status, oxidative stress, and cognitive impairment in KA-induced mice. In addition, we also examined its modulation effects on the release of TNF-α, a proinflammatory cytokine[6,16].

2. Materials and methods

2.1. Drugs and chemicals

PA (CAS 79-83-4), KA (CAS 487-79-6), diazepam (CAS 439-14-5), radioimmuno precipitation assay lysis buffer, and dimethyl sulfoxide (Cat. No. 102952) were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Protease inhibitor cocktail (Cat. No. 78430) was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay kits were obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). All other materials were of the analytical grade.

2.2. Animals

Young Wistar albino mice (3 – 4-weeks-old) of both sexes, weighing 20 – 25 g were used in this study. The animals were housed in cages with paddy husk bedding that was changed daily. The mice were kept in a group of six (two males and four females) under room temperature (25 ± 1°C) with polypropylene cages maintained on a 12-h light-dark cycle. Unrestricted access to water and food was permitted for the mice (standard pallet). All protocols for the experiments were approved and implemented according to committee guidelines (approval no. CPCSEA/474/2021-08).

2.3. Experimental design

KA was administered intraperitoneally (i.p.) to mice at a dose of 10 mg/kg in a solution maintained at pH 7.2 ± 0.1. Following KA treatment, behavioral changes (grooming, rearing, and rubbing of the hind paws, urination, defecation, moist dog shakes, jaw motions, salivation, and head nodding), frequency and delay of seizures, and survival were monitored in mice and recorded for a maximum of 4 h[16]. On the last day of the experiment, all mice were euthanized, the blood (serum) was collected, and the tissues were harvested for histological analysis. The levels of a series of biochemical parameters, such as glutathione (GSH), superoxide dismutase (SOD), nitrite, catalase, acetylcholine esterase (AChE), MAO-A, and MAO-B, were determined. The brain TNF-α level was also determined to assess the extent of inflammation.

2.4. Animal grouping

The mice were divided into several groups and treated as indicated.

Group 1 serves as the control group and was given 0.9% normal saline.

Group 2 serves as the vehicle-treated KA group and was given 10 mg/kg KA i.p. The vehicle (saline 10 mL/kg, i.p.) was administered for 7 consecutive days. KA (10 mg/kg, i.p.) was administered on the 7th day 30 min after the administration of vehicle and animals were observed over a period of 4 h for any change in behavioral parameters, incidence, and latency of convulsions.

Group 3 serves as the positive control group. Diazepam (5 mg/kg, i.p.) was administered on the 7th day, followed by

KA (10 mg/kg, i.p.) administration, which was performed 30 min after the administration of diazepam. The mice, thereafter, were observed over a period of 4 h for any change in behavioral parameters, incidence, and latency of convulsions.

In Groups 4, 5, and 6, KA (10 mg/kg, i.p.) and PA at doses of 30, 60, and 90 mg/kg were given to the groups, respectively, for 7 days. The mice, thereafter, were observed over a period of 4 h for any change in behavioral parameters, incidence, and latency of convulsions.

2.5. Behavioural tests

2.5.1. Single-trial passive avoidance test

Memory retention deficit was evaluated by step through passive avoidance apparatus. The apparatus consists of equal sized light and dark compartments (30 × 20 × 30 cm). A 40-W lamp was fixed 30 cm above its floor in the center of the light compartment. The floor consists of metal grid connected to a shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. Twenty-four h after the administration of KA, mice were placed in the light compartment and the time lapse before each animal entered the dark compartment and had all four paws inside it was measured in seconds and termed as "initial latency" (IL). Immediately, after the mice entered the dark chamber with all the four paws inside the dark chamber, the trap door was closed and an electric foot shock (50 V a.c.) was delivered for 3 s. Five s later, the mice was removed from the dark chamber and returned to its home cage. Twenty-four h after the IL, the latency time was measured again in the same way as in the acquisition trial and was termed as the retention latency (RL). However, during the retention trial, no foot shock was delivered, and the latency time was recorded to a maximum of 600 s. To improve the reliability and validity of the foot shock avoidance test, the grid as well as the mice paw was moistened with water before delivering the foot shock as this is known to reduce the wide inter animal variability in paw skin resistance of the mice[20].

2.5.2. Evaluation

The animals were sacrificed by cervical dislocation. The whole brain was removed from the skull immediately after dislocation. For preparation of the homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4, stored at 2 – 8°C). The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for various neurochemical estimations.

2.5.3. Choline esterase enzyme determination

The enzyme level was measured by the method described by Ellman[13] with slight modifications. The sample absorbance was taken at 412 nm for a continued 7-min interval of 1 min. The results were expressed as nM/L/min/gm of tissue.

2.5.4. GSH estimation

The GSH level was measured using the Butler method with minor modifications. The absorption was spectrophotometrically measured at 412 nm (X-Rite 640B spectrophotometer). Different GSH standard concentrations were also treated simultaneously to create a standard curve (1 – 50 µg). Results were reported as GSH nmol/mg protein[19].

2.5.5. Catalase estimation

Catalase activity was measured using a previously described method[21], wherein the breakdown of H_2O_2 was measured. The processed sample homogenate absorbance was taken at 240 nm. The results were expressed as micromoles of H_2O_2 decomposed/min/mg/of protein.

2.5.6. SOD enzyme activity

The SOD activity was measured with the enzyme kit from RANDOX Ransod. This procedure uses xanthine and xanthine oxidase-produced radicals of superoxides that react with the red formazan dye using 2-(4-iodophenyl)-3-(4-nitrophene)-5-phenyltetrazolium-chloride. The degree of inhibition of the SOD activity was measured[22].

2.5.7. Nitrites content estimation

It was measured by previously described method[23]. 0.2 mL of supernatant from the brain homogenate was mixed with freshly prepared Griess reagent solution and the absorbance was measured at 546 nm. The results were calculated as nM/mg of protein.

2.5.8. Determination of MAO activity

MAO activity was assessed by spectrophotometry. The assay mixture contains 4 mm of serotonin as a specific substrate for MAO-A, 250 µL solution of brain homogenate, and 100 mm of sodium phosphate buffer (pH 7.4) up to a final volume of 1 mL. The action was allowed to proceed at 37°C for 20 min and stopped by adding 1 M HCl (200 µL). The reaction product was extracted with 5 mL of butyl acetate. Blank samples were prepared by adding 1 M HCl (200 µl) before the reaction and worked subsequently in the same manner[24].

2.5.9. Histopathology study of the rat brain

The cortex and hippocampus of control and experimental rats were fixed in 4% formalin and embedded in paraffin. Next, they were sliced into 5 µm sections using a section cutter (Leica, Germany). The sections were stained with hematoxylin and eosin and examined under a light microscope.

2.5.10. Statistics and data analysis

The data were analyzed using Graph Pad Prism 7.0 software and the results expressed as mean ± standard error of the mean. followed by a two-way analysis of variance (ANOVA) or one-way ANOVA when applicable; individual comparison was done using with Tukey's multiple comparison test for statistical significance set to $P < 0.001$.

3. Results

3.1. Effect of PA on KA-induced SE in mice

The behavioral signs, seizures, and the latency was found to be increased in each animal with each dose of PA. At doses of 30, 60, and 90 mg/kg, PA improved the latency of behavioral symptoms to 14.25 ± 0.97 ($P < 0.01$), 26.54 ± 0.88, and 29.81 ± 2.09 min ($P < 0.001$), respectively, as compared with KA alone. In addition, the epilepsy latency was also significantly increased to 45.7 ± 1.83 ($P < 0.05$), 78.5 ± 1.77 ($P < 0.01$), and 91.0 ± 1.08 ($P < 0.01$), respectively, with PA treatment at 30, 60, and 90 mg/kg doses as compared to 37.02 ± 1.31 vehicle-treated KA group (Figure 1).

3.2. Effect of PA on cognitive impairment in KA-induced SE in mice

The retention latency appeared lower in the initial phase (48 h) in KA-treated mice ($P < 0.001$) as compared to control group mice, suggesting KA induced major cognitive dysfunction. Dose-dependent changes in retention latencies ($P < 0.05$ at 30 mg/kg, $P < 0.01$ at 60 mg/kg, and $P < 0.001$ at 90 mg/kg, i.p.) were observed, suggesting a reversal in the retention latencies in the PA (30, 60, and 90 mg/kg, i.p.) + KA-treated groups. The control, vehicle-treated KA, diazepam, and 30, 60, and 90 mg/kg PA-treated groups showed change in retention latencies of 287.14 ± 6.28 s, 86.11 ± 1.56 s, 218.7 ± 1.89 s, 107.4 ± 2.65 s, 167.1 ± 2.6, and 272.7 ± 2.19 s, respectively (Figure 2). The mice pretreated with diazepam did not show any behavioral signs and convulsions.

3.3. Effect of PA on other biochemical parameters

The KA-treated mice showed higher levels of the AChE enzyme and nitrite content as compared to the control group mice. The PA-treated mice showed dose-dependent reduction in levels of AChE and nitrite as compared with KA-treated Group 2. The levels of the other enzymes, catalase, and SOD were significantly decreased in the KA-treated Group 2, which was ameliorated by PA treatment. The greatest protection against enzyme reduction is shown at a dose of 90 mg/kg by PA (Table 1).

3.4. Effect of PA on thiobarbituric acid reactive substances (TBARS) in KA-induced SE in mice

The brain TBARS levels were significantly higher in KA-induced group () (3.72 ± 0.32 nM/mg of protein) as compared with control group (1.31 ± 0.05 nM/mg of protein; $P < 0.001$). TBARS levels were 3.09 ± 0.98, 2.68 ± 0.4, and 1.94 ± 0.8 nM/mg of protein in mice treated with PA at 30, 60, and 90 mg/kg (i.p.), respectively. The PA at the tested doses showed significant antioxidant activity compared with the vehicle treatment using KA (Table 1).

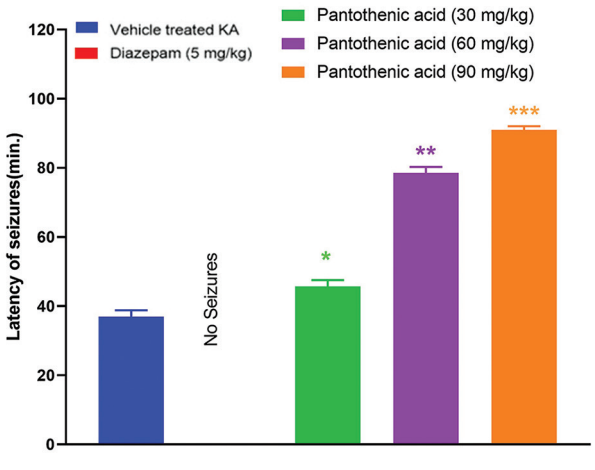


Figure 1. Effect of 7-day pretreatment with pantothenic acid on KA-induced SE in mice**.** Data are expressed as means ± standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, compared with vehicle-treated KA group.

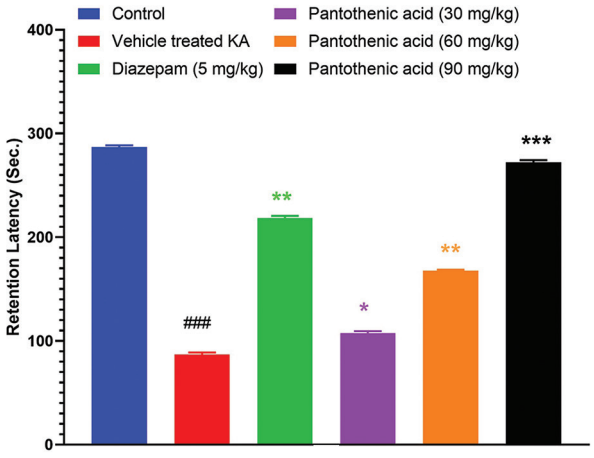


Figure 2. Effect of 7-day pretreatment with pantothenic acid on KA-induced cognitive impairment in mice**.** Data are expressed as means ± standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, compared with vehicle-treated KA group. ###P<0.001, compared with control group.

Table 1. Effect of pantothenic acid on various biochemical parameters in kainic acid-induced epilepsy in mice

Group	AChE (nmol·min^{-1}·mL^{-1}·g^{-1} of tissue)	GSH (nM/mg of protein)	TBARS (nM/mg of protein)	Nitrite (nM/mg of protein)	SOD (IU/mg of protein)	Catalase (μM of H_2O_2 decomposition·min^{-1}·mg of protein)
1	3.2±0.853	7.37±0.13	1.31±0.05	1.19±0.08	37.3±1.05	1.18±0.076
2	6.7±0.73$^{###}$	2.9±0.92$^{###}$	3.72±0.32$^{###}$	3.37±0.91$^{###}$	9.52±1.2$^{###}$	0.36±0.07$^{###}$
3	4.89±0.19***	5.86±0.89***	2.56±0.98***	2.04±0.76***	33.73±2.03***	0.91±0.71***
4	6.55±0.75*	4.67±1.32*	3.09±0.98*	3.17±0.97*	13.72±1.76*	0.47±0.08*
5	5.89±0.7**	5.98±0.7**	2.68±0.4**	3.09±0.3**	21.73±1.8**	0.79±0.04**
6	4.53±0.8***	7.1±0.54***	1.94±0.8***	1.93±0.7***	31.36±2.17***	0.97±0.06***

Data are expressed as means±standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with vehicle-treated KA group (i.e., Group 2). $^{###}P < 0.001$, compared with control group (i.e., Group 1). Group 1: control group, Group 2: Vehicle-treated KA group consisting of untreated KA-induced mice, Group 3: Group of diazepam-treated KA-induced mice, Group 4: Group of pantothenic acid (30 mg/kg, p.o.)-treated KA-induced mice, Group 5: Group of pantothenic acid (60 mg/kg, p.o.)-treated KA-induced mice, and Group 6: Group of pantothenic acid (90 mg/kg, p.o.)-treated KA-induced mice. AChE acetylcholine esterase, GSH: Glutathione, SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances

3.5. Effect of PA on GSH levels in KA-induced SE in mice

Brain GSH levels were measured in the control, vehicle-treated KA-induced, and PA -treated mice. GSH levels were found to be lower (2.9 ± 0.92 nM/mg of protein) in the vehicle-treated KA group ($P < 0.001$) than in the control group (7.37 ± 0.13 nM/mg of protein). GSH levels were 4.67 ± 1.32, 5.98 ± 0.7, and 7.1 ± 0.54 nM/mg of protein in the mice treated with PA at 30, 60 and 90 mg/kg (i.p.), respectively. Furthermore, the levels were found to be maximum in animals treated with the dose of 90 mg/kg as compared to KA-treated Group 2 ($P < 0.001$, Table 1).

3.6. Effect of PA on TNF-α level in KA-induced SE in mice

The brain levels of TNF-α were significantly increased after KA administration as compared to level found in the control group (804.09 ± 19.02 pg/mL and 481.31 ± 7.67 pg/mL, respectively, $P < 0.001$). PA treatment caused a dose-dependent reduction in brain levels of TNF-α (Figure 3), with the highest inhibition occurred at the dose of 90 mg/kg ($P < 0.001$).

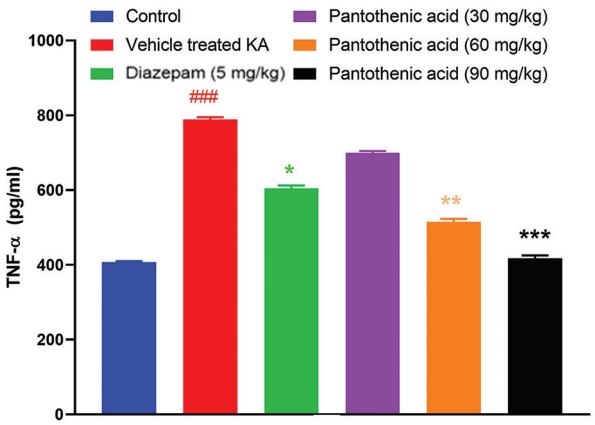


Figure 3. Effect of 7-day pretreatment with pantothenic acid on TNF-α level in KA-induced SE in mice**.** Data are expressed as means ± standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with vehicle-treated KA group. $^{###}P<0.001$, compared with control group.

3.7. Effects on body and brain weight

The mice body weight and brain weight decreased in KA-induced mice, whereas other groups also showed changes in size and body weight. Mice treated with PA improved the hypotrophy of their brains caused by KA induction in the absence of interventional treatment (Table 2).

3.8. Effect of PA on MAO-A and MAO-B levels

The effects of PA on the MAO concentration are shown in Table 3. The MAO-A and MAO-B activity levels of the control group were 26.1 ± 0.32 and 23.2 ± 0.24 nmol/mg of protein, respectively. Oral (p.o.) administration of 60 mg/kg PA inhibited MAO-A and MAO-B by 21% and 35%, respectively, and PA (90 mg/kg, p.o.) resulted in significant inhibition of both MAO isoforms by 35% and 65%, respectively (Table 3).

3.9. Histopathological changes in brain tissue

From the histopathological study, it was observed that the normal control group or Group 1 (Figure 4A) showed a normal brain parenchyma with normal neuronal morphology. KA-treated mice in Group 2 (Figure 4B) showed neuronal degradation in the brain parenchyma, small pyknotic nuclei, and extracellular eosinophilic deposition. In the standard group or Group 3 (Figure 4C), animals showed mild changes in neuronal degeneration, and many nuclei were pyknotic and closely packed. The mice in Group 4 (Figure 4D) and Group 5 (Figure 4E),

Table 2. Effect of pantothenic acid on kainic acid-induced changes in body and brain weight of mice with epilepsy

Group	Change in body weight (BW, g)[a]		Brain weight (g)		Brain/BW ratio/100 g of mice weight	
	Male	Female	Male	Female	Male	Female
1	0.44±0.11	−0.41±0.24	1.06±0.32	1.02±0.16	1.02	1.01
2	1.07±0.19	0.19±0.08	0.73±0.19	0.68±0.13	0.67	0.43
3	−2.53±0.89*	−2.92±0.82*	0.86±0.22*	0.98±0.03**	0.83	0.85
4	−1.76±0.76	−3.95±0.65	0.96±0.43*	0.87±0.88*	0.77	0.65
5	−2.32±0.43*	−3.69±0.76	1.08±0.13**	1.29±0.76**	0.83	0.79
6	−4.51±1.07**	−3.06±0.74*	1.12±0.22***	1.31±0.54***	0.71	1.08

Data are expressed as means±standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with vehicle-treated KA group (i.e., Group 2). Group 1: Control group, Group 2: Vehicle-treated KA group consisting of untreated KA-induced mice, Group 3: Group of diazepam-treated KA-induced mice, Group 4: Group of pantothenic acid (30 mg/kg, p.o.)-treated KA-induced mice, Group 5: Group of pantothenic acid (60 mg/kg, p.o.)-treated KA-induced mice, and Group 6: Group of pantothenic acid (90 mg/kg, p.o.)-treated KA-induced mice. [a]Initial day BW - final day BW

Table 3. Effect of pantothenic acid on monoamine oxidase level in brain tissues

Group	MAO-A		MAO-B	
	Level (nM/mg protein·h)	Inhibition (%)	Level (nM/mg protein·h)	Inhibition (%)
1	22.1±0.32		23.2±0.24	
2	27.4.1±0.82	00	32.1±0.76	00
3	27.1±0.43*	3.89	23.1±0.19*	00
4	25.76±0.36	10	28.76±1.02	17
5	22.8±0.54*	21	18.2±0.54	35
6	21.3±0.33**	35	12.4±1.03*	65

Data are expressed as means±standard error of the mean, $n = 6$. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle-treated KA group (i.e., Group 2). Group 1: Control group, Group 2: Vehicle-treated KA group consisting of untreated KA-induced mice, Group 3: Group of diazepam-treated KA-induced mice, Group 4: Group of pantothenic acid (30 mg/kg, p.o.)-treated KA-induced mice, Group 5: Group of pantothenic acid (60 mg/kg, p.o.)-treated KA-induced mice, and Group 6: Group of pantothenic acid (90 mg/kg, p.o.)-treated KA-induced mice. MAO-A: Monoamine oxidase A, MAO-B: Monoamine oxidase B

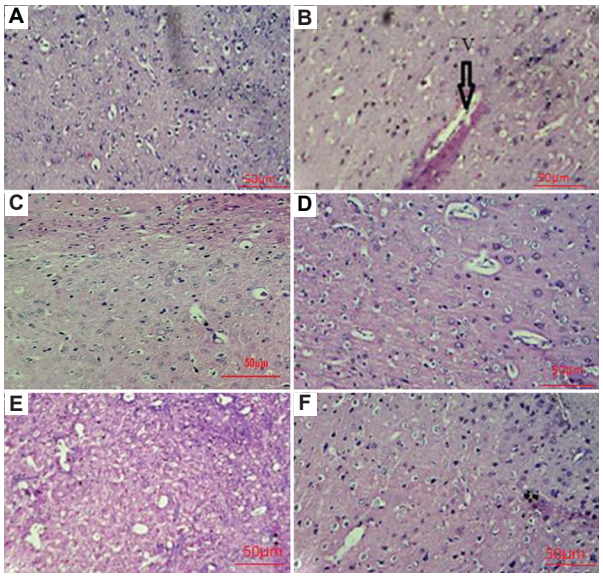


Figure 4. Histopathology of brain tissue (cerebral cortex) showing neuronal degeneration and inflammation. (A) Mice from normal control group. (B) Untreated kainic acid (KA)-induced mice. (C) Diazepam-treated KA-induced mice. (D) Pantothenic acid (30 mg/kg)-treated KA-induced mice. (E) Pantothenic acid (60 mg/kg)-treated KA-induced mice. (F) Pantothenic acid (90 mg/kg)-treated KA-induced mice. Arrow indicates neuronal vacuolation.

which were treated with PA 30 mg/kg and 60 mg/kg, respectively, showed focal areas of cellular degeneration, although certain areas were morphologically normal. The mice in Group 6 (Figure 4F), which were treated with PA (90 mg/kg), showed improvement in the near normal brain parenchyma with minimal degenerative changes.

4. Discussion

The present study found that KA significantly induced the SE state of epilepsy and PA ameliorates the damage and the associated neurodegeneration caused by KA administration in mice. In clinical practice, epilepsy is the most common neurological condition. In the U.S., 2.2 million individuals have epilepsy, while 3% of the population will develop epilepsy later in life[25,26]. At present, significant progress has been made in the diagnosis and treatment of neurological conditions and research is ongoing, especially on neuronal signaling to neighboring cells[27,28]. The basic cause of seizure progression has not yet been established[29-31], but excessive activation of excitatory amino acid receptors or GABAergic system inhibition has been observed in mice[25,26]. Excessive stimulation of excitatory amino acid receptors has been clearly demonstrated to induce prolonged seizures[25,26].

Stress and free radicals have been reported to excite excitatory receptors, leading to a transition between seizure phases[27,28]. In this study, KA induced behavioral changes and seizures in all mice. Compared with KA treatment, pre-treatment with different doses of PA delayed the initiation of seizures and improved memory retention. Furthermore, i.p. pre-treatment with PA (30, 60, and 90 mg/kg) protected mice against KA-induced neurodegeneration. In the KA-treated group, GSH content declined dramatically, indicating the development of oxidative stress.

PA (90 mg/kg) treatment contributed to more significant effects on MDA and GSH levels than KA treatment alone. Elevated MDA content is an indicator of free radical production. The PA-treated groups (Groups 3, 4, and 5) showed a greater increase in the GSH content than the KA-treated group did. GSH interacts with free radicals and can shield cells from the effects of singlet oxygen, hydroxyl radicals, and superoxide. GSH is the most common intracellular thiol and low-molecular-weight tripeptide present in cells[28,29]. The inhibition of KA-induced reduction of GSH levels by PA suggests that it improves the endogenous protective potential of the brain against KA-induced oxidative stress[30].

PA has been hypothesized as an agent for preventing mitochondrial damage by modulating lipid peroxidation, thereby conferring neuroprotection[31,32]. This indicates that PA helps minimize mitochondrial oxidative stress. When the threshold value of excitation is crossed after administration of KA, animals will experience seizures. The administration of KA activates glutamate receptors to boost ROS, thus increasing glutaminergic activity. PA has been reported to modulate glutamatergic activity[33]. The extent to which minimizing the work is difficult to predict, but the disruptive chain is noticeable, which reduces excitotoxicity and, hence, demonstrates a defensive effect[20,34,35].

Therefore, it is difficult to rule out the dual effects of PA as an antioxidant and an anti-excitotoxic agent. The change in neurological conditions is also correlated with the occurrence of inflammation in neuronal cells[20]. especially in the prognosis of epilepsy and SA[36]. Higher levels of cells (such as microglia), effects (such as astrocyte activation), and production of cytokines (such as TNF-α, IL-1β, IL-6, and related molecules) have been reported in the process of inflammation in clinical and preclinical models of epilepsy[37,38]. These cytokines are actively involved in immune protection in the central nervous system. Compounds such as PA and other vitamins have rich antioxidant activities that affect the function of microglia. Cytokines such as TNF-α, IL-1, and IL-1β are directly involved in stress, over-firing of signals in seizures, and prolongation of disease stage[37].

KA-induced animals are an accepted preclinical model for studying neurological disorders, such as epilepsy, Huntington's chorea, and Alzheimer's disease, which involve excitotoxicity and inflammation. PA treatment at 30 mg/kg (i.p.) showed no effect on TNF-α levels, whereas 60 and 90 mg/kg (i.p.) significantly attenuated the increase of brain TNF-α levels ($P < 0.001$) in the PA + KA groups compared with that in the KA-induced group. In the previous studies, PA has also been reported to attenuate TNF-α levels, inhibit inducible nitric oxide synthase (iNOS) expression, and suppress apoptosis through its anti-inflammatory activity[39]. The latter effect could, at least in part, explain the beneficial effects of PA on diseases involving inflammation[40]. These effects are mediated by the inhibition of nuclear factor kappa B (NF-κB) activation[27]. Cyclooxygenase 2 (COX-2), the inducible isoform expressed at the injury or inflammatory sites and the central nervous system, plays a significant role in neurodegeneration linked to increased excitatory activity[28].

Glutamate, an active N-methyl-D-aspartate (NMDA) receptor excitatory neurotransmitter, increases the expression of COX-2 and results in higher prostaglandin synthesis (unpublished observations). This result was confirmed in studies by Vanamala *et al.*[29] who reported that PA decreased the levels of iNOS and COX-2 by inhibiting NF-κB activation[20,33]. In view of the above findings, it may be fair to conclude that the antiepileptic activities of PA are mediated by its significant antioxidant and anti-inflammatory effects.

In this study, KA administration resulted in seizures related to cognitive dysfunction, as shown by the decrease in the passive avoidance behavior of retentive latency. The results were similar to those of the previous studies that demonstrated cognitive damage and epilepsy following KA administration[38-40]. The disease progression of seizures, indicated by increased retention latency in the passive avoidance test following 7 consecutive days of treatment, leads to cognitive deficits. There is uncertainty about the degree of cognitive dysfunction, which is a limitation associated with all major antiepileptic drugs[36].

The present study has shown the beneficial effect of PA on reducing seizures and cognitive impairment induced by KA. Thus, PA has the potential to be utilized in clinical SE management. Furthermore, this study shows that PA could prevent cognitive impairment and serve as a useful conventional antiepileptic treatment.

5. Conclusions

This pharmacological study demonstrated that PA has major anti-inflammatory, antioxidant, and neuromodulator activities against epilepsy induced by KA in mice.

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Conflict of interest

The authors have no conflict of interest to declare.

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ACCSCIENCE PUBLISHING

Advanced Neurology

ORIGINAL RESEARCH ARTICLE

MicroRNA-195 rescues the impaired MS-dCA1 neural circuit and spatial memory in rats with chronic cerebral hypoperfusion

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Abstract

Chronic cerebral hypoperfusion (CCH) is a common risk factor for vascular dementia and Alzheimer's disease (AD). The previous studies have shown that CCH-induced multiple AD-like pathological changes in the hippocampus and cortex through downregulating the *microRNA-195* (*miR-195*) expression. However, whether and how *miR-195* participates in the dysfunction of the medial septum (MS)-dorsal hippocampal CA1 (dCA1) neural circuit following CCH is still obscure. In the present study, we found that *miR-195* was downregulated in the MS region of CCH rats. Moreover, using electrophysiological recording and immunofluorescence staining technique, we found that the knockdown of *miR-195* through the injection of lenti-AMO-195 into the MS region led to a pathological change that mimicked the damage to the MS-dCA1 neural circuit in CCH rats, including the decreased input-output (I/O) curve, increased paired-pulse ratio (PPR), decreased numbers of $ChAT^+$ and PV^+ neurons in MS, and diminished theta rhythm in the hippocampus. More importantly, exogenously supplemented *miR-195* into the MS region rescued the damaged neural circuit in MS-dCA1 of CCH rats by injecting lenti-pre-*miR-195*. Gain-of-function of *miR-195* in the MS region significantly improved the cognitive dysfunction in CCH rats assessed by the Morris water maze test. In conclusion, knockdown of *miR-195* in the MS region can impair MS-dCA1 neural circuit function, while upregulation of *miR-195* can rescue the impaired function of MS-dCA1 neural circuit and spatial memory ability in CCH rats. This provides a valuable reference for future anti-dementia therapy involving *miR-195*.

Keywords: Chronic cerebral hypoperfusion; *MicroRNA-195*; Medial septum-dorsal hippocampal CA1 neural circuit

1. Introduction

With the increasing incidence of cerebrovascular diseases and the aggravation of population aging, vascular dementia (VaD) has become the most common type of dementia after Alzheimer's disease (AD)[1]. VaD is a clinical syndrome of cognitive decline caused by cerebral hypoperfusion due to various ischemic or hemorrhagic cerebrovascular diseases[2]. It is well known that chronic cerebral hypoperfusion (CCH), which usually

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occurs before mild cognitive impairment (MCI), is an early clinical stage shared by both AD and VaD[3]. The previous studies have shown that CCH leads to cognitive decline and induces a series of AD-like pathological changes[4-6], such as Aβ deposition, tau hyperphosphorylation, neuronal death, microglial polarization, and synaptic dysfunction in the hippocampus and cortex[7-11]. Furthermore, our recent study found that CCH also impairs the function of the medial septum (MS)-dorsal hippocampal CA1 (dCA1) (MS-dCA1) neural circuit and reduces hippocampal presynaptic neurotransmitter release in rats[12]. However, the mechanism remains unknown.

There are highly interconnected cholinergic, GABAergic, and a small number of glutamatergic neurons in the MS region of the basal forebrain that can project to glutamatergic pyramidal neurons and GABAergic interneurons in the dCA1 region. This process can modulate the excitability of hippocampal dCA1 neurons[13-15]. The MS-dCA1 neural circuit is essential for various behavioral situations because it controls the rhythm of hippocampal electrical activity by regulating the release of acetylcholine (ACh) in the hippocampus[16]. Importantly, it has been found that the degeneration of nerve fibers in the MS-dCA1 circuit occurs earlier than neuronal death in AD or VaD patients[17]. This phenomenon suggests that activation of MS-dCA1 neural circuit may be a good strategy for treating CCH-induced cognitive impairment.

Clinically, cholinesterase inhibitors are the only class of drugs that treat early AD by blocking hippocampal ACh degradation and provide temporary symptom relief. Finding a drug targeting on multi-targets of AD pathologies has become a consensus. The main characteristic of *microRNAs (miRNAs)* is that they regulate protein expression at the post-transcriptional level by targeting multiple genes[18,19]. Many miRNAs have been found to participate in AD pathological process by regulating classical amyloid precursor protein (APP) and β-site APP cleaving enzyme 1 (BACE1) expressions[20-23]. Of note, it has been reported that *miR-195* is involved in various AD-like pathologies induced by CCH through regulating the expression of multiple proteins, such as APP/BACE1, as well as the N-APP/DR6/caspase pathway and the CX3CL1/CX3CR1 signaling pathway[7-10], and protected against ApoE4-induced cognitive abnormalities and lysosomal deficiencies in mice[24]. Furthermore, *miR-195* attenuates cerebral ischemia-reperfusion injury through the PTEN-AKT and KLF5-mediated JNK signaling pathway in rats with ischemic stroke[25,26]. Recently, Cheng *et al.* reported that *miR-195* could also protect against ischemic and hemorrhagic stroke by protecting the neurovasculature and promoting neurogenesis[27]. However, it is unclear whether and how *miR-195* modulates the function of MS-dCA1 neural circuit under CCH condition.

Here, using the *in vivo* electrophysiological recording and neurotracing technique, we demonstrated for the 1st time that CCH induced the downregulation of *miR-195* expression in the MS region and inhibited hippocampal rhythmogenesis in the dCAl region by reducing the release of ACh. The mechanism is that *miR-195* leads to the loss of cholinergic and GABAergic neurons in the MS region. Upregulation of *miR-195* expression can rescue MS-dCA1 neural circuit damage and decline of spatial memory in CCH rats. Collectivity, the present data provide further evidence that *miR-195* may be a valuable candidate for the *miRNA*-based treatment of dementia.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley (SD) rats (250–280 g) were bought from the Animal Center of the Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China. The rats were accommodated in a rearing room at 24°C, 55% humidity, and a 12-h dark/light cycle with adequate food and water. All animal experiments were approved by the Ethics Committee of Harbin Medical University (Approval No: IRB3199620). All procedures conformed to Directive 2010/63/EU of the European Parliament.

2.2. Permanent bilateral common carotid artery occlusion

The surgical procedure of 2VO rats, which was conducted according to our published work, is briefly described as follows: Rats were anesthetized with chloral hydrate (300 mg/kg) and maintained with isoflurane (0.5–1.0%). The bilateral common carotid arteries of rats were ligated with 5-0 sterile silk thread, and the arteries were cut with ophthalmic scissors 5 min later. The incision was sutured and disinfected at the end of the procedure. After the rats were fully awake, they were put into home cages and kept for another 8 weeks for all subsequent experiments[7].

2.3. Construction of lentivirus vectors

The procedure for constructing lentivirus vectors has also been described in our previous work[7]. Lenti-pre-*miR-195*, lenti-AMO-195, and lenti-NC were created by GeneCopoeia Inc. (Rockville, MD, USA).

2.4. Stereotactic brain injection procedure

The rats were intraperitoneally injected with 10% chloral hydrate after fasting for 8 h. T-shaped incision was made in the rat scalp, and the periosteum was peeled off to

expose herringbone and sagittal suture. The coordinate of the injection site located in MS was referenced by the rat brain atlas as follows: Anterior-posterior (AP), 0.6 mm; mediolateral (ML), 0.1 mm; and dorsal-ventral (DV), 6.0 mm. After drilling a hole in the skull of MS, microsyringes containing lenti-pre-*miR-195*, lenti-AMO-195, and lenti-NC were fixed to the stereotaxic instrument and injected into the MS region of rats. The injection rate was set to 1.5 μl/min. The needle was pulled out slowly after 5 min to prevent the leakage of lentivirus from tissue. The holes in the skull were filled with bone wax. Then, the scalp incisions of the rats were sutured. After surgery, the rats were placed in home cages and irradiated with a warm light source until they were awake.

2.5. Neuro-retrotracing technique

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Then, holes were drilled in the dCA1 (AP, −3.84 mm; ML, ±2.1 mm; DV, 2.7 mm; and AP, −3.84 mm; ML, ±3 mm; DV, 3.3 mm) and DG (AP, −3.84 mm; ML, ±2.1 mm; DV, 3.3 mm) areas of the hippocampus bilaterally according to the rat brain atlas. The green retrobeads were injected at a rate of 0.25 μl/min in each injection site. The needle was pulled out slowly after 5 min to prevent the green retrobeads from spreading. Next, the holes in the skull were filled with bone wax and the scalp incisions were closed with 5-0 medical sutures. The subsequent experiments were performed 7 days later.

2.6. Electrophysiological recording *in vivo*

Following urethane anesthesia (1.2 g/kg) treatments, the rats were put on the stereotaxic frame device (DW-2000, Chengdu Taimeng Software Company Limited, China) for the implantation of electrodes. The rats were put on a heating pad to keep their body temperature at 37°C. Bipolar stimulating electrodes (stainless steel, 0.5 mm) were implanted into the MS area (AP, 0.6 mm; ML, 0.1 mm; and DV, 6.0 mm). The recording electrodes stuffed by 3 mol/L of NaCl were implanted into the pyramidal cell layer of the dCA1 (AP, 3.8 mm; ML, 2.4 mm; and DV, 2.7 mm). To get the best results, specific positions of the stimulating and recording electrodes were varied[28,29]. MS was activated by constant controlled pulses from the BL-420S stimulus generator after an electrode implantation recovery period. A ME-1 preamplifier was used to amplify the field excitatory post-synaptic potential (fEPSP) obtained in hippocampal dCA1 area. The signals were recorded by the BL-420S device after being filtered at 1 Hz–1 kHz and digitalized at 20 kHz. To set up the input-output (I/O) curves, electric stimulation with intensity ranging from 1 to 20 V was applied in the MS. The amplitude and slope of fEPSP were expressed as the average of two responses under each stimulation intensity.

