PHARMACOLOGICAL CHARACTERIZATIONS OF NOVEL ANTI-DEMENTIA NMDA RECEPTOR ANTAGONIST MEMANTINE NITRATES VIA MUTIPLE TARGETS

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Pharmacological Characterizations of Novel Anti-Dementia NMDA Receptor Antagonist Memantine Nitrates via Multiple Targets

MAK Shinghung

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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CERTIFICATE OF ORIGINALITY

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MAK Shinghung

Abstract

Background: Dementia, characterized by the cognitive function impairments, mainly affects the aged population. According to a report from World Health Organization, around 50 million patients with dementia in 2018, and nearly 10 million new cases every year. Although the problems are becoming more and more serious, the causes of dementia remain to be elucidated. Alzheimer's disease (AD) and vascular dementia (VaD) are the two most common forms of dementia, which contributing to 90% of the total cases of dementia today. Currently, only limited drugs are available for treating AD and VaD. Increasing evidences have shown that AD might be caused by multiple factors. Therefore, currently existing single-targeted drugs might only offer limited symptoms relieve effects of patients suffered from dementia.

In the previous studies, our collaborators in Jinan University have designed and synthesized a series of novel compounds derived from memantine, a currently used anti-AD drug. The nitrate moiety has been introduced onto the backbone of memantine, providing multi-functional anti-dementia effects including neuroprotection and vasodilation. Some of the memantine nitrates have been proven to possess neuroprotective effects *in vitro*; however, the detail mechanism(s) is still unclear. My thesis study is aiming to further investigate the neuroprotective effects of these memantine nitrates, as well as the underlying mechanisms both *in vitro* and *in vivo.*

Methodology: Various *in vitro and in vivo* models were employed to investigate the pharmacological characterizations of the memantine nitrates. MTT assays were used to evaluate the cell viability. Hoechst staining was used to differentiate apoptosis and necrosis induced by neurotoxins. Confocal microscopic fluorescent scanning and electrophysiological experiments were performed to explore the involvement of memantine nitrates on the blockage of NMDA receptor. Immunostaining is conducted to show the synapse integrality. Variety of chemical inhibitors and Western blotting were used to investigate the underlying signaling pathways on neuroprotection as offered by the memantine nitrates. Moreover, the 2-vessel occlusion (2VO) rat model was used to evaluate the anti-dementia effects and the improvement of cerebral blood flow (CBF) by memantine nitrates.

Results: The neuroprotective effects of memantine nitrates were firstly evaluated. Three representative memantine nitrates (MN-06, MN-08 and MN-12) have exhibited neuroprotective effects against glutamate-induced excitotoxicity in rat primary cerebellar granule neurons. MN-08, the most promising candidate, has been selected for further mechanistic characterization. Our results revealed that MN-08 prevented glutamate-induced apoptosis through multiple targets. Western blotting assays have shown that 2 h pretreatment of MN-08 could prevent the activation of ERK and inhibition of Akt pathways caused by glutamate. In addition, the neuroprotective effects, as well as the regulations of Akt and GSK3β, exhibited by MN-08 could be abolished with the present of phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002. These

results indicated that MN-08 provided the neuroprotective effects by inhibiting ERK pathway and attenuating the inhibited PI3-K/Akt pathway. In addition, the confocal microscopic calcium imaging demonstrated that the pretreatment of MN-08 prevented the calcium influx. Moreover, by using the patch clamp, MN-08 significantly inhibited MNDA-mediated calcium current in rat primary cultured hippocampal neurons in a concentration-dependent manner, indicating that MN-08 might block the calcium influx through the inhibition of NMDA receptor, which has been further confirmed by molecular docking simulation. Furthermore, MN-08 improved the CBF of the rat at 4th week after 2VO surgery. More importantly, the behavioral tests showed that MN-08 prevented the cognitive function impairments induced by 2VO surgery.

Conclusion: Taken together, the derivatives of memantine nitrates, MN-08, might provide multi-functional anti-dementia effects including neuroprotection and vascular dilation. These data strongly suggest that memantine nitrates might be the potential candidates for the development of a new generation of anti-dementia drugs.

(589 words)

List of publications

1. **Mak SH**, Liu Z, Wu LM, Guo BJ, Luo FC, Liu ZY, Hu SQ, Wang JJ, Cui GZ, Sun YW, Wang YQ, Zhang GX, Han YF and Zhang ZJ. (2019) Multi-functional Memantine Nitrate Attenuated Cognitive Impairment induced by Chronic Cerebral Hypoperfusion Through Neuroprotection and Promotion of Axonal Outgrowth. In preparation.
2. **Mak SH**, Liu Z, Wu LM, Guo BJ, Luo FC, Liu ZY, Hu SQ, Wang JJ, Cui GZ, Sun YW, Wang YQ, Zhang GX, Han YF and Zhang ZJ. (2019) Pharmacokinetic Studies and Neuroprotective Characterizations of Dual-functional Memantine Nitrate. In preparation.
3. Liu Z, Qiu XL, **Mak SH**, Guo BJ, Hu SQ, Wang JJ, Luo FC, Xu DP, Sun Y, Zhang GX, Cui GZ, Wang YQ, Zhang ZJ, Han YF. (2019) Multifunctional Memantine Nitrate Significantly Protects Against Glutamate-induced Excitotoxicity via Inhibiting Calcium Influx and Attenuating PI3K/Akt/GSK3beta pathway. *Chemico-Biological Interaction.s* Under revision.
4. Ke Z, Hu S, Cui W, Sun J, Zhang S, **Mak SH**, Wang J, Tang J, Pang Y, Han YF, Tong K. (2019) Bis(propyl)-cognitin potentiates rehabilitation of treadmill exercise after a transient focal cerebral ischemia, possibly via inhibiting NMDA receptor and regulating VEGF expression. *Neurochem Int.* In press.
5. Luo F, Wu L, Zhang Z, Zhu Z, Liu Z, Guo B, Li N, Ju J, Zhou Q, Li S, Yang X, **Mak SH**, Han YF, Sun Y, Wang Y, Zhang G, Zhang Z. (2019) A dual-functional memantine nitrate MN-08 alleviates cerebral vasospasm and brain injury in experimental subarachnoid hemorrhage models. *Br J Pharmacol.* In press.
6. Weber A*, **Mak SH***, Berenbaum F, Sellam J, Zheng YP, Han YF, Wen CY. (2019) Association between osteoarthritis and increased risk of dementia: A systemic review and meta-analysis. *Medicine (Baltimore)*. 98(10):e14355. *The authors contributed equally to this work.
7. Hu SQ*, **Mak SH***, Zuo X, Li H, Wang Y, Han YF. (2018) Neuroprotection Against MPP^+-Induced Cytotoxicity Through the Activation of PI3-K/Akt/GSK3β/MEF2D Signaling Pathway by Rhynchophylline, the Major Tetracyclic Oxindole Alkaloid Isolated From Uncaria rhynchophylla. *Front Pharmacol.* 9:768. *These authors have contributed equally to this work
8. Chen HX, Xiang SY, Huang L, Lin JJ, Hu SQ, **Mak SH**, Wang C, Wang QW, Cui W, Han YF (2018) Tacrine(10)-hupyridone, a dual-binding acetylcholinesterase inhibitor, potently attenuates scopolamine-induced impairments of cognition in mice. *Metab Brain Dis.* 33(4):1131-1139.

9. Hu SQ, Hu H, **Mak SH**, Cui G, Lee M, Shan L, Wang Y, Lin H, Zhang Z, Han YF. (2018) A Novel Tetramethylpyrazine Derivative Prophylactically Protects against Glutamate-Induced Excitotoxicity in Primary Neurons through the Blockage of N-Methyl-D-aspartate Receptor. *Front Pharmacol.* 12;9:73.

10. Qian S, He L2, Wang Q, Wong YC, **Mak SH**, Ho CY, Han YF, Zuo Z. (2018) Intranasal delivery of a novel acetylcholinesterase inhibitor HLS-3 for treatment of Alzheimer's disease. Life Sci. 207:428-435.

11. Liu K, Lin HH, Pi RB, **Mak SH**, Han YF, Hu YJ. (2018) Research and development of anti-Alzheimer's disease drugs: an update from the perspective of technology flows. *Expert Opin Ther Pat.* 28(4):341-350.

12. Guo BJ, Hu SQ, Zheng C, Wang H, Luo FF, Li H, Cui W, Yang X, Cui GZ, **Mak SH**, Choi TC, Ma ED, Wang YQ, Lee SMY, Zhang ZJ, Han YF. (2017) Substantial protection against MPTP-associated Parkinson's neurotoxicity in vitro and in vivo by anti-cancer agent SU4312 via activation of MEF2D and inhibition of MAO-B. *Neuropharmacology.* 126:12-24.

13. Jiajia L*, **MAK SH***, Jiacheng Z, Jialing W, Dilin X, Shengquan H, Zaijun Z, Qinwen W, Han YF, Cui W. (2017) Assessment of Neuronal Viability Using Fluorescein Diacetate-Propidium Iodide Double Staining in Cerebellar Granule Neuron Culture. *J Vis Exp.* 2017 May 10;(123). *The authors contributed equally to this work.

14. Xu DP, Chen HY, **Mak SH**, Hu SQ, Tsim KW, Hu YJ, Sun Y, Zhang G, Wang YQ, Zhang ZJ, Han YF. (2016) Neuroprotection against glutamate-induced excitotoxicity and induction of neurite outgrowth by T-006, a novel multifunctional derivative of tetramethylpyrazine in neuronal cell models. *Neurochem Int.* 99:194-205.

15. Hu SQ, Wang L, **Mak SH**, Zhang Z, Xu DP, Li H, Li Y, Hu YJ, Lee SM, Wang YQ, Han YF. (2016) Potent Protection Against MPP+-Induced Neurotoxicity via Activating Transcription Factor MEF2D by a Novel Derivative of Naturally Occurring Danshensu/Tetramethylpyrazine. *Neuromolecular Med.* 18(4):561-572.

16. Yu J, Zheng J, Lin J, Jin L, Yu R, **Mak SH**, Hu S, Sun H, Wu X, Zhang Z, Lee M, Tsim W, Su W, Zhou W, Cui W, Han YF, Wang Q. (2016) Indirubin-3-Oxime Prevents H2O2-Induced Neuronal Apoptosis via Concurrently Inhibiting GSK3β and the ERK Pathway. *Cell Mol Neurobiol.* In press

17. Huang L, Lin J, Xiang S, Zhao K, Yu J, Zheng J, Xu DP, **Mak SH**, Hu SQ, Nirasha S, Wang C, Chen X, Zhang J, Xu S, Wei X, Zhang Z, Zhou D, Zhou W, Cui W, Han YF, Hu Z, Wang QW. (2016) Sunitinib, a Clinically Used Anticancer Drug, Is a Potent AChE Inhibitor and Attenuates Cognitive Impairments in Mice. *ACS Chem Neurosci.* 7(8):1047-56.

18. Yan L, Hu Q, **Mak SH**, Lou J, Xu SL, Bi CW, Zhu Y, Wang H, Dong TT, Tsim KW. (2015) A Chinese herbal decoction, reformulated from Kai-Xin-San, relieves the depression-like symptoms in stressed rats and induces neurogenesis in cultured neurons. *Sci Rep.* 6:30014.

19. Zhao Y, Xu P, Hu S, Du L, Xu Z, Zhang H, Cui W, **Mak SH**, Xu DP, Shen J, Han YF, Liu Y, Xue M. (2015) Tanshinone II A, a multiple target neuroprotectant, promotes caveolae-dependent neuronal differentiation. *Eur J Pharmacol.* 765:437-446.

20. Hu SQ, Wang R, Cui W, **Mak SH**, Li G, Hu YJ, Lee MY, Pang YP, Han YF. (2015) Dimeric bis (heptyl)-Cognitin Blocks Alzheimer's β-Amyloid Neurotoxicity Via the Inhibition of Aβ Fibrils Formation and Disaggregation of Preformed Fibrils. CNS Neurosci Ther. 21(12):953-61.

21. Xu ML, Bi CW, Cheng LK, **Mak SH**, Yao P, Luk WK, Lau KK, Cheng AW, Tsim KW. (2015) Reduced Expression of P2Y2 Receptor and Acetylcholinesterase at Neuromuscular Junction of P2Y1 Receptor Knock-out Mice. *J Mol Neurosci.* 2015 Nov;57(3):446-51.

22. Zhao Y, Xu P, Hu S, Du L, Xu Z, Zhang H, Cui W, **Mak SH**, Xu D, Shen J, Han YF, Liu Y, Xue M. (2015) Tanshinone II A, a multiple target neuroprotectant, promotes caveolae-dependent neuronal differentiation. Eur J Pharmacol. 765:437-446.

23. Hu SQ, Cui W, **Mak SH**, Xu DP, Hu YJ, Tang J, Choi C, Lee MY, Pang Y, Han YF. (2015) Substantial Neuroprotective and Neurite Outgrowth-Promoting Activities by Bis(propyl)-cognitin via the Activation of Alpha7-nAChR, a Promising Anti-Alzheimer's Dimer. *ACS Chem Neurosci.* 6(9):1536-45.

24. Chang L, Cui W, Yang Y, Xu SJ, Zhou WH, Fu HJ, Hu SQ, **Mak SH**, Hu JW, Wang Q, Ma Victor PY, Choi CL, Ma Edmond DL, Tao L, Pang YP, Michael J. Rowan, Roger Anwyl, Han YF, Wang QW. (2015) Protection against β-amyloid-induced synaptic and memory impairments via altering β-amyloid assembly by bis(heptyl)-cognitin. *Sci Rep.* 5:10256.

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Shinghung MAK

30 June, 2019

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List of abbreviations

2-VO	2-vessel occlusion
Aβ	beta-amyloid
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
Akt	protein kinase B
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl- 4-isoxazolpropionic acid
APP	beta-amyloid precursor protein
APOE 4	apolipoprotein ε4
ATM	ataxia telangiectasia mutant
BChE	butyrylcholinesterase
BDNF	brain-derived neurotrophic factor
Bcl-2	B-cell lymphoma 2
CAS	catalytic anionic site
CBF	cerebral blood flow
Cdk5	cyclin dependent-kinase 5
CGNs	cerebellar granule neurons
ChAT	choline acetyltransferase

CVD	cerebral vascular disorders
DIV	days *in vitro*
eNOS	endothelial nitric oxide synthase
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
fAD	familial AD
FDA	United State Food and Drug administration
GABA	γ-aminobutyric acid
GSK	glycogen synthase kinase
HSF1	Heat shock factor-1
IGF-1	insulin-like growth factor-1
iNOS	inducible nitric oxide synthase
ISDN	Isosorbide dinitrate
JAK,	janus kinase
JNK	c-Jun N-terminal kinase
KA	kainite acid
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein/extracellular signal-regulated kinase kinase

mGluR	metabotropic glutamate receptors
NFTs	neurofibrillary tangles
NF-κB	nuclear factor-κB
NGF	nerve growth factor
MTDL	multi-targeted directed ligand
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptors
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PAS	peripheral anionic site
PBS	phosphate-buffered saline
PI3-K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PS	presenilin
RNS	reactive nitrogen species
ROS	reactive oxygen species
sAD	sporadic AD
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate

STAT	signal transducers and activators of transcription
TNF-α	tumor necrosis factor-alpha
TREM 2	Triggering Receptor Expressed on Myeloid cells 2
UPS	ubiquitin-proteasome system
VaD	vascular dementia
VEGF	vascular endothelial growth factor
WHO	World Health Organization

Chapter I
Introduction

1. Overview

N-methyl-D-aspartate (NMDA) receptor mediates many important physiological functions in central nerve system, including synaptic plasticity, which is important in learning and memory [1]. However, the overactivation of NMDA receptors has been associated with the excitotoxicity, causing the neurodegenerative disorders, such as Alzheimer's disease (AD), vascular dementia (VaD) and stroke. Hence, NMDA receptor has be implied to be one of the therapeutic targets in treating neurodegenerative disorder.

Dementia, characterized by the impairments of cognitive functions, has been emerged as the fourth leading causes of death worldwide. AD and VaD are the two most common forms of dementia among the elderly. According to World Health Organization (WHO) statistics, 65.7 million people will suffer from dementia, and the estimated medical cost will be around US$600 billion in 2030 [2]. It is no doubt that dementia not only poses a substantial health and economic burden on society; but also severely affect the quality of life of AD patients. Unfortunately, the currently available drugs for treating dementia still limited, since the exact causes of AD and VaD remain to be elucidated. Increasing evidence has shown that AD and VaD could be caused by neurodegeneration in a multi-factorial nature [3]. The existing single target drugs might only offer limited symptoms relieve effects.

AD is currently considered as the most common form of dementia, and accounts for approximately 60% of all dementia cases; whereas VaD is the second most common

cause of acquired cognitive impairments accounting for approximately 30% of all dementia cases [4]. Dementia causes chronic and progressive cognitive decline to the patients and cannot be cured currently. The pathological progression of dementia is slow, making it difficult to be diagnosed, especially during the early stage. The exact causes of dementia are still remaining unknown. However, it has been generally accepted that neuronal loss, induced by variety of factors, might be one of the most important causes, which greatly contributes to the pathological progress of neurodegenerative disorders, including AD and VaD. Thus far, only 5 drugs have been approved by FDA to treat AD, and yet no specific drug for treating VaD.

In the following literature review, AD will be firstly focused from the etiology to the pathogenic mechanisms, as well as the therapeutics. Secondly, the pathological features of VaD will be briefly introduced. Previous studies have further emphasized the important role of excitotoxicity, mediated by NMDA receptor, as the final common pathway in the pathogenesis of these neurodegenerative disorders. Thirdly, the background of the NMDA receptor, including the structure, major functions, modulation, as well as the coupling to excitotoxicity, will be particularly introduced as research background knowledge of the thesis. Finally, the background information associated with nitric oxide (NO), memantine nitrates and cerebral vascular disorders (CVD) will be shown as the foundations of this study followed by the objectives of this project.

1.1 The introduction to Alzheimer's disease

AD is a progressive neurodegenerative disorder characterized by the irreversible impairments of cognitive functions[5] [6]. The prevalence studies show that AD mainly affects the aged population, evidenced by 7 - 10% incidence in the group over age 65 and increased to 40% in the group over age 80. The number of AD patients dramatically increases with 4.6 million new cases worldwide in 2050. As the rapid growth of the aged population in China, the numbers of AD patient starting with a high base of AD patients (6 million) will experience a rapid increase growth (314 - 336%) and reach 26.1 million on 2040 [7]. Besides, AD is also placing a socioeconomic burden on the societies. According to recent estimation, about 13% of the people in the age group over 65 were AD patient, while the figures dramatically raise to 43% in the aged group over 85. On the other hand, the early-onset AD cases were also increased, resulting in the increased of dementia patients in the youth populations. AD is one of the most costly diseases in the world. As AD could affect the quality of the daily life of patients, the medication and care service might require intense attention from Governments and society. The total cost for daily care and hospice services for AD patients were estimated to be $236 billion USD in 2016 [8]. The situation will only be getting worse, especially in the developed countries, as the long-life expectancy and low fertility rate. Thus, the recent prediction of the total cost for AD is estimated to be 9.12 trillion USD in 2050 [9].

AD is not the normal aging. The language skills and cognitive function of AD patients is dramatically decline, as compared to the normal aging population (Fig. 1.1). AD is

usually divided into familial AD (fAD) and sporadic AD (sAD) according to different pathologies [10]. Only 5% of the AD cases is fAD, which might be caused by genetic mutations in β-amyloid precursor protein (APP), Presenilin 1 (PS/1) and/or Presenilin 2 (PS/2) [11]. While nearly 95% of patients with AD are classified as sAD, which are caused by a combination of genetic factors and environmental risk factors. Various risk factors for sAD include insulin desensitization/resistance state, autophagy dysfunction, oxidative stress, lipid metabolism abnormalities, neuroinflammation, gut microbiota disorders, synapse dysfunction, hyperphosphorylation of tau protein, and deposition of Aβ in the brain. The apolipoprotein ε4 (APOE 4) and the Triggering Receptor Expressed on Myeloid cells 2 (TREM 2) have been regarded as two major risk factors for sAD [12].

The exact causes of AD are still unclear. However, phosphorylation of tau protein, accumulation of Aβ, neuronal loss and oxidative stress have been associated with the pathology of AD. The pathological process of AD involves several abnormal changes and degeneration in the brain. Extracellular amyloid plaques accumulation, intracellular neurofibrillary tangles are the most important pathological hallmarks for AD diagnosis. In addition, the abnormal regulations of cellular or molecular pathways, especially in the regions liked hippocampus mediating higher mental functions, might play an important role in the AD pathogenesis. These damages may eventually lead to dementia. However, the exact causes or the underlying mechanisms need to be further elucidated.

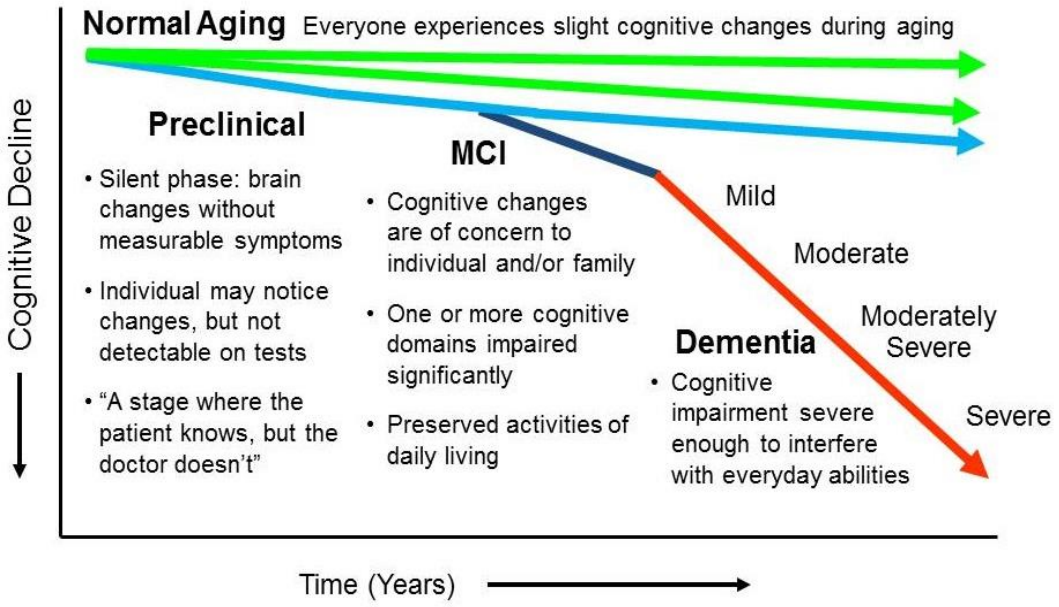

Fig. 1.1 The cognitive function of AD patients dramatically decline compared to normal aging population. (Adapted from the website of *UC Irvine Institute for Memory Impairments and Neurological Disorders)*

The severity of AD can be divided according to the remarkable neuronal loss in the brain and further damage in language ability, emotion expressing and cognitive function [13]. In detail, AD can be divided into four stages, including the pre-dementia, early, moderate and advanced stages [14].

In the *pre-dementia* stage, the most noticeable symptom is memory impairments. For example, the patients may have the difficulty in remembering recently learned facts and inability to acquire new information. This stage is often neglected and regarded as the normal aging. In the *early dementia* stage, the patients may experience obvious symptoms. Patients might frequently show the deficits of cognitive functions, learning and memory in the daily life. In addition, they fail to perform complicate tasks. When the AD patient enter to the *moderate dementia stage*, more severe impairments of short-term memory, but not long-term memory, will be observed[15]. Patients may lose the language ability, as well as the logical thinking, planning and organizing abilities. The patients might not perform even the simple sequence of actions due to the impairments. In addition, the personality of patients might be changed, and become more aggressive and restlessly performing aimless activities. In the *advanced dementia stage*, both short-term and long-term memories of the patients are severely lost. The advanced dementia patients may ultimately loss the daily living skills. They cannot perform the simplest tasks without assistance. Muscle mass and mobility deteriorate to the point where they are bedridden, and they lose the ability to feed themselves[14].

Currently, there is no effective treatment for AD. The only available clinical treatments offer symptomatic relief benefits but not reducing palliative in nature[16]. Furthermore, since the AD patients might lose their daily living skills, the nurse care is very important. AD patients deeply rely on their family or nurse for assistance in their daily life. As the results, AD has brought the great pressures involving social, psychological, physical, and economic elements to the society [17,18].

1.2 Hypotheses for the pathogenesis of AD

The pathological features of AD include excessive phosphorylated tau protein found in cells, neurofibrillary tangles (NFTs) found in the cortex and subcortex, extracellular amyloid plaques, neuronal loss and brain atrophy [19] [20]. The exact causes for AD still remain unclear; however, increasing evidences have shown that AD, as well as the other neurodegenerative disorders, are multi-factorial (Fig. 1.2). There are several hypotheses that have been proposed to contribute the pathogenesis of AD.

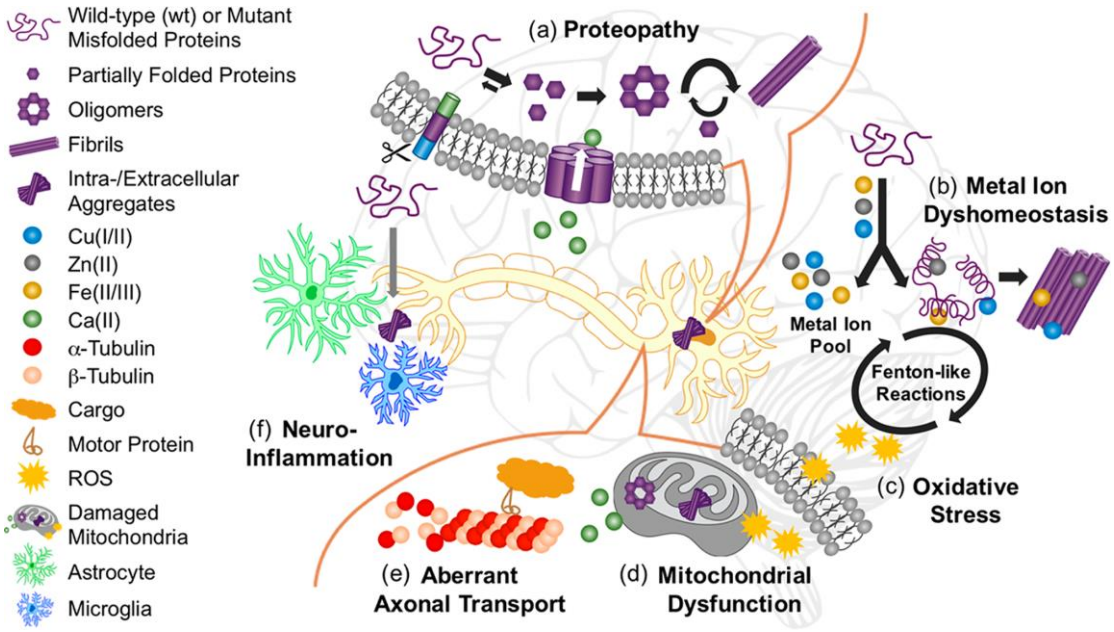

Fig. 1.2 Schematic illustrates the multifactorial nature of neurodegenerative disorders. (Adapted from G Savelieff et al 2019 [3])

1.2.1 Cholinergic hypothesis

The first proposed hypothesis of AD pathology is the cholinergic hypothesis proposed by Davies *et al* in 1976 [21]. This hypothesis proposes that AD might be caused by the selectively degeneration of cholinergic neurons, resulting in the deficit of production of acetylcholine (ACh) (Fig. 1.3). ACh is one the most important neurotransmitters in the brain. In physiological conditions, cholinergic neurons might play an important role in regulating synaptic long-term plasticity, which might be the key for cognitive functions, especially in learning and memory [22,23]. Furthermore, with the progressive degeneration of the cholinergic neurons, the expression of choline acetyltransferase [24], the specific enzyme to produce ACh in the presynaptic neurons, is also downregulated, thus further worsening the cholinergic dysfunctions. To restore the weakening cholinergic transmission, many potent AChE inhibitors have been developed for treating AD. The AChE inhibitors might prolong the duration of ACh remaining in the synapse by preventing the degradation of ACh [25]. Although most anti-AD drugs approved by FDA are AChE inhibitors, the drugs can only relief the symptoms of AD [26 27] . Some studies believed that hyperphosphorylation of tau proteins and synthesis of Aβ might be more important or contribute more to the progress of AD [28]. Furthermore, increasing evidence has shown that intracellular AChE may mediate neuronal apoptosis induced by stimuli[29]. In AD brains, although the total amount of AChE has been decreased, the enzymatic activity of AChE has been maintained around the Aβ plaques, which may be explained by the interaction of Aβ [30 31].