The paired-pulse ratio (PPR) was recorded using the stimulation intensity matching to the 50% peak amplitude of fEPSP. A 5 ms step was used to set the interstimulus interval. The interstimulus interval was set between 20 and 70 ms. The amplitude of the second pulse was divided by the first pulse to calculate the PPR.

2.7. Local field potential (LFP) recording

The LFP of hippocampal dCA1 was recorded as the previous studies[30,31]. In brief, rats were anesthetized by intraperitoneally injecting 20% urethane solution to the rats placed on a stereotaxic frame. The animals were maintained at a level of anesthesia at which spontaneous theta rhythm was not presented but could be elicited by a tail pinch. LFPs were recorded by implanting a monopolar tungsten electrode into the hippocampal dCA1 pyramidal cell layer (AP, 3.8 mm; ML, 2.4 mm; and DV, 2.7 mm). The reference electrode was placed on the skull 2 mm away from the recording electrode. The ground electrode was clamped to the scalp. The LFP of hippocampal dCA1 was amplified by a ME-1 preamplifier (Chengdu Taimeng Software Company Limited, China), and then, the signals were filtered at 0–20 Hz and recorded by BL-420S system. After the LFP waveform became stable, the basic spontaneous LFP was recorded for 2 min. Subsequently, the vicinity of tail base was gently pinched with a metal plastic clamp for 1 min to induce theta rhythm. After recording procedure, the rats were executed for brain tissue or perfused for brain extraction.

2.8. Real-time polymerase chain reaction (PCR)

Utilizing the Trizol reagent to separate the total RNA from the basal forebrain regions, TaqMan MicroRNA reverse transcription kit was used to perform reverse transcription (Applied Biosystems, Carlsbad, CA, USA). The SYBR green core reagent kit's instructions for real-time PCR were followed (Applied Biosystems). Settings for the reactions were 95°C for 10 min, 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. In this experiment, all quantitative PCR data were used to calculate the relative content of target genes by the 2-ΔΔCt method. ΔCt = each group (Ct target gene-Ct housekeeping gene), ΔΔCt = experimental group ΔCt - control group ΔCt.

2.9. Western blot

Total hippocampal proteins were extracted, and the protein content was determined by the BCA Protein Assay Kit. Protein samples fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto nitrocellulose membranes

and incubated with the primary antibodies anti-choline acetyltransferase (ChAT; Catalog. #297013, 1:1,000, Synaptic Systems) and β-actin (Catalog. #8432, 1:1,000, Santa Cruz). The membranes were then incubated with fluorescent secondary antibody. The protein bands were captured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using the Odyssey V3.0 software.

2.10. Assessment of ACh concentration

The guidelines were followed to produce the dorsal hippocampus samples, which were then utilized for the ELISA assay. To obtain the supernatant, frozen tissues were homogenized in pre-chilled phosphate-buffered saline (PBS) and centrifuged at 3000 rpm/min for about 20 min. Following extraction, the concentrations of ACh were assessed using the ACh ELISA Kit (Catalog #MBS774123, MyBioSource) in accordance with the manufacturer's instructions. Then, the standard curve's linear regression equation was fitted using the concentration of the standard sample and the associated optical density (OD) value, and ACh concentration of the test sample was computed using this equation (y=aln(x)+b).

2.11. Immunofluorescence detection

Rats were infused with 0.9% sodium chloride (NaCl) and 4% buffered paraformaldehyde after the electrophysiological and LFP recordings. The brain tissue was preserved in 4% paraformaldehyde for 2 days before being sliced into 30 μm slices with a Leica oscillating microtome. The brain slices were sealed with goat serum for 2 h and then incubated with the primary antibodies (ChAT, #GTX163114, Genetex; GAD67, #MAB5406, Millipore; PV, #MCA3C9, Encor) at 4ºC for 12 h. On the next day, the brain slices were taken out of the freezer and rinsed 3 times in PBS for 15 min followed by secondary antibodies conjugated to Alexa Fluor 594 (1:200) and DAPI (1:50). Then, brain samples were examined under bright-field microscopy using a Zeiss Axio Scope A1 microscope with ×5, ×20, and ×40 objective.

2.12. Morris water maze

The Morris water maze consists of a 2.0 m diameter pool with a black bottom. As hints, circles, triangles, and squares are draped from a black curtain around the pool. In the middle of the first quadrant, there is a hidden escape platform with a 20 cm diameter and a top surface that was 2 cm just under the water. The procedure for preparing 2VO rats is outlined in previous works[7,32]. At 8.00 am, rats were placed in the pool, facing the wall, in the second, third, and quadrants (3-trial per day for 5 days), and they had 120 s to reach the platform. If not, they were led to the platform and allowed to relax for at least 20 s. On day 6, the platform was taken out of the water, and each rat participated in a single 120-s swim probe session. Next, the following parameters were tracked: escape latency, length of swim path, swim speed, frequency of crossing, and percent of swimming distance in target quadrant of overall distance.

2.13. Statistical analysis

The data are presented as mean ± standard error of mean (SEM). MatLab 7.0 was used to examine the power spectra of LFPs. $P < 0.05$ was regarded as statistically significant when comparing two groups using *t*-test. Our statistical analysis was performed using SAS 9.1 software (Institute Incorporation, serial number: 989155).

3. Results

3.1. Downregulation of basal forebrain *miR-195* expression impairs the electrical activity of the MS-dCA1 neural circuits

It has been reported that CCH can induce downregulation of *miR-195* expression in the hippocampi and cortices of rats, which can regulate Aβ deposition, tau hyperphosphorylation, neuronal death, and microglial polarization[7-10]. Here, we found that *miR-195* levels in the MS region were also significantly reduced in 2VO rats (Figure 1A). Our recent study found that CCH can also impair the function of the MS-dCA1 neural circuits in rats[12]. To investigate whether *miR-195* is involved in CCH-induced impairment of MS-dCA1 neural circuit, we stereotaxically injected lenti-AMO-195 into the MS region of rats to establish a rat model of *miR-195* knockdown (Figure 1D). Eight weeks later, the qRT-PCR result showed that compared with lenti-NC group, lenti-AMO-195 injection induced a decrease in the expression of *miR-195* in MS, which was reversed by coinjecting lenti-pre-*miR-195* (Figure 1B).

Subsequently, we assessed the basic electrophysiological properties of the MS-dCA1 neural circuit *in vivo* (Figure 1C and E). Compared with lenti-NC-injected rats, higher stimulation intensity was required to elicit the responsiveness of the MS-dCA1 circuit in lenti-AMO-195-injected rats (Figures 1F). When applying a 1-V-step stimulation to MS, we found that the amplitude of fEPSPs increased with the enhancement of stimulus intensity among lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*-treated rats. However, the fEPSP amplitude in lenti-AMO-195-injected rats was significantly lower than that in lenti-NC-injected rats, which was rescued by coinjection of lenti-pre-*miR-195* in the MS (Figure 1G-I).

To further assess the presynaptic function of the MS-dCA1 neural circuit, we monitored the paired-pulse

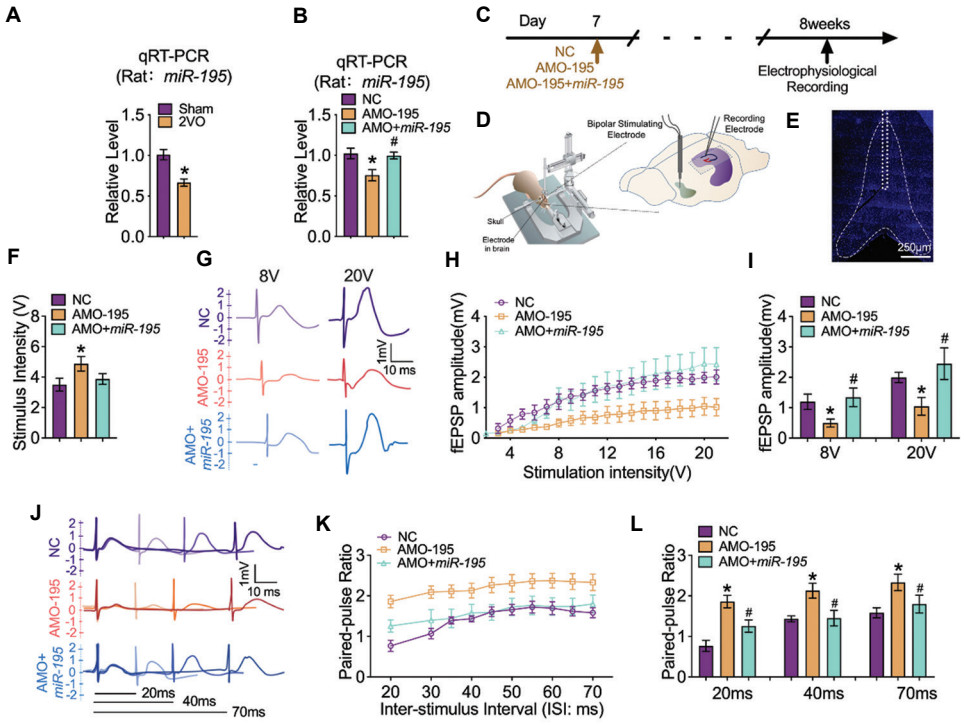


Figure 1. Downregulation of septal *miR-195* expression impairs the electrical activity of the MS-dCA1 neural circuit. (A) The *miR-195* level in the basal forebrain of 2VO rats by qRT-PCR. (B) The level of *miR-195* in the basal forebrain of rats administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*. (C) Schedule of the experiment plan. (D) Diagram of the *in vivo* electrophysiological experiment showing the locations of the recording (dCA1) and stimulating (MS) electrodes. (E) Schematic picture shows the injection site in MS region. (F) Differences of minimum stimulus intensity for triggering a measurable response of MS-dCA1 circuit in rats following lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195* injection. (G) Sample of fEPSP traces in rats administrated by lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195* with 8- and 20-V stimuli. (H) Comparison of input-output curves in the dCA1 region among three groups of rats. (I) Differences of the fEPSP amplitude with 8 and 20 V stimulation in each group. (J) Sample of MS-dCA1 fEPSP traces during paired pulse with 20, 40, and 70 ms of interstimulus intervals for each group. (K) The changes of PPR induced by lenti-AMO-195 injection. (L) The bar graph shows the PPR value in rats from different groups. $n = 6$. $^{*}P < 0.05$ versus NC rats. $^{\#}P < 0.05$ versus lenti-AMO-195 rats.

facilitation (PPF) of the MS-dCA1 circuit using an 8 V paired-pulse stimulus (Figure 1J). PPF is commonly used to evaluate presynaptic plasticity and can be expressed as PPR[33]. An increase of PPR indicates a decreased probability of presynaptic neurotransmitter release[34]. Compared with lenti-NC-injected rats, the PPR value was significantly increased in the lenti-AMO-195-injected rats under paired-pulse stimulus using 20–70 ms interval, which was rescued by coinjection of lenti-pre-*miR-195* into MS of rats (Figure 1K and L). Collectively, the knockdown of septal *miR-195* impairs the basic neurotransmission and presynaptic plasticity of the MS-dCA1 neural circuit.

3.2. Septal loss-of-function of *miR-195* reduced the dCA1-projecting neurons in MS and impaired hippocampal theta rhythmogenesis

The MS-dCA1 neural circuit includes excitatory cholinergic and inhibitory GABAergic projections, in which 66% are excitatory cholinergic neurons which are the major source of ACh release in the hippocampus[35]. In addition, the majority of the GABAergic neurons in the MS-dCA1 neural circuit are parvalbumin (PV)-positive neurons, which are the coordinator regulating the activity of cholinergic neurons in the hippocampus. Therefore, the next question is to clarify whether the cholinergic and GABAergic neurons in MS are involved in the loss-of-function of *miR-195*-induced impairment of MS-dCA1 neural circuits. To this end, we injected green retrobeads, a retrotracer dye, into the dCA1 of rats (**Figure S1A** and **S1B**). The retrobeads can track back from the synaptic terminal to the cell body by axoplasmic transport, thus labeling dCA1-projecting neurons from MS (**Figure S1C**, **S1D**, and **S1E**). One week later (Figure 2A), we compared the number of ChAT^{+}, GAD67^{+}, and PV^{+} neurons that were labeled by retrobeads in MS of rats administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195* (Figure 2B, E, and H). The quantification analysis showed that the numbers of total (Figure 2C, F, and I) and retrobeads-labeled (Figure 2D, G, and J) ChAT^{+}, GAD67^{+}, and PV^{+} neurons were lower in

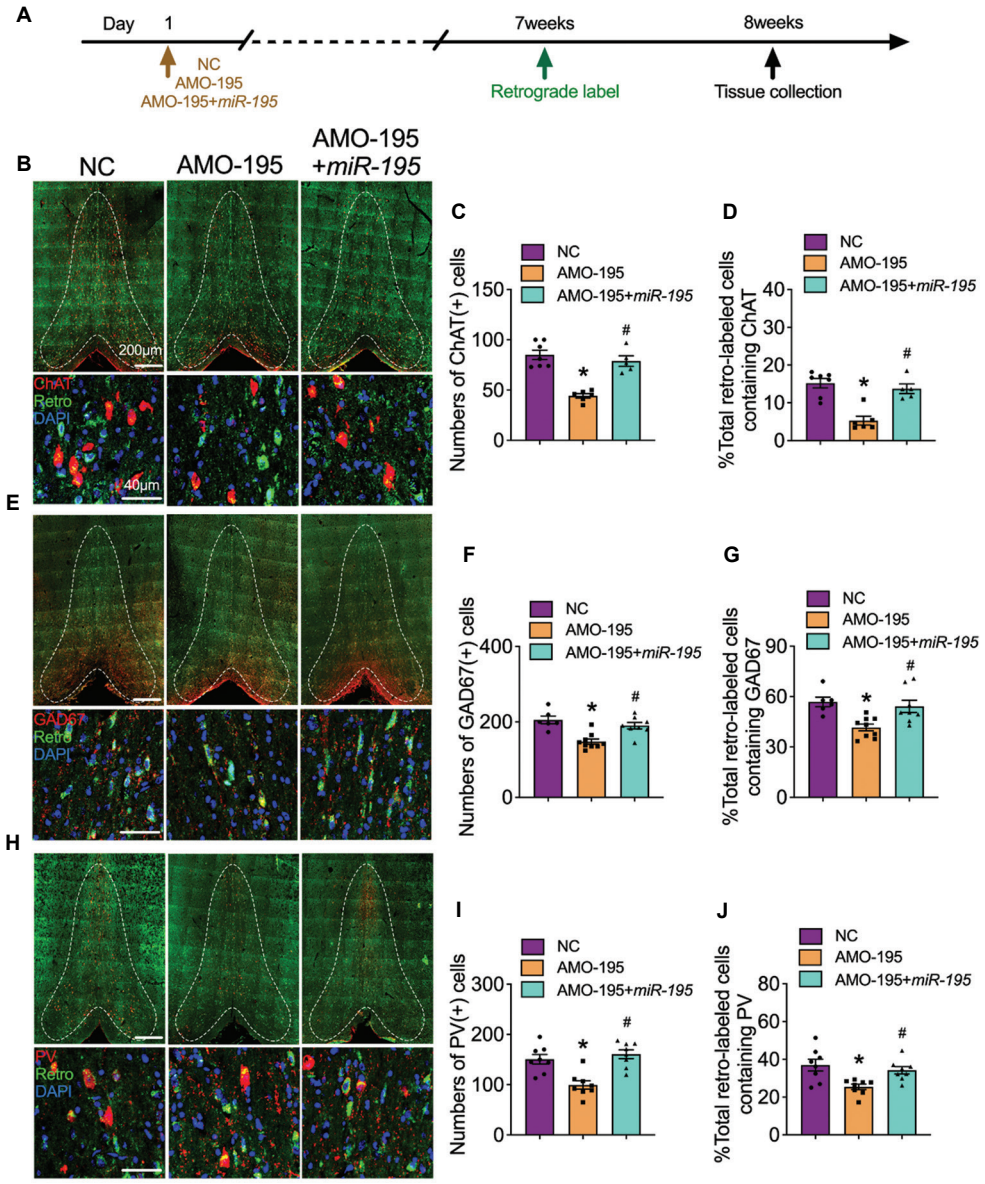


Figure 2. Knockdown of *miR-195* reduces the dCA1-projecting neurons in MS. (A) Schematic illustration of the timeline for the injection of green retrobeads. (B) Representative confocal images of cholinergic neurons (ChAT$^+$) in MS region of rat administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*. (C) Quantitative analysis of the number of neurons double labeled by ChAT and retrobeads. (D) Quantitative analysis of the percentage of retrobead-labeled neuron of the ChAT-marked neurons. (E) Representative confocal images of cholinergic neurons (GAD67$^+$) in MS region of rat administer with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*. (F) Quantitative analysis of the number of neurons double labeled by GAD67 and retrobeads. (G) Quantitative analysis of the percentage of retrobead-labeled neuron of the GAD67$^+$-marked neurons. (H) Representative confocal images of cholinergic neurons (PV$^+$) in MS region of rat administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*. (I) Quantitative analysis of the number of neurons double labeled by PV and retrobeads. (J) Quantitative analysis of the percentage of retrobead-labeled neuron of the PV$^+$-marked neurons. $n = 6$. $^*P < 0.05$ versus NC rats. $^\#P < 0.05$ versus lenti-AMO-195 rats.

lenti-AMO-195-injected rats than that in lenti-NC-injected rats. Moreover, these decreased neurons were all reversed by coinjection of lenti-pre-*miR-195*.

Hippocampal theta rhythmogenesis is controlled by the MS-dCA1 neural circuit depending on the synergistic action of cholinergic and GABAergic neurons[16]. In this process, sufficient ACh release can increase the power of relatively low-frequency theta oscillation[36]. Meanwhile, the activation of PV$^+$ GABAergic neurons in MS can elicit high-frequency firing, which cooperates with cholinergic neurons to maintain normal hippocampal theta rhythm[30]. To further identify the effect of *miR-195* on dCA1-projecting

MS neurons, we recorded hippocampal LFP in the pyramidal cell layer of the dCA1 region of rats (Figure 3A and B). The data showed that the hippocampal LFP changed from delta to theta rhythm following a 1-min tail squeeze in all three group rats (Figure 3C and D). Importantly, we found that the peak frequency and duration of hippocampal theta rhythm were significantly lower in lenti-AMO-195-injected rats than in lenti-NC-injected rats, which were rescued by coinjection of lenti-pre-*miR-195 in vivo* (Figure 3E and F). Taken together, knockdown of septal *miR-195* expression impairs the hippocampal theta rhythmogenesis, which is associated with decreased cholinergic and GABAergic neurons in MS that projects to dCA1 region.

3.3. *MiR-195* rescues the basic electrophysiological properties and MS projection neurons of MS-dCA1 neural circuit in CCH rats

The next issue we explored was to determine whether exogenously supplemented *miR-195* in MS could reverse CCH-induced MS-dCA1 neural circuit impairment. With regard to this issue, we injected lenti-pre-*miR-195* into MS region of 2VO rats. The lenti-pre-*miR-195* injection effectively elevated *miR-195* levels in 2VO rats that were close to sham rats (Figure 4B). We then performed the fEPSP recording *in vivo* as before (Figure 4A). We found that upregulation of *miR-195* expression reduced the increased minimum stimulus intensity that was necessary to record fEPSP in hippocampi of 2VO rats (Figure 4C). In comparison to sham rats, the fEPSP amplitude was noticeably lower in 2VO rats, which was rescued by the upregulation of *miR-195* expression following the injection of lenti-pre-*miR-195* (Figure 4D-F). As expected, upregulation of septal *miR-195* expression successfully inhibited the increased PPR in 2VO rats (Figure 4G-I). Together, upregulation of *miR-195* expression in MS region can reverse CCH-induced electrophysiological function impairment of MS-dCA1 neural circuit.

To clarify whether miR-195 is involved in the loss-of-function of CCH-induced impairment of MS-dCA1 neural circuit, we injected green retrobeads into dorsal hippocampi of rats at 7th week after 2VO surgery (Figure 5A). Subsequently, we assessed the alteration of specific MS-dCA1 projecting neurons by costaining ChAT, GAD67, and PV with green retrobeads among sham, 2VO and 2VO + lenti-pre-*miR-195* rats (Figure 5B, E, and H). Compared with 2VO rats, exogenous supplementation of miR-195 by injecting lenti-pre-*miR-195* into MS of 2VO rats rescued the decreased total number of ChAT$^+$, GAD67$^+$, and PV$^+$ neurons (Figure 5C, F, and I) and elevated the percentage of retrobeads-co-labeled neurons (Figure 5D, G, and J). At the 8th week after lenti-pre-*miR-195* injection into the MS of 2VO rats, we recorded hippocampal dCA1 theta rhythm (Figure 6A). As predicted, the peak theta frequency and duration of 2VO rats administrated by lenti-pre-*miR-195* were improved compared with 2VO control rats (Figure 6B-E). Since ACh release could increase the septal network excitability and highly correlated with the appearance and maintenance of hippocampal theta rhythm[37,38], we next detected ChAT expression and ACh concentration in the dorsal hippocampus of rats. We found that the expression of ChAT protein and the levels of ACh

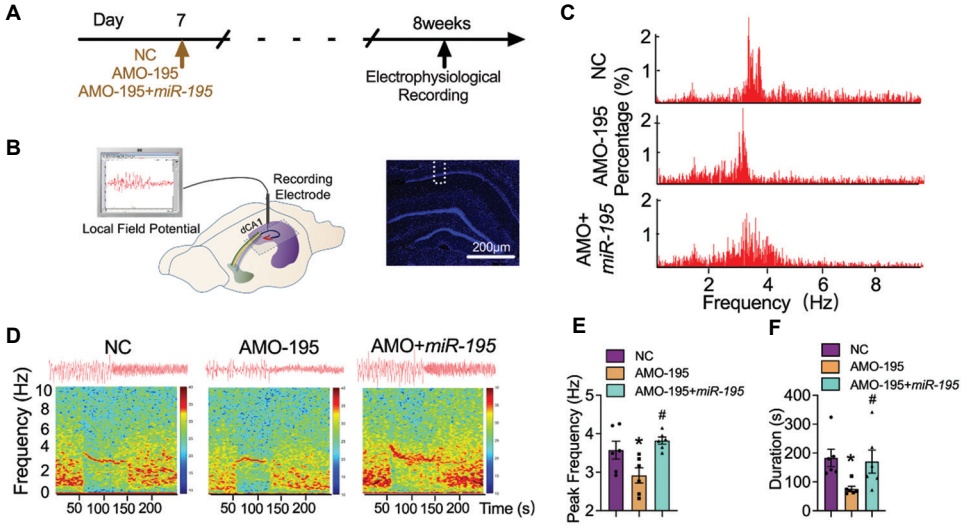


Figure 3. Loss-of-function of septal *miR-195* impairs the theta rhythmogenesis in dCA1 of hippocampus. (A and B) Schematic picture showing theta rhythm recording technique in the hippocampal dCA1 area. (C) Frequency spectrum analysis of LFP in the dCA1of hippocampus in rats. (D) Comparison of LFP of the dCA1 from rats administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-*miR-195*. Upper panel: Samples of dCA1 LFP traces. Lower panel: Heatmaps analysis of LFP in the dCA1. (E and F) The analysis of the theta rhythm's peak frequency and duration among three groups. n =6. $^*P < 0.05$ versus NC rats. $^\#P < 0.05$ versus lenti-AMO-195 rats.

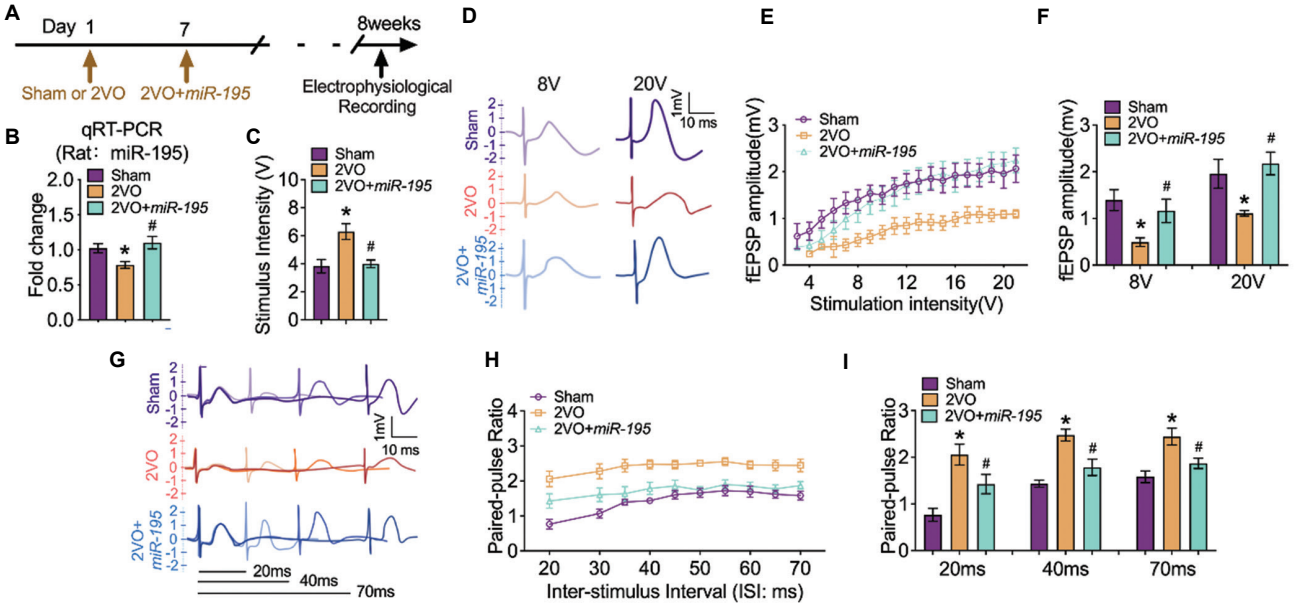


Figure 4. *MiR-195* improves the basic neurotransmission process and presynaptic function of MS-dCA1 neural circuit in CCH rats. (A) Schedule of the experiment plan. (B) The *miR-195* level in the basal forebrain of 2VO rats treated with lenti-pre-*miR-195*. (C) Differences of minimum stimulus intensity for triggering a measurable response of MS-dCA1 circuit in rats among sham, 2VO, and 2VO + lenti-pre-*miR-195* rats. (D) Sample trace of fEPSP in the hippocampal dCA1 with 8 and 20 V stimulations. (E) Comparison of input-output curves in the dCA1 region among three groups of rats. (F) Differences of the fEPSP amplitude with 8- and 20-V stimulations in each group. (G) Samples of MS-dCA1 fEPSP trace after paired pulses stimuli with 20, 40, and 70 ms intervals between in rats from different groups. (H) The changes of PPR induced by lenti- pre-*miR-195* injection. (I) The bar graph shows the largest PPR in rats from different groups. $n = 6$. $^{*}P < 0.05$ versus sham rats. $^{\#}P < 0.05$ versus 2VO rats.

in the hippocampi of 2VO rats were significantly decreased, which were recovered by lenti-pre-*miR-195* injection (Figure 6F and G, **Figure S2**). These results suggest that upregulation of *miR-195* expression can improve the function of MS-dCA1 neural circuit of 2VO rats and prevent the death of ChAT^{+}, GAD67^{+}, and PV^{+} neuron.

3.4. Upregulation of *miR-195* expression in the MS improves the spatial learning and memory ability of CCH rats

Our previous study found that exogenous supplementation of *miR-195* in the hippocampus could prevent 2VO-induced cognitive decline[7]. Therefore, we would like to evaluate whether upregulation of *miR-195* expression in MS has the similar effect using the Morris water maze test. First, we compared the swimming speed among the three groups to exclude the influence of locomotor abnormalities on the experimental results. The data showed that there was no difference among the three groups (Figure 7A). On the 1st day of the cued learning trial, basal forebrain injection of lenti-pre-*miR-195* reduced the latency to the platform of 2VO rats in the third and fourth quadrants (Figure 7B). Exogenous supplementation of *miR-195* also reduced the time spent by 2VO rats to find the hidden platform on the 2nd–5th days after being released into the tank in all of the three non-target quadrants of the localization navigation test (Figure 7C-E). On the 6th day of the spatial exploration test, we found that injection of lenti-pre-*miR-195* reversed the number of platform crossings (Figure 7F) and the percentage of swimming time in the target quadrant in 2VO rats (Figure 7G). The above results showed that upregulation of *miR-195* expression can attenuate the impairment of spatial learning memory ability in CCH rats (Figure 7H).

4. Discussion

With the aging of population and the prevalence of cerebrovascular diseases, the incidence of VaD is increasing year by year. However, the development of therapeutic drugs has not progressed much[39]. At present, many scientists recognize that improving the function of neural circuits is a potential therapeutic approach to treat neurodegenerative diseases[40,41]. Here, we found that loss-of-function of *miR-195* impaired the MS-dCA1 neural circuit in rats, while exogenous supplementation of *miR-195* rescued the dysfunction of the MS-dCA1 neural circuit and declined spatial memory in CCH rats. We thus present in this paper a candidate strategy for alleviating the impaired septohippocampal neural circuit during the pathogenesis of dementia.

As the largest cerebral cholinergic nucleus and theta rhythm "pacemaker," the basal forebrain forms a neural

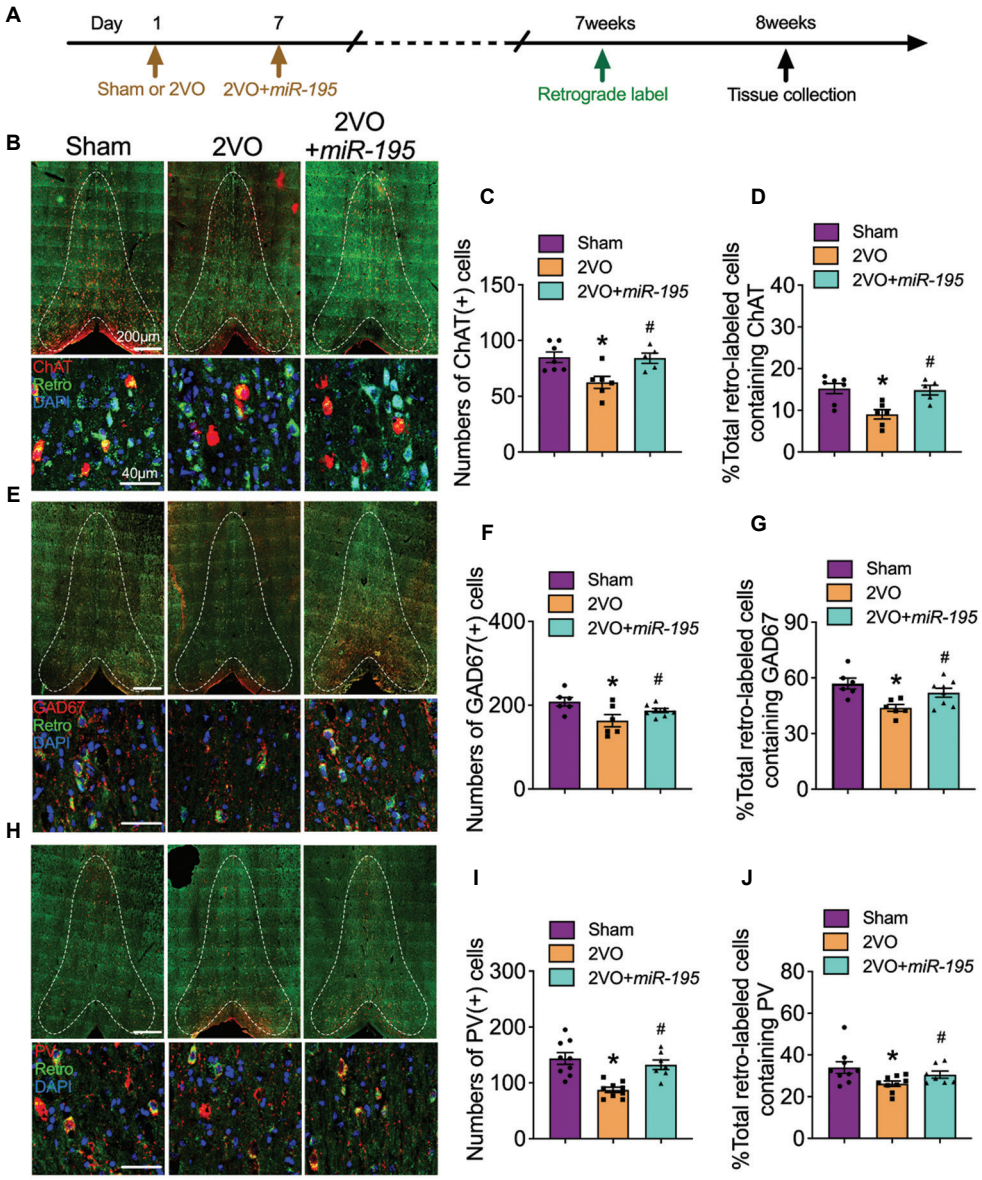


Figure 5. *MiR-195* rescues the decreased dCA1-projecting neurons in MS of CCH rats. (A) Schematic illustration of the timeline for the injection of green retrobeads. (B) Representative confocal images of cholinergic neurons (ChAT$^+$) in MS region in rat from sham, 2VO, and 2VO + lenti-pre-*miR-195* groups. (C) Quantitative analysis of the number of neurons double labeled by ChAT and retrobeads. (D) Quantitative analysis of the percentage of retrobead-labeled neuron of the ChAT-marked neurons. (E) Representative confocal images of cholinergic neurons (GAD67$^+$) in MS region of rat from sham, 2VO, and 2VO + lenti-pre-*miR-195* groups. (F) Quantitative analysis of the number of neurons double labeled by GAD67 and retrobeads. (G) Quantitative analysis of the percentage of retrobead-labeled neuron of the GAD67$^+$-marked neurons. (H) Representative confocal images of cholinergic neurons (PV$^+$) in MS region of rat from sham, 2VO, and 2VO + lenti-pre-*miR-195* groups. (I) Quantitative analysis of the number of neurons double labeled by PV and retrobeads. (J) Quantitative analysis of the percentage of retrobead-labeled neuron of the PV$^+$-marked neurons. $n = 6$. *$P < 0.05$ versus NC rats. #$P < 0.05$ versus lenti-AMO-195 rats.

circuit with the hippocampus that participates in the formation and maintenance of memory. In parallel, the dysfunction of the MS-dCA1 neural circuit is thought to be one of the early pathological features of dementia[17,30,40]. In fact, abnormalities in neural circuit function have repeatedly been recorded before any neurodegeneration or plaque deposition in the brain of transgenic mice overexpressing APP, triple transgenic mice (3 × Tg), and wild-type mice injected with Aβ[42-44]. CCH, a preclinical stage of MCI, has also been shown to be involved in impaired hippocampal theta rhythm and MS-dCA1 neural circuit[12,45]. Previous morphological evidence suggests that the neuronal projection from the MS to the dCA1 pyramidal cells is mainly composed by cholinergic and

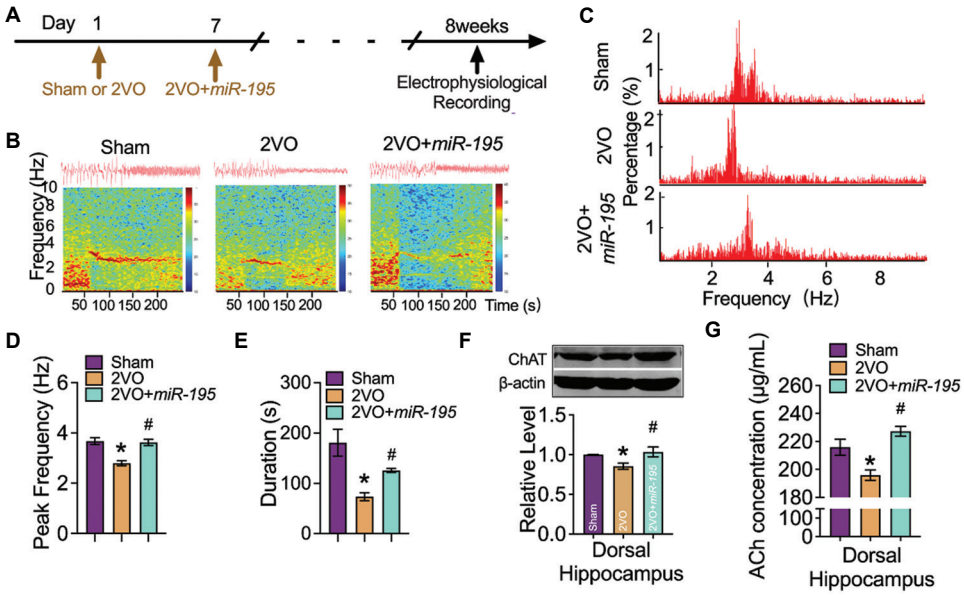


Figure 6. *MiR-195* improves the theta rhythmogenesis of hippocampal dCA1 of CCH rats. (A) Schematic picture showing the timeline for the theta rhythm recording in the hippocampal dCA1 region. (B) Comparison of LFP in dCA1 region from rats of sham, 2VO, and 2VO + lenti-pre-*miR-195* groups. Upper panel: Samples of dCA1 LFP traces. Lower panel: Heatmaps analysis of the dCA1 LFP. (C) Frequency spectrum analysis of LFP in the dCA1 of hippocampus in rats. (D, E) The analysis of the theta rhythm's peak frequency and duration among three groups. (F) Western blot analysis of the expression of ChAT in hippocampal dCA1 region. *n*=4. (G) Comparison of the ACh concentrations in hippocampal dCA1. *n*=6. $^{*}P < 0.05$ versus sham rats. $^{\#}P < 0.05$ versus 2VO rats.