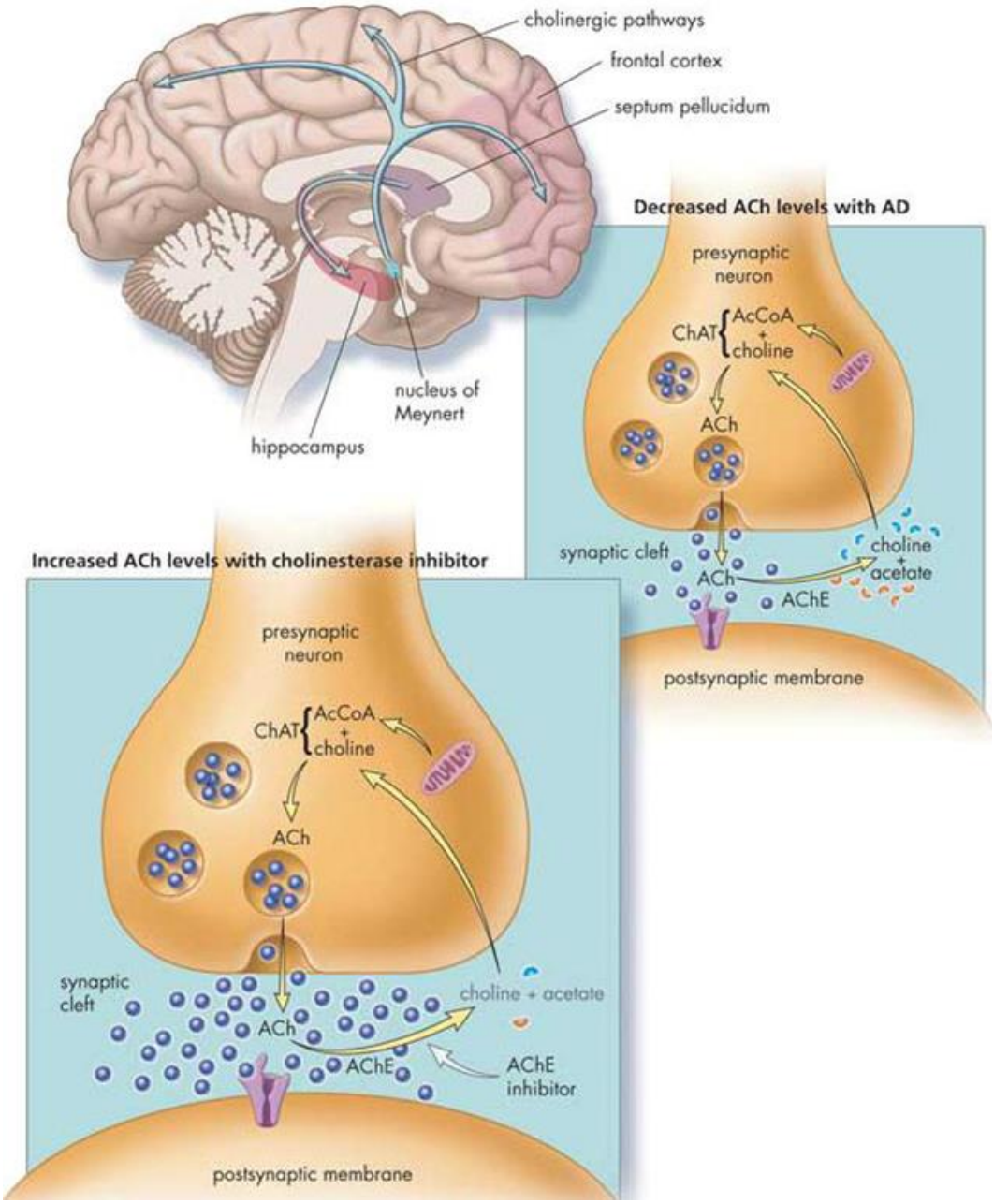

Fig. 1.3 AChE inhibitors could increase ACh in synaptic cleft by inhibiting AChE. Levels of ACh are low in the brains of patient with AD. AChE inhibitors partially correct the deficit by blocking the action of AChE, and thereby increasing the amount of ACh that remains in the synaptic cleft. Adapted from David B. *et al*, 2005[32].

1.2.2 Amyloid β hypothesis

The abnormal plaque of Aβ is the notable pathological change in AD patients, leading to the establishment of Aβ toxicity hypothesis proposed by Glenner *et al* in 1984 [33]. The Aβ hypothesis speculated that AD might be caused by the deposition of Aβ in brain, consequently inducing the cognitive impairments. In the normal conditions, β-amyloid precursor protein (APP) is cleaved by α-secretase, followed by γ-secretase (Fig. 1.4) [34] [35]. Alternatively, in pathological condition, APP is sequentially cleaved by β- and γ-secretases, producing Aβ, which has been widely accepted to play an important role in the pathogenesis of AD [34] [36] [37]. The Aβ hypothesis has been supported by the evidence that abnormal aggregation of Aβ might induce neurodegeneration both *in vitro* [38] and *in vivo* [39-41]. Recently, studies have proposed that the amyloid plaques are less toxic than the other aggregation forms, such as oligomer [42]. Aβ monomers are easily aggregated and form fibrils or oligomers, which is through to be the major source of neurotoxins [42] [35]. The soluble Aβ oligomers, which are proposed to be highly toxic, might initiate the neuronal degeneration by multiple mechanisms. Studies have shown that soluble Aβ oligomers might disrupted the Ca^{2+} homeostasis by interacting with the extra synaptic NMDA receptors [43]. Moreover, *in vitro* experiments also showed that Aβ might self-replicate like prions [44]. The excessive soluble Aβ oligomers might cause synaptic dysfunctions, which have been through to be one of the major contributions to the cognitive function impairment in AD.

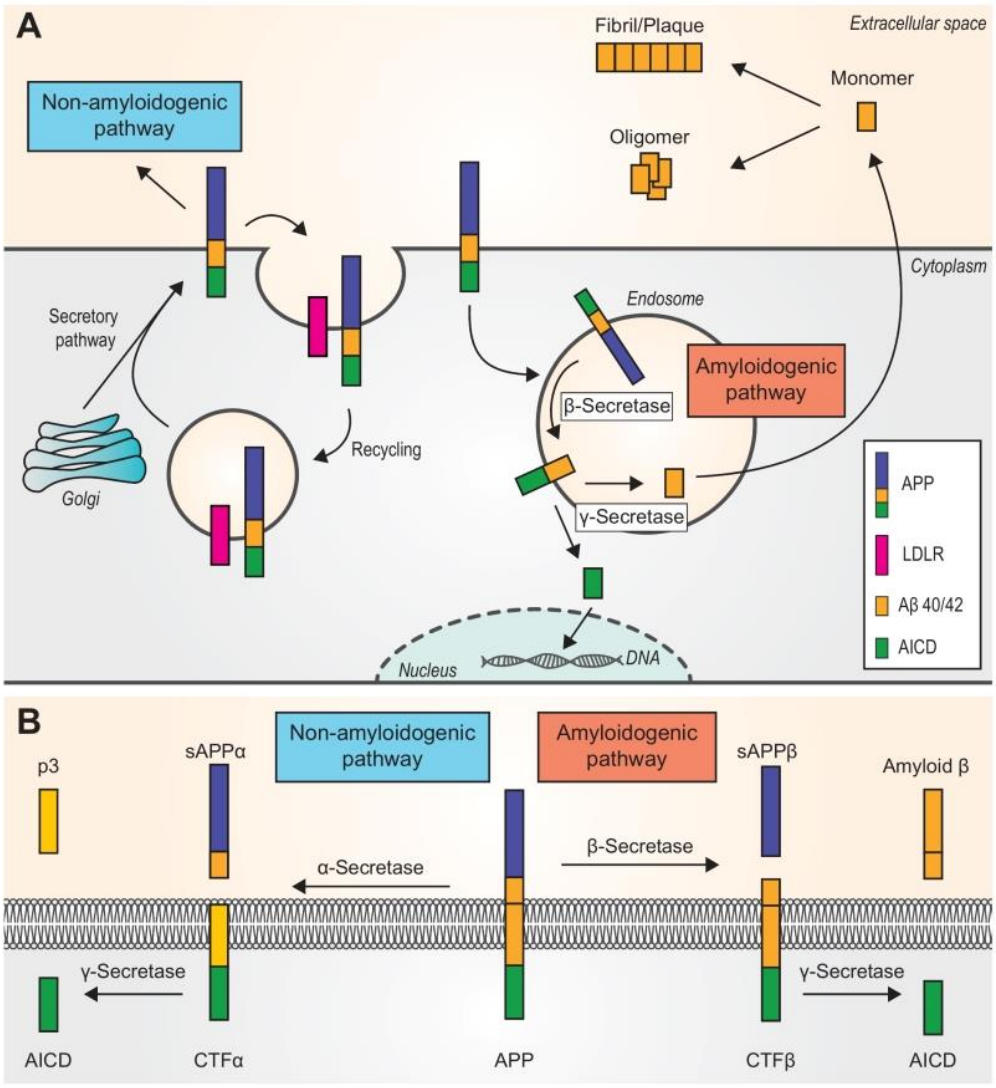

Fig. 1.4 Schematic illustration to show the cleavage of APP by α-secretase, β-secretase or γ-secretase. (A) The majority of APP is processed via the non-amyloidogenic pathway: cleaved by α-secretase and followed by γ-secretase. (B) Alternatively, APP could be processed via the amyloidogenic pathway: cleaved by β-secretase and followed by γ-secretase. As the results, monomeric Aβ40/42 could be produced and further aggregated to form the oligomer and fibril. (Adapted from YX Zhou et al 2018 [45])

1.2.3 Tau protein hypothesis

The hyperphosphorylation of tau protein has been proposed to be another crucial mechanism of AD pathology. Tau protein, mainly found in axon, is responsible for stabilizing the of microtubules (Fig. 1.5). There are 6 isoforms of tau protein that are encoded by the gene located in chromosome 17. As hyperphosphorylated tau protein continues accumulated, it might migrate to the dendrites and insoluble NFTs, that might further deteriorate the cellular trafficking system and hyperphosphorylated the microtubules. The accumulation of dendritic hyperphosphorylated tau protein is believed to enhance the toxicity of Aβ to postsynaptic compartment of neurons. Some studies showed that Aβ accumulation might lead to the hyperphosphorylation of tau protein, resulting in disruption of the microtubules [46]. The increased Aβ toxicity might further stimulate the hyperphosphorylation tau protein, resulting in a vicious circle. Moreover, recent researches have revealed that Aβ and phosphorylated tau protein might cause defective autophagy and mitophagy in AD pathogenesis [47].

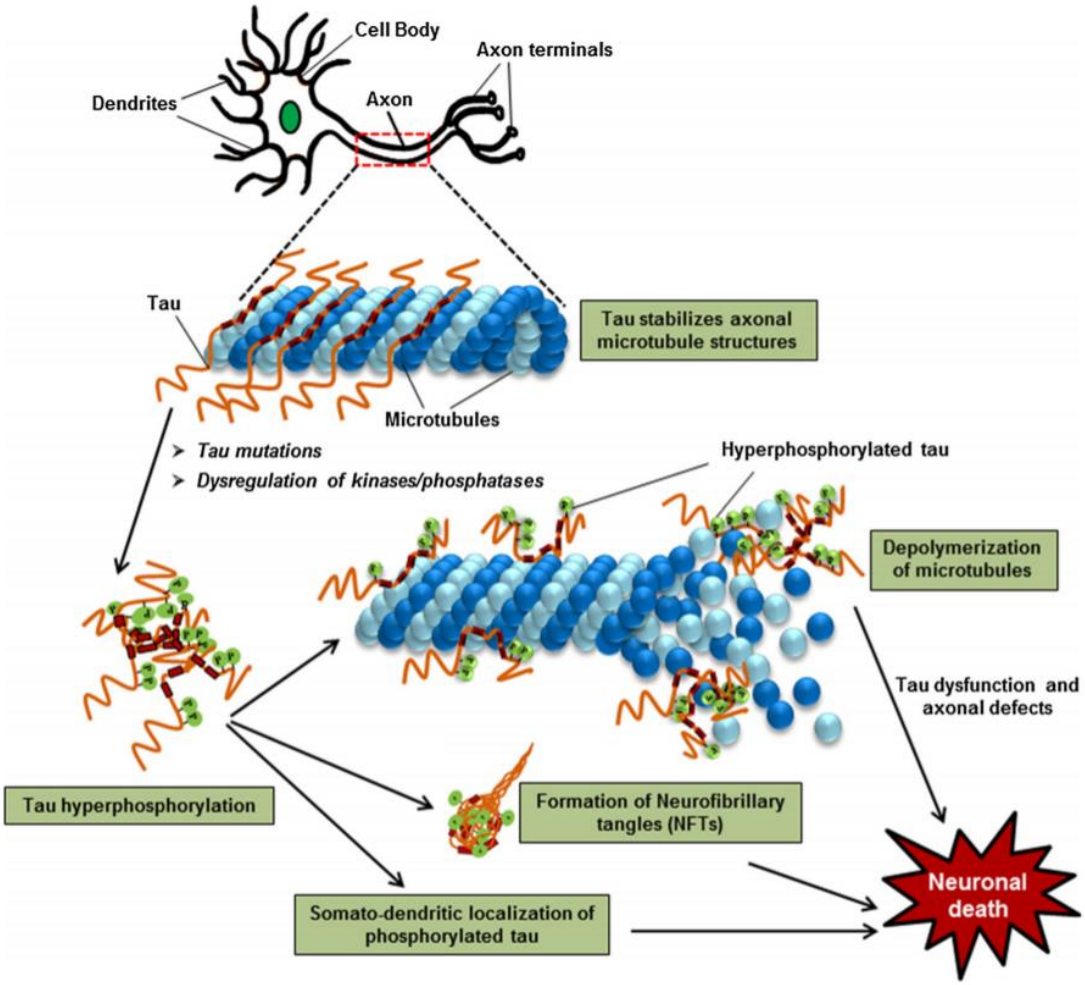

Fig. 1.5 Tau protein hypothesis. Tau protein is mainly found in axon for the stabilization of microtubules. As hyperphosphorylated tau protein continues accumulated, it might migrate to the dendrites and form insoluble NFTs, which might further deteriorate the cellular trafficking system and hyperphosphorylated the microtubules. (Adapted from S Sarkar 2018 [48])

1.2.4 Glutamate-induced excitotoxicity

Many studies indicated that glutamate-induced excitotoxicity might greatly contribute to the pathological progression of AD [49] [50] [51]. The levels of pyramidal cells and NMDA receptors were show by studies to decrease in AD brain, proposing that the selective neuronal loss might has induced by specific neurotoxin. Glutamate was suspected to be the toxin which might cause the specific loss of both NMDA receptors and glutamatergic neurons[51]. Although the GABA receptors can be also found in the large pyramidal cells, they are more distal from the terminals of dendrites; therefore, NMDA receptor and glutamatergic neurons are preferentially lost [52].

Glutamate serves as the excitatory neurotransmitter, playing an important role in signal transmission in neocortex and hippocampus. It has been found that NMDA receptors mediate many physiological functions including learning and memory [53]. Normally, the release of glutamate activates the post-synaptic NMDA receptors, leading to influx of Na^+ and Ca^{2+} through the ion channels (Fig. 1.6). NMDA receptor is a heterotetramer formed by two types of subunits NR1 and NR2 [50]. These components form a superimposed tertiary structure. In the N-terminal of the subunits, there are many binding sites for the specific ligands, such as glutamate, NMDA, glycine and AMPA. Moreover, NMDA receptor regulates the transition of Ca^{2+} and Na^+ across the cell membrane. At the resting state, Mg^{2+} blocks the channel by binding to the Mg^{2+} blocking site, which may dissociated from the biding site in depolarization [54]. The

excitatory signal might be terminated by the highly efficient uptake system for glutamate on membranes of both pre-synaptic and post-synaptic neurons.

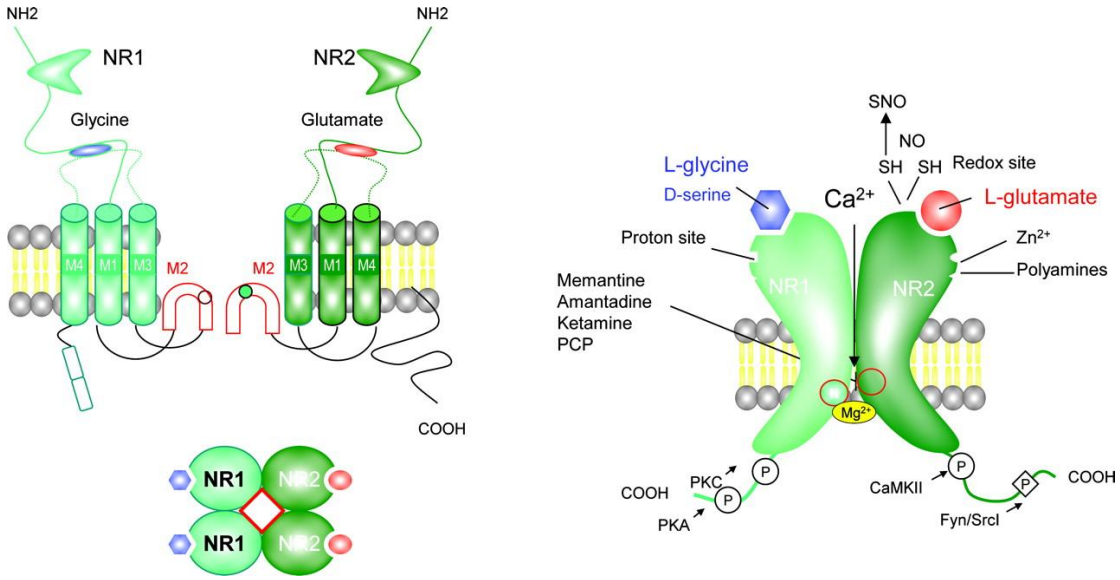

Fig. 1.6 Structure and components of NMDA receptor. The schematic diagram shows the structure and possible binding sites of the agonist (glutamate) and antagonists (memantine and ketamine) of NMDA receptors (Adapted from E. Benarroch 2011 [50])

Glutamate-induced excitotoxicity has been proposed as the major cause for neuronal loss in AD brain [55]. The excessive glutamate could over-stimulate the NMDA receptors and result in the overload of Ca^{2+}. The prolonged influx of Ca^{2+} might cause the dysregulations of the downstream signaling pathways. For examples, the overaction of ERK pathway and suppression of PI3K/Akt pathway are observed by the influx of Ca^{2+}, consequently resulting in apoptosis [55].

The excessive influx of Ca^{2+} might cause the over-activation of ERK, which is coupled with the NR2B subunit directly [56]. The over-activation of ERK pathway has been suggested to promote apoptosis in the response to variety of stimuli [57]. Furthermore, the activation of GSK3β through the suppression of PI3K/Akt pathway by glutamate has been involved in the glutamate insults. Many studies have suggested the phosphorylation of Akt might be inhibited after the exposure of glutamate. As a result, GSK3β, a pro-apoptotic downstream member of Akt might be also activated (Fig. 1.7) [58].

Recent studies suggested that Aβ might stimulate the extra-synaptic NMDA receptor, followed by stimulating the cleavage of APP to Aβ. These findings might provide the linkage between the glutamatergic hypothesis and Aβ hypothesis [55]. It is also suggesting the dysfunction of glutamate transporter 1 (GLT-1) could be caused by oxidative stress, which might provide the linkage of thee glutamatergic hypothesis to oxidative stress hypothesis [59].

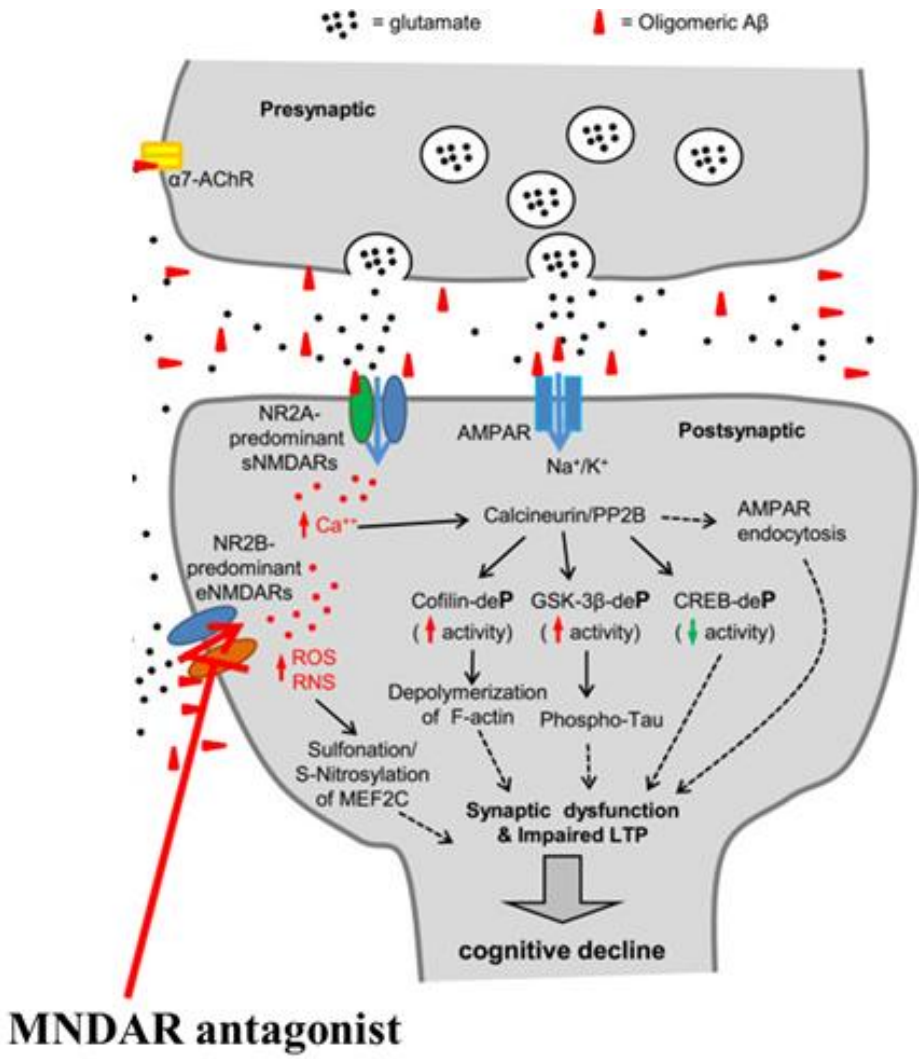

Fig. 1.7 Schematic illustration of some apoptotic pathways mediated by the NMDA receptor. Excess glutamate activates the NMDA receptors, leading to the excess amount of Ca^{2+}, activating several downstream signaling pathways leading to apoptosis, also accompany the oxidative stress. (Adapted from S Tu et al 2014 [42].)

1.2.6 Oxidative Stress

Oxidative stress has been proposed as another important cause of neuro loss in AD pathogenesis [60]. The oxidative stress responded to multiple stimuli contributed mainly to the earlier stage in AD, even earlier than the significant plaque pathology onset (Fig. 1.8) [61]. A variety of oxidative stress markers are found in the AD brain, especially in the plaques and NFTs [59]. Lipid peroxidation has been proposed to be the major source of oxidative stress, occurring in the brain of AD patients [62]. Aβ has been proposed to be the another source, which might induce oxidative stress from its methionine residue, causing neuronal apoptosis by the generation of ROS in hippocampal neurons and cortical synaptosomal membranes [63]. Furthermore, Aβ-induced oxidative stress might promote Aβ deposition, up-regulate genes related to apoptosis and mitochondrial metabolism in transgenic APP mice [64].

Abnormal metal ion homeostasis is another important cause of oxidative stress in AD. It has been reported that higher concentrations of zinc, copper and iron are found in the AD brains [65]. Iron and copper, as the redox-active metals, may cause neurotoxicity by generating hydroxyl radial from the interaction of their reduced transition forms (iron (II) and copper (I)) with H_2O_2. Moreover, the inactivation of ROS scavenger may lead to metal ions-induced oxidative damage [66]. Furthermore, the high affinity of metal ions and Aβ might form the hyper-metalized peptides, which is resistant to be clearance. These hyper-metalized Aβ might consequently induce the relevant high concentration of ROS and cause neuronal apoptosis [34].

Moreover, many signaling pathways might be involved in oxidative stress-induced alterations in gene expression and enzyme activity. The stress-activated protein kinase (SAPK) pathways, which are involved in propagating the stress intracellular signals within the cell. The c-Jun N-terminal kinase (JNK)-SAPK pathway may also induce phosphorylation of tau protein and formation of NFTs.

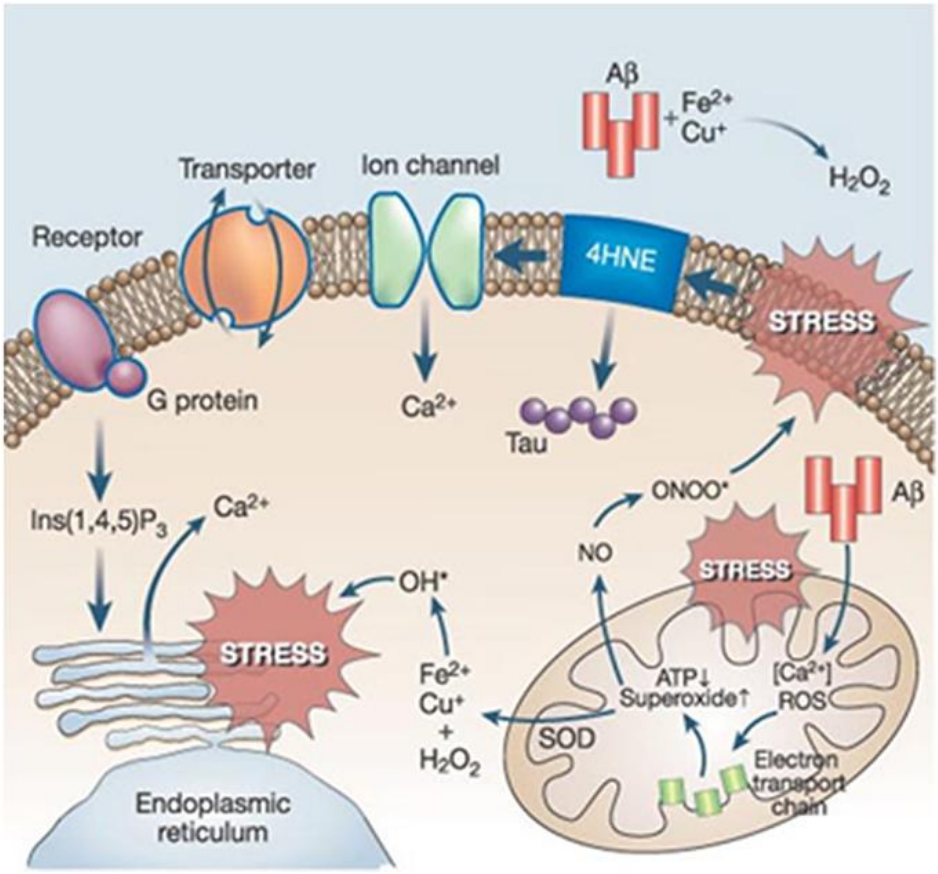

Fig. 1.8 The schematic illustration shows the possible connections between oxidative stress and other key players in AD. Abnormal metal ion homeostasis, hyperphosphorylation of tau protein and aggregation of Aβ have been linked to the elevation of oxidative stress, and consequently leading to neuronal loss. (Adapted from Mattson *et al*, 2004 [60])

1.2.7 Neuroinflammation

Neuroinflammation is important neuropathological changes, which has been correlated to the pathogenesis of AD [67]. The astrogliosis and the inflammation-related markers have been found in the AD brain, especially surrounding with the amyloid plaques [68]. Studies also indicate that the microglia and astrocytes hyperactivation might be strongly associated with amyloid plaques formation. The activated microglia and astrocytes were observed closely with the senile plaques in AD brain [68,69]. The accumulation of Aβ plagues and tau neurofibrillary tangles might further induce the activation of microglia, which produce inflammatory cytokines and cause neuronal cell death (Fig. 1.9).

Inflammatory cytokines might play an important role to promote the inflammation and formation of Aβ. In addition, some studies indicated that the excess tumor necrosis factor-alpha (TNF-α), one of the pro-inflammatory cytokines present in the center nerve system (CNS), might induce the synaptic dysfunctions caused by Aβ oligomers. Furthermore, it was found that the number of receptor of TNF-α was elevated in the AD brain, suggesting that TNF-α plays pivotal roles during the pathological progress of AD [70]. As a result of the activation of TNF-α, the nuclear factor kappa B (NF-κB) might consequently be activated, leading to an increased level of formation of senile plaques in neurites. Based on this hypothesis, inhibition of the inflammatory cytokine TNF-α utilizing by TNF-α inhibitor might significantly improve the cognitive functions [71].

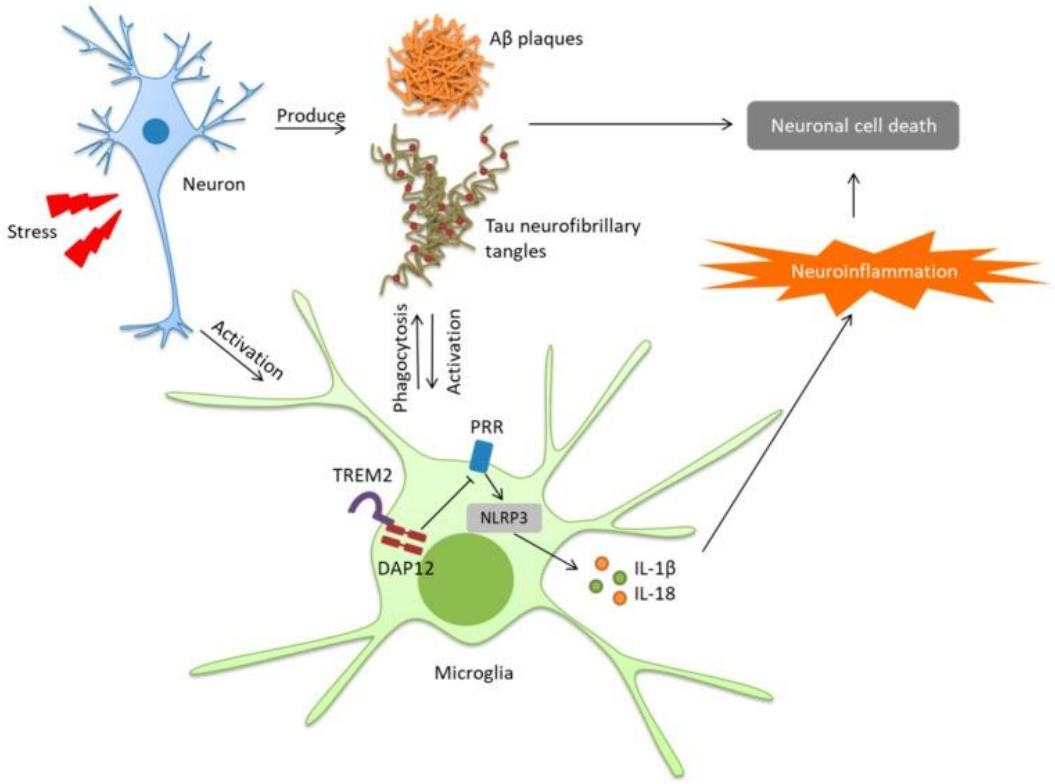

Fig. 1.9 Microglia induced neuroinflammation in AD. Accumulation of Aβ plagues and tau neurofibrillary tangles might induce microglial M1-like activation, which produce inflammatory cytokines and cause neuronal cell death. Meanwhile, M2-like microglia is able to reduce Aβ plagues and tau neurofibrillary tangles accumulation by phagocytosis. (Adapted from Dong Y *et. al.* 2019 [72])

1.3 Current drugs for Alzheimer's disease

Based on the hypothesis mentioned above, many potential anti-AD drugs have been developed; however, the choice for effectively treating AD still limited. Currently, there are only 5 drugs classified into 2 groups have been approved by FDA to ameliorate cognitive symptoms of AD. The first group is AChE inhibitors and the second group is NMDA receptor antagonist [73]. Unfortunately, the existing anti-AD drugs can only manage the symptoms of AD, but not delay or stop the pathological process.