GABAergic components[35]. Cholinergic projections from the MS region coordinate hippocampal pre- and post-synaptic activity and temporally modulate the synaptic plasticity[46,47]. GABAergic afferents from the MS mostly innervate PV-positive interneurons in the hippocampus, and these interneurons inhibit the activity of peripheral pyramidal neurons[48,49]. Accordingly, rhythmically activated GABAergic neurons in MS may eventually transform this activation into the rhythmic disinhibition of hippocampal pyramidal neurons and provide the synchronization between hippocampus and MS[14,50]. Sufficient ACh production in the hippocampus increases the power of low-frequency theta rhythm but suppresses the opposing non-theta activities[36]. While GABAergic projection in the MS-dCA1 neural circuit provides the driving force for theta rhythm in the hippocampus[14]. Thus, combined with the decreased I-O curve, the increased PPR of the MS-dCA1 neural circuit, and the diminished hippocampal theta rhythm, we speculate that the CCH-induced injury may be the result of neuronal loss in the MS-dCA1 neural circuit. The previous studies have reported the decreased cholinergic and GABAergic functions in septal-related neural circuits in AD transgenic models[17,47,51]. Our previous study demonstrated that CCH could induce neuronal loss in the hippocampi of CCH rats by targeting the gene of DR6 protein[8]. In the present study, we found that CCH can also induce neuronal loss in MS region, which was assessed by costaining the signal of ChAT/GAD67/PV antibody and retrotracer dye from dCA1. The results suggested that the neuronal loss in MS also participates in CCH-induced cognitive decline.

A large number of studies reported that many miRNAs, which act as crucial modulators, participate in the AD pathological process by regulating APP and BACE1 expressions[20-23]. Our previous studies reported that CCH led to the reduction of *miR-195* expression in the hippocampus, and downregulation of *miR-195* expression in hippocampal region impaired the spatial cognitive ability by inducing Aβ deposition, tau hyperphosphorylation, microglial polarization, synaptic dysfunction, and neuronal death[7-10]. Here, we also found that CCH could downregulate *miR-195* expression in MS region. Similar to 2VO rats, by injecting lenti-AMO-195, a lentivirus-mediated antisense nucleotide of *miR-195*, into the MS region of sham rats to decrease the level of *miR-195*, we found a significant decrease in fEPSP amplitude and an increase in the minimum stimulus intensity of the neural circuit response as well as impaired presynaptic neurotransmitter release probability. To clarify the action of *miR-195* on neurons in MS region and avoid the influence of neuronal loss in the hippocampus induced by CCH, we first injected lenti-AMO-195 specific into the MS region but not the hippocampus of normal rats and

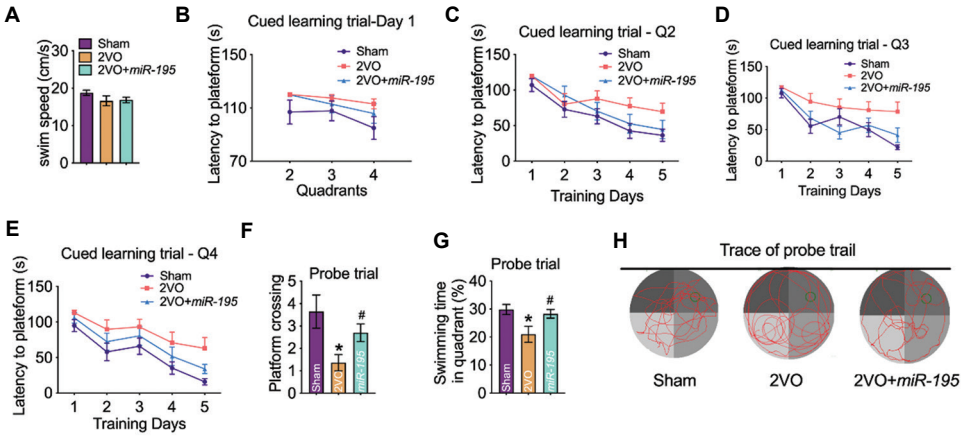


Figure 7. Upregulation of septal *miR-195* expression improves the spatial learning and memory ability of CCH rats. (A) Comparison of swimming speed among sham, 2VO, and 2VO + lenti-pre-*miR-195* rats. (B) Comparison of the latency to hidden platform in the second, third, and fourth quadrant on the 1st day from rats of the three groups. (C–E) Comparison of the latency to hidden platform in the second, third, and fourth quadrant during the training phase from rats of the three groups. (F and G) Differences of the platform crossing (F) and the percentage of swimming in the goal quadrant (G) during the investigation trials from rats of the three groups. (H) Samples of swimming traces of the rats from the three groups in probe trial. $n = 6$. $^{*}P < 0.05$ versus sham rats. $^{\#}P < 0.05$ versus 2VO rats.

induced the loss of Chat^{+}, GAD67^{+}, or PV^{+} neurons in MS. Next, we injected lenti-pre-*miR-195* specific into the MS region but not the hippocampus of 2VO rats and found that upregulation of *miR-195* in MS region but not hippocampus prevented the loss of neurons in the MS following CCH. Taken together, the downregulation of septal *miR-195* expression could impair the function of MS-dCA1 neural circuit that was associated with the neuronal losses in the MS area.

Recent studies using both cellular and animal models demonstrated that *miR-195* has positive effects on anti-apoptosis in injured neurons by suppressing Sema3A/Cdc42/JNK signaling, promotes neural regeneration by promoting neural stem cell proliferation and migration, and has anti-inflammation action by blocking the NF-κB pathway[27]. Furthermore, the cerebral stroke damage of rats was improved by intravenously injecting *miR-195* in the acute stage[27]. These studies suggest that *miR-195* may have the potential in the treatment of cerebral ischemic diseases. Our previous studies found that upregulation of *miR-195* expression in hippocampal and cortical regions rescued the spatial cognitive ability in 8-week 2VO rats by inhibiting Aβ deposition, tau hyperphosphorylation, microglial polarization, synaptic dysfunction, and neuronal death[7-10]. In the present study, we demonstrated that upregulation of *miR-195* expression through the injection of lenti-pre-*miR-195* into MS region successfully reversed the impaired function of MS-dCA1 neural circuit as well as neuronal loss, and even attenuate the declined spatial learning memory ability induced by CCH. All these studies provide solid evidence that *miR-195* may have the potential to be a biomarker and/or therapeutic drug for early AD or VaD. However, the specific molecular mechanism of *miR-195* modulating MS-dCA1 neural circuit function needs to be further investigated.

5. Conclusions

In our study, we found that knockdown of *miR-195* in the MS region can impair MS-dCA1 neural circuit function, while upregulation of *miR-195* can rescue the impaired function of MS-dCA1 neural circuit and spatial memory ability in CCH rats. This provides a valuable reference for future anti-dementia therapy involving *miR-195*.

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Conflict of interest

The authors declare that they have no conflicts of interest to report.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Target molecular-based neuroactivity screening of *Panax ginseng* through network pharmacology integrated molecular docking

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Abstract

Panax ginseng is a medicinal herb that has therapeutic effects against neuronal damage. The main goal of the present study is to investigate the therapeutic potential of *P. ginseng* in treating neuronal damage through *in silico* analysis. The literature review was conducted to create a ingredients-target database for *P. ginseng*. The database was essential in identifying the targets related to neuronal damage, which was followed by the construction of protein-protein interaction network by the STITCH database. The enrichment analysis was performed using Cytoscape (ClueGO) software. The molecular docking analysis was ran using AutoDock and Discovery Studio. The literature search revealed the presence of 66 compounds in *P. ginseng*, which subsequently resulted in the retrieval of 102 potential targets of these compounds. They were screened for their association with neuronal damage. Three proteins, namely, Glutathione S-transferase P1, HSP90, and Mitogen-activated protein kinases (MAPK8), were found to be in prime interaction with the relative compounds. Subsequently, 91 Gene ontology terms were found, of which four genes were associated with neuronal damage. Molecular docking analysis also revealed good binding affinities of various compounds of *P. ginseng* to the selected target, MAPK8. In conclusion, the biological processes linked to the therapeutic action of *P. ginseng* involve the positive regulation of JUN kinase activity, chaperone-mediated protein folding, and MyD88-dependent toll-like receptor signaling pathway.

Keywords: *Panax ginseng*; STITCH; Biological effects; Cytoscape; Neuronal damage; Mechanism of action

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1. Introduction

Plant-derived compounds, also known as natural compounds, have garnered much attention in the recent years due to their therapeutic effects against many diseases and pathological conditions. Their potent activities attributed to their phytochemical and pharmacological properties will certainly benefit many given their widespread use in traditional as well as modern medicine. Therefore, their use has been embraced as

a means of effective and potential therapy for various medical disorders[1]. In many diseases such as neuronal damage, chemotherapy is not always an effective treatment; therefore, complementary and alternative medicine has become an attractive therapeutic option and is gradually gaining traction since this form of medicine commonly involves the use of herbs for their therapeutic applications[2].

Among the herbs used, *Panax ginseng* is a traditional medicinal plant that has been used therapeutically for millennia in East Asian countries such as China, Japan, and Korea. In Traditional Chinese Medicine (TCM), ginseng is considered a potent herb. Interestingly, "*Panax*" means "all-healing", which hails ginseng to be a potent herb in healing all medical aspects of the human body. Among different types of ginseng found all over the world, *P. ginseng* has received much attention and has been the research target of most of the studies surrounding this species[3]. Ginseng has been reported to exert many beneficial effects on the central nervous system as well as endocrine, cardiovascular, and immune systems[4,5], and has a wide range of potential therapeutic advantages against various disorders, such as cancer[6] and ischemic stroke[7]. Furthermore, ginseng has been reported to reduce the side effects caused by chemotherapy and radiotherapy, therefore, improving the overall quality of life and good mental health of patients with neuronal damage[7].

Neuronal damage is one of the most precarious medical conditions affecting the elderly worldwide. The phenotypic features of neuronal damage at the macroscopic level in most cases include headache and seizures, but the pathogenesis differs at both histological and genetic levels, with various predisposing risk factors coming into play[8]. The induction of neuronal damage is facilitated by various events in the cell, along with the generation of reactive oxygen species (ROS), depletion of antioxidative species, and induction of inflammatory phases and cyclooxygenase activity[9]. It is well-established that the development of neuronal damage is prompted by the action of different biological pathways caused by *TP53*, *ERBB2*, *BRAF*, and *KRAS* mutations[10-13]. Moreover, the release of inflammatory mediators, such as heat-shock proteins (HSP), interleukins[6,11,12], and vascular endothelial growth factor[14-17], has been found to significantly accelerate the development of neuronal damage. Various studies have demonstrated the protective effects of ginseng in the treatment of neuronal damage, which also increases the quality of life in patients receiving chemotherapyp[18]. The inhibitory effects of *P. ginseng* and its constituents on neuronal damage mechanisms have been investigated and confirmed in many experiments[19].

In recent years, the concept of network pharmacology has arisen from the systems biology approach, which has resulted in a paradigm shift from one drug and one target to multiple drugs and multiple targets, with their interaction increasing multi-fold[20]. Network pharmacology is an approach that focuses on the biological interactions and their genes counterparts, which effectively play an active role in exerting the therapeutic effect of a herb once it interacts with the target proteins in the host, which is *Homo sapiens*. This approach is, therefore, very potent in deciphering the mechanistic mode of action of several individual herbs or a concoction of herbs that belong to TCM Pharmacopoeia[21].

In this study, the mode of action of *P. ginseng* against neuronal damage was elucidated using a network pharmacology approach. At present, limited data are available for the therapeutic potential of *P. ginseng* against neuronal damage, which further affirms that no study has previously reported on the mechanism of action of the herb against neuronal damage. Furthermore, the molecular docking analysis helps determine the potential action of its active compounds that interact with possible protein targets in human in the treatment of neuronal damage. This study presents a suitable premise to ascertain the potential ability of the herb for its other potentially therapeutic abilities. Therefore, it is necessary to investigate said mechanism of action, with specific attention given to the multi-target interaction of *P. ginseng*. Therefore, a network pharmacology approach was employed in this study along with the analyses of biological pathways and drug interactions to determine the pharmacological action of *P. ginseng* in treating neuronal damage.

2. Materials and methods

2.1. Methodology

The potential of *P. ginseng* in treating neuronal damage was ascertained using the STITCH database[22], which was the first step of the systematic analysis. In the next step, the bioactive compounds were elucidated from an extensive literature search, along with their associated protein targets in human. This step was followed by the construction of protein-protein interaction network (PPIN) and the gene ontology (GO) enrichment analysis (Figure 1).

2.2. Retrieval of chemical ingredients and their targets

This step was performed by screening the compounds of *P. ginseng* on the TCM systems pharmacology database and analysis platform (TCMSP) database[23]. After this step, the herb-associated protein targets were retrieved from the reported literature and TCMSP database, respectively, after which these protein targets were screened for their association in the treatment of neuronal damage. The targets were then analyzed further.

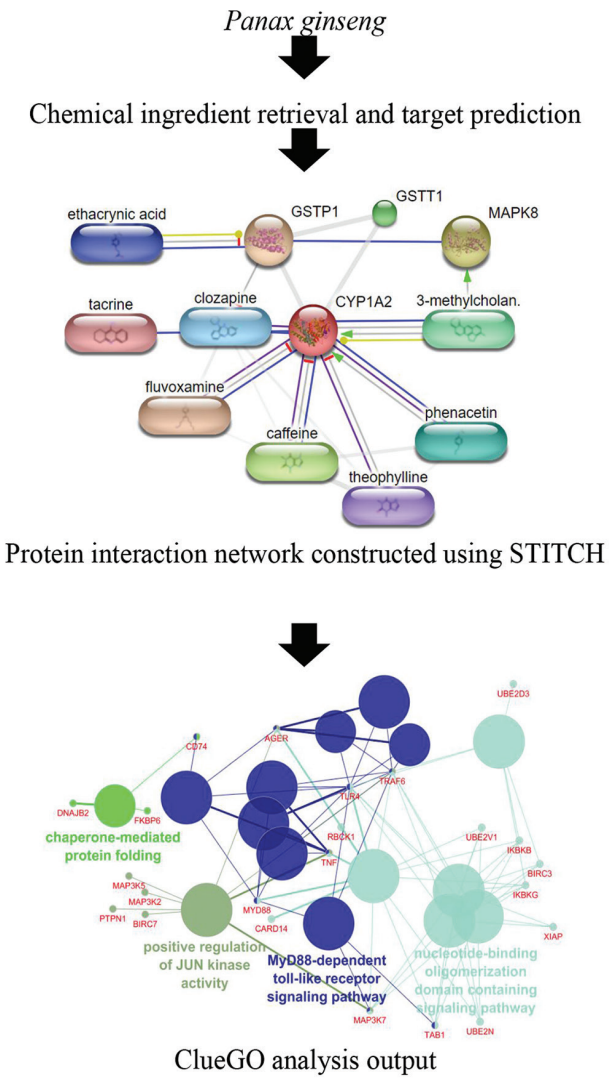


Figure 1. Flowchart of systematic procedure utilized in this study.

2.3. Network construction and analysis

The protective role and pharmacological action of *P. ginseng* and its bioactive compounds with the associated targets and biological pathways were ascertained using the STITCH database version 5.0[24], which is a comprehensive online database containing the knowledge of more than 2000 organisms and around 10 million proteins and their interactions. To investigate the interaction of *P. ginseng* compounds with its target proteins, PPIN was constructed and analyzed.

2.4. GO and pathway enrichment analysis

The therapeutic potential of associated proteins against neuronal damage was analyzed and revealed through GO enrichment analysis, which was carried out using Cytoscape (version 3.4.0) and its plug-in ClueGO[25,26], with the significance level set at 0.05. The parameters set for this analysis were medium network and two-sided hypergeometric test with Bonferroni correction. Eventually, the functional network was analyzed and visualized using algorithmic organic layout.

2.5. Docking study

Four compounds (gensinside, panaxydol, panaxynol, and panaxytriol) were chosen to dock with the selected targets (Glutathione S-transferase P1 [GSTP1], HSP90, and Mitogen-activated protein kinases [MAPK8]) in search of a suitable ligand that could match and favorably bind with the protein[27]. The preparation of ligands was performed by searching the PubChem database, after which the ligand structures were saved in mdl. format (v3000). This file was then visualized in AutoDock (version 4.2). The non-polar H_2 atoms were combined while the torsion value was defined to default. Kollman and Gasteiger charges were affixed with the aid of default parameters. To deduce the function of the acquired protein targets in neuronal damage, three-dimensional structure of the acquired protein targets was downloaded from the PDB database in.pdb format (www.rcsb.org). The structure was visualized in AutoDock (version 4.2), after which the H_2O molecules and the polar H_2 atoms were deleted and added, respectively. The same software was used to dock the compounds with GSTP1, HSP90, and MAPK8. All the compounds were individually docked with the same ligand, after the grid-box for all compounds was assigned before docking, where grid parameters were established to affirm the binding of ligands to the binding sites of protein, and the output was saved in the GPF format. The number of runs was adjusted to 30, while Lamarckian-GA (version 4.2) was chosen for processing the output file. The binding energy was selected as the main mode of result elucidation, which was saved in pdbqt. format. The lowest binding energy scores of ligand-proteins were chosen and saved in.pdb format and were utilized for further analysis. The interaction of ligand-proteins was elucidated further by Discovery Studio 2020 (BIOVIA).

3. Results

3.1. Retrieval of chemical ingredients and their targets

According to the outcomes collected from TCMSP, the enlistment of phytochemical compounds from a literature search revealed 66 compounds in *P. ginseng* (Table 1) and 102 potential targets (Table S1), of which three were found in *H. sapiens*, which were standardized using UniProt.

3.2. Network construction and analysis

STITCH database was employed to construct an intricate PPI network, which consists of the subsequent association

Table 1. The retrieved 66 chemical compounds in *Panax ginseng*

ID	Compound	Mol Wt	OB (%)	DL	ID	Compound	Mol Wt	OB (%)	DL
5318	Dianthramine	289.26	40.45	0.20	4100	N-Salicylidene-salicylamine	227.28	95.46	0.11
1641	Methyl linoleate	294.53	41.93	0.17	422	kaempferol	286.25	41.88	0.24
1818	Methyl palmitelaidate	268.49	34.61	0.12	4492	Chrysanthemaxanthin	584.96	38.72	0.58
1965	Dauricine (8CI)	624.84	23.65	0.37	4498	12-O-Nicotinoylisolineolone	469.63	20.70	0.83
2307	20-Hexadecanoylingenol	586.94	28.20	0.68	5269	(+)-Maalioxide	222.41	55.93	0.13
2669	Campesteryl ferulate	576.94	22.10	0.59	5272	13-Tetradecenyl acetate	254.46	36.76	0.10
2879	Diop	390.62	43.59	0.39	5280	ginsenoside-Rh1_qt	476.82	20.13	0.78
449	Stigmasterol	412.77	43.83	0.76	5282	20(S)-Ginsenoside-Rh1_qt	476.82	20.13	0.78
358	Beta-sitosterol	414.79	36.91	0.75	5285	20(s)-protopanaxadiol	460.82	29.69	0.77
3648	Inermin	284.28	65.83	0.54	5288	20-(S)-Ginsenoside-Rg3_qt	460.82	29.69	0.77
5364	Malonylginsenoside Rd_qt	460.82	29.69	0.77	5292	3-O-beta-D-Glucuronopyranosyl gypsogenin_qt	470.76	23.72	0.75
3902	Methyl (Z)-icos-11-enoate	324.61	29.49	0.23	5301	6'-Malonylginsenoside Rd1_qt1	460.82	29.69	0.77
5302	7-(beta-Xylosyl) cephalomannine	962.15	27.33	0.17	5396	cis-Widdrol alpha-epoxide	238.41	69.04	0.15
5305	Nepetin	316.28	26.75	0.31	5319	Ditertbutyl phthalate	278.38	43.67	0.13
5308	Aposiopolamine	271.34	66.65	0.22	5320	arachidonate	304.52	45.57	0.20
5314	Celabenzine	379.55	101.88	0.49	5321	Frutinone A	264.24	65.90	0.34
5317	Deoxyharringtonine	515.66	39.27	0.81	5327	Gypnoside V_qt	460.82	29.69	0.77
5343	Ginsenoside-Rg3_qt	460.82	29.69	0.77	5353	Ginsenoyne C	276.41	43.38	0.13
5344	Ginsenoside rh2	622.98	36.32	0.56	5355	Ginsenoyne E	258.39	36.53	0.13
5348	Ginsenoside-Rh4_qt	458.80	31.11	0.78	5356	Girinimbin	263.36	61.22	0.31
5351	Ginsenoyne A	258.39	66.22	0.13	5357	Gomisin B	514.62	31.99	0.83
5352	Ginsenoyne B	294.85	39.79	0.13	5360	malkangunin	432.56	57.71	0.63
5376	Panaxadiol	460.82	33.09	0.79	5373	notoginsenoside R2_qt	476.82	20.13	0.78
3845	Folinic acid	473.50	23.60	0.74	5362	Malonylginsenoside Rc_qt1	460.82	29.69	0.77
5366	Malvic acid	280.50	30.99	0.15	5401	ginsenoside Rg5_qt	442.80	39.56	0.79
5378	Panaxytriol	278.43	33.76	0.13	628	darutoside	574.93	21.32	0.63
5384	Suchilactone	368.41	57.52	0.56	66	alloaromadedrene	204.39	53.46	0.10
5386	Vulgarin	264.35	29.21	0.20	676	DBP	278.38	64.54	0.13
749	Linoleic	280.50	41.90	0.14	1312	9-Hexadecenoic acid	254.46	35.78	0.10
5398	Alexandrine	576.95	20.63	0.63	787	Fumarine	353.40	59.26	0.83
5399	Alexandrin_qt	414.79	36.91	0.75	935	Hepanal	204.39	53.83	0.10

Mol Wt, molecular weight; OB, oral bioavailaity; DL, drug likeliness; ID, identity document

of four protein targets, with a medium network probability score of 0.400, with four nodes and four edges. The resultant PPI network comprises functional interactions, where the nodes and edges represented the target proteins and/or their associated genes, and the interactions between different genes, respectively. Details of the constructed network show that its *P*-value is small (0.271), whereas the number of edges for the network was found to be 3 at randomly chosen nodes. Small *P*-value denotes the non-random selection and the significant number of edges in a network. In this PPI network, the values for node degree and clustering coefficient were found to be 2 and 0.833, respectively. Moreover, three hubs were found due to their nodal degree being higher than the average, that is, 2, where *CYP1A2* was found to have the highest node degree, that is, 9. Table 2 describes the functional enrichments in PPIN network (KEGG Pathways). It was followed by other proteins, such as GSTP1, MAPK8, and GSTT1, respectively. These proteins and associated hubs are reported to be significant mediators in depression. The action view of the functional nodes is shown in Figure 2, demonstrating the functions of these proteins,

Table 2. Functional enrichments in PPIN network (KEGG Pathways)

Pathway ID	Pathway description	Count in gene set	False discovery rate
00980	Metabolism of xenobiotics by cytochrome P450	3	0.000824
00982	Drug metabolism – cytochrome P450	3	0.000824
05204	Chemical carcinogenesis	3	0.000824
00480	Glutathione metabolism	2	0.0262

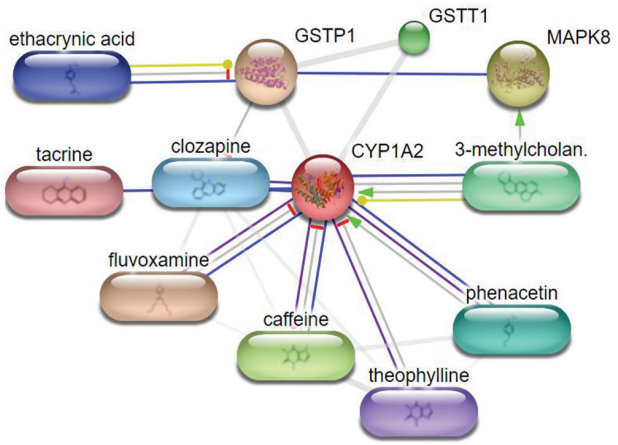


Figure 2. Action view of the protein network of *Panax ginseng* targets. Action type is represented by colored edges, as described here: Activation (•—•), inhibition (•—•), binding (•—•), catalysis (•—•), phenotype (•—•), post-translational modification (•—•), reaction (•—•), and transcriptional regulation (•—•). Action effects are shown by following signs: Positive (•—•), negative (•—•), and unspecified (•—•). Abbreviations: *CYP1A2*, cytochrome P450, family 1, subfamily A, polypeptide 2; GSTP1, glutathione S-transferase pi 1; MAPK8, mitogen-activated protein kinase 8; GSTT1, and glutathione S-transferase theta 1.

including activation, inhibition, binding, catalysis, post-translational modification, and their role in the regulation of transcription.

3.3. GO and pathway enrichment analysis

The output of the network analysis was assessed through Cytoscape software with ClueGO plugin, demonstrating terms that are associated with its therapeutic action. The biological action of *P. ginseng* belonged to 91 GO terms, which were then categorized into four subgroups. These subgroups were associated with positive regulation of JUN kinase activity, chaperone-mediated protein folding, nucleotide-binding oligomerization domain-containing signaling pathway, and MyD88-dependent toll-like receptor signaling pathway (Table 3 and Figure 3). These findings are significant in elucidating the mode of action of *P. ginseng*.

3.4. Docking study

Docking analyses revealed that compounds such as gensinside, panaxydol, panaxynol, and panaxytriol exhibited low binding affinities (−4.37–−8), especially between gensinside and MAPK8, thereby indicating good binding efficiency between them. The quantity of H bonds of these respective four compounds with GSTP1, HSP90, and MAPK8 is given in Tables 4 and 5 and Figure 4.

4. Discussion

The presence and role of various compounds found in *P. ginseng*, such as stigmasterol, beta-sitosterol, inermin, nepetin, aposiopolamine, celabenzine, panaxadiol, panaxytriol, suchilactone, vulgarin, linoleic acid, alexandrin, kaempferol, girinimbine, and ginsenoside, have been associated with the bioactivity of the herb, as reported by various studies over the years. The protective ability of stigmasterol against cellular apoptosis, generation of ROS, and the Ca^{2+} levels in the mitochondria and cytosol in neuronal damage cells was studied, where it was demonstrated that the compound was effective in inhibiting the formation of malignant cells, along with the decrease of the aforementioned activities on cellular level. Moreover, stigmasterol plays a role in stimulating apoptosis through the activation of the estrogen receptor axis in the mitochondria. The migration of malignant cells was also stalled by the activity of stigmasterol[27]. Another study showed that stigmasterol led to a decrease in nuclear factor-erythroid factor 2-related factor 2 (Nrf2) expression in neurodegenerative cells, and increases the sensitivity of these cells to chemotherapy and potent neurotherapeutics[28]. The protective action of beta-sitosterol in neuronal cells has been demonstrated by various cell line studies[29-32]. This protective ability has piqued the interest of many researchers that are now investigating the neuroprotective role of sterol compounds isolated from plants[33]. For instance, a study reported the effective potential of beta-sitosterol in inhibiting the neurodegenerative action in neuronal cells after its oral administration in cancer-induced rat models[34]. Hispidulin and nepetin were observed to decrease the expression of the CD8 cell which was inversely relative to the apoptotic cell death, and inflammation in neuronal cells[35]. Linoleic acid has been reported to demonstrate a somewhat "see-saw" approach to neurodegenerative activity, stimulating proliferation at the cellular level in various neurodegenerative cell lines[36], promoting the growth of neurodegenerative cells in animal models[37], while also exhibiting anti-tumor effects in studies, which contribute to its role as a therapeutic agent in neuronal damage[38]. Conjugated linoleic acid demonstrated potent therapeutic effects in neuronal damage cells. However, cell

Table 3. Retrieval of GO terms and their related genes through ClueGO

GO ID	GO term	Term *P* value (¤)	Group *P* value (¤)	Associated genes
43507	Positive regulation of JUN kinase activity	3.38×10^{-11} (3.71×10^{-10})	3.38×10^{-11} (1.01×10^{-10})	*AGER, BIRC7, MAP3K2, MAP3K5, MAP3K7, PTPN1, TNF, TRAF6*
61077	Chaperone-mediated protein folding	1.10×10^{-3} (1.10×10^{-3})	1.10×10^{-3} (1.10×10^{-03})	*CD74, DNAJB2, FKBP6*
70423	Nucleotide-binding oligomerization domain containing signaling pathway	3.99×10^{-18} (5.98×10^{-17})	1.62×10^{-19} (6.47×10^{-19})	*BIRC3, IKBKB, IKBKG, MAP3K7, TAB1, TLR4, TRAF6, UBE2N, UBE2V1, XIAP*
2755	MyD88-dependent toll-like receptor signaling pathway	9.18×10^{-8} (8.26×10^{-7})	4.33×10^{-7} (8.67×10^{-7})	*MAP3K7, MYD88, TAB1, TLR4, TRAF6*

Corrected with Bonferroni correction using a step-down procedure

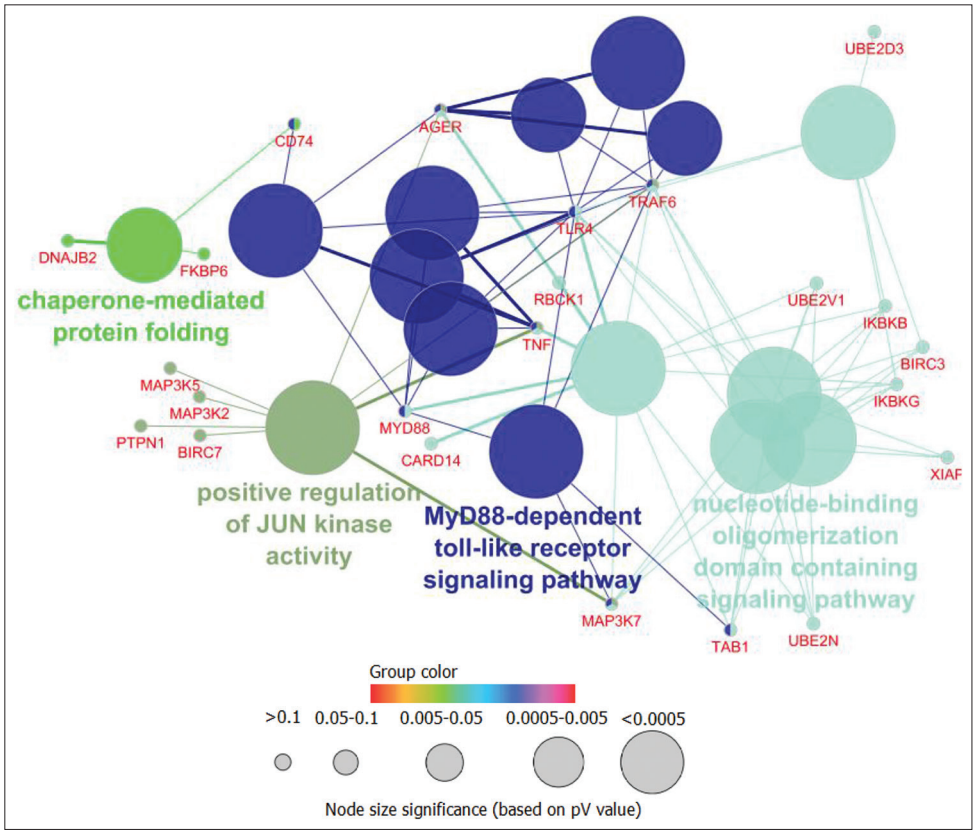


Figure 3. Retrieval of functionally grouped networks for the candidate targets of *Panax ginseng*, obtained through ClueGO, with terms as nodes associated. Each group is represented by only the label of the most significant term. The node size indicates the term enrichment significance. Functionally associated groups partially overlap.

apoptosis was not observed but LC3-II levels were found to be elevated[39].

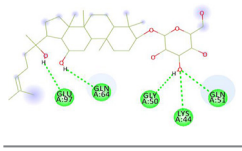
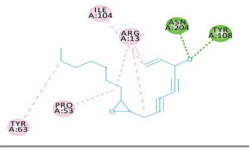
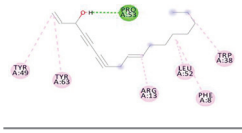
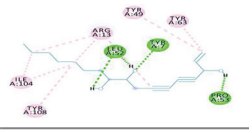
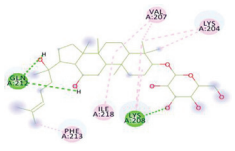
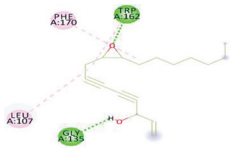
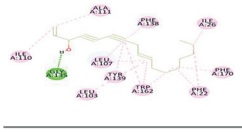
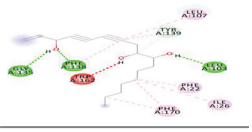
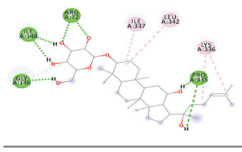
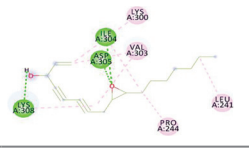
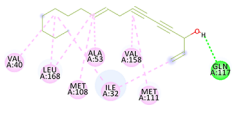
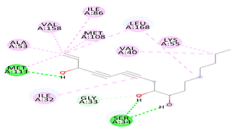
In PPIN, the main protein hubs were found to be *CYP1A2*, GSTP1, MAPK8, and GSTT1, which have already been reported to be involved in the development, and regulation (positive or negative) of neuronal damage. The role of the cytochrome family P450 has been well-reported, as it is primarily involved in major cell functions, particularly in the transportation of electrons[40]. The CYP1 family includes three fundamental proteins, namely, 1A1, 1A2, and 1B1, which reside in human tissues and serve a key role in the development of neurodegeneration and the growth of neurodegeneratice cells[41]. Much of the expression of the CYP1 family is mediated by the AhR signaling pathway, which can result in the dysfunction of the estrogen homeostasis, leading to neurodegeneration[42,43]. *CYP1A2* is one of the important CYP proteins, which is attributable to the action of carcinogens. It was previously believed that the expression of the *CYP1A2* gene largely existed in the liver and was inducible by major environmental stimuli[44].

Table 4. Docking scores of various compounds with disease-related proteins.

Receptor	Ligand	Docking score	H-bonds	H-bonds
GSTP1	Gensinside	−7.63	5	GLU97, GLN64, GLY50, LYS44, and GLN50
	Panaxydol	−6.10	2	ASN204 and TYR108
	Panaxynol	−5.78	1	PRO53
	Panaxytriol	−6.12	3	LEU52, TYR7, and PRO53
HSP90	Gensinside	−7.15	2	GLN212 and LYS208
	Panaxydol	−7.45	2	TRP162 and GLY135
	Panaxynol	−7.79	1	GLY35
	Panaxytriol	−7.21	3	GLY135, PHE138, and LEU103
MAPK8	Gensinside	−8.00	4	ARG72, ILE148, GLY146, and PRO335
	Panaxydol	−4.76	3	ILE304, ASP305, and LYS308
	Panaxynol	−4.93	1	GLN117
	Panaxytriol	−4.37	2	MET111 and SER34

Nevertheless, the latter part remains to be confirmed, while the former has been subject to refutation. Polymorphisms in the encoding gene have been heavily associated with the manifestation of several forms and types of neurodegenerative cells[45,46]. This function of the protein/enzyme has revealed a metabolic pathway that was observed to cause neurodegeneration, with various members of the CYP protein family playing a significant part in the activation of different carcinogenic compounds, which can then contribute to the polymorphisms in the genes, resulting in neurodegenerative cells[47]. Elevated levels of *CYP1A2* have been reported to be associated with increased risk for many types of neurodegenerations[48], with its gene polymorphisms altering its expression that subsequently influence the activation and suppression of signaling pathways[49]. GSTP1 is a protein that has been reported to play an important role in the proliferation of cells by the action of inhibiting JNK activity, which is observed to be inversely associated with GSTP1 activity, thus linking it significantly with the development of neurodegenerative cells in tissues. In patients with neuronal damage, the production of GSTP1 is increased. GSTP1 reportedly targets its production, thereby acting as a GSTP1 target gene, associating the activity of GSTP1 in neurodegenerative as well as healthy cells *in vitro*[50,51].