1.3.1 AChE inhibitors

The present of AChE inhibitors might increase the ACh level in synaptic cleft and maintain the cholinergic transmission by inhibiting the AChE activity. The declined level of ACh production can be compensated, thus boosting the cholinergic transductions [16]. Based on the cholinergic hypothesis, AChE inhibitors have been developed to compensate the deficiency of ACh in the AD brain. Thus far, four AChE inhibitors have been approved by FDA for treating AD, including Cognex® (tacrine), Aricept® (donepezil), Razadyne® (galantamine) and Exelon® (rivastigmine). Tacrine, the first generation of AChE inhibitors, is a tertiary amino compound reversibly binding to the hydrophobic region of AChE. Currently, the production and marketing of tacrine has been discontinued since many studies have reported that long-term uses of tacrine may bring the problem of serious hepatotoxicity [74]. The toxicity is suspected to be caused by CYP1A2, which might convert tacrine into reactive metabolites, inducing hepatotoxicity [75].

Donepezil, galantamine and rivastigmine are the second generation of AChE inhibitors for treating AD. Donepezil is also a tertiary amino compound and mainly bind to the hydrophobic region of AChE, inhibiting the enzymatic activity in a non-competitive manner. The efficacy of donepezil is comparable with tacrine, but not exhibits high hepatotoxicity. Galantamine, a tertiary alkaloid, is the only one marketed anti-AD drug which has been proposed to exhibit dual actions on cholinergic transmission. Galantamine may inhibit AChE in a competitive manner and modulate the nicotinic receptors allosterically [76]. The adverse effect of galantamine is mild. Rivastigmine is carbamoylated complex, which might reversibly inhibit AChE. In addition, rivastigmine cannot be metabolized by P450 in liver. Rivastigmine is very safe with very mild adverse effects [66].

Taken together, the AChE inhibitors can provide transient and limited benefits to the patients with mild to moderate AD. As the progressive degeneration of cholinergic neurons continues during the pathological process of AD, the AChE inhibitors might not be effective. The major reason might be that AChE inhibitors cannot provide protective effects against the neurotoxicity. As the results, production of ACh may be continuously reduced as the number of cholinergic neuron declines. At the late stage of AD, the limited numbers of cholinergic neurons might not produce enough ACh to maintain the cholinergic transmission, even with the present of AChE inhibitors.

1.3.2 N-Methyl-D-aspartate receptor antagonist

Based on the excitotoxity hypothesis, NMDA receptor antagonists might provide neuroprotective effects by blocking glutamate-induced overstimulated extrasynaptic NMDA receptors [77]. Many NMDA receptor antagonists have shown significant neuroprotective effects in several *in vitro* and *in vivo* models associated with AD. However, NMDA receptor antagonists with high-affinity, such as MK-801, cannot be used for treating AD due to the undesirable toxicity and severe psychotropic side effects. The side effects, including psychotomimetic effects and motor dis-coordination, might result mainly from the excessive inhibition of NMDA receptor mediated signal transmission [78]. Lipton *et al* has suggested that the concept of pathological activated therapeutic agent, which might block the NMDA receptors only in the pathological condition, but not the physiological condition [79]. Memantine (Namenda ®), approved by FDA in 2003, is an un-competitive NMDAR antagonist with moderate affinity and fast-off rate [80]. Memantine could provide the neuroprotective effects against glutamate-induced excitotoxicity in the pathological condition [79]. As memantine is an open channel blocker with fast off rate, the NMDA receptor might be blocked in the normal physiological conditions. It is very important that memantine might not affect the physiological functions mediated by NMDA receptor [81]. Compared with the AChE inhibitors, memantine could be benefit for the moderate to severe stages of AD.

1.3.3 Combination of AChE inhibitors and NMDA receptor antagonists

Studies have proposed that the combinations of AChE inhibitors and NNMDA receptor antagonists might provide synergistic therapeutic benefits to the AD patients [82]. With regard to the only existing anti-AD drugs including the AChE inhibitors and NMDA receptor antagonists, it is reasonable to consider combine these two drugs to produce the synergistic therapeutic effects for treating AD. In 2014, FDA has approved a fixed combination of memantine and donepezil in a capsule (NAMZARIC®, 2014) for the treatment of moderate to severe AD [83]. The results from meta-analysis by European Academy of Neurology revealed that Namzaric, concurrently hitting AChE and NMDA receptors, synergistically improved the behaviors and mood, cognitive functions, and global outcomes when compared to those of AChE inhibitors monotherapy used in treating mild to severe AD patients [84,85].

1.4 The potential anti-AD drugs

1.4.1 Drugs targeting Aβ cascades

It was found that extracellular Aβ aggregation might be the key component contributing to the pathogenesis of AD by interfering diverse signaling pathways [86]. In the past decade, huge efforts and resources have been input for the purpose to develop the anti-AD drugs targeting Aβ aggregation. Based on the Aβ hypothesis, the researchers have tried to inhibit the biosynthesis and aggregation or enhanced the degradation of Aβ in order to reduce the accumulation of Aβ in brain.

Aβ can be synthesized by an amyloidogenic pathway resulting from the sequential cleavage of APP, first by β-secretases, and subsequently by γ-secretases. On the other hand, APP can be cleaved by α- and γ-secretases in the non-amyloidogenic pathway. To promote the non-amyloidogenic APP pathway, several α-secretases enhancers or activators have been developed. Acitretin, a retinoid drug for treating dermatologic diseases, and epigallocatechni-gallate (EGCG), a polyphenol from green tea, are proposed to be the α-secretase enhancers, promoting the non-amyloidogenic pathway.

The strategies of the inhibition of the proteolytic activities of β- and γ-secretases could reduce the synthesis of Aβ [62,63]. In the previous studies, some drug candidates, such as Lanabecestat and Verubecestat, targeting β-secretases have been demonstrated to reduce the amount of Aβ in AD patients [87] [88]. However, most of these drugs have failed in large randomized clinical trials conducted for treating mild-to-moderate AD [89]. For

example, Lanabecestat, a novel potent BACE1 inhibitor, has been demonstrated to significantly reduce concentrations of Aβ in cerebrospinal fluid and brain *in vivo* [90]. On June 12 2018, AstraZeneca and Eli Lilly and Company announced that they were discontinuing the phase 3 clinical trial of Lanabecestat. It might be because that Lanabecestat was unlikely to improve the cognitive status in the patient group [89].

Inhibition of γ-secretase might be another strategy to reduce the production of Aβ. Several γ-secretase inhibitors have been developed; however, none of them could pass the clinical trial due the serious adverse effects including weight loss and increased risk of skin cancer [91]. The reason might be that γ-secretase involved in a variety of other substrates and is responsible for many physiological functions.

Studies showed that the amount of Aβ aggregation might be reduced by Aβ inhibitors. For example, tramimprosate, originally found in seaweed, might bind to the β-sheet of Aβ, and subsequently inhibit the aggregation of Aβ. Tramimprosate has shown promising effects to reverse the inhibited long-term potential (LTP) induced by Aβ *in vitro* and *in vivo*. However, tramimprosate failed in the phase 3 clinical trial, similar to the other Aβ aggregation inhibitors.

In the past decade, a huge resource has been input to develop immunotherapies against Aβ for the treatment of AD. Both active immunotherapies (vaccination) with soluble or fibrillar form of Aβ peptides (lees toxic) and passive immunotherapies with monoclonal or polyclonal antibodies against Aβ have been shown to be effective in

reducing the Aβ in transgenic APP mice [92]. The passively administrated monoclonal antibodies, such as Bapineuzumab and Crenezumab, against Aβ might trigger the clearance or inhibit the production of Aβ by binding to the β-secretase cleavage site on APP. Although many of anti-Aβ antibodies have shown promising effects to reduce the amount of Aβ and senile plaques in CSF, hippocampus or brain, most of these drugs have been terminated in Phase III because of a lack of effects to improve cognitive functions.

Previous studies have indicated that the fibrillar form of Aβ greatly contributes to the pathogenesis of AD [5,42]. However, accumulated evidence has suggested that the oligomeric forms of Aβ could be more toxic than the other forms of Aβ including the fibrillar form [5]. The candidates with capabilities to inhibit the formation of soluble oligomeric forms of Aβ have drawn more attention in anti-AD drug development. For example, Aducanumab, a human monoclonal antibody, could inhibit soluble oligomeric forms of Aβ in Tg2576 transgenic mice. Gantenerumab, another monoclonal antibody, has also been shown to reduce the amount of cerebral Aβ in the patients with AD [93]. However, most of the other drugs have been discontinued in clinical trials. Taken together, the disappointing outcomes of immunotherapies suggest the conclusion that further studies should be conducted to confirm the contribution of Aβ during the pathological progress in AD.

1.4.2 Anti-oxidation and anti-inflammation

As mentioned in the previous parts, the neuroinflammation and oxidative stress have been involved in the pathogenesis of AD [34]. Thus, the candidates with the abilities for anti-oxidation and anti-inflammation may be important for the treatment of AD. For example, some studies showed that EGCG from the green tea and vitamin E, might be able to slow down the progression of AD [94]. Ginkgo biloba, an herbal drug with flavonoids and terpenoids, has shown significant anti-oxidative activities, and could reverse the dementia or age-associated memory impairment [95].

Studies suggested that the TNF-α-mediated pro-inflammatory signaling pathways might be involved in the pathogenesis of AD. Thus, the inhibition of TNF-α might be beneficial for the treatment of AD [70]. For example, inhibition of the inflammatory cytokine TNF-α utilizing the peri-spinal administration of etanercept may lead to sustained cognitive improvements in patients with AD [96]. In view of the identification of chronic neuroinflammation as one of the key hallmarks of AD, it is reasonable to use anti-inflammatory therapy, such as Nonsteroidal anti-inflammatory drugs (NSAIDs), cyclo-oxygenase inhibitors, to treat AD. It is also revealed that intake of NSAIDs is associated with decreased risk of AD incidence [97].

1.4.3 Other possible therapeutic approaches

In fact, since the pathogenesis of AD is unclear, the drugs for treating still limited. Many drug candidates with potential anti-AD effects have been developed and try to complete the clinical trial evaluation. Apart from the AChEIs and NMDA receptor antagonists, traditional Chinese medicine (TCM) is another huge resource for the development of multi-functional anti-AD drugs [98]. Huperzine A, an alkaloid extracted from the TCM herbal medicine *Qian Ceng Ta*, has been approved for treating AD in China [99]. Huperzine A is a potent AChE inhibitor and some studies have demonstrated that huperzine A might also provide neuroprotective effects against neurotoxins including glutamate and Aβ [100] [101]. Recently, GV-971, derived from marine glycan, is a multi-targeting oligosaccharide which claimed to offer significant therapeutic effects to the AD patients by regulating the immune system, reducing the neuro-inflammation response and improving the cognition. GV-971 has completed the phase III clinical trial in 2018 [102]. Other drugs including the estrogen, cholesterol lowing drugs, neurotrophic, and metal complexing agents might provide anti-AD effects [103] [82,83]. Furthermore, other medications including antidepressants, anxiolytics, neuroleptics and sedatives are also helpful for improving the quality of lives of AD patients. In addition, good living environment, communications and quality of daily care provided by family and caregivers might also help for the AD patients [104].

1.5 Dementia associated with vascular disorders

VaD is the second common form of dementia, accounting for 20~30 % of the total case in dementia [4]. In the early of 1900s, the researchers have found that the cerebrovascular disease (CVD) might establish dementia since the amyloid plaques were found in the blood vessel of dementia patients. Nowadays, it has been generally accepted that the multiple lesions and infracts caused by CVD might contribute to dementia alone or conjugate with AD [105].

VaD is a heterogeneous disorder mainly caused by vascular and ischemic lesions of brain regions, including cortex and hippocampus [106]. It has been closely associated with the vessel disorders in brain, such as atherosclerosis, cerebral amyloid angiopathy or cerebral small vessel disorders [107]. Moreover, the risks to develop dementia within 1 year after transient ischemic or severe stroke is higher than the people of the matched age-groups in the general population 3 months after the stroke attack [108]. Similar to AD, the common risk factors have been identified for VaD in the absence of stroke, especially aging and vascular risk [107]. The risk of VaD would be doubling every 5.3 years, while AD would be doubling every 4.5 years [109]. In fact, a meta-analysis reported that majority of AD patients might show mixed pathogenies, instead of pure AD. In addition, from group of the age 70 to 90, the prevalence of mixed dementia might be dramatically increased from 2% to 86% [108]. It was found that the cerebral amyloid angiopathy, characterized by the deposition of Aβ in the wall of cerebral blood vessels, might lead to vessel wall rupture and hemorrhage. Furthermore, the risk factors

associated with AD, including hypertension, smoking, ischemic heart disease, atrial fibrillation, raised cholesterol and homocysteine concentrations, diabetes, and obesity, have also been closely associated with VaD. Taken together, the AD patients might have a higher risk to suffer from VaD. Furthermore, it has been recently reported that the damage of cerebral blood vessels damage might cause the reduction of CBF and initiate a cascade of events leading to the Aβ accumulation in brain [110]. The process also further accelerates the aggregations of Aβ, which have been considered to accelerate the pathogenesis of neurodegeneration (Fig 1.10).

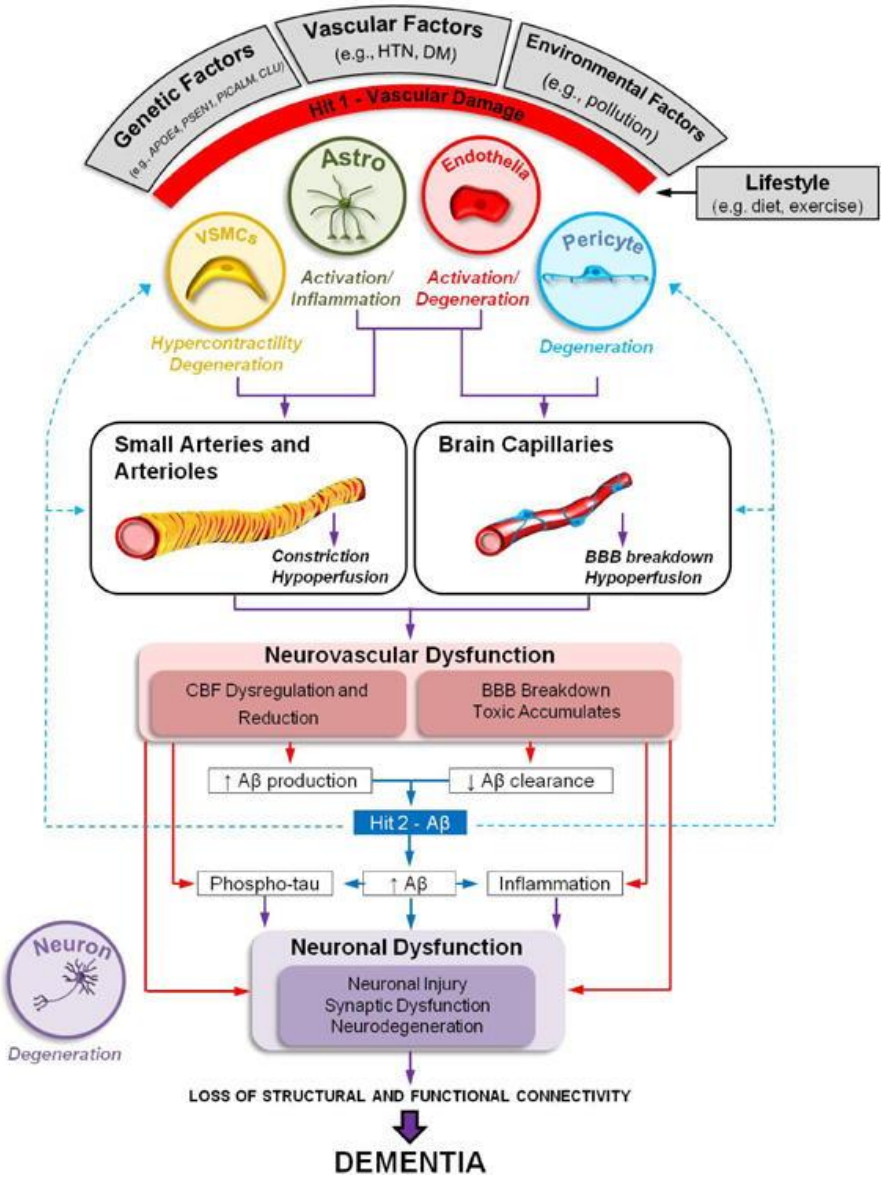

Fig. 1.10 Neurovascular dysfunction in Alzheimer's disease might accelerate the development of dementia. Cerebral blood vessels damage might initiate a cascade of events, which might lead to the Aβ accumulation in brain (hit 1). The process also further accelerates the Aβ-dependent pathway of neurodegeneration (hit 2). (Adapted from Kisler K *et al.* 2017 [110])

1.5.1 Vascular disorder, NO and NMDA receptor

Stroke is the most common cerebral vascular disease found in the aged population. It can be classified into two major categories: ischemic stroke and hemorrhagic stroke. Ischemic stroke is among the leading causes of death and disability worldwide, which results from an interruption of the blood supply [107]. Under these conditions, energy supply is impaired, and neurons undergo depolarization leading to enormous glutamate release and subsequent overactivation of glutamate receptors, especially of NMDA subtype [111]. Prolonged activation of NMDA receptors results in a mass increase of intracellular Ca^{2+} concentration, which in turn triggers cascades that finally lead to cell injury and excitotoxic cell death [112]. Among the consequences of glutamate induced Ca^{2+} overload, the activation of potentially lethal second messengers and enzymes, mitochondrial dysfunction and free radical formation are known to play important roles in executing neuronal cell death. More recently, Ca^{2+}-dependent neurotoxicity has been identified to occur via distinct intracellular signaling pathways, which are likely mediated through physical interaction of cell membrane receptors with specialized submembrane molecules [113]. Following initial lesions, the ischemic process continuously spreads to the periphery and affects the surrounding tissues [114]. As NMDA receptor is the essential mediator of neuronal cell death in ischemic stroke, selective NMDA receptor antagonists are thought to interrupt post-ischemic excitotoxicity. Studies have demonstrated that NMDA receptor antagonists might provide neuroprotective effects against ischemic stroke in the preclinical studies; however, the

majority of them failed in the clinical trials. In addition, two well conducted randomized trials conducted in 2002 reported that administration of memantine could only provide significant but small effects on the improvement for VaD patients [115,116]. The major reason might be due to the NMDA receptor antagonists only showed the therapeutic effects administrated before the onset of ischemic stroke. In addition, NMDA receptor antagonists might induce serious adverse effects, such as the psychotropic side effects [81,117]. We have known that NMDA receptor mediates many important physiological functions including learning and memory. Thus, inhibition of the NMDA receptor by very potent blocker like MK-801 might induce serious adverse effects.

Nitric oxide (NO), found to participates in CNS as a second messenger, can be used for treating cardio/cerebrovascular diseases by vascular dilation [118]. NO could bind to guanylyl cyclase and modulate the synaptic signal transmission through the cGMP-mediate signaling pathway [119]. Additionally, NO is a potent vasodilator, which is important to mediate the coupling between neuronal activity and vascular dilation in brain [110,120]. Interestingly, NO has been regarded as a "double-edged sword", meaning that it could be either neuroprotective or neurotoxic [121]. In the previous studies, NO was considered as a potentially toxic molecule mainly by inducing oxidative stress. However, other opinions point out that NO in the physiological concentrations might elicit anti-apoptotic/pro-survival effects against various neurotoxic challenges through multiple mechanisms. It has been reported that NO donors drugs, such as nitroglycerin, significantly reversed NMDA-induced neurotoxicity in *in vitro* and *in vivo* [122]. The

clinically available NO donor drugs, including nitroglycerin and sodium nitroprusside, are used for treating the cardiovascular diseases. However, the long-term uses of NO donor drugs might circulate in the peripheral blood vessel and systematic release NO, which might be a risk to induce hypotension. Thus, the ideal NO donor for treating VaD should release NO only targeting brain tissue, which might have avoided the risk of hypotension.

Currently, there is no approved treatment for VaD. Only several AChE inhibitors and Ca^{2+} channel blocker such as nimodipine have been proposed for the management of VaD. Although some studies reported that the therapeutic benefits provided by the AChE inhibitors, including galantamine, donepezil and rivastigmine, for treating VaD were significant, the magnitudes of the cognitive improvement were limited [106]. Therefore, the development of new generation of anti-VaD drugs is urgently needed.

1.6 The rationale of the therapeutic uses of memantine nitrates

Memantine is the only one NMDA receptor antagonistic drug approved by FDA for treating moderate to severe AD in 2003 [123]. NMDA receptors play a crucial role for regulating a broad spectrum of physiological functions, including learning and memory in the CNS. Under pathological conditions, excessive glutamate might overstimulate the synaptic and/or extrasynaptic NMDA receptors, resulting in excitotoxicity and leads to chronic neurodegenerative disorders. Lipton *et. al* has proposed that the uncompetitive NMDA receptor open channel blockers with moderate affinity, and fast on/off-rate kinetics might be a well-tolerated neuroprotective drugs for the treatment of various acute and chronic neurodegenerative disorders [80,81,124,125].

Memantine is an example of uncompetitive NMDA receptor antagonist with fast-off rate [126]. Memantine is an open channel blocker on NMDA receptors and blocks the influx of calcium ions only in the pathological condition [127, 128]. More importantly, memantine is well-tolerated and is able to penetrate the blood brain barrier and to reach the CNS. Several studies have reported that the pre-treatment of NMDA receptor antagonists, including memantine, could well prevent the rodent brain after stroke [129,130]. However, several lines of evidence indicated that only a low dose (0.2 mg/kg/day) was able to reduce lesion volume induced by reversible focal cerebral ischemia as well as to improve behavior outcomes. On contrast, memantine at a higher dose (20 mg/kg/day) might significantly increase injury [131]. It could be explained by two possible reasons. Firstly, memantine might inhibit the vascular dilation by blocking α7 nicotinic

receptors [132]. Secondly, memantine might inhibit the NMDA receptors, reducing the production of NO, which plays an important role of maintaining the vessel dilation. Thus, it could be a risk to reduce the CBF and enhance the depletion of the nutrients supply, which might further enhance the cognitive function impairment, if the AD patients conjugated with VaD [111].

To obtain a multi-functional anti-dementia molecule, structural optimization of existing drugs could be one of the most effective approaches. Based on these findings, NitroMemantine, the derivative from memantine, was previously synthesized by Prof. Wang et. al. in 2015 [54]. The novel derivative was obtained by introducing the nitrate group at the 7-position of the amantadine skeleton, and substituting the hydrogens with methyl, ethyl or propyl groups at the 3- and 5-positions, respectively. The preliminary evaluation studies showed that the NitroMemantine exhibited moderate inhibitory effects on NMDA receptors. The previous research revealed at least two hints for structural optimization of memantine. First, the free amino group of memantine skeleton is essential for the penetration of blood–brain barrier and the binding to NMDA receptors; Second, the hydrophobic group(s) on the 3- and/or 5-position of the memantine skeleton might be important for the interaction with NMDA receptors.

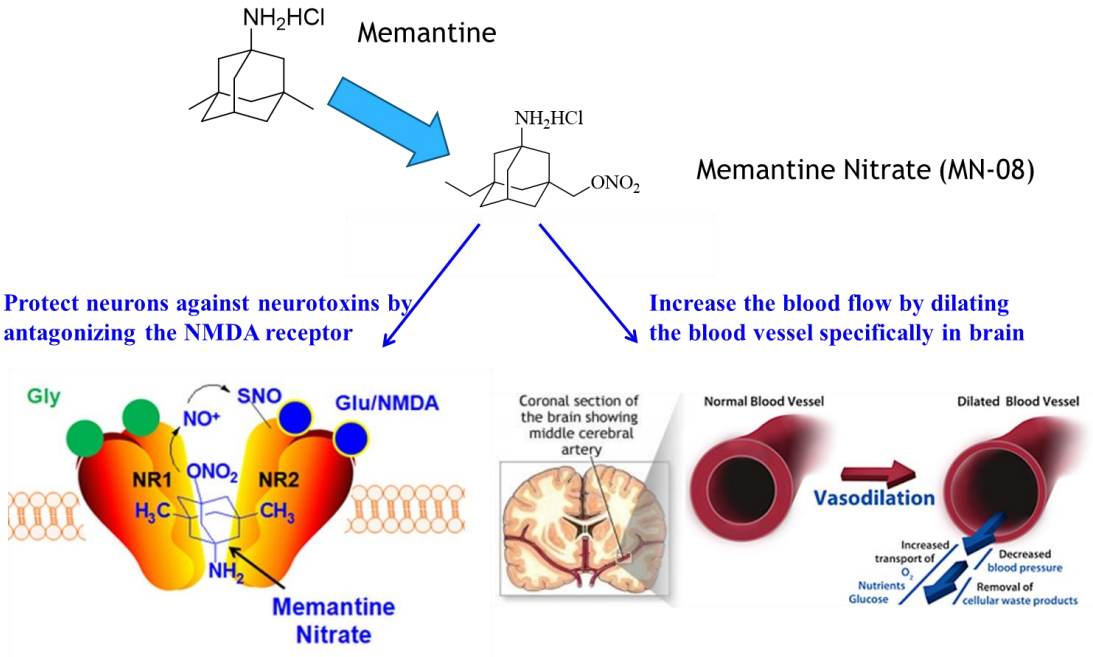

Fig. 1.11 The rationale design of memantine nitrate

Based on these findings, our collaborators Prof. Yuqiang Wang and Prof. Zaijun Zhang have previously designed and synthesized several memantine nitrates by introducing the nitrate moiety onto the backbone of memantine. And the structural optimization of memantine derivatives has been performed. The free amino group at the 1-position and the hydrogen at the 7-position of the memantine skeleton were preserved. The structural modifications of the memantine skeleton were focus on the 3- or (and) 5-position. Different side groups, including methyl or propyl group, were introduced on the 3-position, while the nitrate moiety was introduced on the 5-position of the memantine skeleton (Fig. 1.12) [54]. The length of the carbon chain linking the nitrate group and the memantine skeleton varied from 0–3. In addition, the new compounds may have improved plasma stability due to the replacement of a tertiary nitrate with a primary one [133].

Thus, a series of novel memantine nitrates have been obtained [133]. It is expected that the novel multifunctional memantine nitrates might possess dual anti-dementia effects, possible via the neuroprotection by antagonizing NMDA receptor and improvement of CBF by targeted releasing NO in brain [133].

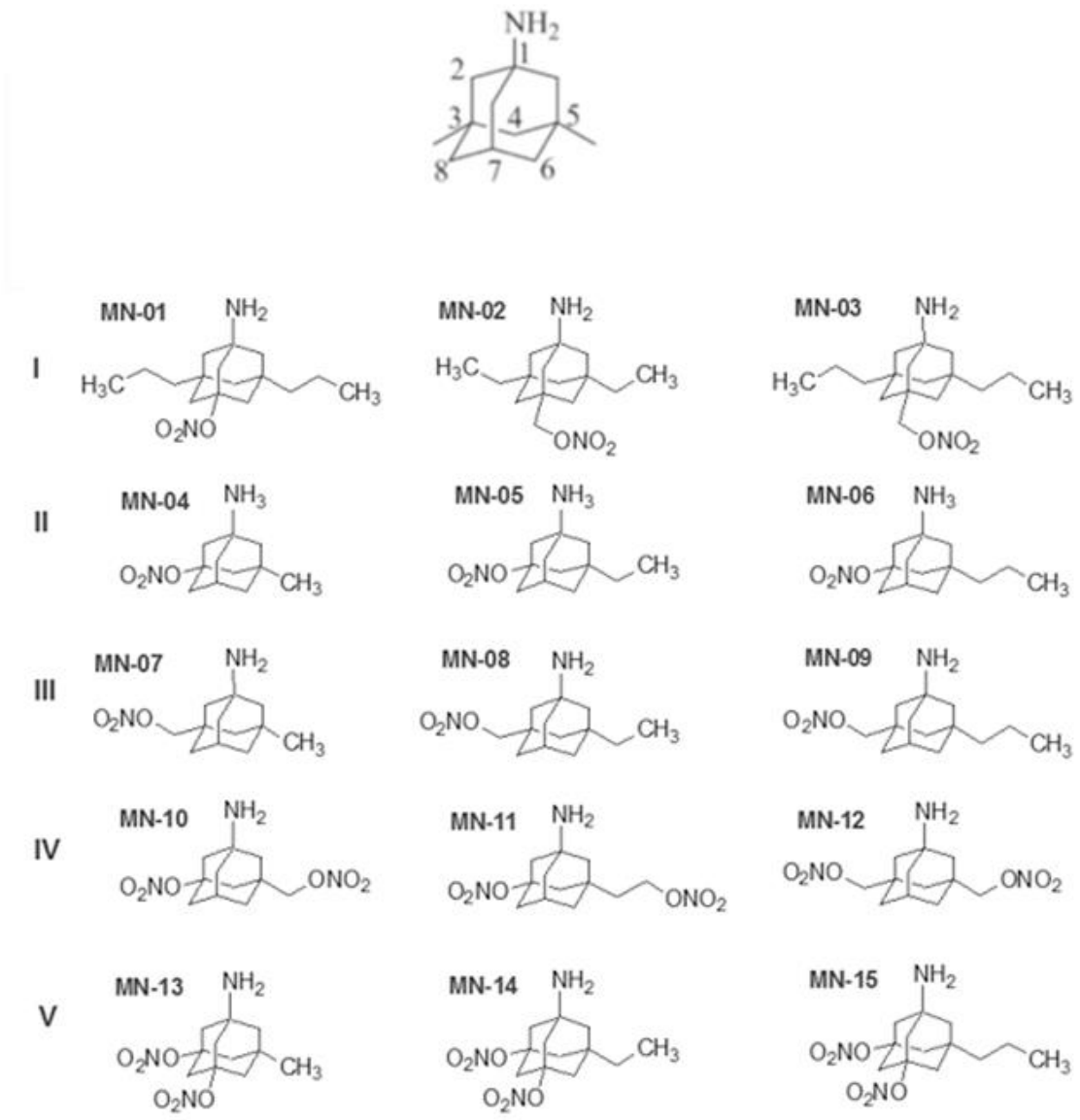

Fig. 1.12 Chemical structure of memantine nitrate

1.6. Research objectives

To achieve the multi-functional anti-dementia effects, a series of memantine nitrates have been designed and synthesized by our collaborators in Jinan University. We have previously shown that some of the novel memantine nitrates exhibited neuroprotective effects against glutamate-induced excitotoxicity; however, the detail mechanisms still remain unclear. We hypothesize that memantine nitrate, in particular, MN-08 as the most representative candidate, might offer multifunctional anti-dementia effects through the multifunctional neuroprotection and the improvement of CBF *in vitro* and *in vivo*. Therefore, in my thesis research, I would like to achieve the following aims:

- To examine the neuroprotective effects of representative candidates (MN-06, MN-08 and MN-12) against glutamate-induced excitotoxicity in primary cultured cerebral granule neurons (CGNs);
- To characterize the underlying cellular mechanisms for the neuroprotection by MN-08;
- To confirm and investigate the direct interaction between MN-08 and NMDA receptor by patch clamp and molecular docking simulation;
- To determine the vessel dilatory effects of MN-08 *ex vivo*;
- To evaluate the vasodilatory and anti-dementia effects of MN-08 *in vivo*;

Chapter II Materials and Methods

2. Methodology

2.1 Chemicals and reagents

The memantine nitrates were designed and synthesized by Prof. Zaijun ZHANG and Prof. Yuqiang WANG from Jinan University [133]. Unless otherwise memtioned, all cell culture media and supplements used were purchased from ThermoFisher Scientific (Waltham, MA, USA). Memantine, glutamate and LY294002 were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). Antibodies against pSer9-GSK3β, pSer473-Akt, pThr202/Tyr204 MAPK (ERK1/2) and bcl-2 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin were obtained from Santa Cruz Biotechnology (Dallars, Texas, USA). Fluorescence dye Fluo-4 acetoxymethylester (Fluo-4 AM) was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Primary rat cerebellar granule neuron cultures

CGNs were prepared from the 8-day-old Sprague–Dawley rats (The Hong Kong Polytechnic University) as described in our previous publication [134]. Briefly, animals were anesthetized and sacrificed, and their cerebella were collected. Then, the freshly collected cerebella were rinsed in cold Kreb's buffer-BSA and digested with 0.025% trypsin incubated in a 37 ^{o}C water bath for 15 min. The reaction was stopped, and the single cell suspension was obtained through pipetting up and down the sedimentary tissue. CGNs were seeded in basal modified Eagle's medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM

glutamine and 100 units/mL penicillin/streptomycin at a density of 1.5×10^6 cells/mL. Cytosine arabinoside (10 μM) was added to inhibit the growth of non-neuronal cells at 24 h after seeding. All experiments were carried out after 8 days in vitro. All experimental procedures were performed according to the institutional animal experimental ethical guidelines at the Hong Kong Polytechnic University.

2.3 Primary rat cultured hippocampal neurons

Rat hippocampal neurons were obtained from 18-day-old Sprague-Dawley rat embryos as previously described with modifications [135]. The hippocampi were obtained and dissected on ice. Then, the hippocampi were digested with 0.25% trypsin at 37 °C for 15 min. The single neurons were mechanically dissociated by using a Pasteur pipette. The neurons were plated at a density of 2×10^5 cells/mL on 35-mm culture dishes, which were pre-coated with poly-L-lysine (10 μg/mL), in neurobasal eagle's medium containing 10% fetal bovine serum, 0.5 mM glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin. The neurons were incubated under a humidified atmosphere of 5% CO_2, 95% air at 37 ℃. In 24 h after the seeding, the culture medium was half-charged with neurobasal Eagle's medium containing 2% B27, 0.5 mM glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin. The culture was half-changed with fresh medium twice weekly. Cells were used for confocal scanning 16 days after plating.

2.4 Determination of cell viability

Neurotoxicity was assessed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) assay as previous publication [127]. Briefly, cells were cultured in 96-well plates. Then, different tested compounds at different concentrations were added and challenged by glutamate. After 24 h incubation, 10 μL of 5 mg/mL MTT was added to the medium. The cells were then incubated at 37 ºC for another 4 h. After the removal of culture medium and then 100 μL of DMSO was added to dissolve the resulted formazon in each well. Cell viability was evaluated by observing colorimetric changes using a CLARIOstar plus Microplate Reader (BMG Labtech, Ortenberg, Germany) at a test wavelength of 570 nm with 655 nm as a reference wavelength. Data were expressed as a percentage of control cultures.

2.5 Measurement of intracellular Ca^{2+} by confocal laser scanning microscopy

A confocal laser scanning microscope was used to evaluate relative changes in intracellular calcium concentrations $[Ca^{2+}]_i$ by monitoring Fluo-4 fluorescence after intracellular cleavage of superfused Fluo-4 acetoxymethylester (1μM, with excitation at 488 nm and emission at 510 nm) [136]. In brief, the neurons were stained with 1μM M Fluo-4 acetoxymethylester for 30 min in a 37 °C incubator and then washed three times with a balanced salt solution containing 130 mM NaCl, 3 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4. The fluorescence images were obtained by Leica TCS SPE Confocal Microscope and analyzed using the LAS AF software (Leica Microsystems Co., Wetzlar and Mannheim, Germany). The data were

obtained by evaluating the fluorescence (F) from selected areas within a cell, following subtraction of background fluorescence, and division by the fluorescence intensity before drug application (F0), expressed as F/F0. Confocal images were taken and stored every 30 s. Drugs were added to the balanced salt solution 30 min prior to glutamate.

2.6 Apoptotic characteristic determination by Hoechst 33342 staining

Chromatin condensation, the apoptotic characteristic feature, was determined by Hoechst 33342 staining as described in previous publications with some modifications [137,138]. CGNs (1.5×10^6 cells/mL) grown in a 6-well plate were rinsed with ice-cold PBS containing 5% glucose. Then Hoechst 33342 (5 μg/mL) was added to the medium and incubated for 5 min. The nuclei were visualized by using a fluorescence Leica TCS SPE confocal (Leica, Wetzlar, Germany) at 200× magnification. In the observation, cells with bright blue fragmented nuclei, which were showing condensation of chromatin, were counted as apoptotic cells. Condensed nuclei were scored by counting at least 500 cells of three randomly chosen fields for each sample in three separated experiments.

2.7 Western blot assay

Western blot analysis was performed as described previously [134,139]. In brief, cells were harvested by cell lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, 1% sodium deoxycholate, 1 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin). Then,

the cell lysates were centrifuged at 14 000 g at 4 °C for 15 min. The whole protein solutions (the supernatant) were collected, and the concentrations were measured by BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The collected cell lysates were diluted in the SDS sample buffer and denaturized by boiling for 5 min. The proteins were firstly separated on a SDS-PAGE, and successively transferred to polyvinylidene fluoride membranes. The membranes were blocking in 5% BSA in TBST solution (20 mM Tris, pH 7.6, 0.1% Tween 20) for 2 h at room temperature. Primary antibodies were diluted in 5% BSA in a TBST solution (1:1000 Bcl-2, phosphor-Ser473 Akt, phosphor-Ser9 GSK3β, total GSK3β, total Akt, 1:2000, ERK, β-actin) and then incubated with the membrane at 4 °C overnight. After that, the membranes were washed with TBST and then incubated with the respective horseradish peroxidase-conjugated secondary antibody (1:2000). Blots were developed by using a Super Enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL) for 5 min. The protein bands were visualized after exposure of the blots to the imaging system Azure C600 (Azure Biosystems, Dublin, CA, USA). Optical density of each band was analyzed using ImageJ Fiji [140].

2.8 Whole-cell patch clamp recording

Whole-cell patch clamp recordings were carried out at room temperature (22-24℃) on the stage of an inverted phase-contrast microscope using an Axopatch 700B patch amplifier (Axon Instruments, Burlingame, CA). Before each experiment, the culture medium was removed, the cells were rinsed completely and continuously superfused

with a solution containing (in mM): 150 NaCl, 5 KCl, 0.25 $CaCl_2$, 10 glucose, 0.001 glycine, 0.001 tetrodotoxin, 0.01 (-)-bicuculline methiodide and 10 HEPES (the pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ~340 mOsm with sucrose). The low concentration of Ca^{2+} was used to minimize the calcium-dependent desensitization of NMDA-activated current. Pipettes pulled from borosilicate glass (TW-150F, World Precision Instruments, Sarasota, FL, U.S.A.) had resistances of 2-4 MΩ when filled with pipette solution containing (in mM): 140 CsCl, 10 EGTA, 10 HEPES, and 5 MgATP with pH 7.3 (adjusted with CsOH) and 315 mOsm in osmolarity (adjusted with sucrose). A small patch of membrane underneath the tip of the pipette was aspirated to form a gigaseal and then a more negative pressure was applied to rupture it, thus establishing a whole-cell configuration. The adjustment of capacitance compensation and series resistance compensation was done before recording the membrane currents. The holding potential was set at –50 mV, except when indicated specially. Data were acquired on a computer using a DigiData interface and the pClamp9.0 software (Axon Instruments). Currents were filtered at 2 kHz and digitized at 5 kHz.

2.9 Measurement of NO *in vitro* and *in vivo*

The concentrations of NO were measured as previous publication with modifications [141]. The samples with different concentrations were applied to the phosphate buffer (PBS, pH 7.4) containing 25 mM L-cysteine and incubated at 37 °C. Griess reagent (500 μL) was added at the different time points (0.5, 1, 2, 4, 6, 8, 12, 16, 24, 32 h) and

incubated for 30 min. The absorbance of each sample was determined by microplate reader (model ELX800, BioTek Instruments) at 540 nm.

2.10 Vasodilatory effect on pre-contracted endothelium-intact middle cerebral artery

The vasodilating assay was performed according to methods previously reported with minor modifications [142,143]. Briefly, Sprague-Dawley rats (300~350 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). Their brains were removed and the middle cerebral artery (MCA) was exposed. The MCA was dissected out and placed in ice-cold oxygenated physiological saline solution. The physiological saline solution contained the following in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 1.5 $CaCl_2$, 24.9 $NaHCO_3$, 0.01 EDTA, and 11 glucose, pH=7.4. After cleaning of the superficial adherent connective tissues, the MCA was cut into ring segments, 3-4 mm in length. The artery rings were then mounted in a Multi-myograph System (Danish Myo Technology A/S, Denmark) to measure tension of the vessels. Artery rings were placed in the standard organ chamber containing Krebs-Henseleit (KH) buffer with the following in mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 11 glucose, 1.2 $MgSO_4$, 25 $NaHCO_3$ and 2.4 $CaCl_2$. The solution was kept at pH 7.4 and gassed with 95% O_2 and 5% CO_2 at 37 ^oC. Arterial rings were set to an optimal tension (4 mN) and stabilized for 60 min. After pretreatment with 60 mM KCl to achieve consistent contraction, MN-08 (1-30 μM) was added cumulatively to the organ chamber and the contraction of arterial rings were recorded.

2.11 Rat vascular dementia model of 2-vessel occlusion (2VO)

2.11.1 Animals and grouping

A total of 36 male Sprague-Dawley rats (300 ± 10 g weight), supplied by Guangdong Medical Laboratory Animal Center, were housed under a 12:12 h light-dark cycle at a constant temperature (19-24 °C) and had *ad libitum* to food and water. The rats were randomly assigned into 6 groups (n = 14), including sham operated group (Control), 2VO with vehicle-treated group (Model), 2VO with MN-08 (7.5, 15, and 30 mg/kg, .ig) treated groups and 2VO with memantine (MEM, 10 mg/kg, ig) treated group. All experimental procedures were conducted according to the guidelines of Jinan University Ethic Committee for the Care and Use of Laboratory Animals (Approval No. SCXK2013-0034).

2.11.2 2VO Surgery and drug treatment

The 2VO surgery was performed according to a previously reported method [4,137]. Briefly, the rats were anesthetized with isoflurane (inhalation, 2.0-2.5%), then the bilateral common carotid arteries (CCAs) were exposed from carotid sheaths and vagal nerves through a midline cervical incision. Then the two CCAs were ligated permanently with silk sutures. The sham operated rats received the same surgical operation but without ligation. The incision was closed, and rats were returned to the cages with food and water *ad libitum*. The body temperature was maintained at 37 °C during the surgery. Three and 6 h after surgery, rats in MN-08 and MEM treated group received MN-08 or memantine at different concentration i.g. From day 2 to day 28, rats administrated MN-08 or memantine twice daily at 9:00 am and 4:00 pm *i.g.*. Rats in control and model group injected saline according to the procedure.

2.11.3 CBF measurement

CBF was measured before surgery, on 5 mins and 28 days after surgery, respectively [144]. The CBF was monitored in the left temporal window using a laser Doppler flowmetry (Perimed, Stockholm, Sweden). The probes were positioned 5 mm left and 2 mm posterior to the bregma (reflecting the change of blood flow in temporal cortex); and in 3 mm left and 2 mm posterior to the bregma (reflecting the change of blood flow in parietal cortex).

2.11.4 Y maze test

The Y-maze test was performed as previously reported [145]. There are three arms of a Y-maze (70 × 15 × 10 cm, length × height × width). In the training session, the animals were trained by placing them at the end of starting arm. The animals were allowed to explore the maze in one arm for 10 min, sequentially. An hour later, the animals were allowed to explore freely three arms for 5 min in the test session. The time of the animals spent in three arms were recorded by a digital camera. Discrimination ratio was calculated as Novel arms / (Novel arms + other arms) for dwell time.

2.11.5 Open field test

Open field test was conducted as previously described [146]. Animals were placed at the center of open-field box (50 × 50 × 50 cm, length × width × height) for 5 min. The chamber was cleaned with ethanol (75%) after each individual animal to eliminate olfactory cues. The total moving distance (cm) was calculated by the locomotive track recorded by digital camera mounted centrally above the chamber.

2.11.6 Novel object recognition test

The novel object recognition test was performed as previously reported [147]. First, rat was sequentially placed into a square box (50 × 50 × 50 cm, length × width × height) without any object for 10 min per day continuously for 2 days to habituate to the environment. Then, two identical objects, A1 and A2, were placed parallel near one wall of the square box. Rat was placed singly in the box and allowed to explore the objects for 10 min. Substituted object A2 with a novel object (B). After 1 h interval, the rat was returned to the box and allowed to explore the objects for 5 min in the test phase. Exploratory behavior was defined as directing the nose at the object at a distance of less than 5 cm and/or touching the object with the nose. Digital camera recorded the time of rat exploring the familiar object (TA) and the novel object Novel and familiar objects were alternated between the left and right positions to reduce potential bias toward any particular location. The objects and the box were cleaned with acetate (5%) after each individual trial to eliminate olfactory cues. The discrimination index (DI) was calculated as (TA-TB)/(TA+TB).

2.12 Statistical analysis

Results are expressed as mean ± SEM. When comprising with two groups, we use paired or unpaired student's t-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used, followed by Dunnett's multiple comparisons test. Levels of $p< 0.05$ were considered to be of statistical significance. All statistical analyses were

performed with Graphpad Prism software version 7.00 (GraphPad Software, Inc., California, USA)

Chapter III
Results

3.1 Memantine nitrates exhibited neuroprotection against glutamate excitotoxicity in CGNs

3.1.1 MN-06 prevented glutamate-induced cell death in CGNs

We have previously reported that glutamate at a concentration of 100 μM might induce around 50% of neuronal loss in CGNs at 8 days *in vitro* (DIV) [135, 148]. CGNs were exposed to the glutamate for 24 h. After the challenge, CGNs were subjected to MTT assay for the measurement of cell viability. To evaluate the neuroprotective effects of MN-06, CGNs were treated with MN-06 at the concentrations from 1 to 50 μM or memantine from 1 to10 μM for 2h, and then exposed to 100 μM glutamate for 24 h. As shown in Fig. 3.1, MN-06 was able to exhibit dose-dependent protection against glutamate-induced excitotoxicity, with the IC_{50} values at 9.28 μM.

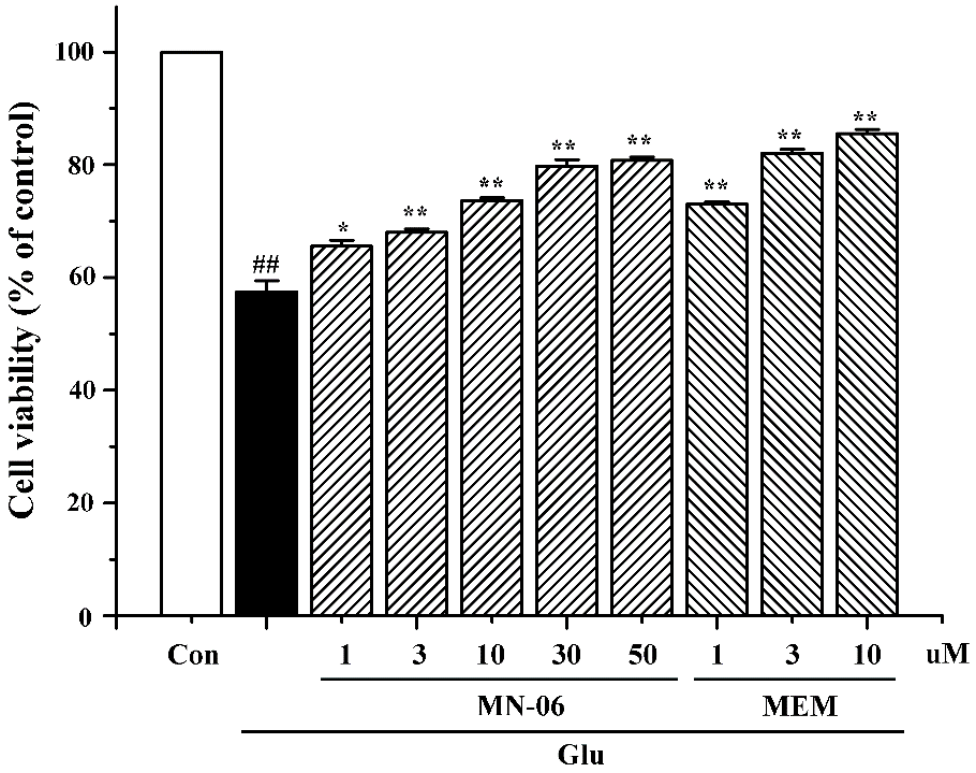

Fig. 3.1 MN-06 prevented glutamate-induced excitotoxicity in CGNs. At 8 DIV. CGNs were pre-treated with MN-06 (1-50 μM) or memantine (1-10 μM) for 2 h, and then incubated with 100 μM glutamate for additional 24 h, and finally subjected to MTT assay. MEM: memantine. Data were the mean ± SEM of three separate experiments. ## $p<0.01$, compared to the control group, * $p<0.05$ or ** $p<0.01$, compared to the glutamate group.

3.1.2 MN-08 prevented glutamate-induced cell death in CGNs

To evaluate the neuroprotective effects of MN-08, CGNs were treated with MN-08 at the concentrations from 1 to 50 μM or memantine from 1 to 10 μM for 2h, and then exposed to 100 μM glutamate for 24 h. As shown in Fig.3.2, MN-08 could concentration-dependently protect against glutamate-induced excitotoxicity, with the IC_{50} values at 7.88 μM (Fig. 3.3).

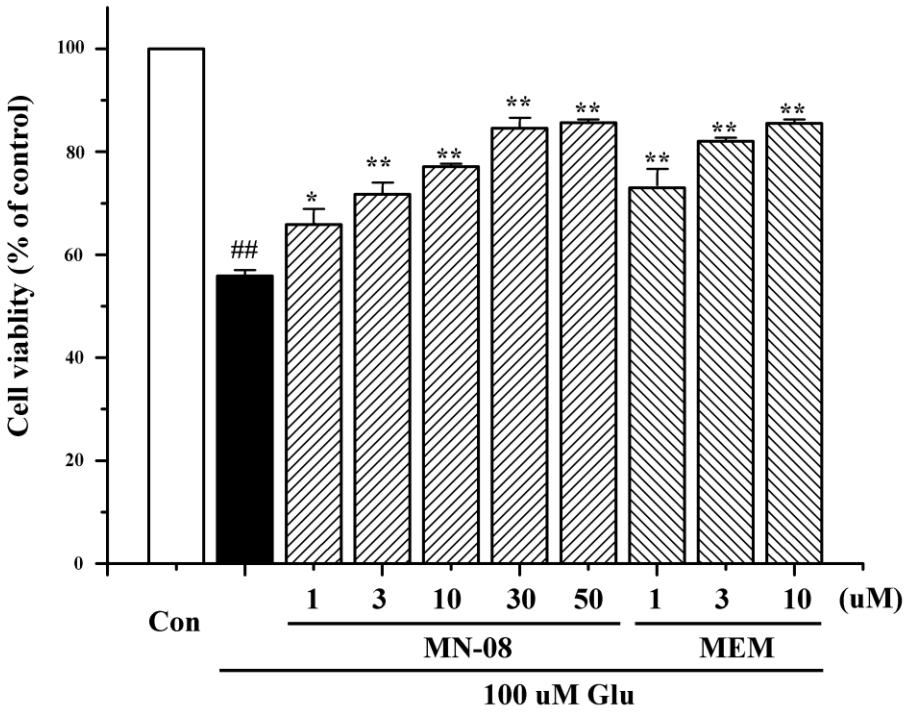

Fig. 3.2 MN-08 prevented glutamate-induced excitotoxicity in CGNs. A, At 8 DIV. CGNs were pre-treated with MN-08 (1 - 50 μM) or memantine (1 - 10 μM) for 2 h, and then incubated with 100 μM glutamate for additional 24 h, and finally subjected to MTT reduction assay. Data were the mean ± SEM of three separate experiments. ##, $p<0.01$, compared to control, **, $p<0.01$, compared to the glutamate group.

3.1.3 MN-12 prevented glutamate-induced cell death in CGNs

To evaluate the neuroprotective effects of MN-12, CGNs were treated with MN-12 at the concentrations from 1 to 50 μM or memantine from 1 to10 μM for 2h, and then exposed to 100 μM glutamate for 24 h. It was found that MN-12 could concentration-dependently protect against glutamate-induced excitotoxicity, with the IC_{50} values at 12.57 μM (Fig. 3.3).

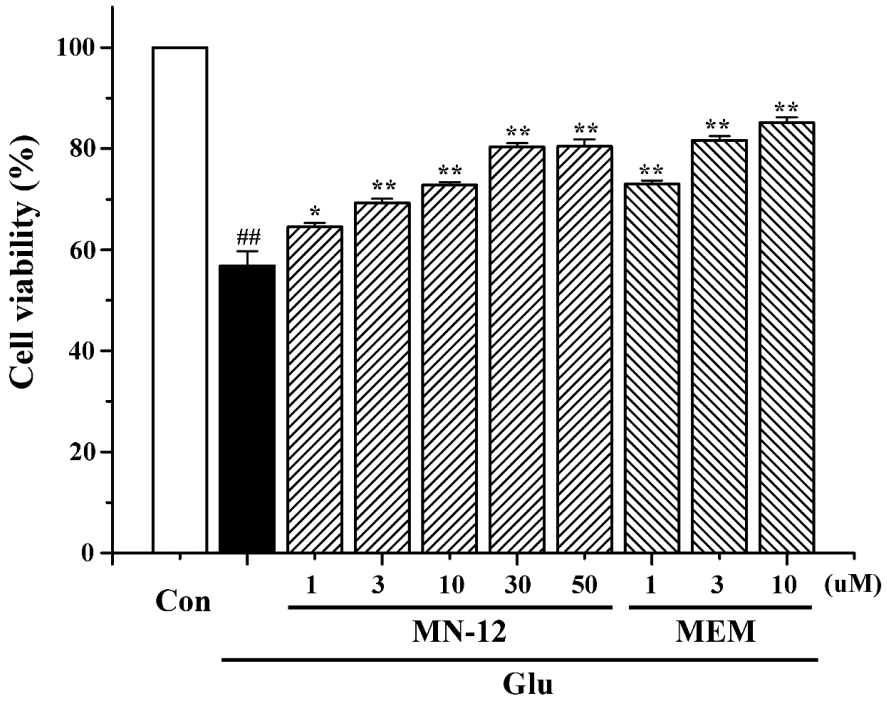

Fig. 3.3 MN-12 prevented glutamate-induced excitotoxicity in CGNs. A, MN-12 protect against glutamate-induced neuronal death. At 8 DIV. CGNs were pre-treated with MN-08 (1 - 50 μM) or memantine (1 - 10 μM) for 2 h, and then incubated with 100 μM glutamate for additional 24 h, and finally subjected to MTT reduction assay. Data were the mean ± SEM of three separate experiments. ##, $p<0.01$, compared to control, **, $p<0.01$, compared to the glutamate group.

3.2 Memantine nitrates blocked the apoptotic hallamrks in CGNs

3.2.1 MN-06 blocked the apoptotic hallmarks caused by glutamate in CGNs

To further demonstrate whether the glutamate-induced neuronal death mainly through apoptosis, the apoptotic bodies were examined by Hoechst staining. As observed from phase contrast microscopy and Hoechst staining (Fig. 3.4A), after 24 h glutamate challenge, the memantine nitrates MN-06 at 30 μM and memantine 3 μM significantly reduced the numbers of apoptotic bodies and reversed the morphological changes, including unhealthy bodies and broken extensive neuritis network. In particular, the counts of apoptotic bodies stained by Hoechst 33342 indicated an apoptotic rate of 46.2 ± 5.6% after the 24 h treatment of glutamate. Our results have shown that the 2 h pretreatment of memantine nitrates and memantine significantly reversed nuclear condensation induced by glutamate (Fig. 3.4B).

In addition, bcl-2, the protein related to anti-apoptosis was determined by western blotting assay. The results shown that 2 h pretreatment of MN-06 significantly reversed the glutamate-induced bcl-2 down-regulation. The bcl-2 was ameliorated to 96.2±5.4% of control by pretreatment of MN-06 (Fig. 3.4C).

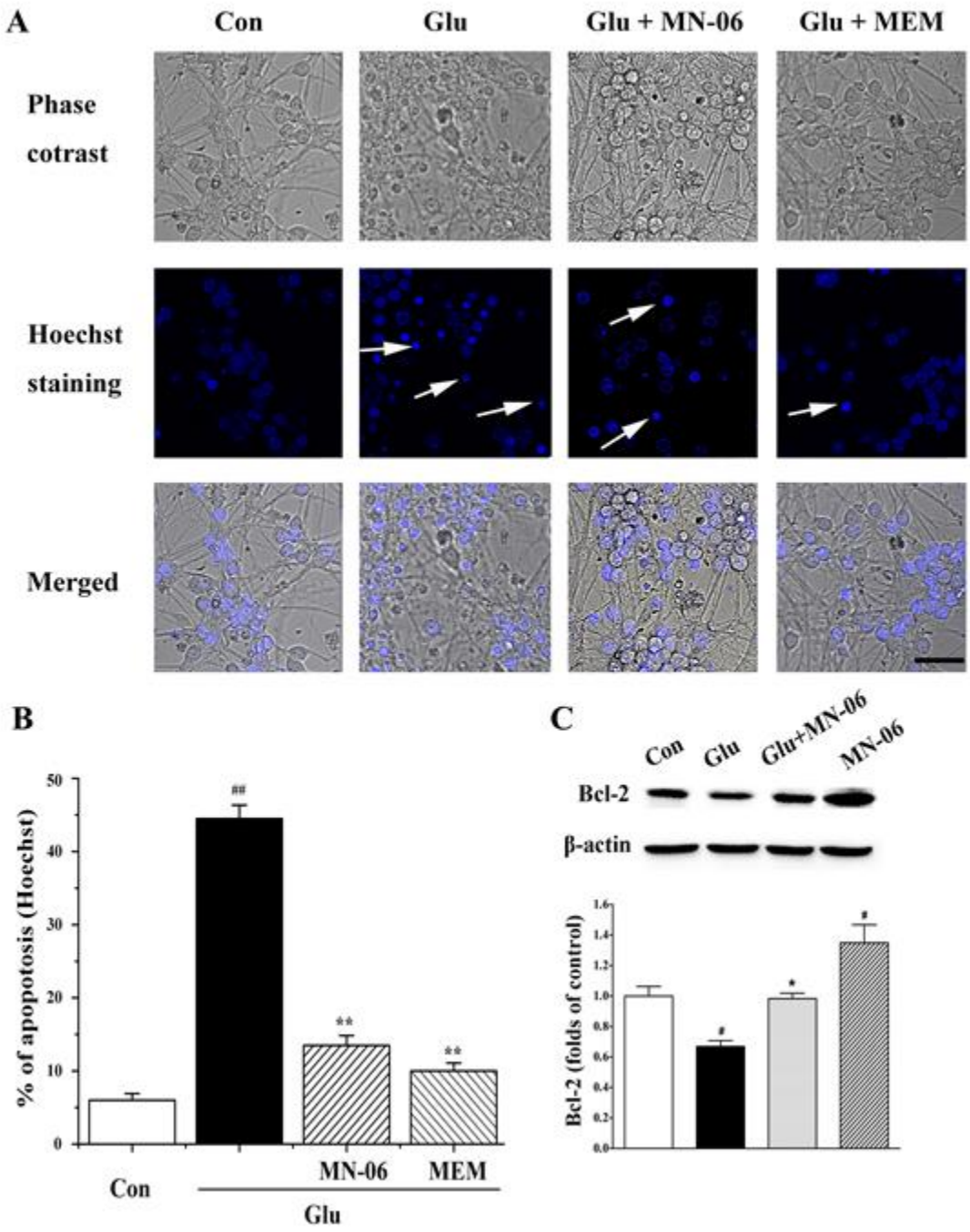

Fig. 3.4 MN-06 attenuated the apoptotic hallmarks induced by glutamate in CGNs. (A) At 8 DIV, CGNs were pre-incubated with or without 30 μM MN-06 or 3 μM MEM, and then exposed to 100 μM glutamate 2 h later. After 24 h glutamate challenge, CGNs were assayed with a phase contrast microscope and Hoechst 33342 staining. The nuclear condensations were pointed by arrows. Scale bar: 10 μm. (B) The counts of apoptotic bodies by Hoechst staining as in A. (C) Western blot of the apoptosis related protein bcl-2. Data were the mean ± SEM of three separate experiments, ## $p<0.01$ compared to control, ** $p<0.01$ compared to the glutamate group.