Table 5. Docking outcomes of various compounds with disease-related proteins.

Receptor	Ligand	
GSTP1		
	Gensinside	Panaxydol
		
	Panaxynol	Panaxytriol
HSP90		
	Gensinside	Panaxydol
		
	Panaxynol	Panaxytriol
MAPK8		
	Gensinside	Panaxydol
		
	Panaxynol	Panaxytriol

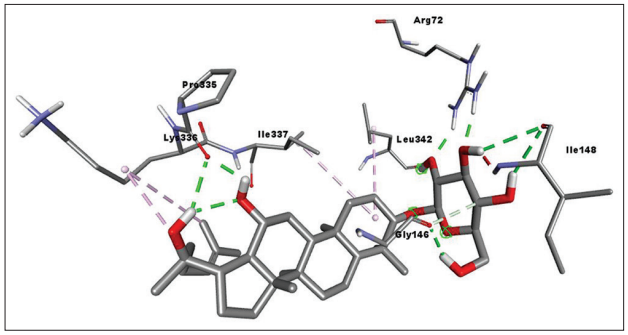


Figure 4. Docking-generated complexes of proteins mitogen-activated protein kinases-8 bonded to gensinside.

MAPKs are well-reported and well-characterized members of the family of Ser/Thr kinases, which actively mediate and regulate various cellular signaling pathways and external stimuli. The activation of the signaling cascade of MAPK is regulated by the help of two disparate types of cell receptors, namely, the RTKs and GCPRs, respectively. This cascade can effectively aid in the cell intermediate which is involved in cell proliferation, differentiation, and survival. The heterogeneous members of the MAPK family are known to serve various biological functions, such as the p-42 and p-44, which are responsive to mitogenic stimuli, whereas the JNK and p-38 MAPK are responsive to external stress stimuli[52,53]. In neurodegenerative cells, various agents and hormonal factors, such as gonadotropin-releasing hormone (GnRH), can lead to the activation of MAPKs, which is indicative of the significant role they tend to serve in the proliferative, apoptotic, and regulatory action of cells in neuronal damage. Several cellular agents, such as ATP, have been reported to be affiliated with G-protein-coupled receptors in neuronal damage cells, where ATP has been found to induce cell growth and elevate calcium levels in neuronal damage[54].

The present study has proposed the pharmacological mechanisms of action that *P. ginseng* fosters for its therapeutic/protective potential neuronal damage. The various biological pathways, as well as the proteins involved in its mechanistic action, shed light on the protective effects *P. ginseng* exerts on human against neuronal damage. Furthermore, the docking analysis shows the efficacious binding of its compounds with the target protein, corroborating the use of *P. ginseng* as a therapeutic agent against neuronal damage.

5. Conclusion

The present study reveals a protective role of *P. ginseng* against neuronal damage through multiple mechanisms. The important biological processes which are involved in the therapeutic action of *P. ginseng* are likely mediated through the positive regulation of JUN kinase activity and MyD88-dependent toll-like receptor signaling pathway. According to the docking analysis, MAPK8 is a potential target site for the action of *P. ginseng* active ingredients in the treatment of neuronal damage.

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None.

Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

Conceptualization: Manal Buabeid

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Resources: Manal Buabeid

Investigation: Mizna Javed, Munaza Ijaz, and Ghulam Murtaza

Formal analysis: Mizna Javed and Munaza Ijaz

Writing – original draft: Mizna Javed, Munaza Ijaz, Manal Buabeid

Writing – review and editing: Ghulam Murtaza

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REVIEW ARTICLE

Neuroimaging associations with spatial navigation impairment in Alzheimer's disease continuum: A narrative review

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Abstract

Identifying individuals with incipient Alzheimer's disease (AD) are critical for early and targeted intervention before the dementia develops as AD progresses. Recently, emerging data have suggested that spatial navigation and neuroimaging could be utilized to identify individuals with prodromal AD. Compared to episodic memory, spatial navigation has fewer cultural and educational discrepancies and could serve as a promising marker for diagnosis and outcome measures in multicenter longitudinal studies with large cohorts. Furthermore, neuroimaging studies have contributed to our understanding of the structural and functional neural basis underlying spatial navigation and provided sensitive and non-invasive neuroimaging markers. The current review summarizes neuroimaging associations with spatial navigation impairment in the AD continuum, their potential pathophysiological mechanisms, and nonpharmacological interventions for spatial navigation impairments. We highlight the promising role of spatial navigation in the early identification of the preclinical and prodromal patients with potential risk of developing AD dementia. Multicenter large-scale longitudinal studies on patients across the AD continuum coupled with a standardized routine assessment of spatial navigation abilities in clinical settings are needed. This review may have implications for clinical practice and future research directions.

Keywords: Spatial navigation; Alzheimer's disease; Neuroimaging; Functional connectivity

1. Introduction

Alzheimer's disease (AD) has long been a global public health concern[1]. Researchers have been diligently searching for sensitive biomarkers for the early diagnosis of AD and interventions to delay disease progression before substantial neuron loss occurs[2].

The previous findings have suggested that spatial navigation impairments, which are superior to episodic memory loss with low sensitivity and specificity, could potentially serve as promising markers for AD-related pathology, even in the preclinical stage of AD[3,4]. Spatial navigation refers to the process of determining or maintaining a trajectory from one place to another and is prone to decline with normal aging; notably, deficits are more pronounced during the progression of AD[5,6]. The navigation system in the brain overlaps substantially with regions that are affected first by AD pathology, and consequently, neurodegeneration in the navigation network results in an inability to create and use cognitive maps[7,8]. Furthermore, neuroimaging provides an opportunity for the early and non-invasive detection of structural and functional alterations in spatial navigation neural circuits in the brain, which not only provides sensitive neuroimaging markers but also contributes to the understanding of the neural basis underlying spatial navigation impairment in AD[9].

This paper reviews the neuroimaging advances regarding spatial navigation in four aspects: (i) Spatial navigation strategies and neural correlates in normal ageing, (ii) spatial navigation impairment and related neuroimaging alterations, (iii) spatial navigation impairment and related pathophysiological changes, and (iv) nonpharmacological interventions for cognitive and spatial navigation impairment.

The current review offers an overview of the neuroimaging advances regarding spatial navigation impairment in AD. We propose that assessment of spatial navigation impairment is crucial for the early identification of subclinical and preclinical AD with potential risk for AD dementia.

2. Spatial navigation function and neural correlates in normal aging

2.1. Spatial navigation: Definition and strategies

Spatial navigation is an essential ability that helps people determine or maintain routes by means of their own and environmental clues when moving between different locations[10]. While navigating the environment, people need to rely on different spatial representations, including two basic navigation strategies, that is, egocentric and allocentric navigation[5,11] (Figure 1).

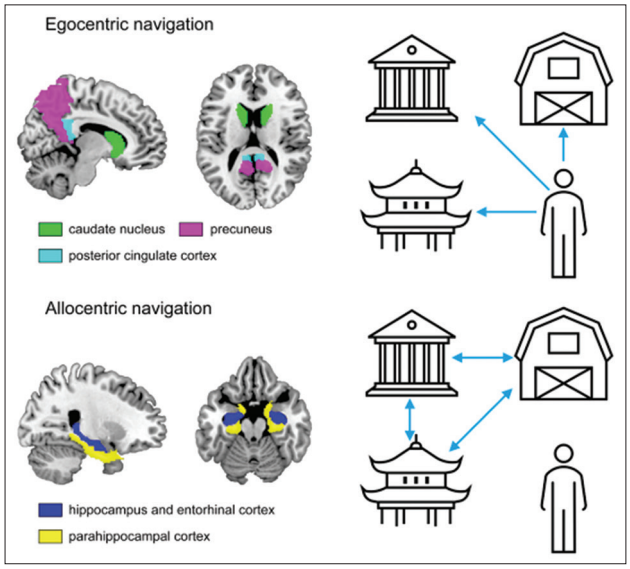


Figure 1. Egocentric and allocentric spatial coding. Egocentric navigation relies on parietal lobes and encodes spatial information with self-to-object relations, while allocentric navigation relies on MTLs and encodes spatial information with object-to-object relations.

Egocentric navigation strategies encode spatial information with its own location as the center to form an internal representation, which maintains the sense of direction when a person is moving. This sequence of body movements (e.g., remembering left, right, and left) facilitates the ability of route learning, which is primarily based on self-motion perception but can also exploit environmental cues by judging self-to-object relations[12]. The temporal sequence of environmental stimuli (e.g., "turn right at the shop") contributes to landmark-based behavioral responses stored in spatial memory.

Allocentric navigation strategies depend on cognitive maps, which require people to memorize and analyze the geographical parts of the whole space from an exploratory perspective, including the location of spatial landmarks and the distance and direction between the starting point and the target location[13,14]. This process requires the recognition of object-to-object relations and is known as path integration[15]. Compared to egocentric spatial encoding, the representation of allocentric information is stable and does not change when people move. Consequently, allocentric spatial information may remain constant as long as the information is saved and retrieved properly.

Notably, strategic integration and translation are more common, and the available evidence may not support a strict functional dissociation of egocentric and allocentric reference frames. It should be emphasized that the two navigation strategies are usually inseparable because a

landmark can be identified by egocentric and allocentric reference frames together. Excellent navigators tend to integrate different spatial information to flexibly translate between egocentric and allocentric strategies to maximize navigation efficiency[16]. In addition, a study has shown that age, sex, and cultural background have potential influences on strategic preference during navigation[17].

2.2. Neural correlates of spatial navigation

The navigation process involves a wide range of brain navigation networks, including the prefrontal lobe, parietal lobe, medial temporal lobe (MTL), caudate nucleus, and thalamus[3]. The parietal lobe mainly includes the posterior cingulate cortex (PCC), precuneus, and retrosplenial cortex (RSC); the MTL mainly includes the hippocampus, entorhinal cortex, and parahippocampal cortex (PHC). The previous studies have shown that MTL structures play prominent roles in allocentric navigation, while the parietal lobe and the caudate nucleus mainly contribute to egocentric navigation[18]. In addition, the RSC and PCC are crucial for the flexible transformation between egocentric and allocentric reference frames[19-21].

Spatial navigation requires effective neural representations that code location, head direction, speed, and boundaries. The discovery of place, head direction, grid, border, boundary-vector, and speed cells identify these representations[22-27]. At the macroscopic level, functional magnetic resonance imaging (fMRI) has also revealed representations[24,28]. The activity of some hippocampal neurons is closely related to their position in the space of animals or humans[22,29]. These cells are called place cells, which fire in a specific location and contribute to the formation of cognitive maps of the environment. Thus, memories of the environment can be stored as a combination of specific activity in place cells. Later, grid cells were identified in the medial entorhinal cortex[25,30]. These cells are regularly activated in a unique space to calculate vector relationships and distances between spatial locations. Grid cells and place cells work together to form a comprehensive positioning system that could function as the global positioning system (GPS) in the brain. Of note, head direction cells are related to the head direction in the horizontal plane. For instance, a specific neuron might fire whenever a person is facing the west, regardless of the location. In this way, head direction cells could function as a compass. In addition, border cells activate nearby a border[26], boundary-vector cells fire at specific distances from a border[31], and speed cells contribute to the firing of grid cells through path integration[27].

Furthermore, neuroelectrophysiological studies have revealed the phenomenon of phase precession during spatial navigation[32]. Phase precession refers to the phase relationship between action potentials and periodic rhythmic field potentials (theta oscillation) of some specific neuron species (e.g., place cells in the hippocampus and grid cells in the entorhinal cortex)[33]. Phase precession is a common and critical neuronal mechanism for the coordination of behavior and cognitive processes[34].

2.3. Spatial navigation and normal aging

Healthy older adults experience a decrease in navigation ability with aging, which is mainly manifested by impaired abilities of movement self-perception and path integration[18,35,36]. In both real and virtual navigation, older adults performed worse than younger adults as the task became more difficult; however, the basic cognitive abilities required for successful performance of object-location memory tasks seemed to be preserved in older adults[37,38].

Aging has different effects on egocentric and allocentric strategies. Research shows that healthy older people tend to take a fixed route to avoid entering unfamiliar environments, indicating a preference for egocentric rather than allocentric strategies[39]. A similar conclusion was reached by the study conducted by Rodgers *et al.*, which showed that older adults overwhelmingly preferred an egocentric strategy, while younger adults had equally distributed egocentric and allocentric preferences[40]. In addition, healthy older adults reached the target location with longer movement paths and spent longer time reaching the destination compared to the young participants in the human version of the Morris Water Maze (hMWM) test. These results demonstrate that aging could affect one's preference for egocentric navigation strategies, which may be partly attributed to worse impairments in the allocentric reference frames. Furthermore, studies have identified age-related impairments in translation between navigation strategies, not only switching from an egocentric to an allocentric frame, but also switching from an allocentric to an egocentric frame[41-43].

A study based on structural MRI data found that caudate nucleus volumes were reduced in older adults and were positively associated with route learning abilities; thus, a diminution in accurate knowledge of egocentric navigation may be a consequence of atrophy in the caudate nucleus during aging[44]. Alterations in the prefrontal and hippocampal functions, which result from atrophy in the prefrontal cortex, hippocampus, and related MTL regions that are crucial for allocentric representations, were also reported to contribute to navigation impairments and strategic preference in healthy elderly individuals during aging[45-48]. Moreover, fMRI studies have shown that the activation of the posterior hippocampus, PHC, RSC, and some parts of the parietal lobe was significantly reduced in

healthy elderly persons compared with young adults during the navigation task, while the activation of the prefrontal cortex was increased[49,50]. In addition, white matter volume in the dorsolateral prefrontal cortex (DLPFC) was positively correlated with navigation performance in the hMWM[51]. Therefore, the structural and functional incompleteness of brain regions responsible for spatial navigation during aging may result in the gradual decline in egocentric and allocentric navigation abilities in healthy elderly individuals.

3. Spatial navigation impairment and related neuroimaging alterations in the AD continuum

3.1. AD dementia

The previous studies conducted on AD dementia patients have revealed deficits in both egocentric and allocentric navigation reference frames, which may be attributed to the widespread neurodegeneration involving the medial temporal, frontal, and parietal lobes in the late AD stage[52-54]. These findings suggest that AD patients have impaired abilities to create and use cognitive maps of the environment and to compute body-centered information for self-orientation. In addition, AD patients experience difficulties in translating egocentric parietal and allocentric hippocampal representations, which mainly depend on the RSC and PCC[16]. The reduction in the ability to transition between allocentric and egocentric spatial navigation strategies was more evident than pure egocentric and allocentric navigation, which may suggest the priority to evaluate the allocentric to egocentric translation of spatial coding when a medical professional is making an AD diagnosis[55]. In addition, impaired spatial memory, the ability to encode and store information from egocentric and allocentric representations, has been observed in AD patients, which was not a product of generalized cognitive decline but instead may reflect spatial organization dysfunction[56]. AD could also affect spatial navigation strategies, as the preference for egocentric over allocentric strategies increased with AD severity[57]. The preference for an egocentric strategy may indicate compensatory mechanisms of recruiting extrahippocampal navigation strategies as an adaptation to hippocampal neurodegeneration in AD progression. In addition, spatial navigation tests presented better accuracy than conventional cognitive screening tests in distinguishing AD patients from normal controls (NCs)[58]. More recently, AD patients could be identified based on GPS data of their outdoor navigation patterns in the community, highlighting the potential utility of real-world everyday navigation behavior as an ecologically valid digital marker for AD[59].

Before the recent development in neuroimaging alterations regarding spatial navigation in AD patients, cumulative structural MRI, task-based fMRI, and resting-state fMRI (rs-fMRI) studies conducted in NCs have suggested that multiple brain regions work collaboratively to guide navigation behaviors[60-70]. Pertinently, neuroimaging deterioration is more pronounced in neurodegenerative diseases such as AD. Voxel-based morphometry analyses based on structural MRI data in AD patients have revealed significant correlations between scene construction impairment and atrophy in the posterior parietal and lateral temporal cortex[71]. Another structural MRI study revealed that basal forebrain atrophy contributed to allocentric navigation impairment independent of hippocampal atrophy in AD patients[46]. As the basal forebrain is a key structure of cholinergic neurons projecting to the hippocampus, amygdala, and cerebral cortex, this study suggested that allocentric disorientation may be related to the loss of cholinergic neurons in AD patients, which corresponds to histopathological findings[72]. Multimodal MRI analyses of grey matter density, glucose metabolism, and white matter axial diffusion have revealed significant converging correlations of the right RSC and PCC with test performance for virtual route learning in a cohort of patients with mild AD[73]. Collectively, AD dementia patients suffer from spatial navigation impairment, which may result from the widespread neurodegeneration in brain regions associated with spatial navigation.

3.2. Mild cognitive impairment (MCI)

MCI is believed to be a predementia stage, and it has been recognized that MCI patients with the amnestic type are more vulnerable to progression to AD dementia than those with the nonamnestic type[74]. Similar to that found in the AD dementia stage, amnestic MCI (aMCI) patients showed spatial navigation dysfunction compared to age-matched NCs[13,75-78]. In addition, patients in the aMCI multiple-domain group performed worse in all subtests of allo-egocentric, egocentric, allocentric, and delayed allocentric navigation, showing high similarity with those of early AD; the aMCI single-domain group was significantly impaired in the subtests of allocentric and delayed allocentric navigation, and patients in the nonamnestic MCI group performed similarly to NCs[79,80]. Spatial navigation tests could further divide aMCI patients into two subgroups: Memory impairment of hippocampal type (hippocampal aMCI) and isolated retrieval impairment (nonhippocampal aMCI); individuals in the former group showed worse performance on spatial navigation, accompanied by an increased risk of AD, compared to the latter group[81,82]. In a longitudinal study with a 2-year follow-up, allocentric spatial memory performance assessed by the Four Mountains Test

could predict conversion from MCI to AD dementia with an accuracy of 0.93, similar to the accuracy of 0.92 by the Tau/amyloid β (Aβ) ratio in cerebrospinal fluid (CSF)[83]. The study provided initial support for the hypothesis that allocentric spatial memory was predictive of progression from MCI to AD dementia. The aforementioned results suggested that spatial navigation impairment was specific for identifying MCI patients with prodromal AD from those with other etiologies and for monitoring disease progression[10,84].

Several structural MRI studies of spatial navigation in MCI patients have been conducted. Reduced volumes in the medial part of the entorhinal cortex were related to self-reported spatial navigation performance in MCI patients[85]. The aMCI patients had worse egocentric memory performance measured by a virtual maze, with smaller volumes of the right-sided precuneus indicating worse egocentric memory[86]. Significant associations between allocentric navigation errors and right hippocampal volumes in both real-space and virtual versions were observed in a cohort consisting of aMCI, mild and moderate AD patients, and NCs, controlling for demographic variables and total brain and left hippocampal volumes[13]. The participants in the aMCI group exhibited poorer path integration accuracy than those in the NC group, with hippocampal volume and thickness of the entorhinal cortex and inferior parietal cortex accounting for 37%, 36%, and 45% of the differences, respectively[87]. The result was supported by another study showing that the right hippocampal volume explained 26% of the variance in allocentric navigation in participants in the aMCI group[88]. According to a study that investigated the contribution of hippocampal subregions to spatial navigation, there are significant associations between volumes of the right hippocampal tail and navigation skills in aMCI patients, while the CA2/3 region appears to be more relevant to spatial navigation in NCs[89].

In addition, the associations among genetic risk factors, spatial navigation, and structural MRI measures have been investigated in MCI patients[90-92]. Apolipoprotein E (*APOE*) ε4 is the principal known risk gene that could increase the risk of sporadic AD[93]. Both results of real-space and computer-based hMWM have demonstrated poorer performance on both egocentric and allocentric navigation in participants in the aMCI ε4+ group compared to participants in the aMCI ε4− group, with those in the former group performing similarly to those in the mild AD group, while those in the latter group resemble the NC group, and aMCI patients with *APOE* ε4 homozygotes were outperformed by those with heterozygotes[90,92]. The right hippocampal volume could account for the differences in allocentric and delayed allocentric subtests but not in the egocentric subtest, supporting the crucial role of the right hippocampus in allocentric navigation[13]. The brain-derived neurotrophic factor (*BDNF*) Val66Met polymorphism was believed to be relevant to an increased risk of AD. The combination of *APOE* ε4 and *BDNF* Met polymorphisms has been found to be associated with more pronounced egocentric navigation impairments and reduced volumes in the hippocampus and entorhinal cortex in aMCI patients[94]. The very long poly-T variant at rs10524523 of the *TOMMO40* gene, which encodes the translocase of the outer mitochondrial membrane pore subunit, was reported to be related to allocentric spatial navigation impairments and reduced cortical thickness of the entorhinal cortex and PCC among aMCI individuals with *APOE* ε3/ε3 homozygotes[91]. Therefore, spatial navigation is associated with multiple genetic polymorphisms, which may exert an effect through alterations in the MTL and parietal lobe structures of MCI patients.

Navigation task-based fMRI studies have revealed reduced activity in aMCI patients in brain regions that are important for navigation, including the hippocampus, caudate, RSC, and precuneus, as well as increased activity in the prefrontal regions, which may serve as compensatory mechanisms[95]. In rs-fMRI analyses, reduced intrinsic activity measured by the amplitude of low-frequency fluctuation (ALFF), fractional ALFF, and regional homogeneity (ReHo) in the subcortical regions was observed in MCI patients[75]. Interestingly, the MCI versus NC group difference can modulate the activity-behavior relationship. More specifically, the correlation slopes between ReHo and allocentric navigation errors differed significantly between participants in the MCI and NC groups, indicating a stage specificity of the brain activity-behavior relationship. Graph theory analysis of the white matter network has revealed significant correlations between network topological properties and navigation errors, with the lower clustering coefficient in the right inferior parietal gyrus predicting larger allocentric navigation errors[76]. The study provided new insight into the neural basis underpinning navigation impairment in MCI patients from the perspective of disruption of white matter network topology. Cumulatively, patients with MCI have deficits in spatial navigation, which varies in subtypes and disease severity and is related to neurodegeneration in the navigation network mainly involving the MTL and parietal lobes.

3.3. Preclinical AD

Contrary to the strong evidence supporting spatial navigation impairment in patients with AD dementia and MCI patients, its role in the preclinical AD stage has never

been extensively investigated. Therefore, spatial navigation has been considered an overlooked cognitive marker for preclinical AD[3]. Nevertheless, spatial navigation impairments are increasingly shown to be present in individuals with incipient AD, which may be related to the high susceptibility of MTL to AD pathology in the preclinical stage[7].

Individuals with subjective cognitive decline (SCD), a self-perceived worsening of cognitive function without objectively detected deficits, have been widely recognized at a higher risk of incipient AD than normal elderly individuals without cognitive complaints[96,97]. Indeed, abundant evidence has suggested significant associations between amyloid pathology, genetic risk of AD, entorhinal cortical thinning, and SCD[9,98]. Spatial navigation complaints are a frequent symptom not only in patients with AD and MCI, but also in individuals with SCD. In particular, 68% of SCD subjects complained about their spatial navigation, which was significantly higher than the 33% in the NC group[99]. The hidden goal task, which has been suggested to show excellent ecological validity and is strongly correlated with real-world navigation difficulty, was applied to evaluate spatial navigation abilities in SCD individuals[79]. In accordance with that observed in AD dementia and MCI patients, both egocentric and allocentric navigation strategies were found to be impaired in individuals with SCD[100,101]. Spatial navigation performance worsened with increasing severity of cognitive impairment in SCD, MCI, and AD dementia patients[102]. The fact that these weak levels of navigation impairment could not be detected objectively using standard neuropsychological evaluations of episodic memory, language, or executive functions indicates that spatial navigation is a specific cognitive domain that is impaired in the earliest stages of AD. The reduced volumes of the basal forebrain, especially in the Ch4p subfield, may serve as a structural basis for allocentric disorientation in SCD individuals independent of hippocampal atrophy[101]. Since the Ch4p subfield of the basal forebrain is a key structure for cholinergic inputs to the cerebral cortex, the results highlighted the potential role of loss of cholinergic neurons in allocentric disorientation. In the rs-fMRI analyses, alterations in functional connectivity between brain regions associated with spatial navigation were observed in SCD individuals. In particular, two spatial navigation brain networks, ego-network and allo-network, each with 10 selected spherical regions of interest, were defined[100]. Compared with the NCs, participants in the SCD group showed decreased functional connectivity between the right RSC and right prefrontal cortex (PFC) in the ego-network and decreased functional connectivity between the right RSC and right hippocampus in the allo-network. More interestingly, a logistic regression model based on spatial navigation and functional connectivity measures revealed a big area under the curve (AUC) of 0.880 in differentiating SCD individuals from NCs, indicating the promising role of spatial navigation and related neuroimaging measures in the preclinical detection of incipient AD patients.

In preclinical AD subjects with genetic risk factors for AD, investigations have shown that *APOE* ε4 noncarriers outperformed ε4 carriers in spatial navigation tests; this finding may be of use to characterize the preclinical phase of cognitive impairment[103,104]. A recent study observed significantly lower functional connectivity between the right entorhinal *cortex* and PCC in subjects of *APOE* ε3ε4 group than in those of ε3ε3 group, and the reduced connectivity was correlated with navigation discrepancies[105]. Therefore, the early neurodegenerative processes in APOE ε4 carriers may significantly damage the neural networks that are presumed to be crucial for spatial navigation. Furthermore, the classification accuracy of the path integration test, coupled with connectivity strength between the right entorhinal *cortex* and PCC, achieved an accuracy of 0.85 to distinguish the *APOE* ε4 carriers. APOE ε4 could also moderate the indirect effects of basal forebrain volumes on allocentric navigation performance through the MTL and PFC in older adults without dementia[106]. In contrast, *APOE* ε2 is known to be protective against AD and is associated with a reduced risk and delayed onset of AD[107]. Of note, Konishi *et al.* found that *APOE* ε2 carriers tend to use allocentric spatial strategies and have greater hippocampal volumes than *APOE* ε3 homozygous individuals and *APOE* ε4 carriers[108]. These findings suggest that the protective effect of the APOE ε2 allele may be expressed in part through increased use of allocentric navigation strategies and greater hippocampal volumes.

Spatial navigation impairments have also been observed in preclinical AD elderly individuals with abnormal AD biomarkers of Aβ compared to those with normal Aβ levels, suggesting that navigation may be particularly sensitive for identifying the earliest pathologic changes in AD[109]. Furthermore, longitudinal studies have provided strong scientific evidence that spatial navigation is specific enough to predict conversion from normal cognition to MCI or dementia[110,111]. More specifically, during an average follow-up period of 4–5 years, baseline performance on cognitive mapping and route learning, which depends on allocentric and egocentric representation, respectively, were predictors of clinical progression of AD[111]. The AUCs of cognitive mapping, route learning, and episodic memory for discriminating progressors from nonprogressors were 0.894, 0.794, and 0.735, respectively. In particular, cognitive mapping tended to perform better

than episodic memory in identifying progressors. It has been emphasized by the researchers that future work should incorporate neuroimaging and biomarker data to assess whether baseline spatial navigation was related to longitudinal alterations in brain structure, function, and AD biomarkers.

In summary, although spatial navigation was less investigated in the preclinical AD stage, emerging data have suggested that SCD subjects, risk gene carriers, and elderly individuals with abnormal AD biomarkers experience difficulties in spatial navigation compared to the navigation ability of NCs. Decreased functional connectivity measured by rs-fMRI may indicate disruption of navigation networks in subjects with incipient AD.

4. Spatial navigation impairment and related pathophysiological changes

4.1. Spatial navigation impairment and AD pathology of Aβ and Tau

It has been widely recognized that the typical pathological changes in AD are extracellular Aβ deposition and the formation of neurofibrillary tangles caused by Tau hyperphosphorylation. The entorhinal cortex is one of the brain regions first involved in phosphorylated Tau (pTau)[7,112]. Multimodal information from cortices converges in the hippocampus primarily through the entorhinal cortex, with the medial part encoding and transferring spatial information[113]. However, the relationships between spatial navigation impairments and pathological biomarkers of AD in humans have not been clearly illustrated, as most studies have been conducted on animals.

Several studies have been conducted to explore the relationships between Aβ deposits and spatial navigation. A study of APP/PS1 mice found that Y-maze performance worsened before the formation of Aβ deposits; however, despite the increased Aβ load in the hippocampus and cortex, these mice did not show impairment in spatial navigation at 6 or 9 months. This suggested that Aβ deposition alone was not sufficient to cause strong spatial memory impairment in mice of this mixed background ancestry and age[114]. In contrast, a study by Puoliväli *et al.* showed that total hippocampal Aβ levels in transgenic mice were associated with spatial navigation impairments[115]. The APP/PS1 mice were impaired in water maze acquisition and retention only at the age of 11–12 months. Moreover, the levels of total $A\beta_{1\text{-}42}$ in the hippocampus were negatively correlated with the retention score in mice in the impaired older age group. This was the first study in which significant correlations between age-dependent impairment of memory retention in a traditional water maze and total Aβ in the hippocampus in APP/PS1 mice were observed. The discrepant results of the associations between Aβ deposits and spatial navigation in animal studies may be partly due to the various extent of extrahippocampal pathology and different mechanisms that underlie neuronal dysfunction and spatial navigation impairment at different stages of Aβ pathology[115]. After healthy older adults completed an on-road driving test, the Santa Barbara Sense of Direction scale, and the Driving Habits Questionnaire, Allison *et al.* found that CSF $A\beta_{42}$, but not Tau or $pTau_{181}$, was associated with self-reported navigation abilities, which further mediated the relationships between CSF $A\beta_{42}$ and driving range. These findings indicated that brain Aβ deposition could contribute to patients' reduced ability to perceive the environment to navigate, which consequently results in older adults with AD pathology having a limited drive range[116].

Interestingly, other studies have focused on the relationships between Tau hyperphosphorylation and spatial navigation impairments. Using a transgenic mouse model, Fu *et al.* demonstrated that the accumulation of Tau pathology in the entorhinal cortex was associated with excitatory neuron loss, grid-cell dysfunction, and deficits in spatial learning and memory[117]. This was the first study that showed a relationship between Tau pathology and grid cell dysfunction *in vivo*, which may further provide a link between Tau pathology and spatial navigation impairments in patients with early AD. Notably, the pattern in pTau staining across the parietal-hippocampus network is a powerful predictor of spatial learning and memory performance. Stimmell *et al.* demonstrated that female mice at 6 months of age had a Tau pathological pattern identified by independent component analysis in the parietal-hippocampus network, with a higher density of pTau-positive cells predicting poorer spatial learning and memory performance in female 3xTg-AD mice[118]. This indicated that spatial disorientation may be attributed to the early accumulation of pTau in the parietal-hippocampus network in AD. Stancu *et al.* crossed APP/PS1 mice with 5 early-onset familial AD mutation (5xFAD) and TauP301S (PS19) transgenic mice and found that Tau pathology was invariably and robustly aggravated in hippocampal and cortical brain regions[119]. Most importantly, the mice displayed more severe deficits in the spatial navigation task than the controls.

From the above studies, we summarize that spatial navigation impairment may be caused by the deposition of Tau and/or Aβ in the entorhinal cortex, hippocampus, and parietal cortex. More clinical studies are urgently needed to determine the exact relationships between spatial navigation impairments and pathological biomarkers of AD in humans.

4.2. Spatial navigation impairment and reduced grid cell-like representations

Grid cells have unique hexagonal grid-like firing patterns and play crucial roles in the process of spatial navigation. Since the discovery in the rat brain by Hafting *et al.* in 2005, the existence of grid cells has been successively confirmed in bats, monkeys, and other animals[25,120]. Grid cells can receive electrical signals from a variety of neuronal cells involved in spatial navigation and are the primary source of information input to hippocampal place cells[121]. Tau pathology of AD occurs first in the entorhinal cortex, the medial part of which contains grid cells. Therefore, research that focuses on grid cells in the human brain is of great value for a better understanding of the neural basis underlying spatial navigation impairment in AD.

The process of reading self-motion information and integrating the information of each spatial location to obtain the path to the target position is called path integration, which is an automatic and continuous update process during navigation. Grid cells can form stable grid graphs, and their path integration function does not depend on environmental landmarks but is instead based on ontology motion cues[122]. Various other cells that are involved in neural circuits for spatial navigation also have typical firing patterns. Border cells fire significantly when animals approach geometric boundaries[123]; moreover, the firing rate of speed cells increases proportionally with the movement speed of the animal to provide instantaneous speed information[27]. In addition, the firing of head direction cells is related to the direction of the animal relative to landmarks in the environment[124]. These cells transmit distance and orientation information to grid cells, which are integrated into the grid map to provide spatial information, including the position in the environment and distances from starting and ending points, and are continuously updating location information based on action cues[18].

Fu *et al.* found that the reduction in grid-cell activation in aged mice was accompanied by the occurrence of spatial memory deficits, and the authors inferred that pathological changes originating from the entorhinal cortex may cause grid-cell dysfunction and lead to spatial navigation impairment in AD patients[117]. The signals of grid cells have been successfully detected by fMRI, which has greatly facilitated studies of grid cells in humans[24]. Kunz *et al.* found that young adults with *APOE* ε4 exhibited reduced grid-cell activation stability and reduced ability to navigate in virtual scenes[125]. These results support the hypothesis that impaired grid-cell function is related to spatial disorientation. Bates *et al.* believed that spatial navigation obstacles were mainly caused by the inaccurate position representation in the navigation system formed by grid cells and place cells[126]. Carriers of the AD risk gene have a more significant decline in the ability to navigate in the center of the virtual environment than at the border, which may be explained by the reason that navigation in the central area lacks environmental cues that rely more on the path integration function of grid cells[127]. Pertinently, the increased activation of the hippocampus in risk gene carriers may serve as a compensatory mechanism for spatial memory impairment.

Phase precession has been widely observed in hippocampal place cells and entorhinal grid cells in navigating rodents[32,128]. Focal dorsal hippocampal Nav1.1 knockdown altered the temporal coding properties of place cells, such as theta phase precession, which subsequently degraded the spatial accuracy of rats[129]. A recent study also provided evidence for spatial phase precession in the human hippocampus and entorhinal cortex during virtual navigation, which exhibited features similar to those observed in rodents[34]. Thus, we speculate that degradation in the phase precession of grid cells might also contribute to spatial dysfunction in AD patients, which deserves to be further validated.

Emerging evidence has suggested the crucial role of grid cells in spatial navigation, and the application of fMRI in grid cell research has provided a novel entry point and opened a new avenue for the study of neural mechanisms underpinning spatial navigation impairments in AD patients. However, due to the complexity of the relevant data collection and analysis processes, many issues, such as how grid cells encode three-dimensional (3D) spaces and large-scale complex environments in preclinical and prodromal AD patients, remain to be further explored.

5. Nonpharmacological interventions for cognitive and spatial navigation impairment

5.1. Virtual reality (VR)-based training program

Due to the high social burden of AD coupled with the lack of effective treatment, new approaches targeting cognitive and spatial navigation impairment are being sought. VR is defined as a technology that digitally provides a changeable 3D environment in which people can interact with different sensory inputs. VR can be immersive or nonimmersive, and the former allows for a sense of presence in the environment[130]. VR has been widely recognized as a promising technique that can improve treatment outcomes in patients with neurocognitive disorders, such as AD. Oliveira *et al.* observed an improvement in overall cognitive function in patients with AD after VR-based cognitive stimulation, with an effect size corresponding to

a large effect on global cognition[131]. The study suggested that VR-based cognitive stimulation is effective for neurocognitive stimulation in older adults with dementia, contributing to the maintenance of cognitive function in patients with AD. A case study of healthy older adults and MCI patients with unilateral vestibular hypoactivity suggested that participants who played a VR racing game in the treated condition (including those with MCI) showed greater improvements in vestibular function than those in the untreated condition[132]. Another study showed that the cognitive function and balancing ability of MCI patients showed a significant increase after exposure to the VR program, which involved 20 sessions for 4 weeks, 30 min/experiment[133]. The results indicated that the VR program is also an effective intervention for elderly individuals with MCI.

More importantly, the VR technique also offers the opportunity to create complex individualized and natural simulated environments in which spatial navigation impairment could be treated precisely. Kober *et al.* found that different aspects of spatial abilities in neurologic patients with focal brain lesions were improved after VR training in comparison to their spatial performance before VR training[134]. This study showed that guided passive navigation training in VR could enhance general spatial cognition in neurological patients with spatial disorientation as well as in NCs and can thus be used in the rehabilitation of spatial navigation impairments associated with spatial disorientation. Moreover, VR training has been thought of as an advanced embodied tool for the treatment of spatial memory impairment in patients with AD. Serino *et al.* recruited 20 AD patients who were randomly assigned to either the VR-based training group or the control group, and observed a significant improvement in long-term spatial memory after VR-based training[135]. Another study demonstrated that after experiencing a VR environment, the skill of a man at the onset of AD in navigating while driving improved noticeably in a cognitive treatment program based on spatial navigation[136]. Interestingly, another study showed that the spatial navigation of older adults was enhanced after VR-based physical exercise with two-dimensional exergames[137].

In general, VR training could serve as a promising approach to treat cognitive and spatial navigation impairments in AD. A big advantage of VR is that it enables the development of customized cognitive exercises in meaningful environments, which is especially important since cognitive training can be particularly demanding for AD patients[135]. More specifically, in a virtual environment, cognitive training can be implemented based on specific rehabilitation mechanisms. Despite the preliminary studies mentioned above, further research that adopts methodologies from clinical trials and incorporates behavioral and neurophysiological data are required to provide clear evidence on the effectiveness of VR-based training for cognitive and spatial navigation impairments in AD.

5.2. Neuromodulation

Due to the unsatisfactory effect of drug therapy for AD, neuromodulation technology has gradually attracted the attention of neurologists and physical therapists. Neuromodulation is defined as an intervention intended to alter nervous system function using energy fields, such as electricity, magnetism, or both, with the goal of improving psychiatric symptoms or related conditions[138]. Neuromodulation techniques include deep brain stimulation (DBS), transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS), transcranial alternating current stimulation, and transcranial focused ultrasound (tFUS). DBS has been widely used to treat Parkinson's disease and essential tremors, while its application in AD is still under research. In study of DBS targeting the fornix to improve cognition in AD patients, Deeb *et al.* found that a total of 20 patients (48%) spontaneously reported having vivid experiences of ostensible previous events in their lives after DBS[139]. The high-frequency repetitive TMS (rTMS) protocol at the left DLPFC has been approved for treatment-resistant depression therapy in the United States, and its effectiveness in AD has also been investigated. Of note, after treating aMCI and AD patients with neuro-navigated rTMS targeting the left angular gyrus for 4 weeks, Yang *et al.* observed significant improvement in episodic memory of aMCI patients and general cognition of both groups[140]. tDCS is the most common choice for transcranial electrical stimulation for treating AD. Another recent study revealed that tDCS to the left DLPFC can enhance verbal episodic memory performance by modulating the memory reconsolidation process in subjects with SCD[141]. In addition, a study suggested that without opening the blood-brain barrier, the regional cerebral metabolic rate of glucose in the superior frontal gyrus, middle cingulate gyrus, and fusiform gyrus increased, and the memory, executive, and global cognitive function improved in patients with AD who received tFUS[142].

A few studies have assessed the effectiveness of neuromodulation on the neural circuits of spatial navigation. Spatial navigation is a complex process that associates with various navigational skills, such as spatial memory. Fyock *et al.* found that tDCS could modulate spatial memory in cognitively intact adults[143]. The study by Krishnamurthy *et al.* revealed that tDCS enhanced functional connectivity between the medial superior parietal lobule seeds and

several other areas that are involved in spatial processing and navigation[144]. A recent study showed that network segregation of MCI patients increased to levels similar to that in cognitively intact older adults following active tDCS; this finding suggests that tDCS over the right parietal cortex may normalize the segregation/integration balance of association networks during spatial navigation in MCI patients, highlighting its potential to restore brain activity in patients with AD[145]. The study by Suthana *et al.* demonstrated that DBS targeting the entorhinal cortex in patients with pharmacoresistant epilepsy could enhance memory of spatial information during learning[146].

Collectively, neuromodulation technology showed convincing outcomes in cognition and spatial navigation enhancement in AD treatment. Non-invasive brain stimulations could be the next AD treatment. However, the lack of a standardized protocol is the current challenge in nonpharmacological interventions. Future studies with large cohorts are warranted to determine the exact effects of neuromodulation in cognition and spatial navigation and to standardize the parameters, including target sites, frequency, and duration of each session.

6. Conclusions

In summary, spatial navigation impairment is commonly observed in patients across the AD continuum and is associated with different patterns of structural and functional alterations in the neural networks of navigation revealed by neuroimaging, which could be attributed to the pathophysiological changes in AD progression. In this paper, we highlight the great potential of spatial navigation by providing novel insights into the early detection of AD and reviewing a more general and uniform approach toward future prevention and intervention strategies for AD. More specifically, subjects in memory clinics who complained of difficulties in everyday navigation in previously familiar routes, accompanied by impaired navigational function, as demonstrated in the spatial navigation test, should be considered at a higher risk of incipient AD. Early identification and intervention of incipient AD patients might provide a promising opportunity for a favorable prognosis before the occurrence of substantial neuronal death. Furthermore, spatial navigation presents a novel approach for measuring the efficacy of AD therapy across research sites regardless of cultural and educational discrepancies[147]. Thus, it is of great value to incorporate spatial navigation tests into standard neuropsychological assessments in clinical settings[148,149].

The development of a standardized and validated testing system that is suitable for routine assessments of spatial navigation abilities in clinical settings for various patient populations is an urgent need[150]. Despite the promising utility of spatial navigation, the current lack of multicenter large-scale longitudinal studies that could corroborate the role of spatial navigation in the progression of the whole AD continuum is an obstacle that requires further investigation. In future studies, multimodal MRI in larger cohorts is needed to further elucidate the mechanisms by which AD pathology influences spatial navigation performance. Additionally, novel spatial navigation training (e.g., immersive VR training) combined with advanced technologies for early spatial rehabilitation interventions, which could serve as effective safeguarding strategies to prevent AD patients from getting lost, remains to be explored[151].

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REVIEW ARTICLE

NMDA receptors: Biological properties and their roles in neuropsychiatric disorders

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Abstract

Proper signal transmission is the fundamental process of the brain activity. Changes and adaption of neuroplasticity based on the strength of synaptic transmission are essential for the information propagation in the central nervous system, which contribute to cognition, learning, and memory. Being the major excitatory neurotransmitter in the central nervous system, glutamate acts primarily through binding to the glutamate receptors, the glutamate-gated ion channels localized on post-synaptic membrane. The ionotropic glutamate receptors, pharmacologically grouped into α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors, N-methyl-D-aspartic acid (NMDA) receptors, and kainate receptors, have been shown to play distinct roles in excitatory neurotransmission and synaptic plasticity. Due to their high permeability to Ca^{2+}, the NMDA receptors have very unique function in neurotransmission and particular importance in the induction of long-term synaptic plasticity. Dysfunction of NMDA receptors causes impairment in synaptic plasticity and learning and memory. In recent years, with the development of genome-wide association studies and next-generation sequencing technology, mutations of NMDA receptor subunits have been in a variety of neuropsychiatric disorders, such as cognitive impairment, schizophrenia, autism or epilepsy. In clinical practice, NMDA receptors are known as the targets for the treatment of many neuropsychiatric disorders. In current review, we summarize current knowledge of NMDA receptors with different subunit compositions in the context of expression pattern, channel properties, protein trafficking, and synaptic plasticity as well as their roles in neuropsychiatric disorders.

Keywords: NMDA receptor; Ion channel; Protein trafficking; Synaptic plasticity; Neuropsychiatric disorders

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1. Introduction

The ionotropic glutamate receptors play essential roles in excitatory neurotransmission. When action potential propagates through the axons, glutamate released from the vesicles at the presynaptic terminals enters the synaptic cleft, the tiny, and highly organized extracellular space, where the neurotransmission occurs. The glutamate binds to ionotropic glutamate receptors on the post-synaptic membrane and triggers the ion channel function, which gives rise to neuronal signal transfer between neurons.

Pharmacologically, the ionotropic glutamate receptors are grouped into three types[1]: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs), kainate receptors, and N-methyl-D-aspartic acid (NMDA) receptors (NMDARs). AMPARs are composed of homomeric or heteromeric assembly of four subunits: GluA1-4. Kainite receptors are homomeric GluK1-3 (GluR5-7 in old nomenclature), or heteromeric assembly of GluK1/2/3 with GluK4/5 (used to be KA1/2). NMDARs are heterotetramers composed of two subunits of GluN1, the obligatory subunits, and GluN2A-D or GluN3A-B. Virtually, glutamate receptors play essential roles in probably every physiological function in the brain; dysregulation of these receptors causes a variety of neuropsychiatric disorders.

Neuropsychiatric disorders, including neurodegenerative and neurodevelopmental disorders, such as Alzheimer's disease (AD) and Autism spectrum disorders (ASD), are severely impair the self-cognition and social communication of individuals and may also genetically affect their descendants[2-5]. As the growing and aging population, researching on the neuropsychiatric disorders is of particular interest to scientists and clinicians. Due to the complexity of these diseases, it is still very challenging for researchers to understand the underlying mechanisms of these diseases[6-9]. The rapid development of genetic and molecular biotechnology provides an avenue for us to explore the pathogenesis of neuropsychiatric disorders[10,11]. With the support from genome-wide association studies and next-generation sequencing technology, the potential genetic architecture of human psychiatric diseases could be directly investigated[12-14]. In combination with genetic modified mouse, the disease-associated risk genes could be further confirmed in different psychiatric disorders, such as *SHANK* gene that is associated with autistic-like behavior[15] and *GRIA3* gene that are associated with aggressive behavior[16]. As numerous genetic variants have been identified in psychiatric patients, potential molecular mechanism underlying these diseases could be gradually elucidated.

Many gene variants, which may act as the causal factors for these neuropsychiatric disorders, have been uncovered to affect the neuroplasticity[17,18]. In the central nervous system (CNS), precise signal transmission is the cornerstone of human physiological processes, and the proper protein structure and function are required to maintain the fine-tune neuronal communication[19,20]. Therefore, numerous efforts have focused on identifying new gene variants in neuropsychiatric disorders and investigating the related mechanism. Understanding of the physiological and pathogenic function of these genes is essential to guide the treatments of the neuropsychiatric disorders.

In this review, we discuss the molecular properties of the NMDA receptors and their roles in neuropsychiatric disorders. We also extend our discussion to the potential therapeutic effects of NMDAR; specifically, it is possible to reverse the improper synaptic transmission and to further mitigate the clinical symptoms of neuropsychiatric disorders by targeting NMDARs.

2. Molecular properties of NMDA receptors

2.1. NMDARs subunit structure and expression pattern

NMDARs are composed of seven distinct subunits, including GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D), and two GluN3 subunits (GluN3A and GluN3B)[1,21,22]. All NMDARs are heterotetramers, each composed of two GluN1 subunits and two identical or different subunits of GluN2s or GluN3s[1,21,22]. The different combination of GluN2/GluN3 subunits also leads to the diversity of receptor compositions in the CNS.

The GluN1 subunit is encoded by a single gene, but due to alternative splicing, it has eight different isoforms (GluN1-1a-4a and GluN1-1b-4b). Compared to GluN1-a isoforms, GluN1-b isoforms contain exon 5, leading to additional extracellular 21 amino acid extensions (called N1 cassette) that influence the NMDA affinity and pharmacological properties[23,24]. GluN1-1 to -4 were derived from the difference of alternative splicing in exon 21 and exon 22, which alter the C-terminal domain (CTD) length and trafficking capacity[25].

All ionotropic glutamate receptor subunits consist of four domains[1,26,27]. A long N-terminal domain (NTD) in the extracellular domain is mainly involved in subunit assembly and allosteric regulation. A ligand-binding domain (LBD) consisting of two discontinuous fragments (S1 and S2) is the domain for binding of glycine (or D-serine) in GluN1 or GluN3 and of glutamate in the GluN2 subunit. The transmembrane domain (TMD) of NMDAR is composed of three transmembrane regions (M1, M3, and M4) and a reentrant loop (M2), which form the channel pore for the influx of ions. The final structure is the intracellular CTD, the length of which varies a lot and determines the length difference among NMDAR subunits[1]. CTD is involved in receptor trafficking, post-synaptic protein anchoring and protein-protein interactions, as well as many signaling pathways[22,28,29].

The NTD of NMDARs plays a critical role in the assembly of NMDARs. The NTD, formed by 1–350 amino

acids that associate as back-to-side heterodimers between GluN1 and GluN2 subunits, adopts a bilobed structure. Upper R2 lobes of GluN1 and GluN2 subunits interact to form a protein-protein interface, while lower R1 lobes connect to the LBD, thus forming a unique dimer-of-dimer arrangement[30-35]. Moreover, there are binding sites for allosteric modulators in NTD, including the sites for extracellular Zn^{2+} and ifenprodil, the GluN2B-selective antagonist[34,36]; therefore, the NTD also plays a role in regulating NMDAR gating and function.

The LBD is formed by the S1 and S2 segments, which forms kidney-shaped bilobed structures consisting of an upper lobe and a lower lobe with the agonist binding sites in the gap located between the two lobes[37]. Besides, there are three independent contact regions in the LBD heterodimer crystal structures of GluN1 and GluN2A (referred to as sites I, II, and III). Hydrophobic residues of GluN1 and GluN2 form sites I and III, and non-polar interactions between these residues mediate agonist binding domain (ABD) heterodimerization[37]. The site II of the ABD contains the binding sites of positive and negative allosteric modulators, which are highly selective for GluN2A[38-40].

The TMD is formed by M1, M3, and M4 and a reentrant loop (M2). The M2 is in the intracellular of the ion channel pore, and the M3 forms the extracellular region of the channel pore. The residues of pore region are highly conserved, which indicates the importance of the region. Normally, M3 forms the helical bundle and blocks the pore of channel so that ions cannot pass through the channel when the M3 helical changes its position[41-43]. The agonist binding to the LBD is the first step leading to M3 rearrangement[30,31,34,35,44], followed by multiple short-lived, intermediate conformations, and eventually channel opening[45]. NMDARs are widely distributed throughout the CNS, though the expression of NMDAR subunits varies in different brain regions and developmental stages. Consistent with a broad CNS distribution, the expression of GluN1 subunits generally begins from embryonic E14 and continues into adulthood[46-48]. Among the GluN1 splicing isoforms, GluN1-2 is widely distributed. The GluN1-1 and GluN1-4 expression distribution is complementary; the former is distributed in more rostral regions (including cerebral cortex and hippocampus). GluN1-a and GluN1-b subtypes have largely overlapped expression patterns, but their relative abundance varies from region to region. It is noteworthy that GluN1-a is expressed in all principal neurons in the hippocampus, while GluN1-b is mainly confined to the CA3 layer[49].

Expression of the GluN2 subunit varies in different brain regions during development. In rodents, the GluN2B and GluN2D subunits are highly expressed in the embryonic brain[47,48,50]. GluN2B expression remains high in the postnatal period, but only in forebrain regions. GluN2D expression is significantly reduced in adults; remaining GluN2D is mainly expressed in midbrain structures, including diencephalon and midbrain. The expression of GluN2A starts from birth and gradually increases over time, eventually becoming abundant throughout the CNS. Thus, NMDAR composition of GluN2B changes to predominantly GluN2A during development in the cerebral cortex and hippocampus[51]. The expression of GluN2C begins in the 2nd week after birth, but is limited to the cerebellum and olfactory bulb. The shift from GluN2B to GluN2C occurs in cerebellar granulosa cells during development, resulting in a sharp decrease in the GluN2B expression in adulthood[48].

GluN3A and GluN3B subunits also show different expression patterns[52,53]. GluN3A expression is the highest in the early postnatal period and then begins to decline gradually. In contrast, GluN3B expression is increased during development, with high levels of expression in motor neurons in adulthood. GluN2B, GluN2D, and GluN3A subunits are highly expressed in the early development, suggesting that these subunits play important roles in synaptic maturation and synaptogenesis[52,53]. GluN2A and GluN2B are major subunits in the CNS of adult, especially in hippocampus and cortex, suggesting that they play a role in synaptic function and plasticity[46-48].

2.2. Dynamic characteristics of NMDAR

2.2.1. Activation of NMDAR

Glycine and glutamate are required for activation of NMDA receptors consisting of GluN1/GluN2 subunits[54-58]. The activation of NMDAR containing of GluN1/GluN3 requires only glycine[53,59]. In the nervous system, glycine is naturally present in the extracellular environment (4.2 ± 1.6 μM of glycine in cerebrospinal fluid)[60]. Other molecules can also activate GluN1/GluN2 receptors as coagonists, such as D-Serine, L-Serine, D-alanine, and L-alanine. In recent years, D-Serine has been proposed as the main coagonist of synaptic NMDARs, while glycine is the main coagonist of NMDARs at extrasynapse[61]. Glutamate, the excitatory neurotransmitter in the CNS, is the native agonist of GluN1/GluN2 NMDARs. Glutamate (L-glutamic acid or D-glutamic acid) can activate NMDARs by binding to the LBD of GluN2 subunit. NMDA, N-methyl-L-aspartic acid, D-aspartic acid, and L-aspartic acid are also the agonists of NMDARs[62].

Unlike the conventional NMDARs, glycine binding to the GluN1/GluN3 receptor only produces a small excitatory current. Indeed, glycine binding to GluN3 activates GluN1/GluN3 receptors, but binding to GluN1

inhibits the channel[59,63-67]. The physiological function of GluN3 NMDARs remains largely unknown.

2.2.2. Gating function of NMDAR

NMDARs composed of different subunits have different channel characteristics. The GluN2A and GluN2B-containing NMDARs have higher conductance, higher Ca^{2+} permeability, and higher Mg^{2+} sensitivity compared to GluN2C and GluN2D-containing NMDARs, which are regulated by the Ser632 in GluN2A and S633 in GluN2B site in M3 region[68]. Besides, the channel gating properties are different among NMDARs, including the open probability, deactivation kinetics, and agonist potency. The open probability of GluN2A-containing NMDARs is higher than that of other GluN2-containing NMDARs, and the deactivation of GluN2A-NMDAR is faster too[69,70]. Therefore, the channel of GluN2A closes earlier after activation by glutamate, leading to a fast decay time. Interestingly, GluN1 splicing isoforms also affect NMDAR gating kinetics, that is, the NMDARs with GluN1-a deactivates slower than those with GluN1-b[23,24]. For the major NMDAR components in the cortex of adult, the GluN1/GluN2A receptors have the faster decay time, while the GluN1/GluN2B receptors have higher Ca^{2+} permeability and charge transfer[71].

2.3. NMDARs trafficking

NMDAR trafficking is mainly mediated by intracellular CTD. The difference in CTD sequences of NMDAR subunits leads to subunit-specific regulations on receptor transport, localization and signal transduction[72-74]. The synaptic transmission and escape from the endoplasmic reticulum (ER) of NMDARs are regulated by the C-terminal splicing of GluN1[25], a process that appears to be driven by neuronal activity[75]. The different motives in the CTDs of GluN2 and GluN3 diversify the trafficking procedures of NMDARs[76].

2.3.1. Receptor biogenesis

Typically, NMDARs are first assembled in ER and matured by glycosylation in the Golgi apparatus before being transported to the plasma membrane through vesicles. Cells have strict mechanisms to prevent unassembled or misfolded NMDARs from being transported to the cell surface[77]. The previous studies indicated that only in the form of GluN1/GluN2, NMDAR could escape from the ER[78]. *Grin1* gene deletion causes the retention of GluN2 subunit in the ER of hippocampus[79]. Both GluN1 and GluN2 subunits contain ER retention signals, which can be masked by the co-assembly of GluN1 and GluN2 subunit[80]. For example, the CTD of the GluN1 subunit contains positively charged ER retention signals, including lysine-lysine-lysine (KKK), and arginine-arginine-arginine (RRR)[25,81,82]. The overexpression of GluN2A and GluN2B in cerebellar granular neurons leads to a significant increase in the number of NMDARs and synaptic targeting, probably through the co-assembly with extra GluN1[83]. An ER retention sequence (HLFY) has been proposed in the CTD of GluN2B subunit[84]. However, subsequent studies showed that HLFY motif was required in the CTD-oriented structure of GluN2B, but its might not serve as an ER retention signal[85]. In a recent study, the KKK879-881 of GluN2A was proven to be an ER retention signal[17], regulating the surface expression of GluN2A-NMDAR.

GluN2 subunits interact with the proteins of membrane-associated guanylate kinases (MAGUK) family, such as synaptic associated proteins-102 kDa (SAP102) and synaptic associated proteins-97 kDa (SAP97), which is necessary for NMDAR secretion[86,87]. SAP102 is highly expressed in the hippocampus on the 2nd day after birth, and its PDZ region interaction with GluN2A and GluN2B subunits of NMDARs makes a difference[88,89]. Moreover, SAP102 is also widely present in the cytoplasm and ER[88]. In addition, SAP102 interacts with mPins through its SrC-homology 3 (SH3)/guanylate kinase domain to stabilize the SAP102-exocyst-NMDAR complex in ER. This process plays an important role in promoting NMDAR trafficking and membrane targeting[86,87].

NMDARs also have a trafficking pattern that bypasses the traditional somatic Golgi network. In this pattern, these receptors mix directly within the dendrite Golgi[90]. This strategy can promote more efficient insertion of NMDARs at the post-synaptic density (PSD). Because they contain large protein complexes including scaffold molecules, vesicles produced by this pathway are highly mobile (0.76 μm/s). Mlin7 binds GluN2B with the motor protein KIF17, which promote the long-distance transport of NMDAR-containing vesicles on microtubules along dendrites[90-92]. Studies have shown that KIF17-mediated NMDARs trafficking is critical in long-term potentiation (LTP), long-term depression (LTD), learning, and memory[93]. Deletion of *kif17* leads to NMDAR degradation due to enhanced ubiquitination, resulting in partial synaptic GluN2A and GluN2B receptors loss. It is interesting that the interaction of CASK leads SAP97 to preferentially bind NMDARs[94]. Meanwhile, SAP97 is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) at two key sites, Ser-39 (in the L27 domain) and Ser-232 (in the PDZ1 domain)[95,96]. Phosphorylation of SAP97 at Ser-39 leads to translocation of SAP97 from the ER to the post-synaptic compartment, while phosphorylation at Ser-232 disrupts binding of SAP97 to GluN2A. In

addition, CaMKII-dependent phosphorylation of KIF17 also facilitates unloading of cargo from microtubules[97]. Therefore, successive phosphorylation of SAP97 and KIF17 may result in the NMDAR escape from the ER and promote the insertion of NMDAR at synapses.

Recent studies have shown that palmitoylation has an important regulatory role in NMDARs post-Golgi trafficking[98,99]. Activity-dependent palmitoylation occurs in two distinct clusters in the CTD of the GluN2A and GluN2B subunits[99,100]. Palmitoylation of GluN2A and GluN2B at the second cluster of cysteines leads to retention of NMDARs in the Golgi apparatus, resulting in decreased surface expression of receptors[100]. Thus, mutation of the cysteines promotes surface expression of NMDARs, although the levels of synaptic NMDARs are not altered[101].

2.3.2. Receptor exocytosis

Exocytosis of NMDARs occurs primarily at extrasynaptic sites[102], where receptors spread to synapses through lateral diffusion[103]. In general, a family of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) proteins mediate the fusion of vesicles to the plasma membrane, including target SNARE syntaxin, synaptosome-associated protein (SNAP), and vesicular SNARE vesicle-associated membrane protein[104]. Although the role of the SNARE complex in NMDAR exocytosis is critical, the precise role of specific members of this protein family remains controversial.

SNP25 plays an important role in mediating NMDAR exocytosis in CA3 pyramidal neurons and *Xenopus* oocytes[105,106]. In these studies, botulinum toxin A, which inactivates SNAP25, could block mGluR1- and PKC-dependent NMDAR exocytosis. SNAP25 is a substrate of PKC; mutation of the Ser-187 phosphate site inhibits PKC-mediated NMDAR insertion[107]. Meanwhile, knockdown of SNAP25 with shRNA also reduces NMDAR transmission[108]. Studies have also shown that another member of the SNAP family, SNAP23, is highly expressed at excitatory synapses and regulates NMDAR exocytosis[109,110]. Reducing SNAP23 by shRNA knockdown or gene deletion impairs surface expression of NMDAR[110]. Taken together, both SNAP23 and SNAP25 are important in regulating the exocytosis of NMDARs.

2.3.3. Receptor lateral diffusion

Lateral diffusion of NMDAR on the membrane is along extrasynapse and synapse[111]. GluN2A-NMDAR and GluN2B-NMDAR appear to be differentially located on the neuronal surface. GluN2A-NMDAR is preferentially expressed in the synapse, while the expression of GluN2B-NMDAR tends to be higher in extrasynapse during development phase[112-115]. The underlying mechanism seems to be that the interaction between CTD of GluN2A with PDZ domain of post-synaptic density protein-95 (PSD-95) is relatively stabler[116,117]. Furthermore, the peptides mimicking GluN2A or GluN2B PDZ binding motif only causes a decrease in synaptic GluN2A-NMDAR but not in synaptic GluN2B-NMDAR, indicating that the PDZ-domain dependent regulation is subunit-related.

Furthermore, the protein phosphorylation of the CTD of NMDARs regulates the surface trafficking of receptors. GluN2B is phosphorylated on Ser-1480 by casein kinase 2 (CK2), which disrupted the interaction with MAGUKs and resulted in decreased synaptic GluN2B expression[117-119]. Other factors that regulate the diffusion of NMDARs include secreted proteases[120], extracellular matrix,[121] and hormones[122,123].

2.3.4. Receptor endocytosis

The surface receptors are also regulated through receptor internalization. NMDAR endocytosis is dependent on development and neuronal activity; the rate of internalization decreases gradually as neurons mature[124]. The CTD of GluN2A and GluN2B contains endocytosis motifs, but GluN2B subunits have relatively higher endocytosis rates in mature neurons[125].

The phosphorylation of Y1472 by Fyn kinase induces GluN2B binding with MAGUKs, which stabilizes GluN2B in the synaptic membranes. Reduction in Y1472 phosphorylation induces the interaction between YEKL1472-1475 endocytic motif and adaptor protein-2 (AP-2), resulting in increased internalization of GluN2B[124,126,127]. On the other hand, Ser-1480 phosphorylation by CK2 contributes to increased GluN2B internalization[83,128]. Therefore, the phosphorylation/dephosphorylation plays an important role in receptor internalization. In GluN2A subunit, the endocytosis is mediated by the di-leucine LL1319-1320[125].

GluN1 subunit also affects the endocytosis of NMDARs containing GluN1/GluN2BΔCTD[129]. Studies have shown that there are two motifs of internalization in the C0 cassette in GluN1: YKRH838-841 and VWRK858-861. Similar sequences have been found in GluN2A (YWKL841-844) and GluN2B (YWQF842-845).

GluN3 endocytosis has been relatively poorly studied. In the GluN3A subunit, PACSIN1 binds to the CTD of GluN3A through its asparagine-proline-phenylalanine (NPF) domain, thereby regulating the internalization of the GluN3A receptor. Moreover, this regulation mode is activity-dependent, and destruction of the function of PACSIN1 leads to the accumulation of GluN3A receptors at the synapse[76].

Thus, the NMDARs undergo various regulation of protein synthesis, trafficking, and internalization. These regulations also ensure the dynamic abundance and composition of NMDARs on the synaptic membrane, thereby allowing physiological functions regulated by neuronal activity and plasticity.

2.4. NMDARs in neuroplasticity

Synaptic plasticity refers to the long-lasting change in morphology and function of synapses caused by the neural activity induced by experience[130]. Two classic types of synaptic plasticity, LTP and LTD, have been studied at hippocampal excitatory synapses; both requires the activation of NMDARs[131]. LTP strengthens synapse function and is induced by high-frequency presynaptic stimulation, while LTD weakens synapse function and requires low presynaptic stimulation to induce. NMDAR-dependent synaptic plasticity is the basis of learning and memory in hippocampus. Blocking hippocampal NMDARs before training impairs rodent learning ability[132,133], but memory is enhanced after hippocampal-dependent task training[134,135].

In hippocampal synapses, AMPARs and NMDARs involve the forms of LTP and LTD. AMPARs and NMDARs are glutamate receptors that are permeable to Na^+ and K^+, while all NMDARs and part of AMPARs are also permeable to Ca^{2+}. When the post-synaptic membrane is at resting potential, NMDARs cannot conduct currents because Mg^{2+} blocks the channel pore. When AMPAR current causes local membrane depolarization, Mg^{2+} is removed from the NMDAR channel, allowing Ca^{2+} flow into the cell. LTP and LTD require NMDARs-mediated influx of Ca^{2+}; LTP requires a large elevation of Ca^{2+} in spine while that it is much less for LTD. When NMDARs mediate a large influx of Ca^{2+}, protein kinases, especially CaMKII acting on receptor proteins, accessory receptors, or transcriptional regulators increase glutamate receptor activity and/or levels in the synapses, thus inducing the induction and maintenance of LTP[136,137]. When NMDAR-mediated Ca^{2+} elevation is modest, Ca^{2+}/calmodulin-dependent protein phosphatase, protein phosphatase 1, and calcineurin are phosphorylated and activated, thereby inducing the dephosphorylation of AMPAR that results in the internalization of AMPAR from the synapses and LTD[138-140].

Synaptic plasticity requires Ca^{2+}, and the composition of NMDARs has different permeability to Ca^{2+}, which could couple with the phosphatase pathway and downstream signaling to regulate the plasticity[140]. However, how specific subunit composition determines synaptic plasticity remains unclear and controversial.

2.4.1. LTP

To investigate the possible specific role of GluN2 in synaptic plasticity, both genetic and pharmacological approaches have been applied.

NMDARs containing GluN2B showed a greater current[48] and carried more Ca^{2+}[71]. Besides, GluN2B preferentially interacts with CaMKII[141], and it is speculated that the GluN2B subtype is more likely to induce LTP than the GluN2A subtype. For example, ifenprodil, the GluN2B antagonist, blocks the pairing-induced LTP in hippocampal slices[142] and in the barrel cortex[143]. Moreover, the GluN2B antagonist could block the pairing- and theta burst-induced LTP in the anterior cingulate cortex at 6–8-week-old mice[144]. The tetanus induced-LTP in hippocampal CA3 synapses was abolished in mice with conditional GluN2B knockout[145]. Lacking GluN2B in the Cornu ammonis 1 (CA1), the LTP is impaired[146]. Another study showed that LTP is deficient by pairing protocol of GluN2B knockout in the forebrain[147]. LTP is enhanced when GluN2B is overexpressed in hippocampal neurons of 4–6-month-old mice[148]. Disruption of the interaction between GluN2B and CaMKII by overexpressing the CTD of GluN2B eliminates the LTP of 3–4-month-old mice[149]. Genetic deletion of GluN2A has no effect on the LTP in P28 mice, indicating that GluN2B is essential for the LTP[150-152]. Other genetic methods that influence the level of GluN2B also impairs the induction of LTP. For instance, knockout of the KIF17, a protein that transports GluN2B to the synapses, reduces synaptic GluN2B and abolishes LTP[93]. Besides, Cdk5 knockout mice show increased GluN2B and enhanced LTP[153]. Recently, it has been found that enhanced GluN2A surface and synaptic expression causes LTP impairment, likely through compensatory GluN2B decrease[17]. Hence, GluN2B-containing NMDARs play a role in the induction of LTP.

However, some studies have shown that GluN2A is important for LTP[153,154]. In pharmacological experiments, blocking of GluN2A by NVP-AAM077 could prevent tetanus- and pairing-induced LTP in 3–4-week-old rats, while the ifenprodil and Ro 25-6981, the antagonists of GluN2B, have no effect on LTP but could block the induction of LTD[154]. Another study that used the same antagonist concentration and rats of the same age found that NVP-AAM077 completely blocks LTP, and ifenprodil and Ro 25-6981 partially block LTP[155]. Low Zn^+ which selectively inhibited GluN2A impairs LTP[72]. GluN2A knockout mice[156] and deletion CTD of GluN2A mice[72] show impaired LTP in hippocampal synapses.

For LTP, a possibility is that both GluN2A and GluN2B subunits mediate Ca^{2+} influx, so both subunits are involved in the induction of LTP[157,158]. However, the exact extent of involvement of these two subunits in LTP remains unclear.

2.4.2. LTD

There are also many contradictory results on the LTD with the composition of NMDAR subunits. One study suggested that ifenprodil blocks LTD in hippocampus[154], while the results of other research groups indicated that ifenprodil does not affect LTD[159] or even enhances LTD[160]. Disruption the interaction of GluN2B and PSD95 has no effect on LTD, although the level of synaptic GluN2B is reduced[161]. Moreover, the overexpression of GluN2B does not affect the LTD[162], while the LTD is deficient in GluN2B knockout mice and KIF17 knockout mice, due to the reduction of synaptic GluN2B[93,146]. Thus, these conflicting findings point to the ambiguous function of the GluN2B subunit in LTD, and to the fact that the experimental conditions are important for the induction of LTD.

On the other hand, some studies found that NVP-AAM077 not only impaired LTP but also blocked LTD[155,163]. By contrast, the NVP-AAM077 only affected LTP but did not impair LTD in slices[154] or *in vivo*[164]. Besides, overexpression GluN2A induced decreased LTD[152] and GluN2A knockout mice displayed no impairment of LTD[165]. However, the LTD could be induced by the 0.5 Hz stimulation in GluN2A knockout mice[165].

More experiments are warranted to clarify the role of NMDAR subunits in the LTD.

2.5. NMDARs pharmacology

NMDARs play a role in the neuropsychiatric disorders. The dysfunction of NMDARs involved in many disease, such as AD, Parkinson's disease (PD), epilepsy, and schizophrenia[166]. Therefore, in the past decades, a great deal of money has been spent to develop NMDAR antagonists and agonists to cure the diseases associated with NMDAR. For example, the ketamine and rapastinel (GLYX-13) are used for the treatment of depression[167-169]. Memantine, a NMDAR blocker, has been proven for use in treating AD and increasing cognitive behavior of AD patients[170,171]. Unfortunately, due to the off-target or side effects of excessive inhibition of NMDAR, many drugs have failed in most clinical trials[166,172]. Despite the challenges, there are still opportunities to develop NMDAR drugs.

Many positive and negative allosteric modulators (PAMs and NAMs, respectively) have been found recently. In addition to the antagonists and agonists of NMDAR, the allosteric modulators can positively and negatively regulate the NMDAR activity. Compared with antagonist or channel blocker, the allosteric modulators have many potential advantages in therapeutics development. The binding domain of allosteric modulators with NMDARs is not highly conserved ligand-binding site or channel pore. Therefore, allosteric agents have better subunit selectivity, which reduces side effects and off-target. Besides, the inhibition of NAMs is <100%, so partial function of NMDARs can be preserved, so as to avoid excessive blockade of receptors. This partial inhibition of NAMs has a better safety profile than competitive antagonists and channel blockers. In schizophrenia caused by NMDAR hypofunction, or other cognitive disorders, PAMs only enhanced the activity of weakly activated NMDAR-mediated signals to restore normal function. This is unlike the NMDAR agonists, which could activate all receptors and lead to side effects and excitotoxicity. Therefore, a better understanding of these allosteric modulators can improve their usage in clinical practice.

2.5.1. The antagonists of NMDARs

The previous studies have shown that most NMDAR antagonists basically interact with NMDARs by glutamate binding site, glycine binding sites, NTD binding sites, or ion channel pores. The glutamate is an activator of NMDARs. Agonists and antagonists that interact with glutamate binding sites were first identified[56], such as D-α-amino adipic acid and D-AP5. Since glutamate binding affinity is the strongest in GluN2D followed by GluN2B/2C, and the lowest in GluN2A, the competitive antagonists generally have the strongest inhibition on GluN2A, then on GluN2B/2C. For example, CPP is more sensitive to GluN2A subunit than to other subunits[173]. Quinoxalinedione derivative (1*RS*,1'S)-PEAQX, a GluN2A competitive antagonist, has no effect on GluN2B[174]. At present, the NVP-AAM077 ([1R,1'S]-stereoisomer) purified from (1*RS*,1'S)-PEAQX is widely used to inhibit GluN2A-NMDARs.

Antagonists against the glycine binding site have been found[175,176]. Glycine binds to GluN1 subunit of NMDARs, so these antagonists show no selectivity for the subunits. The antagonists that act through glycine site include HA-966[177], 7-chloro-5-iodokynurenic acid[176], L-701,324,[178] and MDL 105,519[178].