3.2.2 MN-08 blocked the apoptotic hallmarks in CGNs

To further demonstrate whether the glutamate-induced neuronal death mainly through apoptosis, the apoptotic bodies were examined by Hoechst staining. As observed from phase contrast microscopy and Hoechst staining (Fig. 3.5A), after 24 h glutamate challenge, the memantine nitrates MN-08 at 30 μM and memantine 3 μM significantly reduced the numbers of apoptotic bodies and reversed the morphological changes, including the unhealthy shrieked cell bodies and broken neuritis network. In particular, the counts of apoptotic bodies stained by Hoechst 33342 indicated 47.2 ± 2.2% apoptosis rate after the 24 h treatment of glutamate. As shown in fig. 3.5A, the 2 h pretreatment of memantine nitrates and memantine significantly reversed nuclear condensation induced by glutamate (Fig. 3.5B). In addition, as shown in Fig. 3.5C, bcl-2, the protein related to apoptosis were determined by western blot assay. The results shown that 2 h pretreatment of MN0-08 significantly reversed the bcl-2 down-regulation caused by glutamate.

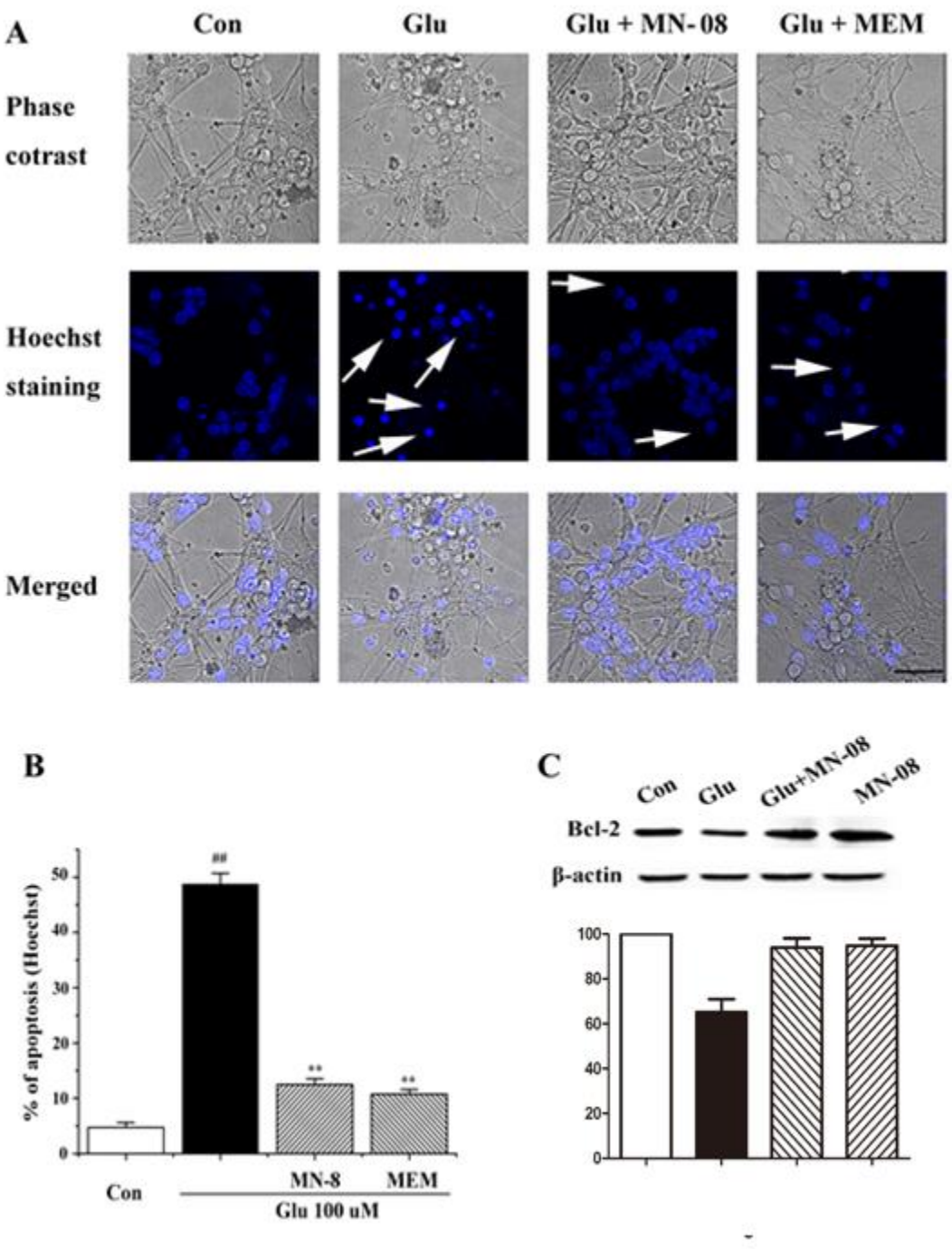

Fig. 3.5 MN-08 attenuated the apoptotic hallmarks induced by glutamate in CGNs. A, At 8 DIV, CGNs were pre-treated with or without 30μM MN-08 or 10 μM MEM for 2 h, and then exposed to 100 μM glutamate. After 24 h glutamate challenge, CGNs were assayed with a phase contrast microscope and Hoechst 33342 staining. The nuclear condensations were pointed by arrows. Scale bar: 10 μm; B, The counts of apoptotic bodies by Hoechst staining as in A. C, Western blot of the apoptosis related protein bcl-2. Data were the mean ± SEM of three separate experiments. ## P<0.01 compared to control, ** P <0.01 compared to the glutamate group.

3.2.3 MN-12 blocked the apoptotic hallmarks in CGNs

To further demonstrate 100 μM glutamate-induced neuronal death mainly through apoptosis, the apoptotic bodies were examined by Hoechst staining. As observed from phase contrast microscopy and Hoechst staining (Fig. 3.6A), after 24 h glutamate challenge, MN-12 at 30 μM and memantine 3 μM significantly reduced the numbers of apoptotic bodies and reversed the morphological changes, including unhealthy bodies and broken extensive neuritis network. In particular, the counts of apoptotic bodies stained by Hoechst 33342 indicated 47.22 ± 3.6% apoptosis rate after the treatment of glutamate for 24 h. Our results have shown that the 2 h pretreatment of memantine nitrates and memantine significantly reversed nuclear condensation induced by glutamate (Fig. 3.6B). In addition, the protein related to anti-apoptosis was determined by western blot assay. The results shown that 2 h pretreatment of MN-12 significantly reversed the glutamate-induced bcl-2 down-regulation (Fig. 3.6C).

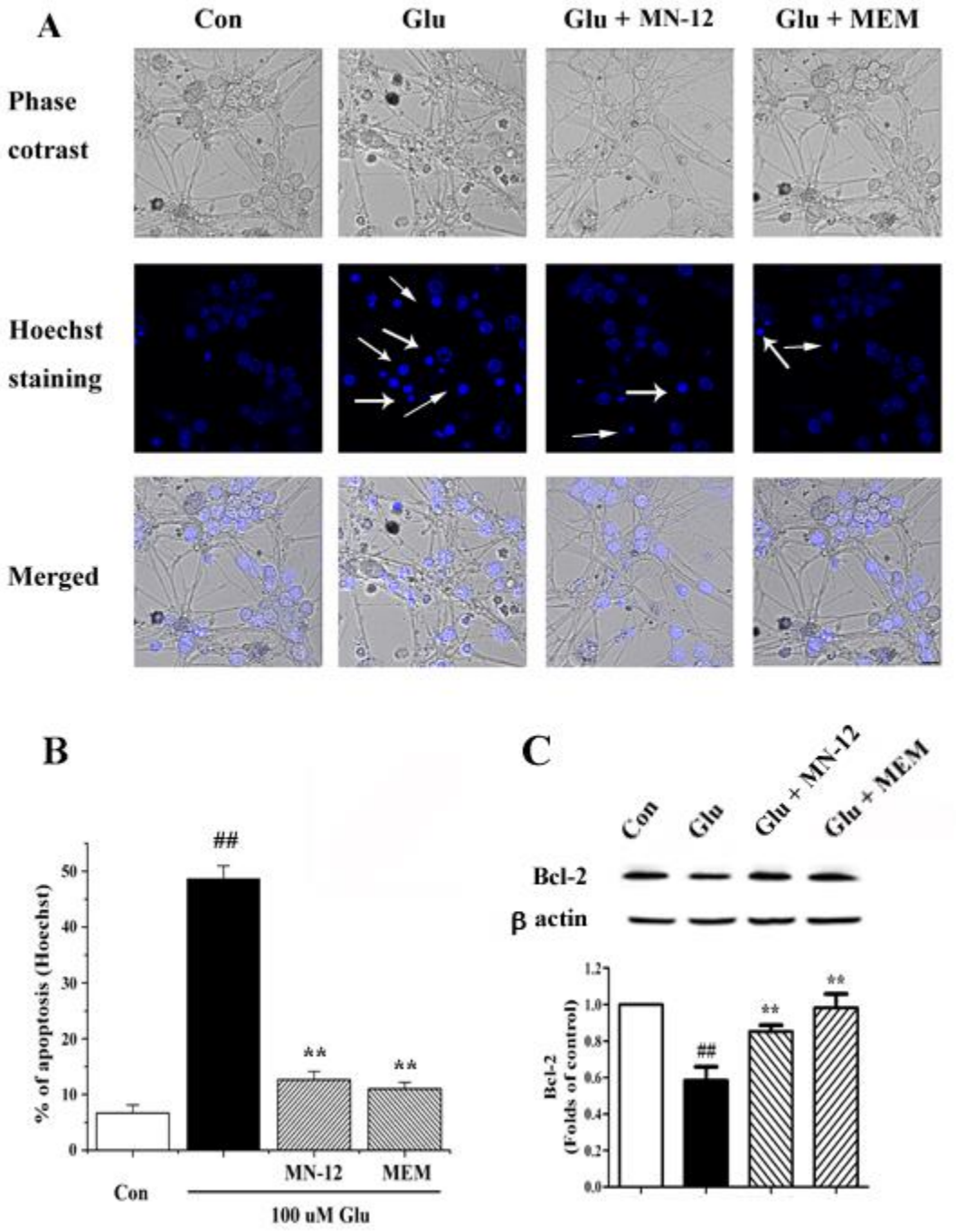

Fig. 3.6 MN-12 attenuated the apoptotic hallmarks induced by glutamate in CGNs. (A) At 8 DIV, CGNs were pre-incubated with or without 30 μM MN-12 or 3 μM MEM for 2 h, and then exposed to 100 μM glutamate. After 24 h glutamate challenge, CGNs were assayed with a phase contrast microscope and Hoechst 33342 staining. The nuclear condensations were pointed by arrows. Scale bar: 10 μm (B) The counts of apoptotic bodies by Hoechst staining as in A. (C) Western blot of the apoptosis related

protein bcl-2. Data were the mean ± SEM of three separate experiments. ## P<0.01 compared to control, ** P <0.01 compared to the glutamate group.

3.3 MN-08 protected against glutamate-induced excitotoxicity through multiple targets

3.3.1 MN-08 Inhibited glutamate-induced activation of ERK pathway in CGNs

The extracellular signal-regulated kinases (ERK) are important members of the signaling pathway associated with glutamate-induced excitotoxicity. EAK1/2 are the two homologous isoforms belonged to the ERK family, sharing the same phosphorylation sites. Therefore, there are double bands appeared in the western blotting. Several studies have indicated that ERK was activated by the glutamate insults in CGNs and reversed the activation of ERK might contribute to neuroprotection [134]. To investigate whether MN-08 had any effects on the regulation of ERK pathway, the levels of phosphorylation of ERK1/2 (p-ERK) were analyzed by Western blotting assay. The results demonstrated that 100 μM glutamate insult significantly enhanced the expression of p-ERK in a time-dependent manner. As shown in Fig. 3.7 A, 30 mins after glutamate incubation, the expression of p-ERK was significantly increased almost 3 times compared that of the control group. Pretreatment of MN-08 (30 μM, 2 h) or memantine (3 μM, 2h) significantly inhibited the up-regulation of ERK1/2 phosphorylation on Ser-42/44 residues induced by glutamate, the expression levels of p-ERK were reduced by half with the treatment of MN-08 (Fig. 3.7 B). Memantine showed more potency than MN-12, which is in accordance with the results of cell viability assay.

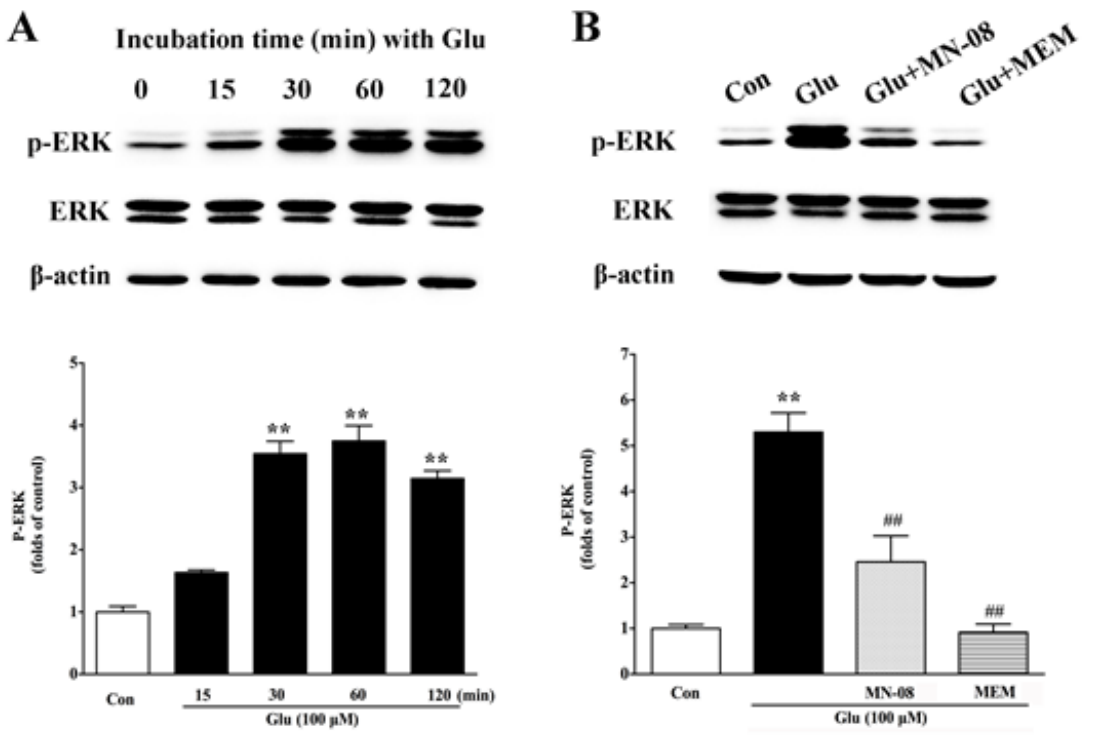

Fig.3.7 Inhibition of glutamate-mediated activation of ERK pathway by MN-08. CGNs were incubated with drugs at the times indicated. Cells were harvested and subjected to Western Blotting assay by using the anti-bodies against the targeted proteins. (A) Glutamate 100 μM upregulated the expression of phosphor-ERK in a time-dependent manner. Data were the mean ± SEM of three separate experiments, **, $p < 0.01$ compared with the control group; (B) CGNs were pre-treated with 30 μM MN-08 and 3 μM MEM for 2 h, and then exposed to 100 μM glutamate for 0.5 h, and the total proteins were extracted for Western Blot with the specific phosphor-ERK, total ERK and β-actin. Data were the mean ± SEM of three separate experiments, **, $p <$ 0.01 compared with the control group; $^{\#\#}$,$p<0.01$, compared to the glutamate group.

3.3.2 MN-08 reversed the inhibition of PI3-K/Akt pathway caused by glutamate

It was reported that PI3-K/Akt pathway and the downstream target GSK3β play important roles to regulate the neuronal death during the glutamate insult [149]. We have previously reported that the dimers derived from tacrine might protect against glutamate-induced excitotoxicity by reversing the inhibition of PI3-K/Akt pathway [150]. To further evaluate the effects of MN-08 on the PI3-K/Akt pathway, the expression level of p-Ser473-Akt and p-Ser9-GSK3β were examined by using Western blotting assay. As shown in Figs. 3.8 A and B, glutamate might significantly downregulate the expression of p-Ser473-Akt and p-Ser9-GSK3β in a time-dependent manner. It was found that the expression of p-Ser473-Akt was downregulated to 28.3 ± 5.3 % of control at 0.5 h after the glutamate challenge. And the level of p-Ser9-GSK3β were reduced to 48.6 ± 11.3 % of the control. As shown in Fig. 3.8 C, the pre-treatment with MN-08 reversed expression levels of both p-Ser473-Akt and p-Ser9-GSK3β with the challenge of glutamate (Fig.3.8 C). Since Akt is an important downstream member of PI3-K, to further investigate whether the PI3-K/Akt pathway was associated with the neuroprotection of MN-08 against glutamate-induced excitotoxicity, specific PI3-K inhibitor LY294002 was applied and incubated with MN-08. It was found that 50 μM of LY294002 significantly abolished the upregulation of both p-Ser473-Akt and p-Ser9-GSK3β by the treatment of MN-08 (Fig. 3.8 C). In addition, the MTT assay showed that the neuroprotective effects of MN-08 against glutamate-induced apoptosis

were cancelled by LY294002, which might further confirm that PI3-K/Akt were involved in the neuroprotective mechanism of MN-08 (Fig.3.8 D).

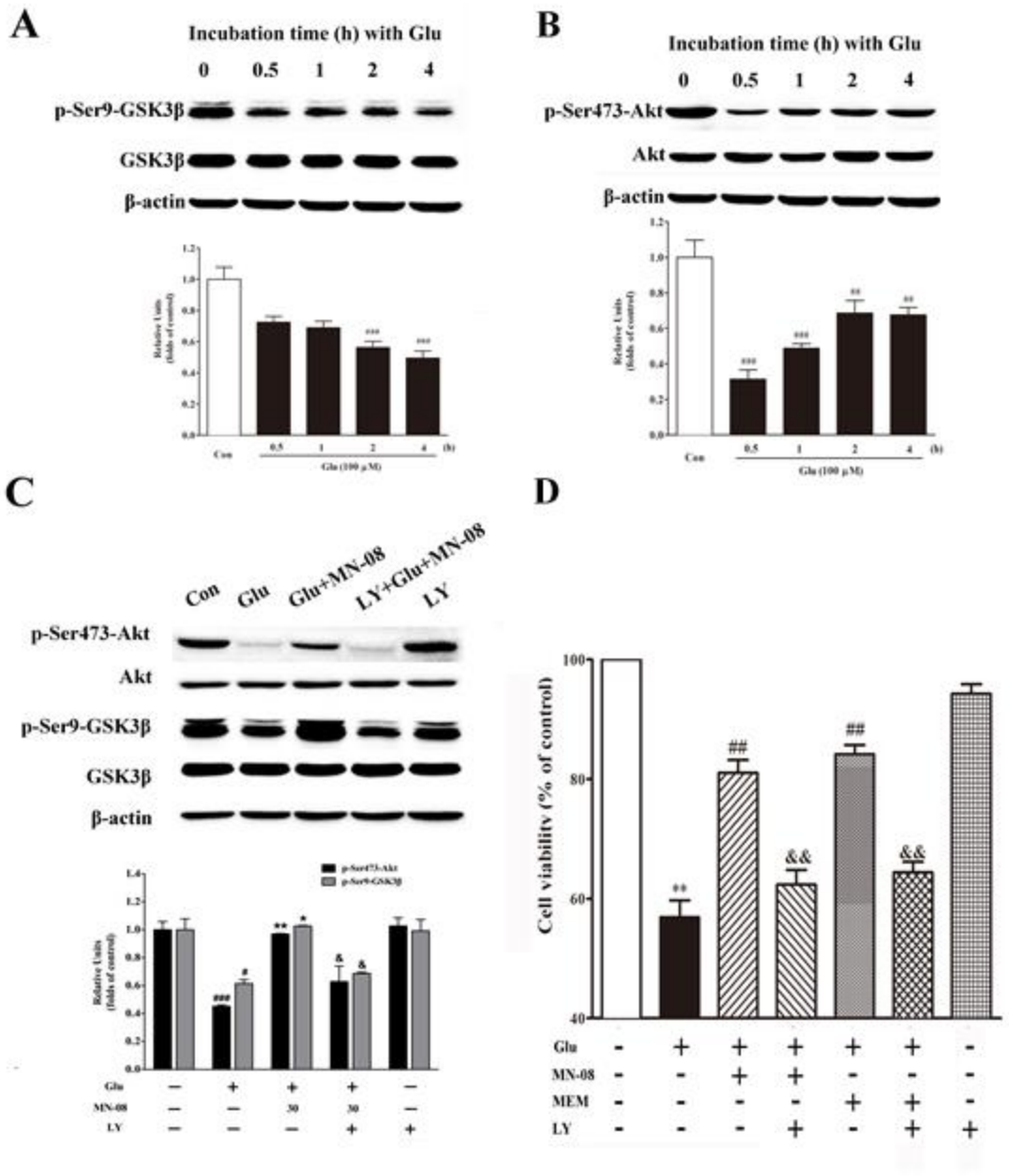

Fig. 3.8 MN-08 attenuates glutamate-induced excitotoxicity by maintaining the activation of PI3-K/Akt pathway in CGNs. (A) Glutamate significantly downregulated the expression of phospho-GSK3β. (B) Glutamate suppressed the levels of phospho-Akt in CGNs in a time-dependent manner. Data were the mean ± SEM of three separate experiments, ##, $p < 0.01$, compared with control; **, $p < 0.01$, compared with glutamate group; &&, $p < 0.01$, compared with glutamate plus MN-12 group; (C) MN-08 reversed glutamate-induced suppress of phospho-Akt and phospho-GSK3β. PI3-K specific inhibitors LY partially abrogated the upregulation of both phospho-Akt and phospho-GSK3β by MN-08 on glutamate-induced excitotoxicity. (D) LY, the PI3-K specific inhibitor abolished the neuroprotection of MN-08 through the inhibition of PI3-K/Akt pathway. Cell viability was examined by using MTT assay. Data were the

mean ± SEM of three separate experiments. **, $p < 0.01$, compared with control; ##, $p < 0.01$, compared with the glutamate group, &&, $p < 0.01$, compared to glutamate plus MN-08 group.

3.3.3 MN-08 reduced the glutamate-induced intracellular Ca^{2+} in rat primary cultured hippocampal neurons

Studies shown that the elevated intracellular Ca^{2+} might be the important mediator of glutamate-induced excitotoxicity by interfering the downstream pathways, including the activation of ERK and inhibition PI3K/Akt pathway. According to the above results, MN-08 might reverse the activation of ERK pathways and inhibition of PI3K/Akt pathway induced by glutamate. Thus, we further investigate whether MN-08 could interfere with the intracellular Ca^{2+}, the common upstream modules of ERK and PI3K/Akt pathways. To investigate whether MN-08 has any effect on the intracellular Ca^{2+} influx, Fluo4-AM fluorescence probe was used for determining the Ca^{2+} levels in primary cultured hippocampal neurons. The results demonstrated that the treatment of glutamate (100 μM) caused a rapid intracellularly influx of Ca^{2+} in hippocampal neurons. (Fig. 3.9 A and B). With the treatment of 30 μM of MN-08 or 3 μM of MEM, the intracellular Ca^{2+} influx caused by glutamate were inhibited, evidenced by the reductions of fluorescence intensity. Compared with the rapid increase of intracellular Ca^{2+} induced by glutamate, MN-08 significantly inhibited the intracellular Ca^{2+} influx (Fig. 3.9 A and B). In addition, there was no significant difference in both magnitude and kinetics of the resting intracellular Ca^{2+} levels between the groups treated with the vehicle, MN-08 or MEN.

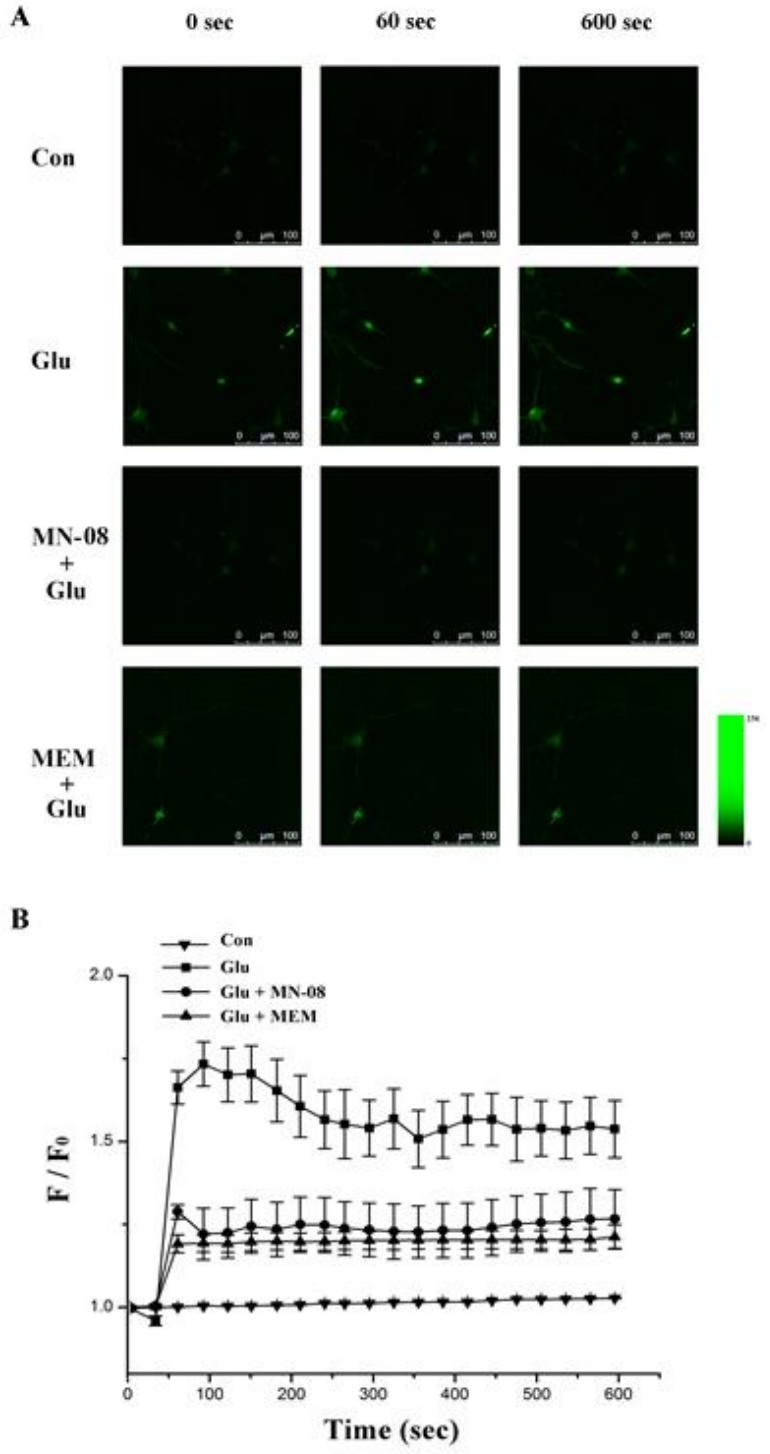

Fig. 3.9 MN-08 inhibited glutamate-induced $[Ca^{2+}]_i$ influx in primary cultured hippocampal neurons. (A) the representative figures of live fluorescence intensity were taken by a confocal microscope from cultured hippocampal neurons exposed to 100 μM glutamate with 30 μM MN-08 or 3 μM MEM at different times (0 s, 60 s, or 600 s). Scale bar: 100 μm (B) The time course curves were based on the fluorescence intensity determined by confocal microscope from cultured primary hippocampal

neurons exposed to the respective treatments. (F, the fluorescence value after exposure to glutamate in 10 min; F0, the fluorescence value just before exposure to glutamate). The data represent the means ± S.E.M of three independent experiments.