The channel sequence and structure of NMDARs are highly conserved, so the selectivity of channel blocker is little. The NMDAR blockers, such as ketamine and phencyclidine (PCP), act as the anesthetics that bind the ion channel pore[179,180]. However, when the Mg^{2+} are present, the memantine appears to be more sensitive to GluN1/GluN2C and GluN1/GluN2D receptors. Besides, the MK-801 is also a NMDAR channel blocker, which binds to channel pore when the NMDARs are activated[181].

2.5.2. NAMs

The binding sites of NMDAR subunits with NAMs are not a highly conserved ligand binding site or channel

pore, so the subunit-specificity of NAMs is greater than the antagonists. Due to the noncompetitive glutamate and glycine binding site and independent NTD regulation to activity, the NAMs are safer to be used in the clinical settings. Many NAMs have been found in recent years, including sulfonamide series, quinazoline-4-one series, and phenanthroic and naphthaloic acid NAMs. Ifenprodil, CP101, and RO25 also belong to the category of NAMs.

Ifenprodil was studied in treatment in stroke and then was proven a GluN1/GluN2B receptor antagonist, which binds the NTD of the subunit[182,183]. Subsequently, CP101, 606[184] and Ro 25-6981[185] were developed as GluN1/GluN2B antagonists, which have been studied for clinical use.

The TCN-201, one of sulfonamide series, was first found as a noncompetitive GluN2A NAM[186], which binds with dimer interface between the LBD[40]. TCN-201 has a higher affinity with GluN2A over other GluN2 subunits[186,187]. Studies indicated that the TCN-201, which reduces the potency of glycine and D-serine, is a noncompetitive antagonist[187,188]. The inhibition of TCN-201 is abolished at high concentrations of glycine[186].

The quinazoline-4-one series inhibitor has been identified to be more sensitive to GluN2C and GluN2D compared with GluN2A and GluN2B[189]. QNZ46, one of this type of noncompetitive NAMs, exerts differential effect on GluN2A and GluN2D. Point mutations and chimeric molecules were used to identify the binding site of QNZ46, and S2 domain of GluN2 is found to be critical for QNZ46 activity[190]. Unlike TCN-201, QNZ46 increases the potency of glutamate but not glycine, although QNZ46 is a NAM. The feature of QNZ46 inhibition is needed in the binding with glutamate. After QNZ46/glycine preincubation, glutamate plus QNZ46/glycine application produced a transient peak response followed by homeostasis inhibition. Thus, the glutamate binding is essential for the inhibition of QNZ46. The QNZ46 inhibits NMDAR function by binding in S2, and the binding site of QZN46 with GluN2 is exposed by glutamate binding.

Phenanthroic and naphthaloic acid NAMs exhibit different subunit selectivity and have enhanced or inhibited activity in different subunits. UBP512 not only is a potentiator of GluN2A but also is an inhibitor of GluN2C and GluN2D-containing NMDARs[191]. Meanwhile, the UBP551 showed PAM activity on GluN2D at high concentration of 30 μM, but inhibited GluN2A, GluN2B, and GluN2C subunits at IC50s around 10 μM[191]. Besides, the UBP618 is an inhibitor of all GluN2 subunits, and the CTD is critical for the inhibition[192]. In addition, the UBP608 shows inhibitory effects on GluN2A-NDMA receptor[192].

2.5.3. PAMs

The function of PAMs is to enhance the NMDAR function. Endogenous PAMs include ATP[193], histamine[194], Mg^{2+}, polyamines such as spermine[195], and pregnenolone sulfate (PS)[196]. The enhancement of histamine and ATP is both enhanced at high glutamate concentrations. The low concentrations of ATP can enhance the GluN2A-, GluN2B-, and GluN2C-containing NMDARs[193]. The effects of spermine on NMDARs are different; it can either increase the potency of glycine, or allosterically interact with GluN2B- and GluN1-lacked exon 5, that is, the glycine-independent[194,195,197]. PS potentiates GluN2A- and GluN2B-containing NMDARs at micromolar concentrations, while inhibits the GluN2C and GluN2D subunits[198]. The mechanism of the potentiation of PS is the increased open probability of NMDARs through phosphorylation[199,200]. Similar results have been found by single channels analysis that the frequency of channel openings is enhanced by PS[201,202]. Besides, the studies have proven that the S2 domain of GluN2A may be the interaction site of PS with GluN2A[200], while the S2 and M4 domain of GluN2B play an important role in the potentiation[203].

Other PAMs of NMDARs, such as phenanthrene, naphthalene, and coumarin derivatives, have been reported in many studies[204]. UBP512, a phenanthroic acid, enhanced the GluN2A-contaning NMDAR, but not the GluN2B-containing NMDAR, and inhibited the GluN2C and GluN2D subunits. The UBP710 enhanced both GluN2A- and GluN2B-containing NMDARs, while inhibited the GluN2C and GluN2D subunits. The UBP551, a naphthoic acid NMDAR PAM, potentiated the GluN2D subunit, while had no effect on the other three GluN2 subunits[191]. Another naphthoic acid NMDAR PAM is the UBP684, which could enhance the effect on all GluN1/GluN2 receptors by increasing the open probability and mean open time[205,206]. The UBP714, a coumarin derivative, slightly potentiated the GluN2A, GluN2B and GluN2D[207]. The CIQ is a potentiator of GluN2C- or GluN2D-containing NMDAR[208,209]. Studied indicated that the linker between NTD and LBD and T592 of GluN2D is the important sites of the GluN2D to activate the CIQ[208].

Recently, the GEN family has been reported as the PAMs of GluN2A. According to the analysis of the GluN2A binding with GEN-6901, we understand that the V783 of GluN2A is a necessary site for binding with GEN-6901 specifically[38]. The potentiation of GEN-8324, instead of GEN-6901, in GluN2A increased the potency of glutamate, but both of them had no effect on the sensitivity to glycine. Meanwhile, they also decreased the NMDAR deactivation[38]. However, there are differences between

the two potentiators. The GEN-8324 has an impact on the inhibitory neurons only, while the GEN-6901 plays a role in both inhibitory and excitatory neurons[38]; however, the underlying principle remaining to be explored. The GEN-0723 had been shown to enhance GluN2A not only *in vitro* but also *in vivo*[39]. The GEN-9278 potentiated not only the GluN2A-NMDARs but also the GluN2B-, GluN2C- and GluN2D-NMDARs[210]. The GEN-9278 increased the glutamate and glycine potency and reduced the deactivation of glutamate.

3. NMDAR in neuropsychiatric disorders

NMDARs play an important role in neuronal development. NMDARs dysfunction in subunits expression, localization, and trafficking may cause brain disorders. Therefore, both NMDAR antagonists and enhancers have the potential to be utilized as CNS treatment agents. In this section, we focus on some neuropsychiatric disorders with NMDAR dysfunction, with special emphasis on subunit specificity.

3.1. AD

AD is a dementia, which is characterized by increased beta-amyloid (Aβ) peptide and the intracellular tangles composed of tau protein in hyperphosphorylation. Aβ may aggregate to form soluble oligomers composed of multiple polypeptides or insoluble amyloid plaques. Hyperactivation of NMDAR is also associated with AD (Figure 1). Memantine, which is an NMDAR channel blocker, is currently used to treat patients with AD. Moreover, the antagonists of NMDAR, such as APV and ifenprodil, as well as the channel blocks, ketamine, and MK-801 can abolish the synaptic depression induced by Aβ[211]. Some studies, further, suggested that GluN2B is essential for the harmful effects of Aβ. Synaptic loss[212], impairment of LTP[212-214], and increased LTD induced by Aβ[215] can all be rescued by GluN2B antagonists. Some of these mechanisms may be the activation of extrasynaptic GluN2B receptors induced by changes in glutamate uptake[215]. Phosphorylation of the GluN2B by the tau and tyrosine protein kinase FYN trafficking to the dendritic spine enhances the interaction with PSD95, which leads to downstream excitatory toxicity[216]. Besides, the induction of Aβ is needed for the activation of extrasynaptic GluN2B-containing NMDARs[217]. A synthetic peptide that interferes with the interaction between GluN2B and PSD95 enhances memory and longevity in AD mouse models[216]. However, this peptide did not impair NMDAR-mediated synaptic currents, but reduced ischemic cell death in stroke model[216,218]. In addition, it should be noted that much of the evidence supporting the role of GluN2B in mediating Aβ-induced excitatory toxicity is obtained through synthetic Aβ preparations *in vitro*. Therefore, it remains to be clarified whether the GluN2B antagonists can prevent or reduce synapse loss and improve cognition in AD models.

3.2. PD

PD is a neurodegenerative disease, which is characterized by the degeneration of nigral dopaminergic neurons and the loss of dopamine in the striatum. Dopaminergic and glutaminergic signaling are involved in controlling motor function. Studies have shown that the NMDAR is impaired in the disorder (Figure 1). For example, in the PD model, the expression of GluN1 and GluN2B, but not GluN2A, was reduced, which can be rescued by L-DOPA[219]. In addition, after L-DOPA treatment in dyskinetic animals, GluN2B was transferred from the synaptic to the extrasynaptic region,

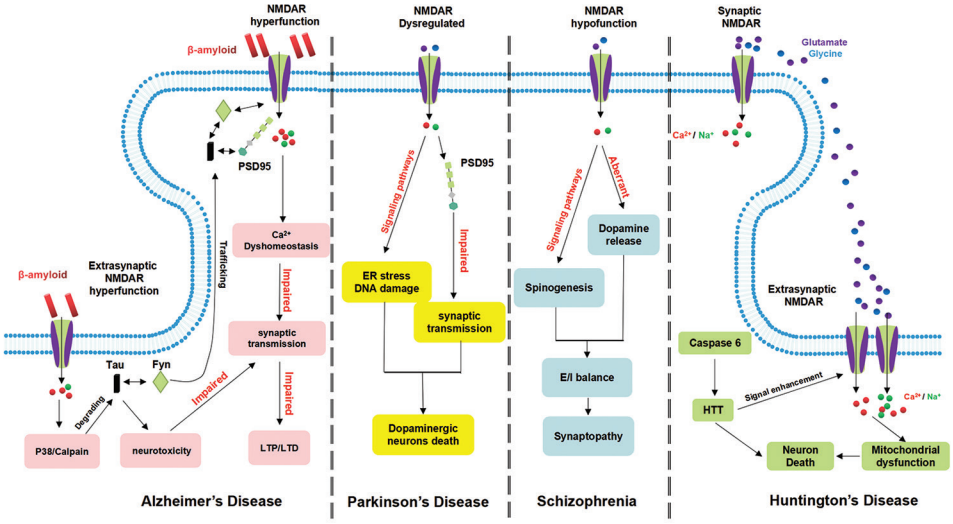


Figure 1. NMDAR and its related signaling pathways in different neuropsychiatric disorders. NMDAR: N-methyl-D-aspartic acid receptors.

and GluN2A was increased in the synaptic region[220]. In addition, the expression of MAGUK protein was decreased, which is probably due to the impaired interaction between MAGUK with GluN2B in the PD models[220]. However, the GluN2B selective antagonists have inconsistent effects on PD models and human PD patients[221,222]. However, in the rat model of PD, specific interference of GluN2A in synaptic localization can reduce motor dysfunction[223].

Although these studies help us further understand the pathophysiology of PD, the disease involves many changes in organs and brain regions. Therefore, a complete cure for PD remains a major challenge.

3.3. Schizophrenia

Schizophrenia is psychiatric disorder with hallucinations and delusions (positive symptoms), aversion and anhedonia (negative symptoms), and cognitive dysfunction[224]. Studies have shown that excessive release of dopamine in striatum is the reason of the positive symptoms[225], but the mechanism of negative symptoms and cognitive dysfunction remains elusive. Changes in glutamate signal may be a pathological basis for the schizophrenia[226], especially dysregulated NMDARs may induce the imbalance between excitation and inhibition in PV neuron (Figure 1).

Studies have indicated that reduction in NMDAR expression causes schizophrenia-like behavior in mice[227]. Impaired dopamine release is found in mice with GluN1 deletion in PV neurons[228]. Recently, other studies in clinic proved the NMDAR hypofunction in schizophrenia. The noncompetitive NMDAR antagonists, PCP, and ketamine could induce schizophrenic phenotypes in people[229-231]. Furthermore, the MK-801 also induced the PCP-like behavior in many species[232,233]. The autoantibodies against NMDARs, which are found in patients with the anti-NMDAR encephalitis, cause NMDAR internalization. These patients show the symptoms similar to schizophrenia[234].

These studies suggested that the NMDAR hypofunction may cause schizophrenia, providing new ideas for developing treatment of schizophrenia in the future.

3.4. Huntington's disease (HD)

HD in human results from an inherited huntingtin (*HTT*) gene defect, which induces progressive degeneration of neurons in the brain and impairs movement, cognitive functions, and emotions. HD is defined by repeated CAG mutation in *HTT* gene[235]. Some studies have indicated the NMDAR function is increased in HD mouse models[236], and the balance of synaptic and extrasynaptic NMDAR plays an important role in HD (Figure 1). The data found that the balance of synaptic and extrasynaptic NMDAR is disrupted in mice model of HD[237]. The experiments also showed that in the HD model, caspase-6 cleavage, a process of the production of toxic mutation *Htt* fragments, increased extransynaptic NMDA receptor-induced currents, and signaling pathways[237]. In addition, the HD mice were treated with memantine, which only antagonized extrasynaptic NMDAR at low dose and rescued impaired learning as indicated by a test on rotarod motor[237]. Another study also presented the similar results that the activation of synaptic NMDAR rendered *Htt* mutation-expressing cortical cells resistant to cell death, while the activation of extrasynaptic NMDAR rendered the cell more vulnerable to cell death[238]. Clinical trials have shown that treating HD mice with low doses rather than high doses of memantine rescued adverse neuropathological and impaired behaviors[238].

Binding of β-amyloid to synaptic NMDAR increases post-synaptic Ca^{2+} level, which impairs synaptic transmission and further affects LTP/LTD. The stimulation of extrasynaptic NMDAR could activate calpain which degrade tau protein to a toxic peptide and also phosphorylate p38-MAPK signaling pathways and leads to cell death. Moreover, tau protein is required to transport kinase Fyn to the post-synaptic localization, and to further promote the interaction with PSD95 and phosphorylation of NMDAR, which lead to the impaired LTP/LTD in AD. Dysregulated NMDAR could induce ER stress and DNA damage, probably through the overloading of Ca^{2+} ions and related signaling pathways. The impaired interaction between PSD95 and NMDAR may also affect the synaptic transmission and lead to dopaminergic neurons death in PD. Hypofunction of NMDAR affects the spinogenesis and also reduces the release of dopamine, resulting in an unbalance between excitatory and inhibitory transmission and schizophrenia. Increased level of extrasynaptic NMDAR attracts more Ca^{2+} ions, which induce mitochondrial dysfunction and further lead to neuron death. In addition, caspase 6 cleavage of HTT is essential to increase the extrasynaptic NMDAR current and subsequent excitotoxicity in HD.

3.5. Major depressive disorder (MDD)

MDD is currently one of the most common mental disorders in the world and frequently occurs as a complication in other diseases[239]. The efficacy of drug treatment for MDD patients usually lasts from weeks to even years. In addition to its severe effect on the social communication and cognitive functioning, the comorbid MDD and anxiety symptoms are also a challenge for pharmaceutical approaches[240,241].

In 1960s, ketamine, a nonselective NMDAR antagonist, was originally developed as an anesthetic[242]. In 2000,

ketamine, which was tested in MDD patients, displayed potential clinical effectiveness[167]. Two enantiomers of ketamine, arketamine and esketamine, show antidepressant activity with different degree of affinity to NMDAR[243]. Recently, the treatment of esketamine for MDD has been approved by the USA and Europe FDA[244]. It has been suggested that the dysfunction of glutamatergic transmission may associated with MDD, and NMDAR attracts considerable attention with respect to its biological function in the CNS[245]. Studies with MDD patients showed significantly reduction of GluN2A and GluN2B subunits of NMDAR but did not change GluN1 protein level compared to the controls, along with reduced level of PSD-95 in prefrontal cortex, indicating an abnormal signaling in the synaptic transmission within MDD[246]. However, in the lateral amygdala and locus coeruleus of depressed subjects, increased levels of GluN2A and GluN2C expression have been detected[247,248]. A recent research showed that α7nAChR-NMDAR complex may play a role in the MDD, and disruption of this complex with a peptide exhibits antidepressant effects[249]. Moreover, another study suggested that the antidepressant mechanism of ketamine is NMDAR-independent but related to AMPAR[250]. Taken together, these studies reveal a crucial role of NMDAR in MDD, underlining the significance of NMDAR in the development of next-generation antidepressants.

3.6. Stroke and traumatic brain injury (TBI)

In stroke and TBI, extracellular glutamate is continuously elevated, causing excitotoxicity and acute neuronal death[251]. The NMDAR-mediated excitotoxicity is a major cause of acute neuronal death after ischemia or injury. Studies have suggested that NMDAR antagonists could inhibit ischemic cell death[252,253]. NMDAR antagonists protect neurons against ischemic cell death if they have been applied before[254-256], but not 30 min[254] or 3 h[255] after stroke in animal models. However, it has also been reported that GluN2B antagonists applied 2 h poststroke could reduce brain infarct volume[257]. Intriguingly, NMDAR-mediated excitotoxicity appears to be subunit-dependent: GluN2A antagonist aggravates but GluN2B antagonist blocks the ischemic cell death[255,256]. Probably due to the intolerable side effects of NMDAR antagonists and the level of elevated extracellular glutamate that last less than an hour, the clinical trials concerning the application of NMDAR antagonists in stroke have so far been unsuccessful[218]. In addition to the direct inhibition of NMDAR activity, neuronal protection can be achieved by disrupting the interactions between NMDARs and their scaffold proteins, including PSD-95, phosphatase and tensin homolog, and associated signaling molecules in stroke animal models[258,259] and in humans[260]. This could be a new strategy targeting NMDAR-associated signaling in stroke.

The clinical trials concerning NMDAR antagonists, including selective GluN2B antagonists, for use in the treatment of TBI were also unsuccessful[261]. Although overactivation of NMDARs is toxic, functional recovery after TBI requires physiological activation of NMDARs[262].

3.7. Epilepsy

Epilepsy is a very common neuropsychiatric disorder that causes abnormal brain activity, seizures, unusual behavior sensations, and sometimes, loss of consciousness. Glutamate-mediated excitability changes could be involved in the pathogenesis of epileptic discharge[263]. NMDAR may be involved in the seizure-induced excitotoxic cell death of hippocampal neuronal populations, as NMDAR antagonists provide protection against such damage[264]. Many animal models of epilepsy have been developed, including chemical induction models (such as kainic acid, pilocarpine, picrotoxin, or bicuculline), physical models (such as hyperthermia, or photic or auditory stimuli), genetic models (such as mutations, transgenes, or knockouts), electrical stimulation models, and spontaneous seizure models (such as post-kindling). Due to the differences in animal models, brain regions, and NMDAR subunits examined, the results of these studies vary. A study found that seizure enhances expression of GluN1 mRNA and protein in rat cerebral cortex[265]. Another study indicated that the mRNA of GluN1 is continuously increased in cortex of amygdaloid kindled rat[266]. However, application of picrotoxin (500 μM) caused a decrease in mRNA levels of GluN2A and GluN2B, while the mRNA level of GluN1 remained unchanged[267]. Besides, a study demonstrated that mRNA and protein levels of GluN2A and GluN2B were increased in spontaneous seizure, but not in kindled seizure[268]. Kainic acid-induced seizure reduces the mRNA level of GluN1 in CA1 and CA3 pyramidal cells, but not dentate gyrus[269].

NMDAR antagonists are proven to be anticonvulsant in several animal models of epilepsy. Felbamate (Felbatol®) is used in patients with intractable partial seizures, infantile spasms, or Lennox-Gastaut syndrome[270,271]. Some studies reported that felbamate inhibits the NMDAR by binding to the glycine site[271-273], while others showed that felbamate binds with the site of channel pore[274]. Therefore, the binding site remains unsolved. Ifenprodil, a GluN2B-selective antagonist, has been reported to have effects on many animal models of seizure[275-277], except for seizure induced by imipenem or pefloxacin in DBA/2 mice[278]. Memantine, a blocker of NMDARs, exerts anticonvulsant effects in seizures[279-281] by blocking the NMDAR ion channel pore. However, many clinical trials have failed due to the side effects. In experimental models of epilepsy, the combination of conventional antiepileptic drugs with

low-dose NMDAR antagonists has shown significant efficacy but minimal side effects[282], although the potential advantage also requires further validation.

3.8. Neuropathic pain

Neuropathic pain is a type of chronic pain induced by the damage of neurons or nerves in the nervous system. During injury and inflammation, glutamate is released to activate NMDARs in the peripheral terminals of primary sensory afferents, leading to pain-related behaviors[283]. During sensitization of the pain, GluN2B expression is increased in the nociceptive neurons in the dorsal horn of the spinal cord, a process that NMDARs activity appears to be involved[283,284]. In animal models of peripheral nerve injury, NMDA-activated whole-cell current and calcium influx in spinal lamina II neurons appear to be increased in nerve-ligated rats[285]. The phosphorylation of GluN1 subunit in the dorsal horn is increased in partial ligation of the sciatic nerve[286]. However, the total protein levels of GluN1 and GluN2A-D subunits were unchanged in the spinal cord after nerve injury[286,287]. Therefore, increased NMDAR phosphorylation may increase surface NMDAR function and is critical for central sensitization induced by nerve injury.

Blocking NMDARs could reduce the hypersensitivity of spinal dorsal horn neurons in neuropathic pain models. Memantine, ketamine, and MK-801, which are the NMDAR antagonists, attenuate evoked responses of dorsal horn neuron in spinal nerve-ligated rats[288]. Besides, a study showed that ifenprodil, a GluN2B subunit-specific antagonist, reduces the amplitude of NMDAR currents in nerve-ligated mice[289]. In clinic, intravenous infusion of ketamine can relieve pain in patients with refractory complex regional pain syndrome, but does not prevent chronic neuropathic pain caused by thoracotomy[290-292]. Intravenous infusion of amantadine, another NMDAR antagonist, reduces persistent neuropathic pain in cancer patients after surgery, but often causes intolerable side effects[293,294]. Therefore, there could be additional mechanisms underlying the enhanced NMDAR activity after nerve injury. Further studies along this direction may facilitate the development of neuropathic pain therapies.

3.9. Post-traumatic stress disorder (PTSD)

PTSD is a complex and chronic neuropsychiatric disorder characterized by recurrent appearance of unpleasant or even painful memories resulted from traumatic events, especially for the military members and also people who have recently been infected with COVID-19[295-297]. The symptoms severely impair physical and mental health and lower the quality of life[298].

It has been suggested that over-response of fear function in the amygdala and a combination of weakened inhibitory effects of the projection from prefrontal cortex to amygdala may lead to PTSD[299]. The functional brain circuits require the proper synaptic connectivity which can attribute to glutamate with its role in maintenance the neuronal activity[300]. Ketamine plays a crucial role in repairing the synaptic network and also shows positive effects on the PTSD[301,302]. The benefits of ketamine in PTSD may result from its anti-depressant and anti-inflammatory effects that improve the brain-derived neurotrophic factor levels and further re-establish the neuronal connectivity[303]. High activity of NMDAR could impair formation of spontaneous intrusive memories that may contribute to the development of PTSD[302]. A study on rat models of PTSD-induced by contextual fear showed that the level of brain-derived neurotrophic factor was strongly reduced, which could be reversed by ketamine treatment, and NMDAR inhibition interrupted the consolidation of fear conditioning related to hippocampus and hypothalamus-pituitary-adrenal axis[303-305]. Taken altogether, the hyperactivity of NMDAR is tightly linked to PTSD and may lead to synaptic abnormalities and decreased expression of brain-derived neurotrophic factor especially under traumatic event. Ketamine is currently an effective pharmaceutical intervention for PTSD. In the future, the studies should focus on optimization of ketamine with appropriate therapeutic dose and duration, and on reducing the side effects.

3.10. NMDARs mutations in diseases

Mutations in NMDARs are related with neuropsychiatric diseases. Nowadays, the pathogenesis of and therapy development for brain diseases can be explored using genomics approaches following the emergence of whole-exome/genome sequencing and targeted therapy. Human genetics studies indicated that more than 500 variants of NMDARs subunit mutations have been found in patients with brain disorders, such as intellectual disability, hyperactivity disorder, ASD, schizophrenia, or epilepsy. These mutations are more prevalent in the GluN2A and GluN2B subunits of the amino terminal domain (ATD), ABD, TMD, and CTD regions. Discovering and analyzing the function of these mutations can help determine the role they play in these neuropsychiatric disorders. In addition, functional analysis of these mutations can advance the understanding in the etiology of the disease and facilitate the formulation of treatment plans in clinic.

3.10.1. The GluN1 mutations

GluN1 subunit is encode by *GRIN1*, which is located in human chromosome 9q34. Many mutations in GluN1 have

been found to be related to many diseases, including the ID, autism spectrum, and epilepsy; the function of some of the mutations has been studied, while others remain unclear. The function of mutations, including D227H, R306E, A349S, S549R, P557R, M641I, and N650K, has not been studied by biological techniques[306,307]. The mutations of D552E, Q556*, S560dup, P557R, G620R, Y647S, G815R, F817L, and G827R are loss-of-functions. The S560 is located in pre-TM1 region, and the mutation of S560dup decreases the activity of NMDARs and changes the structure of the pore region[308]. The G815R and F817L mutations are adjacent to each other, thus their functions are similar, that is, they decreased sensitivity to glutamate and glycine[309]. However, the E662K, A645S, and R844C mutations did not change the function of NMDARs[309-312]. On the contrary, the mutations of Y647C, R659W, and R794Q enhanced the function of NMDARs[306]. The R659W and R794Q enhance the potency of glutamate and glycine, while the mutation of Y647C increases potency of glycine, but not glutamate.

3.10.2. The GluN2A mutations

GluN2A subunit is encoded by *GRIN2A* in the human chromosome 16p13. More than 240 mutations have been found in *GRIN2A*. Many patients had normal delivery at birth, with good appearance, facial expression, and vital signs scores. However, the patients at the 1st year after birth may begin to exhibit neurological abnormalities, such as abnormal electroencephalogram and myoclonic convulsions[313], and then progress to epilepsy, which is possibly due to a gradual increase in GluN2A expression during development[47]. Some studies indicated that the patients carrying GluN2A mutations were more likely to show epilepsy, such as benign focal epilepsy with centrotemporal spikes and Landau-Kleffner syndrome[314-316]. Moreover, the mutations of GluN2A can produce different effects: gain-of-function and loss-of-function.

Some mutations are gain-of-function. The GluN2A_N447K is found in a male patient with Rolandic epilepsy and is located in the S1 domain of ABD. The GluN2A_N447K enhanced the current density of NMDAR by electrophysiological recording. The potency of glutamate was increased, while the inhibition of Mg^{2+} was decreased by GluN2A_N447K. Lamotrigine and valproate treatment could rescue the epilepsy in patient[317]. The patient is a child with impaired cognitive and epileptic encephalopathy. Another mutation, GluN2A_L812M, is located in the linker between S2 of ABD and M4 of TMD. Studies have shown that the GluN2A_L812M and M817V enhanced the open probability and potency with glutamate and glycine of NMDAR, and decreased the inhibition of Mg^{2+}[318,319]. The patient with GluN2A_L812M was treated by memantine, which attenuated epilepsy[313]. The other mutation GluN2A_N615K is in the channel pore of M2 TMD domain. The patient was a 3-year-old female with abnormal electroencephalogram, developmental delay, and early-onset epileptic encephalopathy[320]. The GluN2A_N615K mutation changed the channel characteristics, including the decreased Ca^{2+} permeability and Mg^{2+} sensitivity[321-323]. The reduction of Mg^{2+} sensitivity to NMDARs increased the NMDAR current.

Meanwhile, the mutations of GluN2A are loss-of-function. The GluN2A_D731N was found in a child with developmental delay and epilepsy. The missense mutation is in the ABD of GluN2A. The results indicated that the mutation decreased the NMDAR current, glutamate sensitivity, and enhanced the potency of NAM[324]. The GluN2A_V685G is located in the S2 of ABD region and caused the developmental delay and epilepsy. The mutation caused a low glutamate potency with NMDAR and decreased NMDAR current with reduced surface expression[325].

Besides, the mutations also lead to impairment in trafficking. The GluN2A_S1459G, which is located in the CTD-related to the protein trafficking of GluN2A, was found in the epilepsy patients[314]. The GluN2A_S1459G induced the impaired binding with SNX27 and PSD95, which led to deficits in NMDAR trafficking and spine density, and synaptic transmission in excitatory neurons[326].

From the studies, we understand that the mutations may exist in any domains of GluN2A to cause the disorders. Since each mutation is specific, we need to better understand the pathogenesis and changes in function of the mutation so as to increase the efficacy of targeted therapy in the patients.

3.10.3. The GluN2B mutations

GRIN2B gene encodes the GluN2B subunit and is in the human chromosome 12p13. Over 200 mutations have been found in *GRIN2B* in the patients not only with epilepsy, epileptic encephalopathy[325,327], intellectual disability[320,325,327,328], and ASD[320,325,327], but also with the AD[329] and schizophrenia[330-333]. The mutations exist in all regions of GluN2B. Some of the mutations have been functionally analyzed.

Many GluN2B mutations are loss-of-function in patient with intellectual disability or ASD. The GluN2B_E413G is located in the ATD domain, which is adjacent to the glutamate-binding site. The E413G reduced the potency of glutamate and deactivation, resulting in the loss-of-function of NMDAR[334]. Another study reported the function of GluN2B_C456Y mutation. They constructed the heterozygous GluN2B_C456Y mutation mice and

found that the protein levels of GluN2B was decreased. Besides, the NMDAR current was reduced and the LTD was impaired, while the LTP was normal. The behavior data showed that the mice represented the anxious behavior with normal social interaction. In rescue experiments, the D-cycloserine had an effect on the NMDAR currents and LTP and recued the impaired behavior in adult mice[335]. The mutation GluN2B_C461F is located in the S1 domain of ABD in patient with Lennox-Gastaut syndrome and autism. Functional analysis described the C461F mutation decreased the glutamate potency[327]. In the same study, the P553L was found in the patient with intellectual disability. The mutation, which is located in the pre-MI domain, decreased the glutamate potency and NMDAR current in the neurons[327]. Another study found the mutation, which, however, mutated to the threonine (P553T), at the same site. The mutation was found in a 5-year-old patient with Rett-like syndrome. The P553T reduced the potency of glutamate, NMDAR currents and spine density in the neurons. Furthermore, the D-serine could restore the deficits induced by GluN2B_P553T[336].

The mutations of N615I and V618G are gain-of-function and found in the patients with West syndrome and intellectual disability[337]. The two mutations reduced the inhibition of Mg^{2+}, although the glutamate potency was unchanged. Furthermore, the mutation of R540H increased the sensitivity to glutamate and Ca^{2+} permeability[338]. The gain-of-function mutations may lead to neuronal hyperexcitability, resulting in the disorders. Therefore, we can treat the diseases by inhibiting NMDAR function in gain-of-function mutations.

3.11. The challenge and opportunity in treatment of diseases

In biological experiments, the addition of glycine, D-serine, and D-cycloserine can enhance the function of NMDAR, which can provide a new drug choice for clinical use as NMDAR potentiators[336]. Besides, in some studies, patients carrying GluN2A and GluN2B mutations were treated with drugs, such as memantine and the blocker of NMDARs[319]. Likewise, the other drugs, such as ketamine, magnesium, and TCN-201, have effects on the inhibition of NMDAR current *in vitro*[318,319,339]. Since different patients respond differently to the drug, personalized treatment is needed for patients with NMDAR mutations.

However, due to the wide distribution and function of NMDARs, drugs targeting NMDARs may cause severe adverse effects limiting their clinical potential. The competitive NMDAR antagonists, which prevent glutamate-mediated neurotoxicity by inhibiting NMDAR function, could cause extensive inhibition of NMDAR function. Inhibition of NMDAR with non-competitive antagonists is the most attractive therapeutic intervention, because the effect of antagonists requires pre-activation of the NMDA receptor[340]. However, NMDAR hypofunction has an influence on the brain function. Even low doses of NMDAR antagonists can cause decreased excitability of NMDAR, leading to memory dysfunction and learning disabilities. Besides, enhancing the function of NMDARs may cause NMDAR hyperexcitability due to the inability to distinguish between extrasynaptic and synaptic NMDARs. Comprehensive weighing of the benefits versus adverse effects need be carried out during the clinical development of drugs targeting NMDARs.

4. Summary

NMDAR plays an important role in many processes, including learning and memory, development, and neuroplasticity. Many functions of NMDAR depend primarily on the GluN2 subunit. GluN2 subunits are mainly expressed in the cortex and hippocampus, and they differ in expression patterns, protein trafficking, channel dynamics, synaptic plasticity, and neuropsychiatric disorders. This difference also determines the different functions of NMDAR. In addition, the composition of the NMDAR subunit is also regulated by neural activity, which, further, indicates that the composition of NMDARs is consistent with neural function. However, it is still difficult to distinguish the functions and effects of individual subunits, which also leads to the difficulty in clinical treatment of neuropsychiatric disorders. Therefore, in addition to using new drugs and genetic approaches to specifically target different subunits and related signaling pathways in the treatment of neuropsychiatric disorders, it would be also interesting to identify the auxiliary subunits of NMDAR, which is involved in the regulation of the ions accessibility, and how this interaction affects the downstream signaling pathways may contribute to the understanding of the cellular mechanism in different brain diseases. The double-sided function of NMDAR poses great challenges for clinical application, so we still need to make continuous efforts in the treatment of neuropsychiatric disorders targeting NMDARs.

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Conflict of interest

The authors declare no competing interests.

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REVIEW ARTICLE

Neurological complications of coronavirus disease 2019 and the underlying mechanisms

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Abstract

The recent global pandemic of coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although respiratory symptoms are the primary manifestation of the majority of COVID-19 patients, an increasing number of neurological symptoms and manifestations of COVID-19 have been observed. In this review, we detail the neurological complications of COVID-19, such as gustatory and olfactory dysfunctions, stroke, memory decline, muscle injury, and seizures. Furthermore, we introduce neural invasion mechanism underlying SARS-CoV-2 infection and, further, explain the occurrence of these complications. This review offers insights into the neurological signs and symptoms of COVID-19, which may help improve the prognosis of the infected patients.

Keywords: COVID-19; Neurological complications; Clinic characteristics; Neural invasion

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1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in a global pandemic[1]. At present, there are over 100 million confirmed cases of COVID-19 worldwide, with more than 6 million deaths. Most patients with COVID-19 present with respiratory symptoms, such as cough and dyspnea[2]. COVID-19 patients also exhibit manifestations of other systems, which do not receive much attention despite their clinical significance. With further investigations, neurological manifestations are found to be instrumental in the diagnosis, treatment, and prognosis of COVID-19 patients; these manifestations include

headache, memory decline, cerebrovascular event, and taste and smell disorders. In this review, we summarize the neurological complications in COVID-19 patients and the underlying mechanisms, which might help facilitate early diagnosis and early treatment so as to improve prognosis of COVID-19 patients.

2. Neurological complication of COVID-19

2.1. Alterations in taste and smell

The loss or diminished senses of taste (ageusia) or smell (anosmia) are the frequent symptoms and the early neurologic manifestations in COVID-19 patients[3]. The occurrence rate of these symptoms is reported to range between 38% and 74% across different cohorts[4-8]. In addition, in a multicenter study, around 38% and 41% of mildly infected patients have been reported to show gustatory and olfactory impairment, respectively[7]. Further, a meta-analysis of 15 studies consisting of a total of 3739 participants with confirmed COVID-19 reported that around 1354 and 1729 of infected patients showed symptoms of taste and smell impairment, respectively[9]. The similar study also demonstrated that the loss of taste and smell occurred in 49.0% (95% confidence interval [CI] 34.0–64.0) and 61.0% (95% CI 44.0–75.0) of the COVID-19 patients, respectively. Another meta-analysis on patients in North America reported that the rate of taste disorder was 38.6% (95% CI 7.9–75.3), while the rate of smell disorder was 54.5% (95% CI 39.3–69.3), and around 5977 patients manifested symptoms of both smell and taste disorders, accounting for 31.3% of the patients (95% CI 13.6–52.2)[10]. Taken together, the loss of smell and/or taste is a common neurologic symptom of COVID-19 patients.

However, the occurrence rate of smell and taste disorders in COVID-19 patients varies among different populations as well as gender and age groups. Compared with male COVID-19 patients, female patients were more likely to have trouble in smelling and tasting[11]. On the contrary, another study reported that gender did not significantly influence the olfactory and gustatory function in patients[12]. In addition, younger people have been reported to have more severe smell disorders and take longer time to recover[8]. It is worth noting that in a study on 841 COVID-19 patients, olfactory and gustatory disorders were more common in patients with mild COVID-19[13], suggesting that olfactory and gustatory changes may be an important marker for the early disease diagnosis.

The recovery of smell and taste disorders in the infected patients is another concerning topic. The recovery time of the olfactory and gustatory disorder depends on the severity of COVID-19 and the disorders themselves[14]. A follow-up study reported that after 4 weeks of infection, patients with mild COVID-19 (89%) underwent a complete resolution or improvement of olfactory and gustatory functions[4]. According to another study, the recovery time of infected adolescents aged 10–19 years varied from 2 days to 2 weeks, with an average of 5.7 days[15]. Besides, it has been reported that 12% of smell disorders preceded the onset of other symptoms, 22% of these disorders occurred in conjunction with other symptoms, and 65% occurred after other symptoms[16].

However, at present, the mechanism of smell and taste disorders in patients with COVID-19 is still not clearly defined. Angiotensin-converting enzyme 2 (ACE2) receptors are functional receptors of SARS-CoV-2[17]; therefore, the olfactory epithelium cells, which have high ACE2 expression, become easily infected by SARS-CoV-2[18]. Anosmia is caused by the injury of olfactory epithelium, instead of sensory neurons. Furthermore, there is a positive correlation between olfactory and gustatory disorders[19]. Most COVID-19 patients gradually recover from olfactory and gustatory disorders, and some interventions such as olfactory training may be helpful during the recovery process[20]. The interventions for loss of taste are less common and deserve more investigations.

2.2. Brain function decline

The COVID-19 affects the brain functions, such as cognitive ability and consciousness, in a variety of ways, which result in acute and long-term effects on the infected individuals. Acute brain dysfunctions include delirium, coma, confusion, and somnolence. It has been reported that delirium was present in 81.7% of COVID-19 patients admitted to intensive care units[21]. In addition, these acute symptoms are associated with higher risk of long-term cognitive decline[22], which explains why longitudinal studies on long-term changes in cognitive function have attracted much attention. In a longitudinal study of 3232 COVID-19 patients in China, it was found that the occurrence rate of cognitive impairment in COVID-19 survivors was 12.45% after 12 months. The cognitive function of severe patients was lower than that of non-severe patients and control group[23]. High risk of the early-onset (95% CI 3.30–7.20) and late-onset (95% CI 3.58–16.03) cognitive impairment was associated with severe COVID-19. Another follow-up study on home-isolated COVID-19 patients reported that around 44% of young patients had cognition impairments, such as memory problems and impaired concentration, 6 months after infection, which were associated with increased convalescent antibody titers, indicating the severity of underlying disease[24].

The reduction in brain function caused by COVID-19 is governed by a number of factors, such as lung damage and the resulting hypoxemia caused by COVID-19. It has been

reported that patients with chronic hypoxemia, such as obstructive sleep apnea and chronic obstructive pulmonary disease, often experience cognitive decline, and chronic hypoxemia is also common in patients with COVID-19[25]. To fight against chronic hypoxemia, sedatives are often used to facilitate invasive mechanical ventilation; however, the use of sedatives is commonly associated with high risk of acute phase brain decline. A multi-center study found that excessive sedation in response to COVID-19 was associated with an increase in incidence of delirium in the infected patients[21].

Inflammation often leads to brain dysfunction with the aid of pro-inflammatory cytokines and inflammatory mediators, which are abundant in the blood and brain[26]. The pro-inflammatory cytokines disrupt the blood-brain barrier permeability by increasing the cyclooxygenase-2 expression and activating matrix metalloproteinase[27]. In addition, the microglial activation and oxidative stress caused by the pro-inflammatory cytokines lead to short-term delirium and severe long-term cognitive impairment. An electroencephalogram (EEG) study found that higher regional current density at delta band was correlated with worse executive performances in COVID-19 patients[28]. A follow-up study also found that lower EEG delta band at baseline predicted worse cognitive performance. Further, a neuroimaging study found that COVID-19 patients showed poorer hypometabolism predominantly in the frontoparietal network, and this neuroimaging patterns showed a high correlation (R^2 = 0.62) with the Montreal Cognitive Assessment performance[29].

2.3. Muscle injury and movement disorders

Muscle injury and movement disorders are among the complications associated with COVID-19. Some patients showed fatigue, muscle soreness, and elevated muscle enzyme levels[30]. Another study also reported that the creatine kinase level in 15.7% of hospitalized COVID-19 patients increased dramatically[31]. In addition, a meta-analysis on 1100 studies found that around 34.7% of COVID-19 patients showed symptoms of myalgia or fatigue (95% CI 26.0–44.4)[32]. In addition, a meta-analysis involving 3062 hospitalized COVID-19 patients reported that the patients suffered from muscle soreness, which was the third most common symptom after fever and cough[33]. Besides, the associated symptoms in discharged patients are also noteworthy. In a longitudinal follow-up study, fatigue and muscle weakness were the most common sequelae (63%, 1038 of 1655)[34]. Furthermore, a comprehensive health assessment after 3 months of rehabilitation found that 22% and 64% of COVID-19 survivors had poor exercise capacity and muscle fatigue, respectively[35].

A number of possible mechanisms underlying muscle injury in COVID-19 patients have been proposed, such as: (i) increased oxidative stress caused by COVID-19; (ii) the abuse of medications; and (iii) being bedridden and lack of exercises. Malnutrition or inflammation is the main cause of increased secretion of cytokine in COVID-19 patients, and excessive cytokines can lead to skeletal muscle damage and rhabdomyolysis[36,37]. These cytokines can also reduce the production of testosterone, a hormone which promotes muscular integrity[38]. Some antiviral drugs such as hydroxychloroquine and medications, which are used to treat critical patients with COVID-19, were also found to damage muscles[39], and this effect was more pronounced in inactive patients. In addition, intubation and mechanical ventilation used in the treatment of COVID-19 patients as well as long hours of bed rest could contribute to significant muscle loss[40]. A few reports have suggested that SARS-CoV-2 can directly induce muscle toxicity in the infected patients[39]. A Chinese study on 1014 hospitalized COVID-19 patients showed that those with rhabdomyolysis were susceptible to a higher risk of deterioration and had a higher mortality rate than those without rhabdomyolysis[41]. COVID-19 is believed to exacerbate pre-existing motor impairments, such as those in infected patients with Parkinson's disease. In a study on 694 COVID-19 patients, it is found that patients with Parkinson's disease have a higher mortality rate than patients without the disease (95% CI 1.04–1.53)[42]. Therefore, early diagnosis and intervention are necessary in patients with poor baseline underlying conditions.

2.4. Cerebrovascular diseases

Acute ischemic stroke is a dangerous complication of COVID-19. The coincidences of ischemic stroke reported in hospitalized COVID-19 patients range from 1% to 5%, and patients with more severe infection had a higher risk of stroke[43,44]; however, the occurrence rate of hemorrhagic cerebrovascular disease was lower than 1%. A meta-analysis reported that ischemic stroke occurred in 1.5% of COVID-19 patients (95% CI 0.9–3.7%), and the in-hospital mortality rate was 34.4% (95% CI 27.2–42.4%)[45]. Furthermore, a study on influenza (n = 1486) or COVID-19 (n = 1916) patients found that COVID-19 patients had a higher risk of stroke (95% CI 2.3–25.2) compared to influenza patients[46]. The pathogenic pathways underlying the development of ischemic stroke vary between patients with and without COVID-19, and COVID-19 patients had worse prognosis. Another report also showed that stroke patients with COVID-19 took longer time for recovery and were more likely to have higher mortality rate and worse baseline neurological test scores[47]. It is noteworthy that the risk factors of ischemic stroke in COVID-19 patients

may be associated with hypercoagulability under hypoxia state. In concordance with the above, a study demonstrated that a considerable part of COVID-19 patients have abnormal coagulation parameters, such as elevated levels of d-dimer and C-reactive protein as well as positive lupus anticoagulant test[48]. Elevations in these factors may be a sign of COVID-19-mediated damage to the nervous system. This suggests that COVID-19 may directly increase the risk of stroke through cerebral vasculitis. It has been found in another cohort that the patients were negative for lupus anticoagulant but positive for anti-cardiolipin and anti-B2-glycoprotein antibodies[49]. These abnormal coagulation parameters may predict the risk of cerebrovascular disease in COVID-19 patients, but, further, investigations on the mechanisms underlying these abnormalities are warranted.

The treatment with prophylactic anticoagulation may be beneficial to patients with COVID-19. According to the recommendation of The American Society of Hematology, hospitalized COVID-19 patients should be treated with standard thromboprophylaxis[50]. Based on the findings of randomized clinical trials, a full-dose anticoagulant therapy may benefit the critically ill patients with COVID-19, but at the same time, this treatment may increase the risk of other complications[51]. Thus, the prophylactic use of anticoagulants as a treatment for critically ill patients is still up for debate. Of note, the occurrence rate of arterial or venous thromboembolism in hospitalized COVID-19 patients has been reported to be between 8% and 31% despite the fact that prophylactic anticoagulant therapy was adopted[52,53]. A multicenter study conducted in Italy has reported that COVID-19 patients to whom stroke occurred despite the use of anticoagulant therapy had a worse clinical outcome, along with 64.7% of fatality rate[54]. The treatment for stroke is similarly administered to patients with or without COVID-19; however, there are few studies about the safety and feasibility of thrombectomy treatment in COVID-19 patients with acute ischemic stroke.

Despite its rarity, cerebral venous thrombosis is also a complication of COVID-19. Most patients with cerebral venous thrombosis present with only nonspecific symptoms, such as headache, fever, and visual problems, which implies elevated intracranial pressure[55]. A study found that the median duration of onset in hospitalized COVID-19 patients across different countries was three days from COVID-19 diagnosis, and the superior sagittal sinus is the most common site of thrombosis[56]. The diagnosis of cerebral venous sinus thrombosis is based on clinical and radiological findings obtained from magnetic resonance imaging (MRI) and venography. Heparin anticoagulant therapy, either at therapeutic doses of low molecular weight heparin or unitary heparin, is preferred for patients suffering from this complication[57]. It is important to note that RNA vaccination may increase the risk of cerebral venous sinus thrombosis. Moreover, a German study has reported that the occurrence rate of cerebral venous sinus thrombosis was 0.55 (95% CI = 0.38–0.78) per 100,000 person-months, which was higher than that in the general population[58]. Therefore, the vaccination against COVID-19 is essential, given that it can reduce the risk of serious complications of COVID-19.

2.5. Other neurological complications

In addition to the neurological complications mentioned above, there are many other complications associated with COVID-19, such as epilepsy, meningitis, and demyelinating diseases.

A growing number of case reports point out that COVID-19 could exacerbate epilepsy in patients who already have the nervous disorder and, unfortunately, trigger epilepsy in patients who do not at the same time suffer from the disorder[59,60]. Metabolic disorders, high fever, or electrolyte disarrangements caused by COVID-19 may be the cause of epilepsy[61]. Therefore, electroencephalography monitoring of severe COVID-19 patients is helpful for early diagnosis and prevention of epilepsy, and early monitoring of patients with symptoms of encephalopathy is also beneficial[62]. COVID-19 patients may develop symptoms of epilepsy before the onset of respiratory symptoms, and the symptoms may include both generalized tonic-clonic seizures and focal seizures[63]. It is important to note that cases of epilepsy in pediatric COVID-19 patients have been reported, although the exact cause of epilepsy is unknown[64]. In short, epilepsy and seizures should be considered the underlying manifestations of COVID-19 in the pediatric patients.

Symptoms of encephalitis have also been reported in COVID-19 patients. SARS-CoV-2 has been found in the cerebrospinal fluid (CSF) of COVID-19 patients[65,66]; however, CSF of some COVID-19 patients with acute meningoencephalitis was found to be free from SARS-CoV-2 or other viral pathogens[67]. This indicates that meningitis in the COVID-19 patients is not caused directly by brain infection, but by other factors, such as inflammation during acute COVID-19 phase. Encephalitis can be complicated by intracerebral and subdural hematoma, and it is either severely disabling or life-threatening; therefore, infected individuals should be wary of this possible complication following COVID-19.

Besides, the rare connection of COVID-19 with temporary visual impairment has been reported, and the visual loss may be associated with cerebral vasculitis[68]. In addition, demyelinating diseases such as Guillain–Barre syndrome

(GBS) are also increasingly well-recognized as one of the complications of COVID-19. Recently, a study described the development of GBS in COVID-19 patients, who first developed weakness in the lower limbs and then progressive quadriplegia on the 5–10 days after the onset of COVID-19 symptoms[69]. However, no SARS-CoV-2 was detected in the CSF of these patients, and the CSF protein levels were normal in 40% of infected patients. However, another study reported the isolation of conalbumin from CSF in COVID-19 patients with GBS, and CSF analysis showed normal white blood cell counts and elevated protein levels[70]. In general, GBS responds well to intravenous immunoglobulin therapy, which brings about significant improvement in nervous system, suggesting that neuropathy is immune-mediated[71], but the long-term outcomes are still unclear.

3. Mechanisms underlying the neurologic complications of COVID-19

In this section, we summarize the underlying mechanisms of how COVID-19 affects the nervous system (Figure 1). Early in the outbreak of COVID, researchers had attempted to detect the SARS-CoV-2 RNA in the CSF of infected patients with neurological symptoms[65]. However, the virus has never been successfully detected in most COVID-19 patients[72], most likely due to the insufficient viral load in the CSF or the fact that the virus does not enter the CSF.

The COVID-19-related neurological complications are mainly observed in the central nervous system (CNS), the peripheral nervous system (PNS), and the musculoskeletal system. Possibly, different mechanisms, such as neuronal retrograde dissemination and systemic hematogenous spread, are in place to allow viral entry and damage to the nervous system. Although the exact mechanism of SARS-CoV-2 invasion remains unclear, multiple lines of evidence showed that the virus can destroy the neurons by either of the two mechanisms[73]. In addition to destroying the blood-brain barrier and causing central neuron death through viremia, SARS-CoV-2 can also infect olfactory bulb (OB) and subsequent transport to neurons[74].

SARS-CoV-2 could cause viremia to destroy neurons by binding to ACE2. ACE2 receptors are widely expressed in the CNS systems, including neurons, astrocytes, oligodendrocytes, and OB[17]. Binding of the transmembrane protease serine 2 to the ACE2 receptor results in protein cleavage and activation of the spike protein, therefore allowing the virus to enter the host cell[75]. Thus, SARS-CoV-2 has the potential to infect neurons and glial cells throughout the CNS.

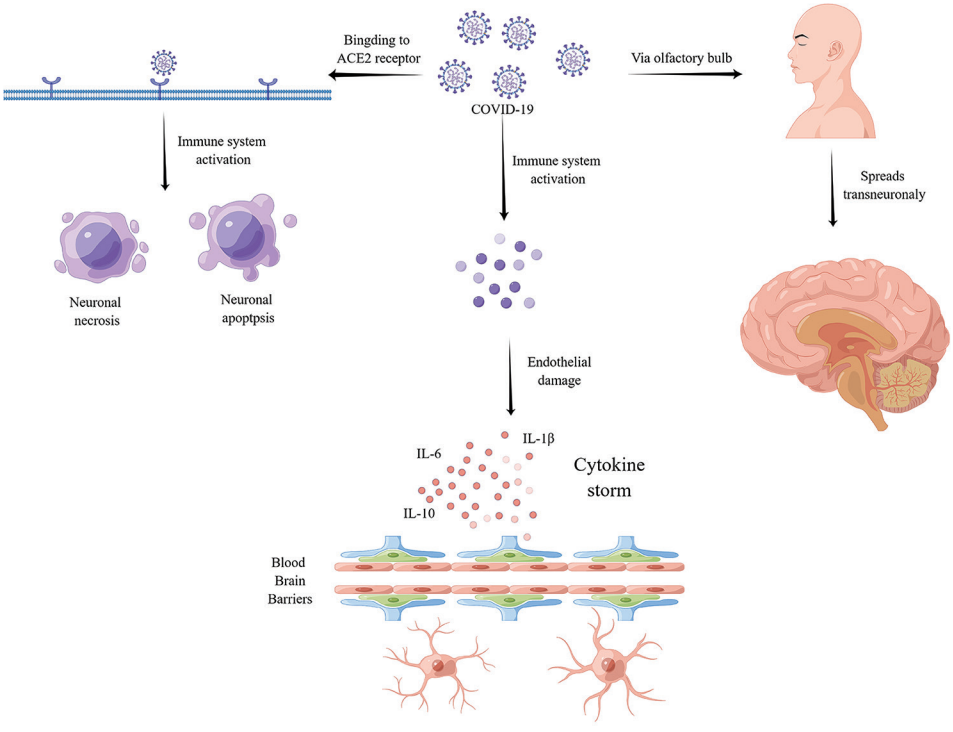


Figure 1. Mechanisms underlying the damages to the central nervous system mediated by SARS-CoV-2. The binding of SARS-CoV-2 to the ACE2 receptor leads to neuronal apoptosis or death. In addition, SARS-CoV-2 not only disrupts the blood-brain barrier by triggering cytokine storm, but also spreads retrograde through neurons by infecting the olfactory bulb, thus disrupting the normal function of the central nervous system. The schematic is illustrated with Figdraw (www.figdraw.com).

Table 1. Neurological complications associated with COVID-19 and their prevalence

Neurological complications	Occurrence rate
Gustatory dysfunctions[9]	49% of COVID-19 patients
Olfactory dysfunctions[9]	65% of COVID-19 patients
Stroke[45]	1.5% of COVID-19 patients
Memory impairment[23]	12.5% of COVID-19 patients
Muscle injury[13]	16% of COVID-19 patients
Epilepsy[51]	<1% of COVID-19 patients
Headache[13]	15% of COVID-19 patients
Dizziness[85]	9% of COVID-19 patients
Nausea and vomiting[86]	5% of COVID-19 patients

On the one hand, after infecting the respiratory system, SARS-CoV-2 can enter the bloodstream by infecting endothelial cells in the choroid plexus blood-brain barrier or blood-CSF barrier in the choroid plexus. Furthermore, SARS-CoV-2 can infect leukocyte and cross the barriers to invade the CNS so as to spread throughout the body with leukocyte[76]. In this process, leukocytes release pro-inflammatory factors, such as tumor necrosis factor, interleukin (IL)-1β, IL-6, IL-12, and even cause cytokine storm, which can destroy oligodendrocytes and neurons[77]. The integrity of the blood-brain barrier may be compromised by cytokine-driven damage and immune-mediated toxicity[78]. Taken together, SARS-CoV-2 binds to the ACE2 receptor, which, in turn, induces an inflammatory cascade that ultimately destroys the neurons.

On the other hand, SARS-CoV-2 could also enter the CNS through OB. OB is often considered an immune organ that can prevent viruses from entering the CNS, but its dysfunction may lead to an infection of the CNS[79]. A previous study has confirmed that virus can penetrate the olfactory epithelium into the brain and cause death in the mice[80]. Meanwhile, a recent study showed that olfactory epithelial cells express ACE2, allowing viruses to infect the cells through a replacement receptor[18]. OB MRI studies showed that MRI signal changes were consistent with virus invasion of OB and cortical regions[80], suggesting that SARS-CoV-2 may invade human CNS from the external environment through OB.

Fecal-oral route is hypothesized as a potential route of transmission for SARS-CoV-2 on the grounds of the high expression of ACE2 in gastrointestinal system[81], and the nervous system injury caused by indirect immune-mediated cytokine storm could be another possible mechanism of nervous system complications[82]. Further and more detailed research is needed to investigate the mechanism of how SARS-CoV-2 affects the nervous system and causes neurological complications. It is worth noting that there are many subtypes of COVID-19, and these subtypes differ in many aspects, such as virulence and transmissibility, which explain the variability in the mechanisms. A study found that convulsions could be a sign of the Omicron variant in children with COVID-19[83]. Another study on children with COVID-19 found that the Omicron variant is more likely to be associated with neurologic manifestations such as seizures compared with other COVID-19 virus variants[84]. However, there is still a lack of multicenter and forward-looking research on the neurological complications caused by specific COVID-19 virus variants.

4. Conclusion

Despite being a disease primarily affecting the respiratory system, COVID-19 has gradually attracted attention as a disease accompanied by the unexpected neurological complications, evidenced by a growing number of studies (Table 1). In this review, we summarize the symptoms, characteristics, and mechanisms underlying the neurological complications of COVID-19. The neurological complications of COVID-19 patients include CNS complications, such as brain function decline, cerebrovascular disease and dysgeusia, as well as PNS complications. Some of these complications are reversible, but others could be life-threatening. The SARS-CoV-2 infection in the nervous system is mediated by the binding of the virus to the ACE2 receptor or its replacement receptor, which leads to a chain of inflammatory responses. This review provides reference contents for understanding the heterogeneous neurological symptoms and manifestations as well as potential neurological complications of COVID-19. A deeper understanding of these manifestations and complications can help make appropriate treatment decisions that would directly reduce morbidity and mortality among the patients. Finally, more investigations should be carried out to determine the broad spectrum of neurological symptoms and the underlying pathophysiological mechanisms of COVID-19.

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Conflict of interest

The authors have no competing interest to declare.

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REVIEW ARTICLE

Implications of age-related changes in the blood-brain barrier for ischemic stroke and new treatment strategies

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Abstract

Ischemic strokes are prevalent across all age groups. Recent research has highlighted the importance of better understanding ischemia-induced damage of the blood-brain barrier (BBB) because it is related to both the severity of ischemic injury and neurological outcomes. The influence of advancing age on the structure and function of the BBB and the potential influence of these changes on ischemic stroke injury have received little consideration to date. Therefore, the present review outlines how ischemic injury influences the structure and function of the BBB at the anatomical, cellular, and molecular levels, and how these changes differ between adult and elderly populations with and without age-related comorbid diseases. This review further discusses how age-dependent changes and features of the BBB, and the corresponding alterations in response to ischemia, can affect the efficacy and delivery of current and future treatment options. Current research efforts are underway to develop prospective stroke treatment strategies that target the restoration of BBB functionality. This review also discusses the importance of considering the unique properties and characteristics of the BBB in elderly individuals for developing new stroke treatment strategies.

Keywords: Ischemic stroke; Blood-brain barrier; Inflammation; Drug delivery; Diabetes; Alzheimer's disease

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1. Introduction

Ischemic strokes can occur at any point in life, independent of age. Nevertheless, the blood-brain barrier (BBB) changes with age[1], and changes in BBB integrity and functionality may significantly affect the brain. With a growing line of evidence focusing on the effect of BBB integrity on ischemic injury and recovery[2-4], it is of increasing importance that age-dependent characteristics of the BBB be considered. In a healthy state, the BBB acts as a physical and enzymatic barrier that protects the neuronal environment from blood-borne toxins, inflammation, and potential fluctuations in ion and water homeostasis to maintain neuronal health and activity[5]. This is accomplished by a single-cell layer of specialized endothelial cells connected together through tight junctional complexes made of occludin, zona occludens (ZO), and claudin proteins. This microvascular barrier of the brain limits paracellular and transcellular movement from the blood into the brain[5].

Shortly after an ischemic stroke, the BBB undergoes significant damage[6]. As a result of ischemic injury to neural tissue, oxidative stressors accumulate and induce rapid degradation and functional breakdown of tight junctions, thereby opening the BBB. This enables inflammatory chemokines to enter the brain and cause disruptions in both ion and water homeostasis[7]. These localized actions on the BBB cause further immune cell activation and infiltration in response to ischemic injury, leading to the release of inflammatory mediators and other molecules that further degrade endothelial tight junction complexes[7]. The loss of BBB integrity leaves the brain susceptible to greater inflammation and edema, which may worsen ischemic injury and contribute to neuronal loss far beyond acute injury[3,7]. Interestingly, due to variations in cellular and molecular mechanisms, the BBB in elderly patients undergoes far more extensive damage following ischemic injury than that observed in the younger adult brain; that is, the BBB additionally deteriorates only in patients with age-associated health conditions[8,9]. Exacerbated degradation of the BBB leaves brains in elderly patients vulnerable to greater ischemic injury and poorer prognosis.

The current review outlines the cellular and molecular mechanisms of BBB damage following stroke with an emphasis on how age influences the nature of these degradative processes. Furthermore, the BBB influences the delivery and efficacy of ischemic stroke treatment. Thus, the influence of age-dependent BBB degradative mechanisms on treatments and how the BBB itself may be a key therapeutic target to restore or maintain BBB function and integrity in the elderly population are discussed.

2. Age and ischemic injury

Ischemic stroke, although much more commonly studied in young-adult-to-middle-aged animal models and human populations, is prevalent across all age groups. The incidence of stroke in the elderly population is higher than in the young-adult population, and the presentation and clinical outcomes of stroke are highly age-dependent. Importantly, aging populations are vulnerable to ischemic injury and may receive significantly worse prognoses.

2.1. Common etiologies and risk factors

The incidence of stroke increases with age, and thus younger adults are generally at a lower relative risk. It is estimated that 10 – 20% of ischemic events occur in those aged 18 – 50 years[10]. In addition, compared with other age groups, young-to-middle-aged adults differ quite significantly in both the etiology and presentation of ischemic stroke. For instance, compared with elderly patients, young-adult patients presenting with ischemic stroke are more likely to be male and experience a stroke resulting from cervical vessel dissection[11-13]. Furthermore, clinical data demonstrate that in comparison to elderly patients over the age of 80 years, younger adults are more likely to present with hyperlipidemia and obesity and have a history of smoking, drinking, or heavy drug use, all of which are considered to act as major risk factors for stroke in this age group[11,12,14,15]. Aside from the aforementioned hyperlipidemia and obesity, comorbid disease is much less common in younger ischemic patients[11,12,15].

Ischemic stroke is much more prevalent in the elderly population than in young or middle-aged adults. Specifically, 80% of ischemic stroke occurs in the elderly (over the age of 65 years), with 25% of these cases attributed to those over the age of 80 years[16]. Furthermore, these cases occur in a larger proportion of female patients, in contrast to what is observed in younger patients[12,14]. Older patients (over the age of 50 years) tend to have more physiologically based risk factors for ischemic stroke, including numerous vascular-related comorbidities that can influence the basal structure and function of the BBB. Older patients more frequently report hypertension, cardiac failure, coronary heart disease, cardiac arrhythmia, and atrial fibrillation in addition to high cholesterol[12,14].

2.2. Prognosis

Unlike elderly populations, young adults have a relatively good prognosis following ischemic injury. Young ischemic patients aged ≤50 years had a 100% survival rate during 1-, 3-, and 6-month follow-ups, and this survival rate only decreased to 96.8% by the 3-year follow-up[11]. Moreover, recurrent stroke only occurs in 2% of those under the age of 50 years[11]. With increasing age, the incidence of ischemic stroke increases and clinical outcomes worsen. Ischemic tissue surrounding the infarct following injury is much more likely to convert into an infarction as age increases in both men and women[17,18]. In comparison to younger patients, those aged >50 years exhibit significantly lower post-stroke cognitive function and higher rates of disability and social impairments[12], which may be attributed to more severe injury. Middle cerebral artery occlusion (MCAO) animal models using 18-month-old Sprague-Dawley rats (roughly equivalent to humans over the age of 45 years)[19] exhibited infarctions that were up to twofold greater than those observed in 3-month-old rats (roughly equivalent to young adults)[19], following stroke[9]. In addition, unlike younger rats, and aged rats experienced more severe behavioral deficits and did not significantly recover after a 2-week period[9]. A brief overview of the differing etiologies and prognoses of adult and elderly ischemic stroke patients is illustrated in Figure 1.

Age-related comorbidities may further worsen prognoses in elderly patients. Diabetes and Alzheimer's

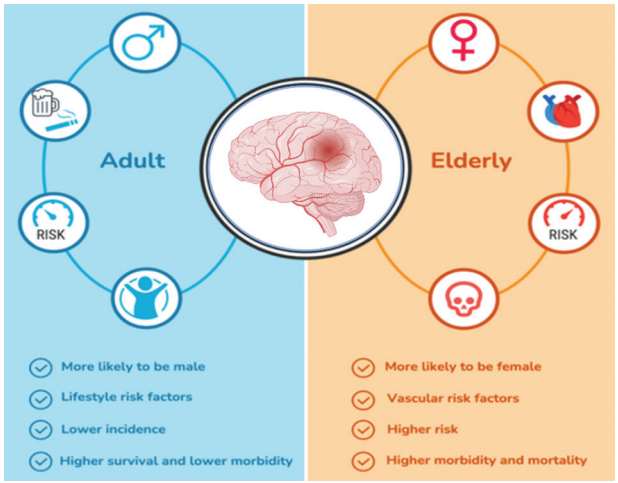


Figure 1. Risk factors and prognoses following stroke in adult and elderly patients. A brief comparison of the commonly reported risk factors of ischemic stroke in adult and elderly patients and later prognosis and survival. The illustration was created with BioRender.com.

disease (AD) significantly worsen the clinical outcome of stroke in both humans and animal models[20,21]. The incidence of stroke increases in AD patients[22], along with the increased risk of a worse ischemic outcome. Dementia patients experience a two- to three-fold increased risk of death following ischemic stroke compared with non-dementia patients, with the risk of death increasing with the severity of dementia[23]. Mouse models of AD further demonstrate a significant increase in ischemic damage compared with non-AD controls, with amyloid precursor protein overexpression resulting in significantly larger cerebral infarcts and greater reductions in cerebral blood flow[20,24]. Diabetic mouse models also demonstrate greater ischemic injury and the development of more severe neurological deficits post-stroke[21,25], potentially linked to greater inflammatory responses[21,26]. Overall, the observed vulnerability of the aged brain is due to a variety of age-related physiological factors that may contribute to a weak BBB, including inflammatory mechanisms and a naturally aging neurovascular unit.

3. BBB injury following ischemic stroke

Opening of the BBB after ischemia is observed in all age groups but may vary in timing and degree of opening, which is largely thought to correlate with the degree of injury severity and prognosis. Elevations in BBB permeability following injury leave the brain susceptible to blood-borne toxins, greater immune cell infiltration, inflammatory mediators, and water influx, which can further worsen neuronal damage and brain edema. Several key cellular and molecular events correspond to BBB injury following stroke, and these vary in degree and form between adult and elderly patients (Figure 2). Maintaining BBB integrity clearly may diminish neuronal damage and thus lead to better clinical outcomes after ischemic stroke. Therefore, understanding the characteristics and underlying mechanisms of BBB injury in different age populations is crucial.

3.1. BBB structure and function following ischemic stroke

Adults experience significant opening of the BBB shortly after ischemic stroke, which contributes to ischemic injury. Despite previous assumptions of a biphasic opening of the BBB following ischemia[27], more recent data support a continuous opening of the BBB that can last few days to few weeks post-injury. For instance, adult Wistar rats that underwent MCAO experienced continuous BBB opening with the use of small and large tracers that were observable beginning 25 min post-injury and lasting up to 4 weeks[28]. Similarly, in humans, a continuously disrupted BBB was observed in 33% of adult patients in the first week following stroke[2], which began around 12.9 h post-ischemia[2] and was reported to last up to 90.7 h[29]. Notably, early BBB disruption is associated with poorer clinical outcomes that are significantly correlated to hemorrhagic transformation, stroke severity, and long-term neurological scores assessed up to 90 days post-injury[2-4]. Thus, any age-related variations in the timing and degree of BBB injury following stroke may considerably affect both acute and long-term outcomes.

Elderly patients are more susceptible to ischemia-induced BBB damage than younger adult patients; this is also worsened by comorbid conditions prevalent in elderly patients. Compared with a younger BBB, an aged BBB exhibits numerous qualities that make it more susceptible to ischemic injury and result in a more severe prognosis. This includes a large age-dependent reduction in barrier function, which is observed in both aged animal models[9,30] and humans[1]. Consequently, the aged BBB undergoes earlier and more severe disruption after injury, which precedes a large portion of neuronal damage[9,31]. In fact, aged rats experience up to a two-fold greater increase in BBB permeability after ischemia compared with younger adults[9]. Diabetes further exacerbates ischemic BBB opening, contributing to edema and poorer clinical outcomes[32]. Diabetes significantly affects basal BBB integrity, as evident in diabetic mouse models exhibiting a 2.4-fold greater BBB permeability than non-diabetic controls[33]. This translates to significantly greater BBB leakage after ischemic stroke[34]. Similarly, the elevated susceptibility to ischemic damage associated with AD is thought to be a result of vascular dysfunction, which is significantly associated with BBB disruption. Cerebral amyloid angiopathy (CAA), which is observed universally

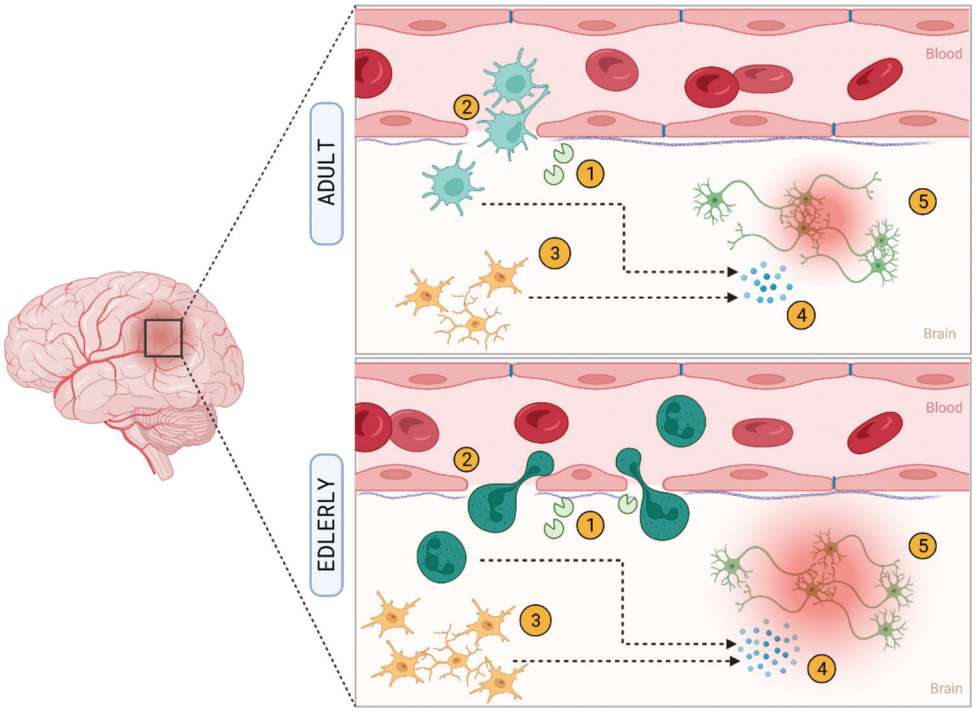


Figure 2. An overview of the cellular and molecular events contributing to BBB damage and consequential neuronal injury in ischemic adult (top) and elderly (bottom) stroke patients. Compared with ischemic stroke in the adult brain, in elderly patients, it is accompanied by greater tight junction loss and increased BBB permeability due to amplified matrix metalloproteinase (MMP) enzymatic activity (1). This leads to greater immune cell infiltration (2), which is characterized by a more neutrophilic response in the elderly brain compared with a monocytic response in the adult brain. Infiltrating immune cells coalesce with activated resident microglial cells (3), which are activated to a greater extent in the elderly brain, producing a myriad of reactive oxygen species (ROS) and inflammatory cytokines (4). This ultimately contributes to neuronal injury (5), which is amplified in the elderly compared with the adult brain. The illustration was created with BioRender.com.

in AD patients and progresses with age[35], has been suggested to be the driver of the observed heightened vulnerability to injury and larger infarct volumes. CAA exerts profoundly deleterious effects on cerebrovascular function and is associated with significantly greater elevations in BBB permeability, up to 1.5-fold greater than that observed in the absence of CAA[35-37]. Taking the above into consideration, the elderly BBB can be inferred to be susceptible to greater ischemic damage both under physiological and pathological conditions.

3.2. Cellular mechanisms of BBB degradation

The large-scale changes to BBB function in response to ischemia are attributed to a variety of cellular transformations within the BBB and its supporting cells, including endothelial cell degeneration and immune cell activation and infiltration. Notably, these cellular mechanisms of BBB degradation vary with age.

In general, oxidative stress within the brain due to acute ischemic events leads to damaged endothelial cells and loss of tight junctions. This reduction in barrier integrity increases paracellular transport of water and disrupts ion homeostasis, contributing to cerebral edema. In comparison, aging is associated with significantly enhanced endothelial degradation and tight junction disruption in the BBB after ischemic stroke. Aging is naturally associated with a loss of tight junction proteins in the BBB, with older subjects expressing significantly less occludin-1 and ZO-1 compared with those of younger age[30]. This phenomenon is only worsened by ischemic events beyond that observed in younger patients. Older mice experience a significantly greater loss of tight junction proteins compared with younger adults following ischemia, which is apparent up to 3 days post-injury[38]. Moreover, those with additional health conditions can experience an even more dramatic ischemia-induced loss of tight junctions. High cholesterol induces greater reductions in tight junction protein expression, specifically occludin-1, after ischemic injury[39]. Diabetes is also associated with a 1.3-fold greater reduction in occludin, ZO-1, and claudin-5 expression following stroke[32-34]. Furthermore, AD-associated CAA is linked to endothelial cell degradation[36,37] and subsequent reductions in tight junction protein expression within the BBB. Compared with that in controls, the expression of claudin-1 and -5 in CAA patients is reduced by 84% and 43%, respectively; this is compounded by the already

greater loss of tight junctions following ischemia in the aging population[37]. Therefore, endothelial barrier degradation following ischemia clearly varies with age and appears to occur to a greater extent in the elderly and to an even greater extent in the presence of pre-existing conditions.