3.4 MN-08 blocked the NMDA receptor

3.4.1 MN-08 inhibited NMDA-mediated current in primary cultured hippocampal neurons

As mentioned above, the results of confocal microscopic Ca^{2+} imaging revealed that MN-08 might protect against glutamate-induced excitotoxicity by reducing the intracellular Ca^{2+} elevation. Since glutamate might induce the rapid increase of intracellular Ca^{2+} and NMDA receptors are highly permeable to Ca^{2+} in hippocampal neurons, we speculated that MN-08 might inhibit the glutamate-induced Ca^{2+} influx by interacting NMDA receptors. Next, we test the possibility that MN-08 might interact with NMDA receptors by using electrophysiological technique. As shown in Fig. 3.10, it can be observed that MN-08 at 1 – 30 μM inhibited NMDA-activated current in rat hippocampal pyramidal neurons by whole-cell patch-clamp recording. To determine the IC_{50}, different concentrations of MN-08 have been applied till the inhibitory effect reached steady state. The concentration responding curve constructed for MN-08 is shown in Fig 3.10B; the calculated IC_{50} value of MN-08 on the inhibition of MNDA-mediated current is 7.32 ± 0.23 μM at holding potential of -50 mV (Fig. 3.10). Moreover, as shown in Fig, 3.11, 30 μM of NMDA was applied to induced NMDA-mediated current, followed by MN-08 at 4 different concentration co-applied with NMDA. It was found that the inhibitory effects were increased as the concentration of MN-08 increased. Moreover, when the application of MN-08 was washed out, the NMDA-

activated current was recovered rapidly, suggesting that MN-08 might be disassociated with NMDA receptors in a fast-off rate.

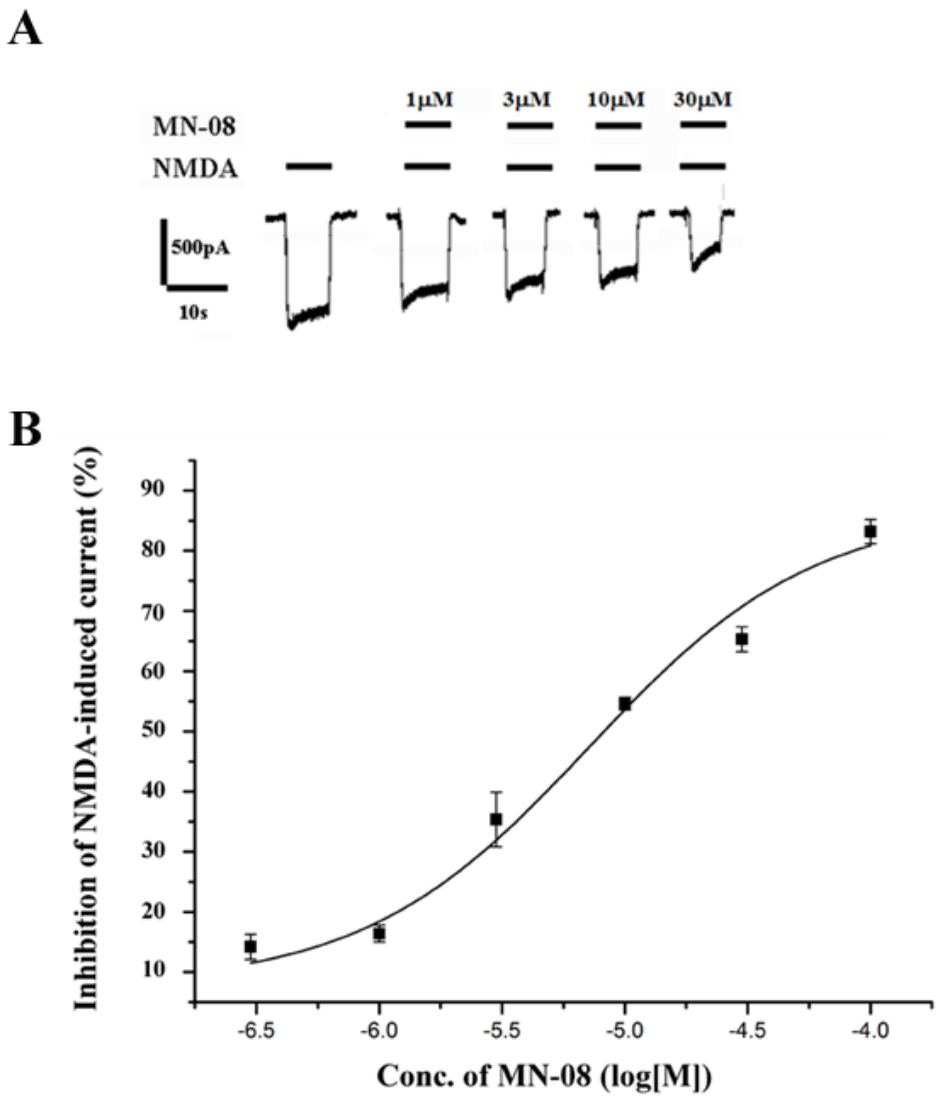

Fig.3.10 MN-08 inhibited the NMDA-mediate current in primary cultured hippocampal neurons. (A) The representative current traces reveal the inhibition of NMDA-mediate current by MN-08 at different concentration from 1 μM to 30 μM. **(**B) The concentration-response curve shows the inhibitory effect by MN-08 on current mediated by 30 μM NMDA. Amplitude of current activated by 30 μM NMDA serves as the control value for the calculation of percentage inhibition. The data represent the means ± S.E.M of three independent experiments.

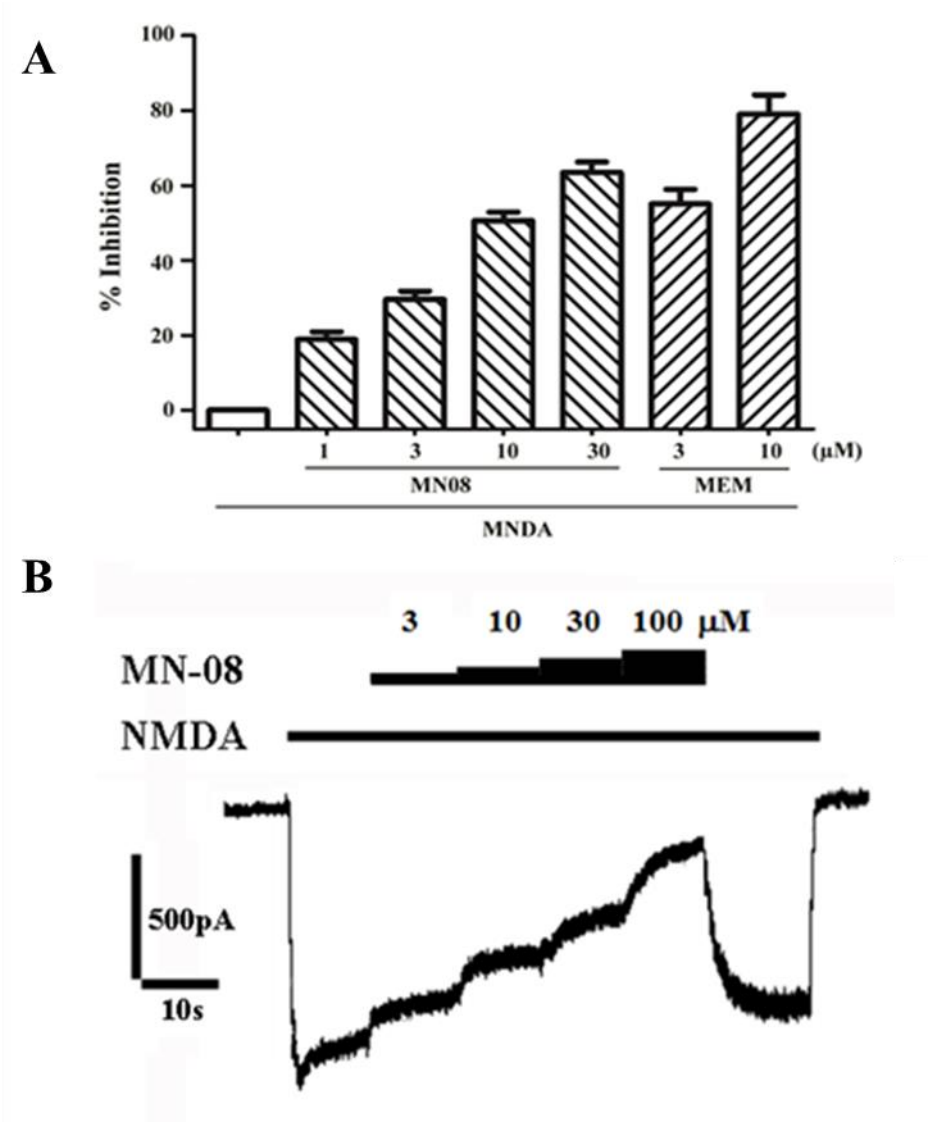

Fig. 3.11 Channel block of NMDA-mediated currents by MN-08. The representative current trace was recorded by whole-cell recording mode from the same patch-clamped neuron. (A) MN-08 inhibited the NMDA-mediate current in primary cultured hippocampal neurons. Dose-response of MN-08 and MEM on the inhibitory effects of MNDA-activated current at -50mV. (B) Current traces show the control current induced by NMDA (30 μM) and the cumulative inhibitions incurred by four consecutive co-applications of NMDA and MN-08 (3-100 μM) at -50mV. When the NM-08 was washed out, the NMDA-activated current was recovery. The data represent the means ± S.E.M of three independent experiments.

3.4.2 MN-08 interacted with NMDA receptor with the binding site similar to memantine

To gain insight into the acting site (s) of MN-08 and the NMDA receptor, molecular docking simulation was performed. The result showed that MN-08 might interact with NMDA receptor at the ion channel, with free energy of binding of -6.3 kcal/mol. As a reference, the free energy of binding of memantine with NMDA receptor is -5.4 kcal/mol, which is similar to that of MN-08. Furthermore, as illustrated in fig. 3.12, MN-08 (yellow) interacted with NMDA receptor at the Ser132 residue, which is similar to memantine.

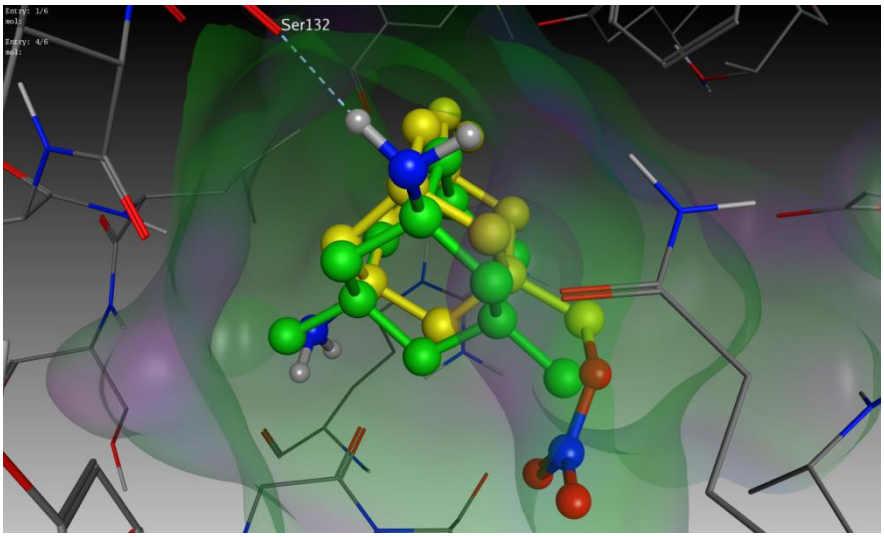

Fig. 3.12 Acting site of MN-08 and memantine on the NMDA receptor. A close-up view of the low energy pose of MN-08 (yellow) and memantine in the NMDA receptor channel pore generated by molecular docking.

3.5 MN-08 reversed the cognitive function impairment in VaD rat possible through the vessel dilation and improvement of the CBF.

3.5.1 MN-08 released NO *In vitro in vivo*

MN-08 was designed to release NO with the nitrate group by an enzymatic or non-enzymatic mechanism. To investigate whether MN-08 release NO *in vitro* (in PBS with L-cysteine), the concentration of NO was evaluated by the NO assay kit. It was found that MN-08 might release NO in a time-dependent manner. At 24 h after incubation, the concentration of NO released by MN-08 was 24.5 μM (Fig. 3.13A). We further evaluated the NO release by MN-08 *in vivo*. The blood samples were collected from rats by jugular vein via a PE50 catheter at various times (0, 0.25, 0.5, 1, 2 and 4 h) after administration with either MN-08 (25 mg/kg, iv) or Isosorbide dinitrate (ISDN, 3 mg/kg, iv). It was found the MN-08 released NO in a time dependent manner. At 4 h after the injection, the NO concentration has been elevated to 23.8 ± 1.89 μM. ISDN, a drug used for treating heart failure, was used as the positive control for the evaluation of the concentration of NO. (Fig, 3.13B)

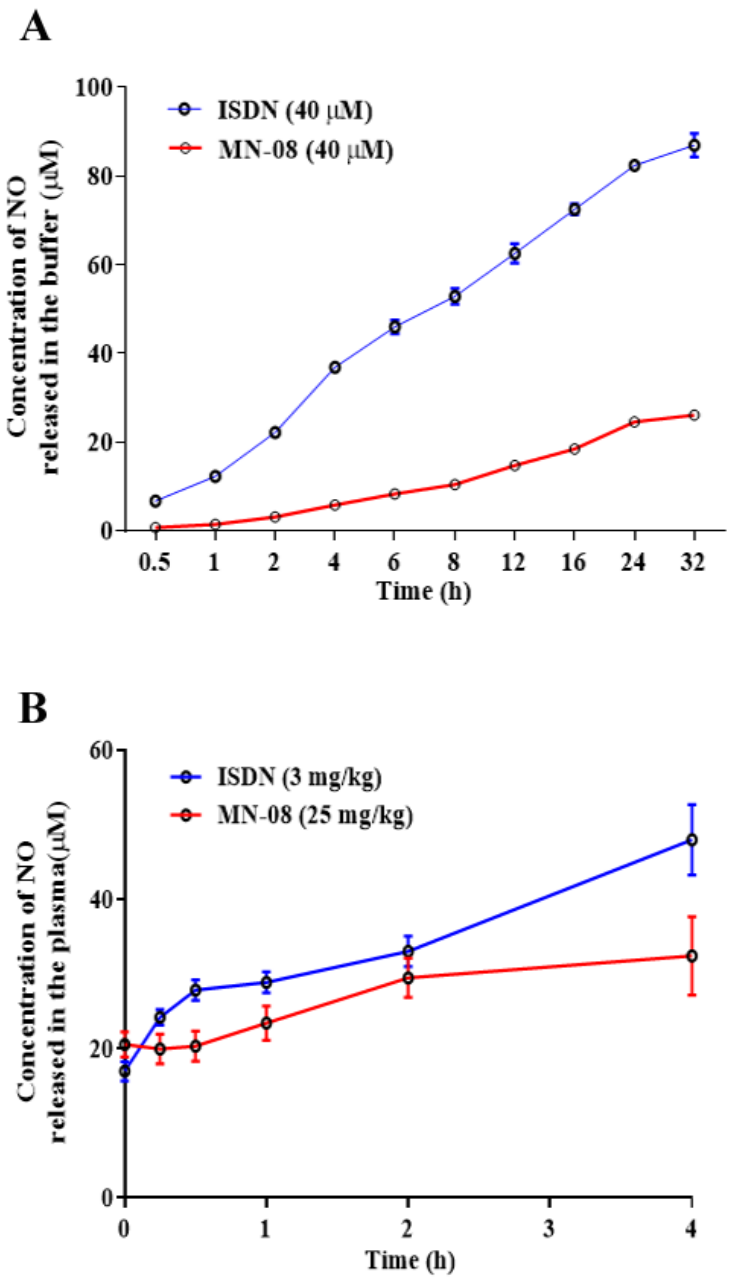

Fig. 3.13 MN-08 released NO in plasma of normal rats. (A) Formation of total NO by MN-08 (40 μM) and ISDN (40 μM) incubated in PBS buffer with 25 mM L-cysteine. (B) NO levels released in rat plasma. After MN-08 (25 mg/kg) or ISDN (3 mg/kg) was given intravenously through the tail vein, the rat's blood was drawn from the jugular vein at 0, 0.25, 0.5, 1, 2, and 4 h after drug administration. The MN-08 and ISDN released NO was reacted with Griess reagent. The absorbance was measured at 540 nm by microplate reader. Data were expressed as mean ± SEM (n=6).

3.5.2 MN-08 dilated the rabbit middle cerebral artery ring

To examine the vessel dilatory effects by MN-08, the rabbit middle cerebral artery rings pre-contracted by KCl (60 mM) were used to perform the experiments. The relaxation percentage and maximum relaxation (E_{max}) of the pre-contracted artery ring were recorded. Our results shown that MN08 (3 – 30 μM) significantly caused the relaxation of the artery ring in 5 mins. In particular, MN-08 at 30 μM might cause 57.32% of the relaxation (Fig. 3.14). Notably, memantine at 10 μM has shown no effects on the vessel dilation. Nimodipine was used as the positive control.

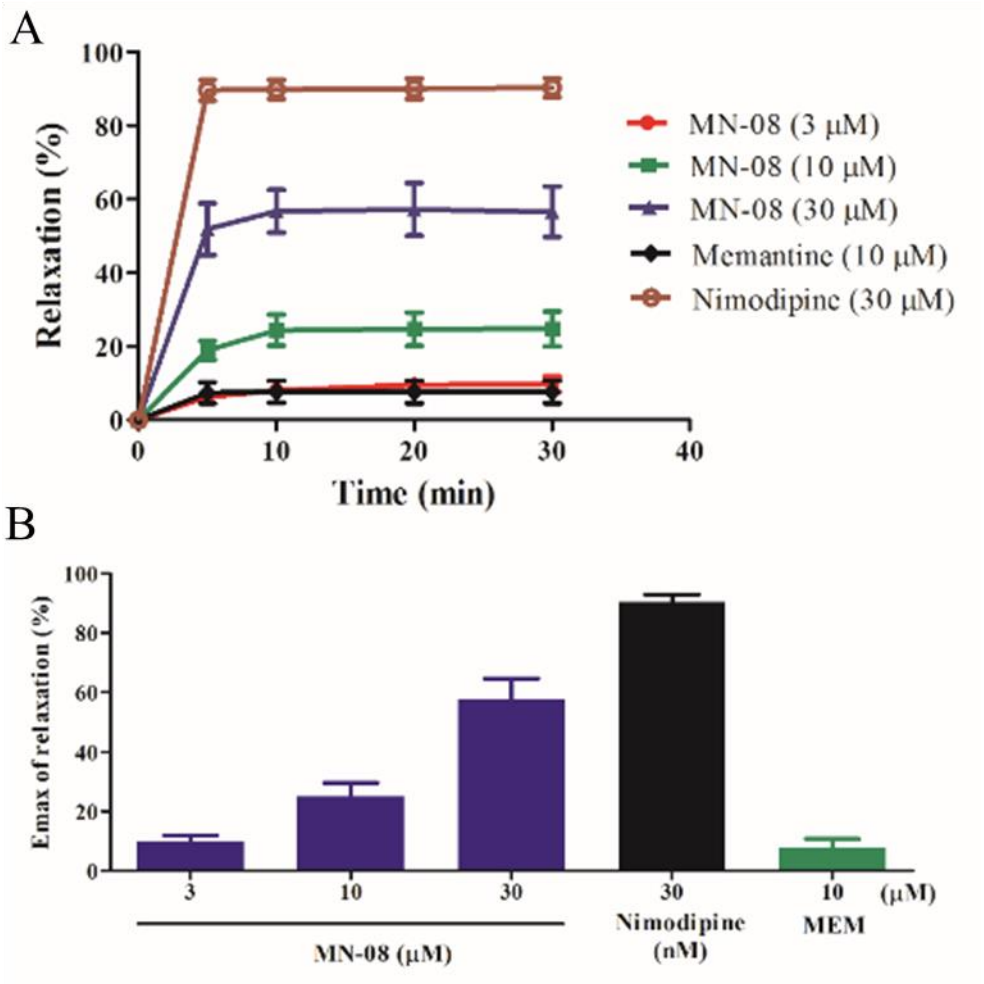

Fig. 3.14. Vasodilatory effect of MN-08 on KCl (60 mM) pre-contracted the middle cerebral artery with endothelium. The percentage (A) and E_{max} (B) of relaxation responses of middle cerebral artery induced by different treatments were expressed as the percentage of maximal contraction induced by KCl (60 mM). Nimodipine is the positive control. Data were expressed as mean ± SEM (n = 4).

3.5.3 Prediction of ADMET properties of MN-08

To investigate the ADMET properties and preliminary pharmacokinetics of MN-08, in silico predictions was carried out by using ACD/Percepta v14.1.0. A series of ADMET properties. In detail, the logarithmic ratio of the octanol - water partitioning coefficient (clog P), molecular weight (MW), solubility, Coca2 permeability, CNS penetration scores were calculated. In addition, preliminary profiles of toxicity of MN-08 were also predicted in terms of calculating the median lethal dose (LD_{50}) by oral administration.

As shown in Table 1, MN-08 was predicted to reach the brain by penetrating the blood-brain barrier, being characterized by adequate lipophilicity values. Notably, MN-08 exhibited very good Coca2 permeability and an acceptable toxicity profile, with the estimated LD_{50} being in the range of 50 – 2000 mg/kg for mouse, after oral administration. Memantine was also included in the prediction as a reference.

Table 1. Physical properties and ADMET prediction of MN-08

Comp.	LogP	MW	Solubility (mg/mL)	Caco2 permeability (Pe)	CNS penetration (Score)	LD_{50} ⩽ (mg/Kg)	LD_{50} ≥ (mg/Kg)
MN-08	3.26	254.33	79/6 - **S**	87E-6 - **HP**	-2.53 - **P**	2000	50
Memantine	3.48	179.30	244 - **S**	89E-6 - **HP**	-2.03 - **P**	2000	300

Note: MW: molecular weight**; LogP:** logarithm of the octanol-water partition coefficient; **S:** Soluble; **Hp :** Highly permeable; **P:** penetrant.

3.5.4 MN-08 increases the CBF in temporal and parietal cortex of VD rats

CBF reduction usually occurs in the early stages of vascular dementia [151]. It has been considered to be one of the main causes of cognitive impairment in VaD patients [152]. As shown in Fig. 3.15, the decline of CBF could be observed in both parietal and temporal cortex of the rats in 5 mins after the 2VO surgery. The CBF in temporal and parietal cortex significantly declined to approximate 30% compared to the sham group. In addition, the CBF would gradual be restored to approximate 70% 4 weeks after 2VO surgery. Our result shown that, MN-08 (6 and 12mg/kg, ig) significantly improved the CBF in both parietal and temporal cortex compared with those in the model group 4 weeks after surgery. In particular, MN-08 at 6mg/kg has exhibited the greatest improvement of CBF in both temporal and parietal cortex. The NO scavenger carboxy-PTIO significantly abolished the improvement induced by MN-08 (6 mg/kg) in CBF, indicating that NO signaling pathway might be involved in the anti-dementia effect by MN-08. Notably, the memantine treatment at 5mg/kg has also shown effects to improve the CBF in parietal and temporal cortex. But the efficacy was smaller compared to the MN-08 treatment (6 and 12 mg/kg) groups.

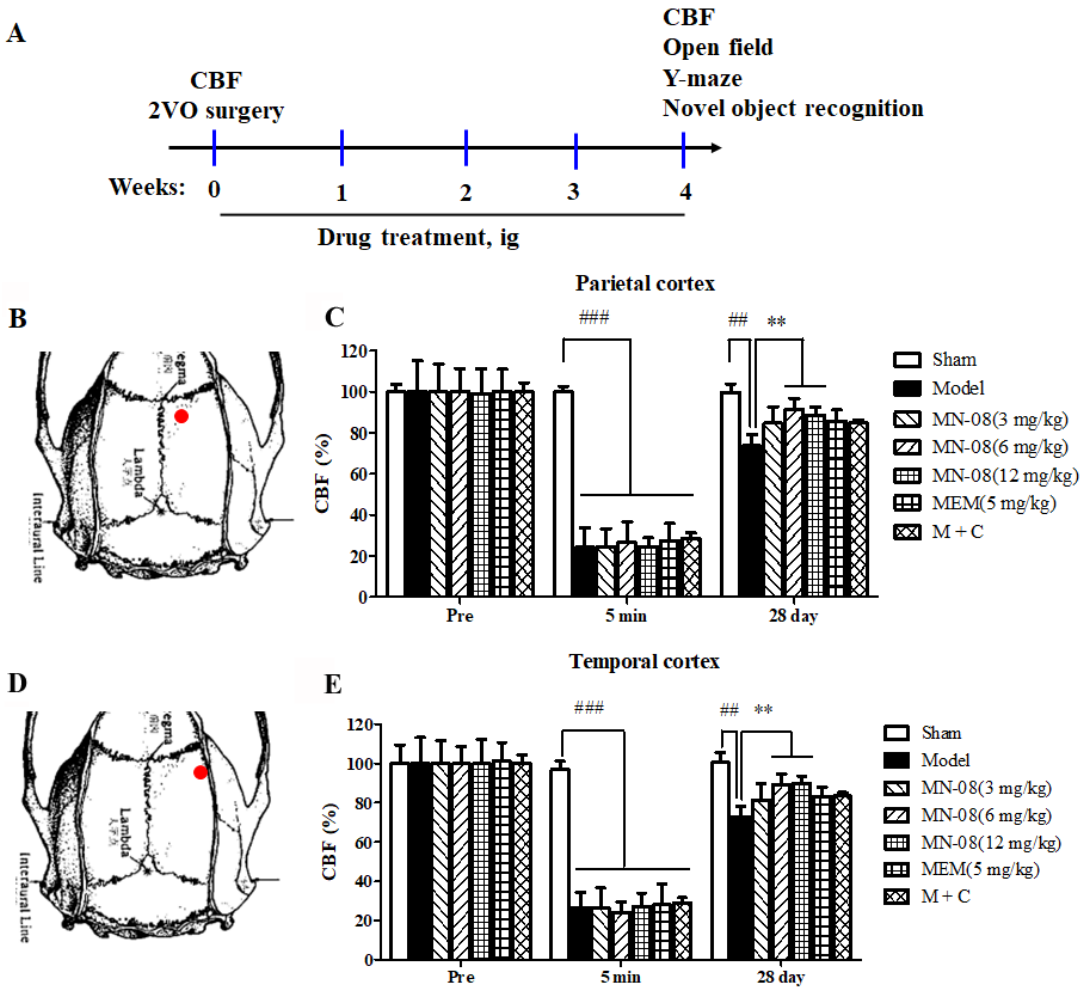

Fig. 3.15 MN-08 increases the CBF in temporal and parietal cortex of VD rats. The diagram shows the experimental design of the animal study (A). The schematic illustrations of the monitory sites, (B) parietal cortex and (C) temporal cortex, of CBF indicated by the red dots. The changes of CBF monitored by a laser Doppler in parietal cortex (D) and temporal cortex (E) at pre-operation (Pre), 5 min after surgery and 28 days after MN-08 treatment. Results were expressed as means ± SEM (n=16 per group). MEM: memantine; M + C: MN-08 (6 mg/kg) + carboxy-PTIO (0.3 mg/kg); Statistical significance was analyzed by one-way ANOVA. ###$p < 0.001$, ## $p < 0.01$ vs. sham group; **$p < 0.01$ vs. model group.

3.5.5 MN-08 improves the cognitive function impairments and spontaneous activities of VD rats

To evaluate the effects to improve the cognitive function impairment and spontaneous activities of MN-08 *in vivo*, open field test and novel objective recognition were performed. The spontaneous activity of the rats with or without drug treatments after 2VO surgery were evaluated by open field test (Fig. 3.16 A). After 4 weeks of 2VO surgery, the total moving distance of rats in model group were significantly decreased to 7,822 ± 824.8 cm. Our result shown that both MN-08 (3, 6 and 12 mg/kg, ig) and memantine (5 mg/kg, ig) groups remarkable increased the total moving distance of rats compared to those of the model group. Furthermore, the improvement by MN-08 (6 mg/kg) on locomotor activities were significantly abolished carboxy-PTIO (0.3 mg/kg), indicating that NO released from MN-08 might be involved in the improvement of spontaneous activity in VD.

The cognitive abilities of the rats after 2VO surgery were examined by Y maze test and the object recognition test. Specifically, the spatial memory could be evaluated by Y maze test and the non-spatial working memory related to the frontal-subcortical circuits might be evaluated by the novel object recognition test. As shown in Fig. 3.16 B and C, the rats in the model group spent lesser dwell time compared to those of the sham group in the novel arm in Y maze test. In addition, the discriminative ability in the object recognition test of rats in the model group was also markedly decline. Our result demonstrated that MN-08 (6 mg/kg) significantly improved cognitive memory

compared to the model group in Y maze test and object recognition test respectively. Notably, memantine treatment also showed the improvement in the behavior tests, but the efficacy was lower than MN-08. Furthermore, the improvements by MN-08 (6 mg/kg) on spatial memory were significantly abolished carboxy-PTIO (0.3 mg/kg).