Ischemia in adults also activates resident microglia immune cells and increases the degree of infiltrating leukocytes, including macrophages, which contribute to greater destructive inflammatory responses in the brain, including BBB degradation and resulting hemorrhagic transformation[40]. While aging is similarly associated with an elevated immune response after ischemia that further disrupts BBB integrity and in turn worsens clinical outcome[9], the cellular profile of this inflammatory response is quite distinct from the young adult response. In mice subjected to MCAO injury, older mice express more $CD68^+$ cells in the peri-infarct region up to 14 days post-ischemia than younger mice, indicating greater macrophage and microglia activation[38]. The disproportionately large microglial response is thought to be a key contributing factor to exacerbated ischemic BBB injury in aged models[41]. In addition to an intensified resident immune response, aged mice demonstrate a strong neutrophilic response to ischemia that appears to be one of the more differentiating features of ischemic events in this age group, as younger adults are subject to a stronger monocytic response[41]. The neutrophilic response to ischemic stroke in the aging population correlates with more severe ischemic injury outcomes and hemorrhagic transformation, according to both animal and human data[41]. In addition, recent evidence suggests that a disproportionately elevated level of neutrophils correlates with greater cognitive impairment post-stroke, affecting both memory and visuospatial abilities up to 3 months post-injury[42]. Subjects with diabetes also experience this strong neutrophilic reaction, which may be compounded by age, demonstrating earlier, and larger neutrophil infiltration in cortical and subcortical regions of the ipsilateral hemisphere 4 and 24 h post-stroke, which is strongly associated with greater infarct volume[34,43]. Although it is yet to be elucidated, AD patients may also be at a further disadvantage because of their natural predisposition for greater neutrophil reactivity[44]. However, the role this plays in ischemia and the consequences on the BBB remain to be explored.

3.3. Molecular mechanisms of BBB damage

Activation of the cellular mechanisms of BBB degradation is accompanied by the release of numerous molecular factors that can disassemble tight junction complexes and increase BBB permeability. These molecular mechanisms contribute to the age-dependent changes within the BBB that influence the degree of ischemic insult and clinical outcome. The key molecular players in stroke-induced BBB degradation are MMPs, inflammatory cytokines, and ROS. Comparable to cellular changes, the molecular mechanisms described below differ in their characteristics and the degree to which they contribute to BBB degradation after stroke.

As reviewed by Lakhan *et al.*, MMPs, specifically MMP-2 and MMP-9 secreted by resident and infiltrating inflammatory cells, are largely thought to regulate BBB integrity after stroke by degrading tight junction and basal lamina proteins[45]. MMP-induced BBB degradation contributes to further leukocyte infiltration, edema, and hemorrhage. MMP-2 and MMP-9 were both elevated in adult ischemic stroke patients on admission[46]. MMP elevation is correlated with both injury severity and prognosis[46,47]. MMP-9 expression in response to stroke is two-fold greater in elderly patients than that in younger adults[41]. MMP-2 further contributes to ischemia-induced BBB breakdown, and the activities of both MMP-9 and MMP-2 were aggravated in aged-related comorbidities. Patients with diabetes exhibit significantly greater and earlier MMP-2 activity and a seven-fold increase in MMP-9 activity after stroke, as compared with those without diabetes[33,34,48]. The observed elevation in MMP-9 activity after stroke in the context of diabetes is thought to be the result of both a greater neutrophilic response, which is already elevated in older patients, and reductions in MMP-9 inhibition[34,43]. In addition, AD-associated CAA was also associated with significant elevations in MMP-2 and MMP-9 expression, with a significant 2.4-fold increase in MMP-9 activity compared with controls[37]. Therefore, while MMPs play a role in both adult and elderly BBB degradation, the timing and degree to which they do so vary across age groups with and without additional medical conditions.

Certain pro-inflammatory cytokines, namely, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1b, are thought to contribute to ischemic injury by degrading the BBB and are associated with poorer clinical outcomes[49-51]. After stroke, microglial, endothelial, and infiltrating immune cells release such cytokines, which wreak havoc on the integrity of the BBB, beginning hours after injury and lasting days post-stroke[50,51]. Patients with higher TNF-α and IL-6 levels had significantly lower neurological scores, both acutely and 30 days after stroke[49], which correlated to significantly elevated BBB permeability through reductions in claudin-5, occludin, and ZO-1 expression as a consequence of these inflammatory cytokines[52]. Aging is associated with elevated inflammatory responses to ischemia, correlating

with severe BBB disruption and subsequent injury. Aging is naturally associated with elevated inflammatory mediators, such as IL-6 and TNF-α, resulting in a natural leakiness of the BBB via the loss of tight junctions[30]. This is more exacerbated during ischemic events in the elderly compared to younger adults. Compared with 2-month-old adult mice, aged (12-month-old) mice that started to experience senescence[53] had significantly higher IL-1b and IL-6 expression after ischemia, correlating to more severe and longer-lasting behavioral deficits and infarction due to greater BBB leakage[38]. Thus, similar to MMPs, inflammatory cytokines play a greater role in vulnerable elderly brains (with greater damage inflicted on the BBB post-stroke) than in the brains of younger adults.

Finally, oxidative stress caused by ROS and other free radicals released by reactive and infiltrating immune cells plays an important role in ischemic injury progression. It contributes to the loss of tight junctions and promotes MMP-9 degradation of the BBB, ultimately leading to a loss of barrier integrity[50]. Microglia and neutrophils have been shown to produce more ROS in response to ischemia in elderly compared to younger adult brains[41]. This may be further observed under age-associated comorbid conditions, such as hyperglycemia, which is associated with greater oxidative stress, superoxide production, BBB degradation, and increased MMP-9 activity after stroke[48].

4. The BBB in ischemic treatment and the influence of age

Not only can the BBB influence the timing of administration and effectiveness of ischemic stroke treatments and strategies but also it is itself considered to be an increasingly attractive target for future therapeutic development. As outlined throughout the current review, the degree of ischemic injury and later recovery depend on the degradation of the BBB. Thus, maintaining the BBB after stroke may be the key for achieving better long-term clinical outcomes. However, due to the above-mentioned age-dependent mechanisms of BBB degradation after stroke, considering these cellular and molecular variations is crucial for developing new potential therapeutics.

4.1. Drug delivery and efficacy

The time frame for drug delivery and treatment efficacy are largely dictated by post-ischemia BBB dynamics and age-specific BBB characteristics and comorbidities. At present, tissue plasminogen activator (tPA) is the only therapy for ischemic stroke in the mature brain approved by the U.S. Food and Drug Administration. However, tPA has a short time frame of delivery of <3 h, largely due to the increased risk of hemorrhagic transformation because of delayed tPA-induced BBB degradation. This is especially relevant in older patients who are already at a greater risk of hemorrhage after treatment[31]. Along these lines, the elderly BBB undergoes greater damage following tPA treatment, which is correlated to the greater likelihood of hematoma formation compared with the young brain[31] and can limit the potential benefits of tPA treatment. tPA-induced damage to the BBB and the corresponding risk of hematoma and death are suspected to be linked to the neutrophilic response that the elderly brain experiences during ischemia[41,54]. The elderly neutrophilic response is associated with increased MMP-9, which is a predictor of hemorrhagic transformation and death following tPA administration[55]. Furthermore, the efficacy of tPA treatment may be reduced in elderly patients with comorbid health conditions. For example, diabetes significantly reduces tPA efficacy in infarction reduction, which is associated with increased hemispheric swelling and hemorrhage due to greater BBB degradation[56], conceivably through even greater microglial proliferation and increased MMP-9 activity.

Appreciating the different timelines of BBB disruption following ischemic injury is critical for the development and administration of new potential neuroprotective drugs in both adult and elderly populations[57]. Most research is conducted using adult rodent models, even though, as discussed, BBB opening can occur to a greater extent and at a much earlier time in elderly patients compared with young and middle-aged adults. Thus, unlike with younger patients, for elderly patients (with a higher level of BBB permeability), it may be useful to develop potential neuroprotectants with a longer time frame for delivery and larger composition. To this end, understanding the timing, extent, and mechanisms of BBB degradation in vulnerable patients with and without comorbid conditions could facilitate the development of new and more effective treatment options that take advantage of the timelines of BBB permeability in different age groups.

4.2. Targeting the BBB in ischemic stroke treatment

BBB degradation is associated with more severe ischemic injury, subsequent edema, and hemorrhage, and therefore protecting the BBB from further damage following ischemia has been a goal to improve clinical prognosis for all age groups[58,59]. Such treatments are largely suggested to target the inflammatory cytokine and MMP proteolytic mechanisms underlying BBB degradation, as discussed in the current review. In addition, targeting the BBB may allow effective treatment to be delivered during a more clinically relevant time frame and may widen the time frame for delivery of current therapeutics[60,61]. However, the above-mentioned age-specific characteristics of the BBB under ischemic conditions must be taken into consideration.

As discussed, older patients experience significantly increased MMP-9 activity following ischemic stroke compared with younger patients. Because MMP-9 activity plays a crucial role in BBB degradation (which leads to poorer clinical outcomes), it is pertinent that future therapies aim to reduce this proteolytic activity. The mechanisms of MMP-9 inhibition in the context of ischemic stroke treatment are reviewed by Chaturvedi and Kaczmarek[59]. Altogether, MMP-9 inhibition seems to be a viable target for adult and especially elderly stroke patients, given their comparably robust MMP-9 response to ischemia. However, MMP-9 has also been linked to critical angiogenic mechanisms[62] and neurological recovery in the late stages of stroke[63]. Thus, targeting MMP-9 activity would require a highly controlled mechanism and time frame of inhibitory action because a long-term therapeutic strategy to reduce MMP-9 activity may interfere with the beneficial actions and effects of this enzyme during the later recovery stages after stroke.

Inhibition of inflammatory processes successfully mitigates injury severity in older patients. Older stroke patients are subject to greater inflammatory BBB damage after stroke, and thus drugs that restrict stroke-induced inflammation seem to be a viable avenue to use in this cohort of patients. Inhibiting inflammation in the aged brain significantly diminishes stroke volume and improves neurological function by reducing TNF-α- and MMP-9-mediated BBB degradation and corresponding inhibition of neutrophil infiltration[64]. Because the aging population experiences a strong neutrophilic response to ischemia, which largely contributes to BBB degradation and poor clinical outcomes, a drug that could inhibit neutrophil infiltration and reduce systemic neutrophil levels[42] would be ideal for improving acute and chronic stroke outcomes in aging patients.

In fact, the discrepancy in the cellular profile of the immune response after ischemia between adult and elderly brains may provide key insight into the optimal treatment of post-stroke patients by taking into consideration age-dependent disease characteristics. The strong neutrophilic response in elderly stroke patients, as reviewed, contributes to both greater ischemic injury in terms of infarct volumes as well as secondary injury due to BBB damage, and has further been linked to worsened cognitive impairment months after injury[42,65]. Interestingly, this inflammatory event may be one of the easier targets to address, as it begins systemically rather than beyond the BBB. Furthermore, extensive knowledge has accumulated regarding the subsets of neutrophils that respond to injury, essentially classifying the helpful from the harmful based on extracellular proteins[65]. Using this knowledge, efforts should be made to identify clinically relevant methods that may disarm the damaging response of the hyperinflammatory neutrophils in systemic circulation before entry into the brain. This would reduce MMP, ROS, and inflammatory cytokine loads, while still allowing anti-inflammatory subsets to permeate the BBB. Thus, leveraging the heterogeneity of the nucleophilic response and avoiding global suppression of critical immune function may effectively support ischemic injury recovery and suppress inflammatory destruction of the BBB.

Additional therapeutic avenues that maintain BBB integrity and can be delivered in conjunction with current treatment options to improve clinical outcomes are also under investigation. Adjuvant therapeutics in conjunction with tPA treatment that stabilize the BBB not only reduce injury severity and risk of hemorrhagic transformation but also increase the time frame for treatment in elderly patients. Delivery of tPA outside the recommended 3-h time frame after initial injury may increase the likelihood of hemorrhagic transformation because of tPA-induced MMP-9 activation[55]. Because, as previously discussed, elderly patients are at a greater risk of hemorrhagic transformation after tPA treatment, providing an MMP-9 inhibitor in conjunction with tPA may improve clinical outcomes in this population. Treatment with minocycline (an MMP inhibitor) in combination with tPA significantly reduces 24-h infarct size and ameliorates hemorrhagic transformation, allowing tPA treatment to successfully delay ensuing ischemic injury as a direct consequence of MMP-9 inhibition[66]. However, how MMP-9 inhibition affects later stages of recovery and angiogenic events still needs to be elucidated.

Neural stem cell transplantation in conjunction with tPA treatment has also been shown to reduce infarct volume in the aged brain after stroke through reduction of pro-inflammatory cytokines (e.g., TNF-α and IL-6) and MMP-9, thereby reducing BBB damage and maintaining tight junctions, while allowing a longer time frame of successful tPA treatment (6 h)[67]. Stem cell administration in animal models of ischemic stroke has further been associated with improved neurological function as a result of reductions in post-stroke ischemic injury[68,69].

Neutrophil activity inhibits the efficacy of tPA treatment through tPA-induced formation of neutrophil extracellular traps, which largely contributes to an increased risk of hemorrhage[70]. Thus, efforts should be made to identify the means to selectively inhibit inflammatory neutrophil activity in conjunction with tPA treatment. Knecht *et al.* further outline specific pharmacological and non-drug interventions that target molecules contributing to BBB degradation in ischemic stroke; these may represent potential adjuvant therapies to tPA, increase the time frame

of delivery, and improve clinical outcomes[71]. Accordingly, elderly ischemic stroke patients would greatly benefit from therapeutics that reduce the inflammatory degradation of the BBB and boost the efficacy of current treatment options (such as tPA) by supporting BBB integrity.

5. Conclusions

Ischemic stroke is prevalent in all age groups. Ischemic stroke contributes to severe BBB degradation, which is associated with worse prognoses, edema, and hemorrhagic transformation. Age not only influences the features of ischemic stroke but also how the BBB is affected after injury. This can be explained by age-specific cellular and molecular mechanisms of BBB degradation, such as increases in the loss of tight junctions, immune cell infiltration, neutrophilic responses, MMP-9 activity, release of inflammatory cytokines, and oxidative stress. Elderly populations are at a greater risk of more substantial BBB damage after ischemic injury due to ischemia-induced cellular and molecular mechanisms of BBB degradation that occur to a greater extent than that observed in younger adults. Such degradation is exacerbated by age-related comorbid health conditions, such as diabetes and AD. As such, with the BBB mediating the delivery and efficacy of ischemic stroke treatment, new stroke therapies that may be more beneficial to vulnerable patient populations, with special emphasis on selectively inhibiting the neutrophilic response, are required. Current research is highlighting the potential of BBB maintenance following ischemic stroke as a therapeutic avenue. Therefore, age-dependent post-stroke BBB characteristics should be taken into consideration to better improve current and future ischemic stroke therapeutics.

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Conflict of interest

The authors declare no conflict of interests.

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ACCSCIENCE PUBLISHING

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SHORT COMMUNICATION

Associations of common variants in *TAAR5*, *OR6C70,* and *GBA* with hyposmia in Han Chinese individuals with Parkinson's disease

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Abstract

Hyposmia is one of the cardinal symptoms of Parkinson's disease (PD), with a considerably high prevalence rate in PD individuals. However, some individuals still retain normal olfactory function. Recent studies have shown that genetic factors may play a role in such a phenomenon. This study aimed to explore the potential genetic factors underlying the variation of olfactory function among PD individuals. Two hundred and three Han Chinese individuals with PD were recruited into this study. All the individuals underwent detailed clinical assessment conducted by experienced neurologists. High-throughput sequencing was performed to identify gene variants associated with PD. *TAAR5*, *OR6C70,* and *GBA* were included in the association analysis. In our study, 85 out of 203 individuals (41.9%) reported normal olfaction, and the other 118 (58.1%) reported hyposmia. Genotype and allele logistic regression models were applied to association analysis. We did not find any significant association of *TAAR5* and *OR6C70* with hyposmia. However, we found that *GBA* rs762488 was associated with increased hyposmia risk (P = 0.036, OR = 3.05, 95% CI = 1.08–8.63), while *GBA* rs1800438 was associated with decreased hyposmia risk (P = 0.032, OR = 0.47, 95% CI = 0.24–0.94). In conclusion, this study revealed the association of *GBA* with hyposmia, indicating the genetic involvement in PD hyposmia variation. However, we did not replicate previous results (*TAAR5* and *OR6C70*) in this study. Further studies with larger sample sizes in different populations are warranted.

Keywords: Parkinson's disease; Olfaction; *TAAR5*; *OR6C70*; *GBA*

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1. Introduction

The olfactory system is the bodily structures that serve the sense of smell and enable animals to identify the environmental information from specific odors[1]. Hyposmia is characterized by the loss of odor detection, identification, or memory[2] and is a common symptom in a plethora of neurodegenerative diseases[2,3].

More than half of Parkinson's disease (PD) individuals reported hyposmia[4], which precedes the occurrence of motor symptoms. However, olfactory function remains

normal in some cases long after motor symptoms onset[5]. The detailed mechanism that contributes to the differences in olfactory function in individuals is yet to be clarified; therefore, numerous studies focusing on the underlying genetic variations of olfactory function in PD patients have been conducted[6-8].

Interestingly, a recent whole-genome sequencing analysis revealed that variants in *OR6C70* (encoding the olfactory receptor family six subfamily C member 70) and *TAAR5* (encoding the trace amine associated receptor 5) affect the different perceptions of specific odors between different individuals. Genetic variations in these two genes may partially underlie the olfactory recognition differences[6]. To date, these conclusions have not been replicated in other studies or populations. To our concern, PD is a disease well associated with olfactory dysfunction. Whether the aforementioned gene variants contribute to the differences in olfactory function of PD individuals remains unclear. In our study, we aimed to explore the potential association of *OR6C70* and TAAR5 with hyposmia in Han Chinese individuals with PD. In addition, *GBA* (glucocerebrosidase gene, a common PD causative gene) was also included in this study since previous studies have demonstrated that some PD individuals with mutations in *GBA* have reported hyposmia[8,9].

2. Materials and methods

2.1. Subjects

Two hundred and three Han Chinese individuals with PD, who fulfilled either the 2015 Movement Disorder Society clinical diagnostic criteria[10] or the UK Brain Bank criteria[11] for PD, were recruited consecutively from the Department of Neurology of the Second Affiliated Hospital of Soochow University (Suzhou, China) from January 2013 to December 2019. Individuals with severe cognitive impairment who cannot cooperate with the assessment were excluded from the study. Our study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University (JD-LK-2018-061-01). Detailed clinical data and blood samples were collected after written informed consent were obtained from all the participants.

2.2. Clinical assessment

The demographics, including age, age at onset, duration and gender, of enrolled PD individuals were recorded. Disease severity was assessed by the Unified Parkinson Disease Rating Scale (UPDRS) Part III[12] during the "on" state. Prescribed levodopa equivalent doses of dopaminergic medication were calculated according to the protocol[13]. Non-motor symptoms (NMSs) were assessed by the Non-Motor Symptoms Questionnaire (NMSQ)[14]. We defined hyposmia by the item in NMSQ, "Loss or change in your ability to taste or smell"[15]. PD individuals with taste dysfunction were excluded from the study.

2.3. High-throughput sequencing

Genomic DNA of 203 individuals was extracted from the peripheral blood using the QIA ampDNA Blood Maxi Kit (QIAGEN, Valencia, CA, USA). Then, whole-exome sequencing was performed as described in our previous study[16,17]. *TAAR5*, *OR6C70,* and *GBA* were included in our genotype analysis. Single nucleotide polymorphisms (SNPs) and insertions/deletions (Indels) located within the exons and exon-intron boundaries of these three genes were then identified as described previously[17]. After variants calling and annotation, the data were compared to our in-house data to distinguish between common and rare variants[16]. To achieve adequate statistical power, we excluded rare variants[16]. Linkage disequilibrium was estimated using SHEsis (offline version)[18], and SNPs with $r^2 \geq 0.80$ were defined as being in linkage disequilibrium.

2.4. Statistical analysis

In univariate analysis, Kolmogorov–Smirnov test or Shapiro–Wilk test was used to test if the variables were normally distributed in 203 PD individuals. Differences in age, duration, UPDRS-III score, and other variables between hyposmia and non-hyposmia groups were compared using Student's *t*-test (when they were normally distributed) or non-parametric Mann–Whitney U-test (when they were not normally distributed). Gender and other dichotomous variables were compared with Chi-square test or Fisher's exact test. Binary logic regression to hyposmia was used to explore the potential associated factors.

Associations were quantified with the odds ratio (OR) and 95% confidence intervals (CIs). All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. All tests were performed using IBM SPSS Statistics, version 25.0 (IBM Corporation, NY, USA).

3. Results

Of 203 PD individuals, 118 (58.1%) reported hyposmia, and 85 (41.9%) reported normal olfactory function. According to the demographic analysis, male individuals with and without hyposmia were 76 (57.6%) and 49 (64.4%), respectively. We did not find any significant differences in the demographic profiles between PD individuals with and without hyposmia; these demographic parameters include gender, age, age of onset, duration, disease severity, and prescribed dopaminergic medication dose (Table 1).

In our genotype analyses, we found 91 SNPs and 0 Indel within the exons and exon-intron boundaries of *OR6C70*,

TAAR5, and *GBA*. We did not find the previously reported rare *TAAR5* p.Ser95Pro mutation in any of the individuals. Eighty-three of the 91 SNPs were rare variants (minor allele frequency < 0.10) and were excluded from subsequent analysis for better statistical power. Finally, eight common SNPs (*TAAR5* rs3813355, rs3813354; *OR6C70* rs60683621; *GBA* rs1800473, rs762488, rs2009578, rs2974923, and rs1800438) and 0 Indel were included in our association analyses and none of them was in linkage disequilibrium. In our genotype logistic regression analyses to hyposmia, considering age, gender, duration, motor symptoms and other demographics, we found that none of the SNPs was associated with hyposmia (Table 2).

In our allele logistic regression analyses to hyposmia, considering age, gender, duration, motor symptoms and other demographics, we did not find the three common SNPs in *TAAR5* and *OR6C70* being significantly associated with hyposmia. However, we found that *GBA* rs762488 was associated with increased hyposmia risk (P = 0.036, OR = 3.05, 95% CI = 1.08–8.63), and *GBA* rs1800438 was associated with decreased hyposmia risk (P = 0.032, OR = 0.47, 95% CI = 0.24–0.94) (Table 3).

Table 1. Demographic profiles of PD individuals with/ without hyposmia

	Non-hyposmia (n=85)	Hyposmia (n=118)	P*
Gender (male, %)	49 (57.6%)	76 (64.4%)	0.381
Age (year)	60.59±11.78	63.00±9.22	0.212
Age of onset (year)	56.18±12.74	57.85±9.18	0.598
Duration (month)	52.58±42.42	61.47±53.66	0.471
LED	420.36±250.73	446.71±255.68	0.535
UPDRS-III	20.26±10.40	23.25±13.81	0.153

LED, Levodopa equivalent dose; PD, Parkinson's disease; UPDRS-III, Unified Parkinson's Disease Rating Scale – Part III. *Mann–Whitney U-test

Table 2. Genotype logistic regression analysis of hyposmia in 203 PD individuals

	Regression coefficient	P*	OR	95% CI
Gender (male, %)	−0.382	0.220	0.68	0.37–1.26
Age (year)	−0.028	0.949	0.97	0.41–2.28
Age of onset (year)	0.054	0.902	1.05	0.45–2.47
Duration (month)	0.008	0.834	1.01	0.94–1.08
LED	0.000	0.711	1.00	1.00–1.00
UPDRS-III	0.016	0.250	1.02	0.99–1.04
rs3813355	0.344	0.202	1.41	0.83–2.39
rs3813354	−0.134	0.655	0.87	0.49–1.57
rs60683621	0.109	0.603	1.12	0.74–1.68
rs1800473	−0.378	0.340	0.69	0.32–1.49
rs762488	0.779	0.073	2.18	0.93–5.11
rs2009578	−0.390	0.244	0.68	0.35–1.31
rs2974923	0.074	0.724	1.08	0.71–1.62
rs1800438	−0.412	0.105	0.66	0.40–1.09

CI, Confidence interval; LED, Levodopa equivalent dose; OR, Odds ratio; PD, Parkinson's disease; UPDRS-III, Unified Parkinson's Disease Rating Scale - Part III. *Logistic regression adjusted for gender, age, age of onset, motor symptoms, LED, and UPDRS-III

4. Discussion

PD is the second most common neurodegenerative disease with many motor and non-motor symptoms[19,20]. Hyposmia is one of the core non-motor symptoms of PD and is part of the critical clinical diagnostic criteria for PD[10]. Nevertheless, hyposmia is a condition with incomplete penetrance and different PD individuals with hyposmia may have a varying degree of olfactory loss[21-23]. The detailed mechanism of hyposmia occurrence remains undetermined. So far, there have been limited studies unraveling the specific genetic background of hyposmia in PD. Since hyposmia is a prevalent non-motor symptom in PD, increasing attention has been devoted to investigating olfaction in PD. Recently, Gisladottir *et al.* found that *TAAR5* p.Ser95Pro mutation is associated with a reduced intensity rating of fish odor containing trimethylamine[6]. *TAAR5* is a G protein-coupled receptor, which shares 35% of the sequence with 5-HT-1D receptor[24] and 33% with dopamine D2 receptor[25], which may contribute to PD pathology[26]. Besides, Gisladottir *et al.* also found that *OR6C70* p.Lys233Asn (rs60683621) is associated with increased intensity and identification of licorice odor[6]. However, the associations of *OR6C70* and *TAAR5* with PD have not been reported previously. Such results highlighted that sequence diversity in olfactory receptor genes can lead to enhanced olfactory ability.

In our study, the sequencing covered exon and exon-intron boundary regions of *TAAR5* and *OR6C70,* but we were not able to find any significant association between hyposmia and common variants (including the reported rs60683621) in these two genes. We did not replicate the reported association between rs60683621 and olfactory function, which merely a consequence of global hyposmia assessment previously instead of an assessment scale with specific olfactory domain evaluation in our study, which warrants further investigations in future studies.

GBA encodes glucocerebrosidase, a lysosomal protein that cleaves the beta-glycosidic bond of glucosylceramide and contributes to Gaucher's disease and PD. Thaler *et al.* and da Silva *et al.* found that mutations in *GBA*

Table 3. Allele logistic regression analysis of hyposmia in 203 PD individuals

	Regression coefficient	*P**	OR	95% CI
Gender (male, %)	−0.358	0.101	0.70	0.46–1.07
Age (year)	−0.044	0.886	0.96	0.53–1.74
Age of onset (year)	0.068	0.824	1.07	0.59–1.95
Duration (month)	0.008	0.738	1.01	0.96–1.06
LED	0.000	0.679	1.00	1.00–1.00
UPDRS-III	0.015	0.116	1.02	1.00–1.03
rs3813355	0.316	0.235	1.37	0.81–2.31
rs3813354	−0.142	0.629	0.87	0.49–1.54
rs60683621	0.048	0.827	1.05	0.68–1.62
rs1800473	−0.725	0.129	0.48	0.19–1.23
rs762488	1.115	0.036	3.05	1.08–8.63
rs2009578	−0.503	0.208	0.60	0.28–1.32
rs2974923	0.163	0.529	1.18	0.71–1.95
rs1800438	−0.749	0.032	0.47	0.24–0.94

CI, Confidence interval; LED, Levodopa equivalent dose; OR, Odds ratio; PD, Parkinson's disease; UPDRS-III, Unified Parkinson's Disease Rating Scale - Part III. *Logistic regression adjusted for gender, age, age of onset, motor symptoms, LED, and UPDRS-III

were associated with hyposmia in PD[8,9]. In our study, we found that *GBA* rs1800438 was associated with decreased hyposmia risk, while *GBA* rs762488 was associated with increased hyposmia risk. It is possible that rs762488 and rs1800438 are located in different introns, which may lead to different expression levels of *GBA*. This deserves further investigation with functional experiment. In summary, this is the first report concerning the associations between common variants in *GBA* and hyposmia in PD. We consider that associations between PD and *GBA*, including its common and rare variants, should be studied in future.

Our study has some limitations. First, the sample size is limited. The collection of a larger sample size was limited due to the fact that this is a single-center cross-sectional study. Second, the prevalence rate of hyposmia in our study is not comparable to that in some epidemiological studies[4,27]. This may be partially attributed to ethnicity factor. In general, marked differences in allele frequencies had been discerned between different populations. Geological conditions, as well as social customs, may also lead to different odor recognition[28,29]. In addition, we applied a single-question method for screening olfactory dysfunction, which could be further improved. In general, in accordance with the study by Gisladottir *et al.*, more studies with larger sample sizes and standardized assessments of olfactory function are warranted in different populations in the future, which may help us differentiate different subtypes of PD and facilitate precise treatment.

5. Conclusion

In our study, we found that *GBA* is associated with hyposmia in PD, indicating genetic involvement in PD hyposmia variation. However, we did not replicate the previous results (loci in *TAAR5* and *OR6C70*) in this study. More studies with larger sample sizes concerning the above genes need to be implemented to explore the potential association between hyposmia in different populations.

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Conflict of interest

None.

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Contributor role	Definition
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.
Data curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use.
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.
Funding acquisition	Acquisition of the financial support for the project leading to this publication.
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.
Methodology	Development or design of methodology; creation of models.
Project administration	Management and coordination responsibility for the research activity planning and execution.
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.

Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs.
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.
Writing – original draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).
Writing – review & editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.

Authors are advised to follow *Advanced Neurology*'s preferred style of writing the Author Contributions statement. See an example below:

Conceptualization: Ali Jackson, Helen Meyer

Investigation: Ali Jackson, Tom Lewis-Hans, Han Xiang

Formal analysis: Han Xiang

Writing – original draft: Ali Jackson

Writing – review & editing: Helen Meyer, Joshua O'Brien

Supplementary files

This section is optional and contains all materials and figures that are excluded from the manuscript. These materials, figures or additional information are relevant to the manuscript but remain non-essential to readers' understanding of the manuscript's main content. All supplementary information should be submitted as a separate file during submission.

Supplementary figures and tables should be submitted in a single, separate supplementary file, and must be numbered, for example, **Figure S1** and **Table S1**. All tables must be editable (preferably created from Microsoft Word). The acceptable formats of images and illustrations used in figures are JPEG, PNG and TIFF. Citations of these items must be appropriately referenced in the manuscript in chronological manner, for instance, "Additional information can be found in **Table S1**." Note the additional letter **S** helps distinguish the normal from supplementary items.

Data set file are usually prepared using Microsoft Excel (in XLS or XLSX format).

Videos (MP4 format), with a constituent maximum size of 15 MB, can be uploaded as part of the supplementary file.

Revision and response/rebuttal letter

If the editorial decision for a submission is major revision or minor revision, authors are advised to revise the manuscript (and possibly, the supplementary files) as per the review reports and resubmit the revision file, including the manuscript, title page and back matter, cover letter, and response/rebuttal letter, before the due date.

Revisions should be done on the latest version of the manuscript (or in some rare cases, edited manuscript provided by the editor) with the track change on. The revisions made should be described and/or clarified in the response/rebuttal letter; ideally, explanation about the revisions should be made clear with the help of page number and line number. If authors do not agree with reviewers' comments and suggestions, rebut their points with strong evidence and reasonable arguments.

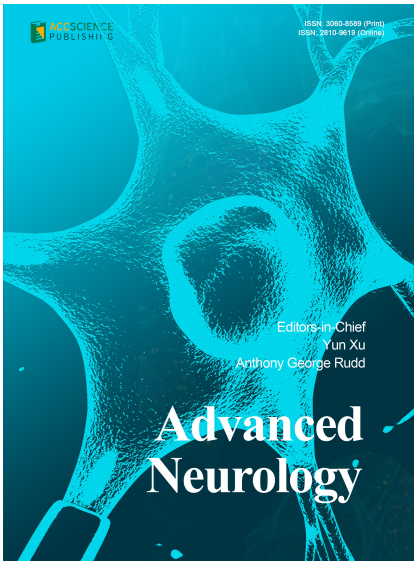
Advanced Neurology

Special Issue Alerts

Invitation for Special Issue Proposals

Organizing and editing for a Special Issue helps Guest Editors gain editorial experience and improve academic profile, in addition to being a part of organizing scientific communication of contemporary topics.

If you are published researcher and have an idea for a Special Issue, please write in via email to our Managing Editor (an.office@accscience.sg). Please provide your CV, professional profile page and a topic of interest in your email. Our colleague will guide you in the process of writing a Special Issue proposal.



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   Yes, all full-length article submissions to a Special Issue will go through the same editorial and peer-review process. The distinct difference here is that the Guest Editors will replace the usual editors and get involved in the making professional decisions on papers after peer review. Note that the specific roles of a Guest Editor could vary across Special Issues.

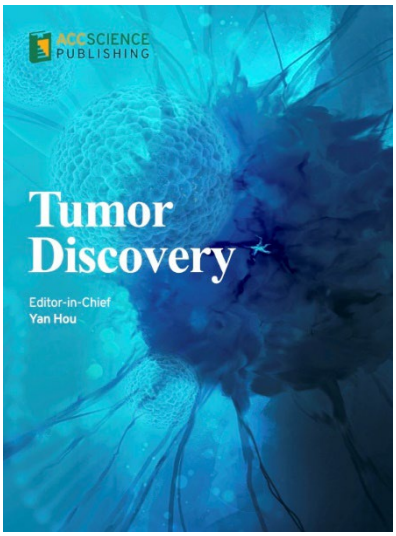
2. **How many Guest Editors are required to organize a Special Issue?**
   There is no fixed number; however, we suggest no more than 4 Guest Editors per Special Issue. More importantly, all Guest Editors should have excellent publication track records and demonstrated expertise in the topic(s) being proposed.

3. **Is the Special Issue governed by important deadlines?**
   Yes.

Benefits of Being A Guest Editor

- A chance to get involved in the conception and development of a specialty, contemporary topic that is of interest to the readers
- A chance to expand your professional network to the scholars and researchers who are similarly involved in the research of specialty topic
- A chance to hone your editorial skills
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- A chance to improve your academic profile and help establish your academic influence within your discipline

Our Journals

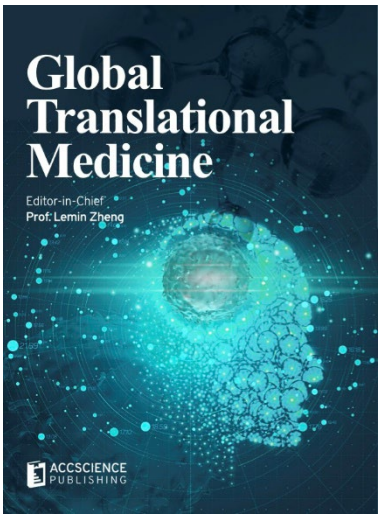


Tumor Discovery is a peer-reviewed and open-access journal that aims to present new cancer research with strong emphasis on fundamental and translational studies. *Tumor Discovery* covers topics, including but not limited to the following:

- Etiology and pathogenesis of cancer
- Mechanisms and molecular pathways underlying cancer initiation and progression
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- Tumor evolution and heterogeneity
- Tumor microenvironment and tumor-host interactions
- Cancer genetics and genomics
- Cancer characterization using omics approaches
- Discovery and validation of cancer biomarker
- Discovery of new therapeutic targets
- New approaches of diagnostic and treatment modalities
- Statistical methods in cancer research

Global Translational Medicine is a quarterly journal that focuses on medicine, biological sciences, and biomaterials engineering. The goal of *Global Translational Medicine* is to provide a platform to researchers for showcasing their latest research works in translational medicine so as to advance the field towards the betterment of human health. Despite the advancement of omics and new technologies, the process of transforming these technologies and scientific research results into effective therapies and putting them into clinical use still has a long way to go. *Global Translational Medicine* provides a platform to fill the gaps in preclinical and inter-disciplinary research, to promote clinical translation of scientific research results, and to contribute to the conception of new and improved preventive measures as well as diagnostic and therapeutic techniques of diseases.

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