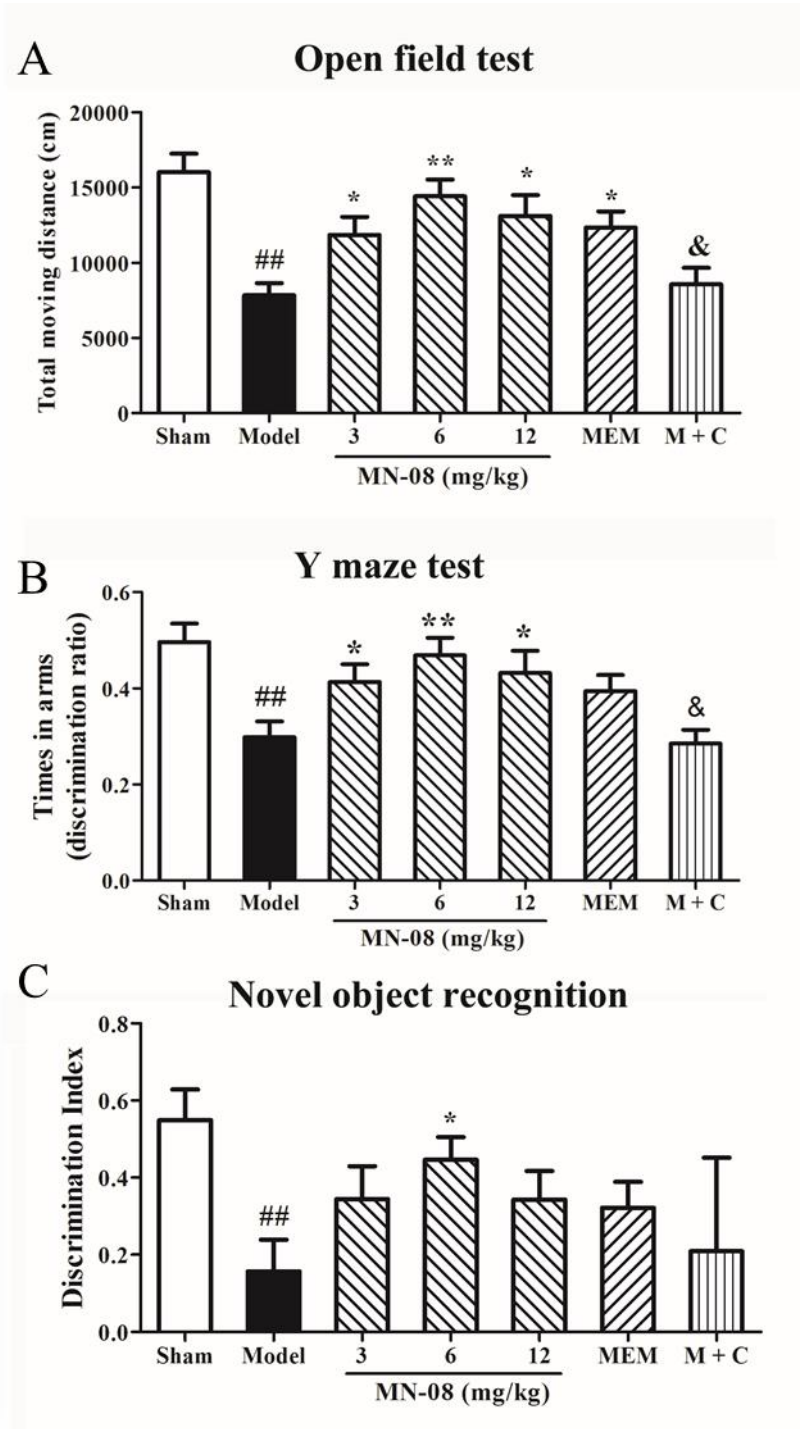

Fig. 3.16 MN-08 improves the learning memory, cognitive abilities and spontaneous activities of rats after 2VO surgery. (A) MN-08 significantly increased the total moving distance of the rat after 2VO surgery in open field. (B) MN-08 significantly improved the discrimination ratio in Y maze test; and (C) the discrimination index in object recognition task. MEM: memantine; M + C: MN-08 (6 mg/kg) + carboxy-PTIO (0.3 mg/kg). Results were expressed as means ± SEM (n=16). $^{\#\#}p < 0.01$ vs. sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. model group; $^{\&}P < 0.05$ vs. MN-08 (6 mg/kg) group.

Chapter IV
Discussion

The etiological studies suggested that the dementia patients might have multiple pathologies, possibly due to the high prevalence of VaD and AD. According to the Religious Orders Study and the Rush Memory and Aging Project, the mixed VaD and AD pathology was predominantly found in dementia patients. In fact, VaD and AD shared the same risk factors among the elderly, such as aging and cerebral vascular diseases. Additionally, the cerebral vascular diseases might enhance the pathological progress of AD possibly by the limitation of cerebral blood supply [110]. Here, we speculated that the memantine nitrates, MN-08 particularly, might be the multi-functional anti-dementia drugs. Firstly, similar to memantine, MN-08 might protect against glutamate-induced excitotoxicity through the blockage of NMDA receptors; and secondary, the nitrate moiety of MN-08 might improve the CBF by vasodilation which might effectively reverse the cognitive function impairments of the patients with VaD. To validate our hypothesis, we have employed various of *ex vivo*, *in vitro* and *in vivo* models in this study. Meanwhile, the underlying mechanisms might also be investigated.

4.1 Memantine nitrates prevented glutamate-induced neuronal loss in CGNs

Although it can be foreseeing that patients with dementia will be rapidly increased in the coming decades, limited drugs can be used to cure the disease. Currently anti-AD drugs can only offer limited symptom relief benefits, but not delay or stop the pathological progress. Our collaborators have designed and synthesized the novel memantine nitrates by adding a nitrate group onto memantine backbone. Some of the memantine nitrates have shown significant protective effects. In the present study, we have further evaluated the neuroprotective effects and characterized the underlying mechanisms on the selected compounds *in vitro* and *in vivo*.

Many studies have revealed that the neuronal loss induced by excessive glutamate might play a crucial role in AD pathology. In previous studies, our laboratory has demonstrated that the multi-functional neuroprotective dimers derived from tacrine might protect against the glutamate-induced excitotoxicity in CGNs [127,150,153]. CGNs are widely employed in the investigations of neuroscience and neuropharmacology [154,155]. Many studies have indicated that the glutamate-induced excitotoxicity in CGNs could be used to evaluate the neuroprotective effects of novel anti-AD candidates [127,138]. In several studies, a high concentration of glutamate was used for induction of neuronal loss in primary cultured rat cortical neurons or cell lines. The major concern is that the high concentration of glutamate might induce cell lost only by excitotoxity but also by non-specific damage to the neurons, such as increased oxidative stress. In this study, we chose to evaluate the neuroprotective effects of the candidates in CGNs. Low

concentration of glutamate (100 μM) might be able to cause 50-60% neuronal loss in CGNs mainly through the excitotoxicity. Based on our well-established protocol, high purity of neurons from the cerebellum might account for the difference of the concentration. In addition, CGNs are easier to acquire and maintain for *in vitro* assays, including MTT and Western blotting [134]. More importantly, it was found that glutamate at a low concentration in CGNs might mimic the signaling cascades as those in hippocampal and cortical neurons [154]. Thus, CGNs might be not only used for evaluating the neuroprotective effects but also investigating the underlying mechanisms of the novel compounds.

The results of MTT assays have demonstrated that the pretreatment of three memantine nitrates, including MN-06, MN-08 and MN-12, significantly protected the neurons against glutamate-induced excitotoxicity in CGNs. We further investigated whether the compounds prevent glutamate-induced apoptosis in CGNs. The results of staining and western blotting indicated that the selected three compounds might reduce the number of apoptotic bodies and increase the level of anti-apoptotic protein bcl-2. The results showed that MN-08 should be the best neuroprotectant with the highest efficacy and most potent among the three compounds. Thus, the detail neuroprotective mechanisms of MN-08, as the representative, were carefully characterized by variety of experiments.

4.2 MN-08 protected against glutamate-induced apoptosis though multiple targets

Previous studies have reported that the glutamate-induced excitotoxicity might cause neuronal death by the activation of ERK pathway and the suppression of PI3-K/Akt pathways [156]. In fact, the activation of ERK and GSK3β have been found in a variety of *in vitro* and *in vivo* models associated with neurodegenerative diseases, indicating that the two pathways might greatly contribute to neuronal degeneration [149,157]. Thus, we speculated that MN-08 might exhibit the neuroprotective effects by regulating the ERK and PI3-K/Akt pathway.

Consistent with the previous studies, glutamate at low concentration might cause ERK phosphorylation on Ser-42/44 site in a time-dependent manner [158]. The results of western blotting revealed that the glutamate insult significantly increased the expression of phosphorylation of ERK at Ser-42/44 site, indicating that the activation of ERK might be involved in the glutamate-induced apoptosis. The pretreatment of MN-08 might effectively reduce the elevation of p-ERK, as caused by the glutamate insult. The result implied that MN-08 might protect against glutamate-induced cytotoxicity by reversing the activation of ERK pathway.

On the other hand, the activation of PI3-K/Akt is one of the important pro-survival pathways. We have previously demonstrated that the attenuation of glutamate-suppressed PI3-K/Akt could offer significant neuroprotective effects [150,159]. GSK3β is an important downstream signaling member of Akt, which can be activated on two

phosphorylation sites (Tyr216 and Ser9) by variety of stimuli [160]. In particular, the phosphorylation of Tyr216 residue might activate GSK3β; while the phosphorylation of Ser9 residue might inhibit the activity of GSK3 [161]. It was found that the pretreatment of MN-08 reversed the glutamate-induced phosphorylation of GSK3β at Ser-9, as well as the suppression of p-Akt at Ser473, suggesting that MN-08 offered neuroprotective effects by inhibiting GSK3β and activating Akt. Furthermore, Akt is one of the important downstream targets of PI3-K, which might activate Akt by the phosphorylation at Ser473. As mentioned above, the activation of Akt might inhibit the activity of GSK3β by inducing the phosphorylation at Ser9 [149,150,162]. Our results of western blotting demonstrated that MN-08 might reverse the glutamate-induced inhibition of Akt and activation of GSK3β. Moreover, LY294002, a specific PI3-K inhibitor might block the effects of MN-08 on Akt and GSK3β, indicating MN-08 is likely to possess neuroprotective effects via the PI3-K/Akt pathway. The findings were also confirmed by the MTT assay, showing that LY294002 significantly abrogated the neuroprotective effects by MN-08 against glutamate-induced neural toxicity in CGNs. Taken together, MN-08 might protect against glutamate-induce cytotoxicity by reversing the dysregulations of ERK pathway and PI3-K/Akt pathway.

As we have previously reported, ERK and PI3-K/Akt pathways are the two important pathways regulating the neuronal cell death during the glutamate insults [134,150]. In particular, ERK pathway could be overactivated, while PI3-K/Akt pathway might be suppressed by treatment of the AD models *in vitro* and *in vivo*. However, only

regulating the dysfunctions of ERK or PI3-K/Akt pathway by using the specific inhibitors might not be able to effectively prevent the neuronal loss. We have demonstrated that the novel neuroprotective dimers might significantly protect against several neural toxicities, such as glutamate and hydrogen peroxide [134,163]. As we have known that the nature of AD and VaD are multifactorial neurodegenerative disorders, single-targeted drugs might not be effective enough to stop or reverse the pathological progress. In this connection, the multiple-targeted neuroprotective candidates might be the answer for dementia [164]. Moreover, in pharmacological screening stage, three memantine nitrates have shown significant neuroprotective effects against glutamate-induce neurotoxicity via multiple targets, suggesting that memantine nitrates might be the potential candidates for treating dementia. More importantly, these results suggested that the structural modifications of memantine might obtain a series of neuroprotectants, which could regulate the dysfunction of signal pathways during the glutamate insults.

4.3 MN-08 reduced the Ca^{2+} influx induced by glutamate in primary cultured hippocampal neurons

As mentioned above, MN-08 might reverse the dysfunctions of ERK and PI3-K/Akt pathways caused by glutamate, which could be very important for the treatment of dementia including AD and VaD. Previous studies have indicated that the rapid influx of Ca^{2+} is crucial to initiate the apoptotic pathways, including ERK and PI3-K/Akt pathways, and subsequently causes synaptic dysfunctions and neuronal loss [134,150,165]. In addition, the overloaded Ca^{2+} subsequently induce oxidative stress, which might damage the cell components and promote neuronal cell death. Furthermore, studies suggested that the extra-synaptic NMDA receptor might be activated by Aβ, subsequently causing the synaptic dysfunctions and neuronal loss [134,150,165]. Moreover, it was found that glutamate might be overexpressed in the ischemic stage of stroke, also elevating the level of excitotoxicity and causing the neuronal loss [50,166].

NMDA receptor is a type of ionotropic receptors, gated by glutamate, which has been closely related to neurodegenerative disorders [167]. The excessive influx of Ca^{2+} might be induced by overstimulation of NMDA receptor [168]. Lines of evidence have indicated that NMDA receptor antagonists could attenuate neuronal cell death against the glutamate insults [169]. We would like to explore whether MN-08 retain the property to inhibit NMDA receptors from memantine.

Memantine is a NMDA receptor antagonist, which prevents the glutamate-induced

neuronal cell death by reducing the Ca^{2+} influx via directly blocking the NMDA receptor. We speculated that MN-08, the memantine nitrate derived from memantine, might also be capable to reduce Ca^{2+} influx by blocking NMDA receptor. Firstly, we examined whether MN-08 might reduce Ca^{2+} influx by using the confocal microscopic scanning. Our results demonstrated that 100 μM of glutamate induced a repaid intracellular influx of Ca^{2+} in primary cultured rat hippocampal neurons. As expected, the results of confocal microscopic scanning showed that MN-08 might inhibit the glutamate-induced Ca^{2+} influx. Moreover, focus on the chemical structure of memantine nitrates, the backbone of memantine has been retained, aiming to keep the interaction with NMDA receptors. To confirm the interaction between MN-08 on NMDA receptor, the molecular docking simulation have been performed. The results shown that MN-08 binds to NMDA receptor on similar binding sites, compared to memantine with similar binding free energy. The results indicated that MN-08 might be the potential NMDA antagonist, as memantine, with moderate affinity with NMDA receptor.

The findings are further confirmed by electrophysiological experiments by patch clamp. We have shown that NMDA at 30 μM might evoke Ca^{2+} current, which could be recorded by using whole cell recording of patch clamp. We have demonstrated that the co-applied of MN-08 (1 - 30 μM) might inhibit the NMDA-evoked Ca^{2+} current in primary cultured hippocampal neuron. Moreover, the inhibitory effects by MN-08 were abolished when we withdraw the application of MN-08, indicating that MN-08 might

also dissociated from the NMDA receptors with fast-off rate. And the IC_{50} of MN-08 on the inhibition of NMDA mediated current is slightly high than memantine.

Based on the glutamate-induced neuronal excitotoxicity hypothesis, the scientists try to discover and develop the NMDA receptor antagonists as the anti-dementia drugs. Several NMDA receptor antagonists have been developed; however, few of them succeeded to pass the clinical trial and become the anti-dementia drugs clinically [8]. One of the major issues might be the serious adverse effects caused by the candidates. It has been revealed that many important physiological functions are mediated by NMDA receptor. Antagonists with high affinity could reduce the Ca^{2+} influx by antagonizing the NMDA receptor tightly; however, the normal physiological signal transductions might be also blocked. For example, MK-801 and phencyclidine, very potent NMDA receptor antagonists with excessively high affinity, cannot be used for treating AD because they might affect the physiological functions of NMDA receptors. Compelling evidence suggests that the antagonist with moderate affinity on NMDA receptors might offer therapeutic benefits for treating dementia without affecting physiological functions. Lipton *et. al.* have proposed that the ideal NMDA receptor antagonist drug should be pathological activated, uncompetitive with moderate affinity and fast-off rate [79]. Memantine might be a good example of the ideal NMDA receptor antagonist for treating AD. Memantine is an open channel blocker of NMDA receptors in an uncompetitive manner with moderate affinity and fast-off rate. These characterizations,

therefore, make memantine to be clinically effective and well tolerated to the AD patients. According to the results obtained in this study, MN-08 has shown moderate affinity for NMDA receptors. In addition, the inhibitory effects of MN-08 were disappearance after the interaction with NMDA receptors. All these results suggested that, similar to memantine, MN-08 might also an ideal NMDA receptors antagonist for treating AD. In the future, the detail electrophysiological studies might be worth to conduct for the characterization of the detail interaction between MN-08 and NMDA receptor.

4.4 MN-08 might dilate the vessel and improved CBF by releasing NO

The brain accounts for 2% of our body mass but may consume around 20% of energy. The relatively huge energy consumption is supported by the cerebral circulation, which responses to the oxygen and nutrient supply. One of the major reasons for large amounts of energy requirement is to regulate the neuronal signal transmission. Therefore, the reduction of CBF by CVD might cause the dysfunctions of brain functions, and finally resulting in VaD. CVD was firstly associated with dementia as "arteriosclerotic dementia" by McMenemey in 1961 [170]. The lesions of cerebrovascular system might enhance the cognitive decline and consequently develop dementia. Many CVD such as ischemia stroke, initiating a cascade of biochemical processes including the release of excitotoxicity, which might be one of the key factors for neurodegeneration [166,171]. In addition, limitation of CBF might further increase the neuronal loss, subsequently resulting in cognitive functions impairment [172,173]. Previous studies had shown that NO donors increased the CBF in stroke models, indicating that the elevation of NO might also be helpful for the treatment of VaD [174-177]. Apart from the neuroprotection, MN-08 might increase the CBF by providing exogenous NO to dilate the cerebral vessel.

NO signaling pathway is important to regulate the CBF. Studies have revealed that NO might affect the CBF in the vasculature and cellular level [178,179]. NO is important to maintain the vasodilation through the activation of NO/cGMP signaling pathway, resulting in increases of cerebral blood supply in brain region [180]. We have

demonstrated that MN-08 might release NO *in vitro in vivo* and wonder whether the NO might cause the cerebral vessel dilation. As shown in Fig. 3.13, MN-08 concentration dependently induces vessel dilation in the pre-contracted rat middle cerebral artery ring *ex vivo*, and the vessel dilatory effects were further confirmed by the improvement of CBF in the 2VO rat 28 days after the surgery. Notably, memantine showed few effects on the vessel dilation *ex vivo* and *in vivo* possibly due to the inhibition of nicotine-induced neurogenic vasodilation [131,181]. Notably, the CBF of the animals in the control group would also be recovery after surgery. The phenomenon could be explained by the angiogenesis, which might compensate the lesions of cerebral vessel circuit induced by 2-VO surgery. That means the reduced CBF might be recovered through the neovascularization. Memantine might provide partial effects to increase CBF, possibly by upregulating the expression of vascular endothelial cell growth factor (VEGF), as memantine might increase the expression of VEGF [182,183]. Constituted with the previous works, the Memantine treatment group also showed effects to reverse the CBF decline and spatial memory impairment after the 2-VO surgery [184,185]. Apart from the vasodilation, these results strongly suggested that memantine nitrates might also enhance the expression of VEGF, which will be further focused in the future investigations.

In the traditional view, NO is a toxic molecule, which might induce cytotoxicity by overexpression of tumor suppressor p53 and DNA fragmentation. As shown in the results, MN-08 might increase the concentration of NO both *in vitro in vivo*. Would it

induce neurotoxicity and enhance the neuronal loss inside the brain? The answer should be no, evidenced by the neuroprotective effects offered by the treatment of mN-08 *in vitro in vivo*. Interestingly, the endogenous NO is a double-edged sword: it could be neurotoxic or neuroprotective. Endogenous NO synthesized by nNOS neuronal nitric oxide synthase (nNOS) is the major source of NO in CNS, responding to the overloaded Ca^{2+}. The overproduction of NO was thought to exhibit neurotoxicity closely associated with neurodegenerative disorders [186,187]. Reduction of the upregulated nNOS activity might be the effective for neuroprotection by inhibiting the nitrosative stress [180,188-190]. In contrast, studies implicated that inhaled NO or administrated sodium nitrite, one of the NO donors, by intravenous injection might show neuroprotective effects in both mice and rats, respectively. Another study also showed that inhalation of NO exhibited neuroprotective effects in nNOS and endothelial nitric oxide synthase (eNOS) knockout mice [191]. Taken together, all these findings indicate that NO might provide the therapeutic benefit to the cerebral vascular disease, as well as the VaD, not only through the vasodilation but also possibly through the neuroprotection.

Studies showed that memantine might significantly inhibit the generation of NO by inhibiting nNOS, which might be one of the possible mechanisms for its neuroprotection [187,188,190]. We have demonstrated that MN-08, different from the dramatically raise of NO derived from nNOS, is able to cause the release of NO continuously and slowly in a long period of time. It might provide two advantages: the neuroprotective effect and vessel dilatory effect. In fact, our latest results have shown

that MN-08 might protect against the oxygen glucose derivation (GOD)-induced neuronal death in primary cultured cortical neuron (data not shown). The protective effects could be abolished by the NO scavenger, indicating that NO mediated the neuroprotective effects provided by MN-08, at least partially.

4.5 MN-08 reversed the cognitive function impairment in rat after 2-VO surgery

The novel memantine nitrates have been designed based on the idea to achieve the targeted release of NO in the desire location, such as the brain. The backbone of memantine have been maintained to retain the pharmacokinetic profile, including quickly passing through the blood brain barrier and distributing to brain tissue. In addition, the memantine nitrates, similar to memantine, preferentially bind to NMDA receptors, which are mainly existing in the brain. NMDA receptor might act as an anchor to retain the memantine nitrates in the brain. As the results, NO can be specifically released in brain and selectively dilate cerebral vessels. Thus, MN-08 may provide therapeutic potential for the treatment of neurodegenerative diseases via neuroprotection and vasodilation.

We have also conducted preliminary pharmacokinetic studies on MN-08. The tissue distribution of MN-08 wa evaluated in rat. MN-08 was quickly removed from the plasma, thus reducing the risk for hypotension. It was also found that MN-08 could be quickly distributed to the major organs including brain, indicating that MN-08 might pass through the blood brain barrier. These results were consistent with our *in silico* prediction. More importantly, the concentration of MN-08 in brain could be reach to 50 μM when the administrative dosage is 60 mg/kg (i.v.) in SD rats. The results suggest that MN-08 might be sufficient to possess neuroprotection and vasodilation in brain.

In the next step, the anti-dementia effects of MN-08 were further investigated in the VaD *in vivo* model. We have selected the 2-VO surgery to induce the movement and spatial memory impairment, which might mimic the symptoms of VaD, in rats. In the acute ischemic stage, the over stimulation of NMDA receptors might be the dominant mechanism, causing the primary neuronal loss. And in the reperfusion stage, the energy and nutrient would be reduced as the reduction of CBF after the surgery. The cognitive ability was examined with both the Y maze test and the object recognition test. Specifically, the spatial memory could be evaluated by Y maze test and the non-spatial working memory related to the frontal-subcortical circuits might be evaluated by the novel object recognition test. The results of behavior tests showed that MN-08 might improve the movement and cognitive function impairments in the 2-VO rats. Moreover, according to the results of in vivo experiments, the NO scavenger Carboxyl-PTIO abolished the improvement offered by MN-08, indicating that NO derived from MN-08 might be involved, at least partially. Thus, these findings indicated that the roles of NO derived from MN-08 could be vasodilation and neuroprotection to reverse the impairments of 2-VO rats. Consistent with the previous works, the Memantine treatment group also showed effects to reverse the movement and spatial memory impairment after the 2-VO surgery [184,185]. But the efficacy was lower than those of MN-08. Apart from the neuroprotection and vasodilation, these results strongly suggested that memantine nitrates might also enhance the expression of neurotrophic factors including VEGF, which will be further focused in the future

investigations. Therefore, comprehensive comparison of the therapeutic effects of MN-08 and memantine deserves an in-depth *in vivo* study in appropriate models.

4.6 Multi-target directed ligand strategy might be the answer for the treatment of dementia.

Dementia, including AD and VaD, is caused by multiple factors, suggesting that there might be multiple therapeutic targets, which could be major reason why the currently available single-targeted drugs can only offer limited benefit to the patients. More importantly, due to the complexity of AD pathology, there was no new anti-AD drug being approved by FDA since 2001, even billions of dollars have been input by the big pharmaceutical companies to the new drug investment. In particular, the antibody targeting the reduction of Aβ have shown very promising anti-dementia effects in variety of animal model; however, none of them could complete the clinical trial. The reality indicates that single-target drug might not be the promising strategy for the treatment of dementia. Alternatively, the multi-target directed ligand (MTDL) has been introduced as the innovative strategy for the development of anti-dementia drugs.

In 2014, the combination of donepezil and memantine have been approved to be used for treating moderate to severe AD. The combination therapy has been borne out by preclinical studies and clinical observations. Furthermore, study has pointed out that the therapeutic effects were achieved not only additive but also synergistic action by donepezil and memantine [82]. Memantine could inhibit the over-activation of NMDA receptor, which might further release the inhibition of the cholinergic transmission [98]. As the results, the cholinergic transmission could be restored synergistically with the inhibition of AChE by donepezil.

The success of donepezil and memantine combination has opened a door for the treatment of multi-factorial neurodegenerative disorders through multiple targets. In the last few years, multi-functional compounds or MTDL paradigm has been proposed as an innovative strategy for developing new drugs [164,192]. The MTDL strategy can be obtained by combining the pharmacophores and functional chemical moieties, making it possess desired multiple functions [193] [194]. The multi-functional single molecules might provide more advantages compared to the combinations, such as more appropriate pharmacokinetic profile and easier for admission for the dementia patients. According to the concept of MTDL, many novel dimers/hybrids have been synthesized based on the exciting anti-AD drugs. For example, a series of tacrine-coumarine hybrids have been designed and synthesized to inhibit AChE, which was shown to reduce the Aβ aggregation and chelate the metal ions [195] [196]. Another example is the donepezil and curcumin hybrids, which were shown significant AChE inhibitory effects, antioxidant effects and metal-chelating activity [197]. Prof. Yifan Han *et al* have designed and synthesized series of homo- and hetero dimers derived from tacrine and/or the fragment of huperzine A [87,153,163]. It has been found that the dimers might provide multi-functional anti-AD effects by inhibiting AChE, antagonizing NMDA receptors, and regulating the dysfunctions of cellular pathways [134,163].

NMDA receptor antagonists have been suggested to be the potential therapeutic agents

for neurodegenerative disorders. Many researchers have tried to develop more potent and safe NMDA antagonist drugs for the treatments of AD, ALS and stroke. However, thus far, memantine still is the only one NMDA antagonist drug approved by FDA for the treatment of AD. Therefore, based on the strategy of MTDL, structural modification by adding the desire pharmacophore onto memantine could be an effective approach to obtain the novel NMDA antagonist with multiple functions. The novel memantine nitrates have been designed and synthesized to achieve the superior therapeutic effects for dementia associated with AD and VaD. Here, we have demonstrated that MN-08 might provide anti-dementia effects by inhibiting NMDA receptors, regulating the dysfunction of PI3-K/Akt pathway and improve CBF through NO releasing. Moreover, we have further found that MN-08 might promote synaptogenesis and reverse the dysfunctions of molecular targets, including MEF2D, indicating that MN-08 might be the promising multifunctional anti-dementia drugs (data no shown). All these results indicate that adding the nitrate group onto the backbone of memantine might not greatly change the safety and pharmacokinetic properties. These data also support that structural modification of an existing drug could be an effective strategy for the development of multi-functional anti-dementia drugs in the future.

Chapter V
Conclusion

5. Conclusion

In the previous studies, our collaborators have designed and synthesized a series of memantine nitrates derived from memantine. In this study, the neuroprotective effects of three novel memantine nitrates, MN-06, MN-08 and MN-12, as well as the underlying mechanisms, have been investigated. Three memantine nitrates, MN-06, MN-08 and MN-12 have shown significant neuroprotective effect against glutamate-induced neurotoxicity in CGNs.

MN-08 has shown the strongest protective effects, in terms of potency and efficacy. MN-08 has been demonstrated to protect against glutamate-induced excitotoxicity by concurrently regulating the dysfunctions of ERK and PI3K/Akt pathways. MN-08 inhibited the Ca^{2+} influx induced by glutamate in the rat primary cultured hippocampal neurons. MN-08 blocked the MNDA-mediated current via inhibition of NMDA receptors. Moreover, MN-08 might dilate the pre-contracted vessel ring *ex vivo* and improve the CBF in VaD rat model. More importantly, the behavior tests showed that MN-08 might improve the cognitive function impairment in the VaD rat model (Fig. 5.1).

In conclusion, memantine nitrates, MN-08 in particular, could protect against glutamate-induced excitotoxicity via blocking the upstream NMDA receptors, inhibit the subsequent Ca^{2+} influx and to the downstream signaling pathways including ERK and PI3-K/Akt/GSK3β cascade. Moreover, MN-08 has also reversed the cognitive

impairments *in vivo* through neuroprotection and vascular dilation. Taken together, MN-08 may be the potential candidate for the further preclinical study aimed at the prevention and treatment of neurodegenerative disorders such as AD and VaD.

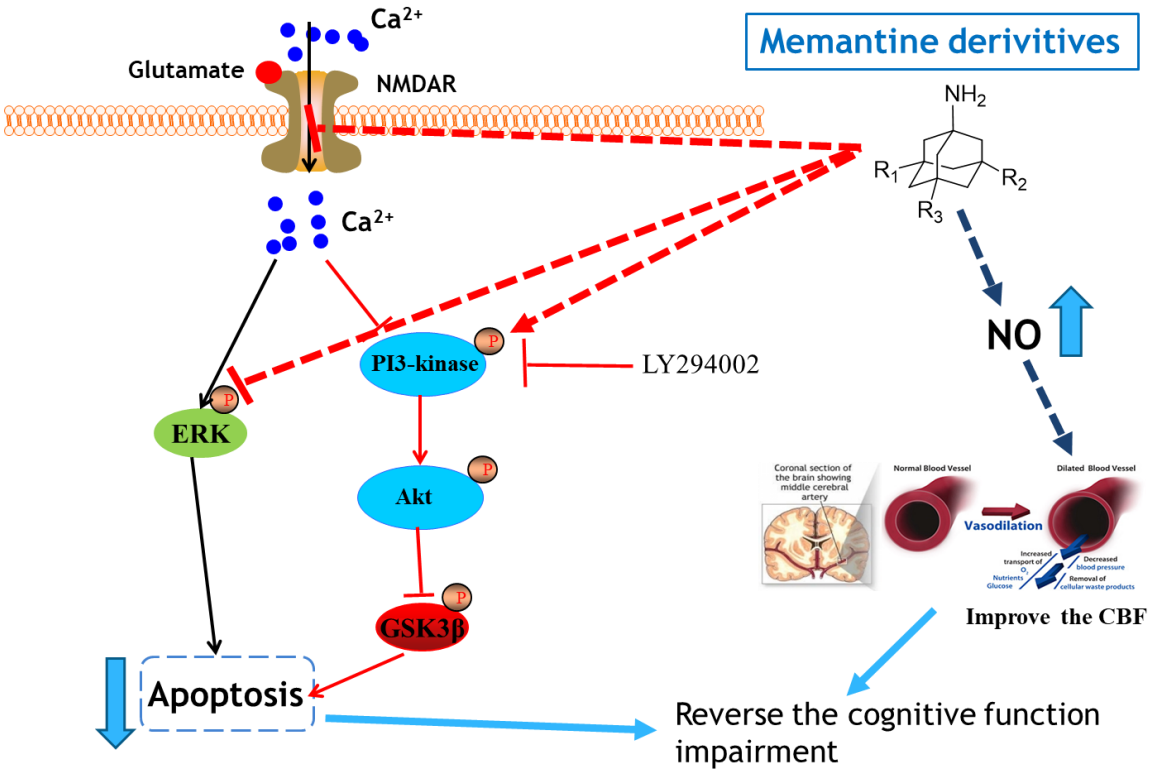

Fig. 5.1 The diagram illustrates the underlying mechanisms of multi-functional memantine derivatives for treating dementia. The multi-functional memantine nitrates exhibited anti-dementia effects via neuroprotection and vasodilation.

Future plans

In this study, we have demonstrated that memantine nitrates exhibited neuroprotective effects, as well as the vessel dilatory effects, in both *in vitro in vivo* models. However, as previously reported, there were several signaling pathways involved in glutamate-induced excitotoxicity. We have examined two of the most dominant pathways; but the others should be also investigated in future. Furthermore, the patch clamp experiments have demonstrated that MN-08 might dose-dependently inhibit NMDA receptor, but the details including the inhibitory pattern and binding site(s) on NMDA receptors needed to be further investigated. In addition, studies showed that memantine might provide angiogenesis by enhancing the expression of VEGF. In fact, the in vivo studies have showed that memantine could improve the CBF as well as the cognitive functions impairments induced by 2-VO surgery. We wonder whether memantine nitrates could regulate the expression of the neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and VEGF. Moreover, as mentioned above, the anti-dementia effects offered by memantine nitrates should be further confirmed in more animal models, including the 3XTg transgenic mice model.

- To characterize the mode of action of MN-08 on antagonizing NMDA receptor by electrophysiological studies.
- To determine the detail mechanisms of vessel dilatory effects of MN-08 by NO releasing;

- To evaluate the anti-dementia effects by MN-08 in more animal models, such as the 3XTg transgenic mice, associated with neurodegenerative disorder;

References

1. Collingridge, G. Synaptic plasticity. The role of NMDA receptors in learning and memory. *Nature* **330**, 604-605 (1987).
2. Scheltens, P.*, et al.* Alzheimer's disease. *Lancet* **388**, 505-517 (2016).
3. Savelieff, M.G.*, et al.* Development of multifunctional molecules as potential therapeutic candidates for Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis in the last decade. *Chem Rev* **119**, 1221-1322 (2019).
4. Gorelick, P.B., Counts, S.E. & Nyenhuis, D. Vascular cognitive impairment and dementia. *Biochim Biophys Acta* **1862**, 860-868 (2016).
5. Dahlgren, K.N.*, et al.* Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *The Journal of biological chemistry* **277**, 32046-32053 (2002).
6. Michaud, J.P., Richard, K.L. & Rivest, S. Hematopoietic MyD88-adaptor protein acts as a natural defense mechanism for cognitive deficits in Alzheimer's disease. *Stem Cell Rev* **8**, 898-904 (2012).
7. Hampel, H.*, et al.* The future of Alzheimer's disease: the next 10 years. *Prog Neurobiol* **95**, 718-728 (2011).
8. Alzheimer's, A. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement* **12**, 459-509 (2016).
9. Jia, J.*, et al.* The cost of Alzheimer's disease in China and re-estimation of costs worldwide. *Alzheimers Dement* **14**, 483-491 (2018).
10. Dorszewska, J., Prendecki, M., Oczkowska, A., Dezor, M. & Kozubski, W. Molecular basis of familial and sporadic Alzheimer's disease. *Curr Alzheimer Res* **13**, 952-963 (2016).
11. Zetterberg, H. & Mattsson, N. Understanding the cause of sporadic Alzheimer's disease. *Expert Rev Neurother* **14**, 621-630 (2014).
12. Shi, Y. & Holtzman, D.M. Interplay between innate immunity and Alzheimer disease: APOE and TREM2 in the spotlight. *Nat Rev Immunol* **18**, 759-772 (2018).
13. Marx, J.L. Lost neurons identified in Alzheimer's disease. *Science* **232**, 1500 (1986).
14. Forstl, H. & Kurz, A. Clinical features of Alzheimer's disease. *Eur Arch Psychiatry Clin Neurosci* **249**, 288-290 (1999).
15. Passmore, M.J., Ho, A. & Gallagher, R. Behavioral and psychological symptoms in moderate to severe Alzheimer's disease: a palliative care approach emphasizing recognition of personhood and preservation of dignity. *J Alzheimers Dis* **29**, 1-13 (2012).
16. van Marum, R.J. Current and future therapy in Alzheimer's disease. *Fundam Clin Pharmacol* **22**, 265-274 (2008).
17. Carling-Jenkins, R., Torr, J., Iacono, T. & Bigby, C. Experiences of supporting people with Down syndrome and Alzheimer's disease in aged care and family environments. *J Intellect Dev Disabil* **37**, 54-60 (2012).
18. Chaufan, C., Hollister, B., Nazareno, J. & Fox, P. Medical ideology as a double-edged sword: the politics of cure and care in the making of Alzheimer's disease. *Soc Sci Med* **74**, 788-795 (2012).

19. Del Villar, K. & Miller, C.A. Down-regulation of DENN/MADD, a TNF receptor binding protein, correlates with neuronal cell death in Alzheimer's disease brain and hippocampal neurons. *Proc Natl Acad Sci U S A* **101**, 4210-4215 (2004).
20. Vijayan, M., Kumar, S., Bhatti, J.S. & Reddy, P.H. Molecular links and biomarkers of stroke, vascular dementia, and Alzheimer's disease. *Prog Mol Biol Transl Sci* **146**, 95-126 (2017).
21. Davies, P. & Maloney, A.J. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* **2**, 1403 (1976).
22. Palacios-Filardo, J. & Mellor, J.R. Neuromodulation of hippocampal long-term synaptic plasticity. *Curr Opin Neurobiol* **54**, 37-43 (2019).
23. Coyle, J.T., Price, D.L. & DeLong, M.R. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* **219**, 1184-1190 (1983).
24. Limapichat, W., Yu, W.Y., Branigan, E., Lester, H.A. & Dougherty, D.A. Key binding interactions for memantine in the NMDA receptor. *ACS chemical neuroscience* **4**, 255-260 (2013).
25. McGleenon, B.M., Dynan, K.B. & Passmore, A.P. Acetylcholinesterase inhibitors in Alzheimer's disease. *Br J Clin Pharmacol* **48**, 471-480 (1999).
26. Hung, S.Y. & Fu, W.M. Drug candidates in clinical trials for Alzheimer's disease. *J Biomed Sci* **24**, 47 (2017).
27. Mufson, E.J., Counts, S.E., Perez, S.E. & Ginsberg, S.D. Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. *Expert Rev Neurother* **8**, 1703-1718 (2008).
28. Francis, P.T., Palmer, A.M., Snape, M. & Wilcock, G.K. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry* **66**, 137-147 (1999).
29. Zhang, X.J.*, et al.* Induction of acetylcholinesterase expression during apoptosis in various cell types. *Cell Death Differ* **9**, 790-800 (2002).
30. Talesa, V.N. Acetylcholinesterase in Alzheimer's disease. *Mech Ageing Dev* **122**, 1961-1969 (2001).
31. Inestrosa, N.C.*, et al.* Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* **16**, 881-891 (1996).
32. David B. Hogan, M., FRCPC. Practical approach to the use of cholinesterase inhibitors in patients with early Alzheimer's disease. *Geriatrics and Aging* **12**, (4):202-207(2009).
33. Glenner, G.G. & Wong, C.W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* **120**, 885-890 (1984).
34. Maynard, C.J.*, et al.* Overexpression of Alzheimer's disease amyloid-beta opposes the age-dependent elevations of brain copper and iron. *The Journal of biological chemistry* **277**, 44670-44676 (2002).
35. Selkoe, D.J. & Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* **8**, 595-608 (2016).
36. Glenner, G.G. & Wong, C.W. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun* **122**, 1131-1135 (1984).

37. Hardy, J. & Selkoe, D.J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-356 (2002).
38. Li, J.*, et al.* Protection of PMS777, a new AChE inhibitor with PAF antagonism, against amyloid-beta-induced neuronal apoptosis and neuroinflammation. *Cell Mol Neurobiol* **29**, 589-595 (2009).
39. Ito, Y., Ito, M., Takagi, N., Saito, H. & Ishige, K. Neurotoxicity induced by amyloid beta-peptide and ibotenic acid in organotypic hippocampal cultures: protection by S-allyl-L-cysteine, a garlic compound. *Brain Res* **985**, 98-107 (2003).
40. Nelson, T.J. & Alkon, D.L. Protection against beta-amyloid-induced apoptosis by peptides interacting with beta-amyloid. *The Journal of biological chemistry* **282**, 31238-31249 (2007).
41. Castellani, R.J., Lee, H.G., Perry, G. & Smith, M.A. Antioxidant protection and neurodegenerative disease: the role of amyloid-beta and tau. *Am J Alzheimers Dis Other Demen* **21**, 126-130 (2006).
42. Tu, S., Okamoto, S., Lipton, S.A. & Xu, H. Oligomeric Abeta-induced synaptic dysfunction in Alzheimer's disease. *Mol Neurodegener* **9**, 48 (2014).
43. Cline, E.N., Bicca, M.A., Viola, K.L. & Klein, W.L. The amyloid-beta oligomer hypothesis: beginning of the third decade. *J Alzheimers Dis* **64**, S567-S610 (2018).
44. Rasmussen, J., Jucker, M. & Walker, L.C. Abeta seeds and prions: How close the fit? *Prion* **11**, 215-225 (2017).
45. Zhou, Y., Sun, Y., Ma, Q.H. & Liu, Y. Alzheimer's disease: amyloid-based pathogenesis and potential therapies. *Cell Stress* **2**, 150-161 (2018).
46. Rafii, M.S. Targeting tau protein in Alzheimer's disease. *Lancet* **388**, 2842-2844 (2016).
47. Reddy, P.H. & Oliver, D.M. Amyloid beta and phosphorylated Tau-induced defective autophagy and mitophagy in Alzheimer's disease. *Cells* **8**(2019).
48. Sarkar, S. Neurofibrillary tangles mediated human neuronal tauopathies: insights from fly models. *J Genet* **97**, 783-793 (2018).
49. Bliss, T.V. & Collingridge, G.L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31-39 (1993).
50. Benarroch, E.E. NMDA receptors: recent insights and clinical correlations. *Neurology* **76**, 1750-1757 (2011).
51. Hynd, M.R., Scott, H.L. & Dodd, P.R. Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. *Neurochem Int* **45**, 583-595 (2004).
52. Prusiner, S.B. Disorders of glutamate metabolism and neurological dysfunction. *Annu Rev Med* **32**, 521-542 (1981).
53. Sheng, M. & Kim, M.J. Postsynaptic signaling and plasticity mechanisms. *Science* **298**, 776-780 (2002).
54. Takahashi, H.*, et al.* Pharmacologically targeted NMDA receptor antagonism by NitroMemantine for cerebrovascular disease. *Sci Rep* **5**, 14781 (2015).
55. Danysz, W. & Parsons, C.G. Alzheimer's disease, beta-amyloid, glutamate, NMDA receptors and memantine--searching for the connections. *Br J Pharmacol* **167**, 324-352 (2012).
56. Krapivinsky, G.*, et al.* The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* **40**, 775-784 (2003).

57. Jiang, Q., Gu, Z., Zhang, G. & Jing, G. Diphosphorylation and involvement of extracellular signal-regulated kinases (ERK1/2) in glutamate-induced apoptotic-like death in cultured rat cortical neurons. *Brain Res* **857**, 71-77 (2000).
58. Chalecka-Franaszek, E. & Chuang, D.M. Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. *Proc Natl Acad Sci U S A* **96**, 8745-8750 (1999).
59. Butterfield, D.A. & Pocernich, C.B. The glutamatergic system and Alzheimer's disease: therapeutic implications. *CNS Drugs* **17**, 641-652 (2003).
60. Mattson, M.P. Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631-639 (2004).
61. Perry, G., Nunomura, A., Lucassen, P., Lassmann, H. & Smith, M.A. Apoptosis and Alzheimer's disease. *Science* **282**, 1268-1269 (1998).
62. Sano, M*., et al.* A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* **336**, 1216-1222 (1997).
63. Tamagno, E*., et al.* H2O2 and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK. *Exp Neurol* **180**, 144-155 (2003).
64. Reddy, P.H*., et al.* Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease. *Hum Mol Genet* **13**, 1225-1240 (2004).
65. Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L. & Markesbery, W.R. Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* **158**, 47-52 (1998).
66. Atwood, C.S*., et al.* Characterization of copper interactions with alzheimer amyloid beta peptides: identification of an attomolar-affinity copper binding site on amyloid beta1-42. *J Neurochem* **75**, 1219-1233 (2000).
67. Heppner, F.L., Ransohoff, R.M. & Becher, B. Immune attack: the role of inflammation in Alzheimer disease. *Nat Rev Neurosci* **16**, 358-372 (2015).
68. Ardura-Fabregat, A*., et al.* Targeting neuroinflammation to treat Alzheimer's disease. *CNS Drugs* **31**, 1057-1082 (2017).
69. Salminen, A., Ojala, J., Suuronen, T., Kaarniranta, K. & Kauppinen, A. Amyloid-beta oligomers set fire to inflammasomes and induce Alzheimer's pathology. *J Cell Mol Med* **12**, 2255-2262 (2008).
70. Tweedie, D., Sambamurti, K. & Greig, N.H. TNF-alpha inhibition as a treatment strategy for neurodegenerative disorders: new drug candidates and targets. *Curr Alzheimer Res* **4**, 378-385 (2007).
71. White, C.S., Lawrence, C.B., Brough, D. & Rivers-Auty, J. Inflammasomes as therapeutic targets for Alzheimer's disease. *Brain Pathol* **27**, 223-234 (2017).
72. Dong, Y., Li, X., Cheng, J. & Hou, L. Drug development for Alzheimer's disease: microglia induced neuroinflammation as a target? *Int J Mol Sci* **20**(2019).
73. Godyn, J., Jonczyk, J., Panek, D. & Malawska, B. Therapeutic strategies for Alzheimer's disease in clinical trials. *Pharmacol Rep* **68**, 127-138 (2016).

74. Watkins, P.B., Zimmerman, H.J., Knapp, M.J., Gracon, S.I. & Lewis, K.W. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *JAMA* **271**, 992-998 (1994).
75. Sameem, B., Saeedi, M., Mahdavi, M. & Shafiee, A. A review on tacrine-based scaffolds as multi-target drugs (MTDLs) for Alzheimer's disease. *Eur J Med Chem* **128**, 332-345 (2017).
76. Lilienfeld, S. Galantamine--a novel cholinergic drug with a unique dual mode of action for the treatment of patients with Alzheimer's disease. *CNS Drug Rev* **8**, 159-176 (2002).
77. Chen, H.S. & Lipton, S.A. The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* **97**, 1611-1626 (2006).
78. Chen, H.S*., et al.* Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. *J Neurosci* **12**, 4427-4436 (1992).
79. Lipton, S.A. Pathologically-activated therapeutics for neuroprotection: mechanism of NMDA receptor block by memantine and S-nitrosylation. *Curr Drug Targets* **8**, 621-632 (2007).
80. Lipton, S.A. Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. *J Alzheimers Dis* **6**, S61-74 (2004).
81. Lipton, S.A. Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx* **1**, 101-110 (2004).
82. Francis, P.T., Parsons, C.G. & Jones, R.W. Rationale for combining glutamatergic and cholinergic approaches in the symptomatic treatment of Alzheimer's disease. *Expert Rev Neurother* **12**, 1351-1365 (2012).
83. Greig, S.L. Memantine ER/Donepezil: a review in Alzheimer's disease. *CNS Drugs* **29**, 963-970 (2015).
84. Schmidt, R*., et al.* EFNS-ENS/EAN Guideline on concomitant use of cholinesterase inhibitors and memantine in moderate to severe Alzheimer's disease. *Eur J Neurol* **22**, 889-898 (2015).
85. Matsunaga, S., Kishi, T. & Iwata, N. Combination therapy with cholinesterase inhibitors and memantine for Alzheimer's disease: a systematic review and meta-analysis. *International Journal of Neuropsychopharmacology* **18**(2015).
86. Pereira, C., Ferreiro, E., Cardoso, S.M. & de Oliveira, C.R. Cell degeneration induced by amyloid-beta peptides: implications for Alzheimer's disease. *J Mol Neurosci* **23**, 97-104 (2004).
87. Li, W*., et al.* Novel anti-Alzheimer's dimer Bis(7)-cognitin: cellular and molecular mechanisms of neuroprotection through multiple targets. *Neurotherapeutics* **6**, 187-201 (2009).
88. Golde, T.E. Disease modifying therapy for AD? *J Neurochem* **99**, 689-707 (2006).
89. Panza, F*., et al.* BACE inhibitors in clinical development for the treatment of Alzheimer's disease. *Expert Rev Neurother* **18**, 847-857 (2018).
90. Eketjall, S*., et al.* AZD3293: A Novel, orally active BACE1 inhibitor with high potency and permeability and markedly slow off-rate kinetics. *J Alzheimers Dis* **50**, 1109-1123 (2016).
91. Folch, J*., et al.* Current research therapeutic strategies for Alzheimer's disease treatment. *Neural Plast* **2016**, 8501693 (2016).

92. Spencer, B., Rockenstein, E., Crews, L., Marr, R. & Masliah, E. Novel strategies for Alzheimer's disease treatment. *Expert opinion on biological therapy* **7**, 1853-1867 (2007).
93. Cummings, J., Lee, G., Ritter, A., Sabbagh, M. & Zhong, K. Alzheimer's disease drug development pipeline: 2019. *Alzheimers Dement (N Y)* **5**, 272-293 (2019).
94. Grundman, M. Vitamin E and Alzheimer disease: the basis for additional clinical trials. *The American journal of clinical nutrition* **71**, 630S-636S (2000).
95. Gertz, H.J. & Kiefer, M. Review about Ginkgo biloba special extract EGb 761 (Ginkgo). *Current pharmaceutical design* **10**, 261-264 (2004).
96. Tobinick, E. Perispinal etanercept for treatment of Alzheimer's disease. *Curr Alzheimer Res* **4**, 550-552 (2007).
97. Szekely, C.A*., et al.* Nonsteroidal anti-inflammatory drugs for the prevention of Alzheimer's disease: a systematic review. *Neuroepidemiology* **23**, 159-169 (2004).
98. Sahoo, A.K., Dandapat, J., Dash, U.C. & Kanhar, S. Features and outcomes of drugs for combination therapy as multi-targets strategy to combat Alzheimer's disease. *J Ethnopharmacol* **215**, 42-73 (2018).
99. Wang, Y.E., Yue, D.X. & Tang, X.C. [Anti-cholinesterase activity of huperzine A]. *Zhongguo Yao Li Xue Bao* **7**, 110-113 (1986).
100. Zhang, H.Y. & Tang, X.C. Neuroprotective effects of huperzine A: new therapeutic targets for neurodegenerative disease. *Trends Pharmacol Sci* **27**, 619-625 (2006).
101. Damar, U., Gersner, R., Johnstone, J.T., Schachter, S. & Rotenberg, A. Huperzine A as a neuroprotective and antiepileptic drug: a review of preclinical research. *Expert Rev Neurother* **16**, 671-680 (2016).
102. Gao, Y., Zhang, L. & Jiao, W. Marine glycan-derived therapeutics in China. *Prog Mol Biol Transl Sci* **163**, 113-134 (2019).
103. Price, K.A., Crouch, P.J. & White, A.R. Therapeutic treatment of Alzheimer's disease using metal complexing agents. *Recent patents on CNS drug discovery* **2**, 180-187 (2007).
104. Teri, L. & Logsdon, R.G. Assessment and management of behavioral disturbances in Alzheimer disease. *Comprehensive therapy* **26**, 169-175 (2000).
105. McKay, E. & Counts, S.E. Multi-Infarct dementia: a historical perspective. *Dement Geriatr Cogn Dis Extra* **7**, 160-171 (2017).
106. O'Brien, J.T. & Thomas, A. Vascular dementia. *Lancet* **386**, 1698-1706 (2015).
107. Vijayan, M. & Reddy, P.H. Stroke, vascular dementia, and Alzheimer's disease: molecular links. *J Alzheimers Dis* **54**, 427-443 (2016).
108. Pendlebury, S.T., Rothwell, P.M. & Oxford Vascular, S. Incidence and prevalence of dementia associated with transient ischaemic attack and stroke: analysis of the population-based Oxford Vascular Study. *Lancet Neurol* **18**, 248-258 (2019).
109. Song, J., Lee, W.T., Park, K.A. & Lee, J.E. Association between risk factors for vascular dementia and adiponectin. *Biomed Res Int* **2014**, 261672 (2014).
110. Kisler, K., Nelson, A.R., Montagne, A. & Zlokovic, B.V. Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer disease. *Nat Rev Neurosci* **18**, 419-434 (2017).
111. Wu, Q.J. & Tymianski, M. Targeting NMDA receptors in stroke: new hope in neuroprotection. *Mol Brain* **11**, 15 (2018).

112. Choi, D.W. & Rothman, S.M. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annual review of neuroscience* **13**, 171-182 (1990).
113. Arundine, M. & Tymianski, M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol Life Sci* **61**, 657-668 (2004).
114. Villmann, C. & Becker, C.M. On the Hypes and Falls in Neuroprotection: Targeting the NMDA Receptor. *Neuroscientist* **13**, 594-615 (2007).
115. Wilcock, G., Mobius, H.J., Stoffler, A. & group, M.M.M. A double-blind, placebo-controlled multicentre study of memantine in mild to moderate vascular dementia (MMM500). *Int Clin Psychopharmacol* **17**, 297-305 (2002).
116. Orgogozo, J.M., Rigaud, A.S., Stoffler, A., Mobius, H.J. & Forette, F. Efficacy and safety of memantine in patients with mild to moderate vascular dementia: a randomized, placebo-controlled trial (MMM 300). *Stroke* **33**, 1834-1839 (2002).
117. Tai, K.K., Blondelle, S.E., Ostresh, J.M., Houghten, R.A. & Montal, M. An N-methyl-D-aspartate receptor channel blocker with neuroprotective activity. *Proc Natl Acad Sci U S A* **98**, 3519-3524 (2001).
118. Dzoljic, E., Grbatinic, I. & Kostic, V. Why is nitric oxide important for our brain? *Funct Neurol* **30**, 159-163 (2015).
119. Wang, H.G*., et al.* Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins. *Neuron* **45**, 389-403 (2005).
120. Girouard, H. & Iadecola, C. Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. *J Appl Physiol (1985)* **100**, 328-335 (2006).
121. Vidwans, A.S., Kim, S., Coffin, D.O., Wink, D.A. & Hewett, S.J. Analysis of the neuroprotective effects of various nitric oxide donor compounds in murine mixed cortical cell culture. *J Neurochem* **72**, 1843-1852 (1999).
122. Godinez-Rubi, M., Rojas-Mayorquin, A.E. & Ortuno-Sahagun, D. Nitric oxide donors as neuroprotective agents after an ischemic stroke-related inflammatory reaction. *Oxid Med Cell Longev* **2013**, 297357 (2013).
123. Reisberg, B*., et al.* Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med* **348**, 1333-1341 (2003).
124. Planells-Cases, R*., et al.* A novel N-methyl-D-aspartate receptor open channel blocker with in vivo neuroprotectant activity. *The Journal of pharmacology and experimental therapeutics* **302**, 163-173 (2002).
125. Lipton, S.A. Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nature reviews* **5**, 160-170 (2006).
126. Lipton, S.A. The molecular basis of memantine action in Alzheimer's disease and other neurologic disorders: low-affinity, uncompetitive antagonism. *Curr Alzheimer Res* **2**, 155-165 (2005).
127. Luo, J*., et al.* Pathologically activated neuroprotection via uncompetitive blockade of N-methyl-D-aspartate receptors with fast off-rate by novel multifunctional dimer bis(propyl)-cognitin. *The Journal of biological chemistry* **285**, 19947-19958 (2010).

128. Nakamura, T. & Lipton, S.A. Preventing Ca2+-mediated nitrosative stress in neurodegenerative diseases: possible pharmacological strategies. *Cell Calcium* **47**, 190-197 (2010).
129. Doeppner, T.R.*, et al.* The indirect NMDAR antagonist acamprosate induces postischemic neurologic recovery associated with sustained neuroprotection and neuroregeneration. *J Cereb Blood Flow Metab* **35**, 2089-2097 (2015).
130. Montagne, A.*, et al.* Memantine improves safety of thrombolysis for stroke. *Stroke* **43**, 2774-2781 (2012).
131. Trotman, M., Vermehren, P., Gibson, C.L. & Fern, R. The dichotomy of memantine treatment for ischemic stroke: dose-dependent protective and detrimental effects. *J Cereb Blood Flow Metab* **35**, 230-239 (2015).
132. Maskell, P.D., Speder, P., Newberry, N.R. & Bermudez, I. Inhibition of human alpha 7 nicotinic acetylcholine receptors by open channel blockers of N-methyl-D-aspartate receptors. *Br J Pharmacol* **140**, 1313-1319 (2003).
133. Liu, Z.*, et al.* Synthesis and biological evaluation of memantine nitrates as a potential treatment for neurodegenerative diseases. *Medchemcomm* **8**, 135-147 (2017).
134. Li, W.*, et al.* Novel dimeric acetylcholinesterase inhibitor bis7-tacrine, but not donepezil, prevents glutamate-induced neuronal apoptosis by blocking N-methyl-D-aspartate receptors. *The Journal of biological chemistry* **280**, 18179-18188 (2005).
135. Subramaniam, S.*, et al.* Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. *J Neurosci* **25**, 2838-2852 (2005).
136. Bkaily, G., Al-Khoury, J., Simon, Y. & Jacques, D. Intracellular free calcium measurement using confocal imaging. *Methods Mol Biol* **1527**, 177-187 (2017).
137. Guo, B.*, et al.* Substantial protection against MPTP-associated Parkinson's neurotoxicity in vitro and in vivo by anti-cancer agent SU4312 via activation of MEF2D and inhibition of MAO-B. *Neuropharmacology* **126**, 12-24 (2017).
138. Jiajia, L.*, et al.* Assessment of Neuronal Viability Using Fluorescein Diacetate-Propidium Iodide Double Staining in Cerebellar Granule Neuron Culture. *J Vis Exp* (2017).
139. Hu, S.*, et al.* A Novel Tetramethylpyrazine Derivative Prophylactically Protects against Glutamate-Induced Excitotoxicity in Primary Neurons through the Blockage of N-Methyl-D-aspartate Receptor. *Front Pharmacol* **9**, 73 (2018).
140. Schindelin, J.*, et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
141. Luo, F.*, et al.* A dual-functional memantine nitrate MN-08 alleviates cerebral vasospasm and brain injury in experimental subarachnoid hemorrhage models. *Br J Pharmacol* (2019).
142. Fukada, S.Y., Tirapelli, C.R., de Godoy, M.A. & de Oliveira, A.M. Mechanisms underlying the endothelium-independent relaxation induced by angiotensin II in rat aorta. *Journal of cardiovascular pharmacology* **45**, 136-143 (2005).
143. Bonaventura, D., Lunardi, C.N., Rodrigues, G.J., Neto, M.A. & Bendhack, L.M. A novel mechanism of vascular relaxation induced by sodium nitroprusside in the isolated rat aorta. *Nitric Oxide* **18**, 287-295 (2008).

144. Harada, H.*, et al.* A novel method of detecting rCBF with laser-Doppler flowmetry without cranial window through the skull for a MCAO rat model. *Brain research. Brain research protocols* **14**, 165-170 (2005).
145. Zhang, T.*, et al.* Neuroprotective and axonal outgrowth-promoting effects of tetramethylpyrazine nitrone in chronic cerebral hypoperfusion rats and primary hippocampal neurons exposed to hypoxia. *Neuropharmacology* **118**, 137-147 (2017).
146. Zuloaga, K.L.*, et al.* Neurobehavioral and imaging correlates of hippocampal atrophy in a mouse model of vascular cognitive impairment. *Translational stroke research* **6**, 390-398 (2015).
147. Jin, G.*, et al.* Silibinin rescues learning and memory deficits by attenuating microglia activation and preventing neuroinflammatory reactions in SAMP8 mice. *Neuroscience Letters* (2016).
148. Du, Y.*, et al.* Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. *Proc Natl Acad Sci U S A* **94**, 11657-11662 (1997).
149. Llorens-Martin, M., Jurado, J., Hernandez, F. & Avila, J. GSK-3beta, a pivotal kinase in Alzheimer disease. *Front Mol Neurosci* **7**, 46 (2014).
150. Hu, S.*, et al.* Bis(propyl)-cognitin protects against glutamate-induced neuro-excitotoxicity via concurrent regulation of NO, MAPK/ERK and PI3-K/Akt/GSK3beta pathways. *Neurochem Int* **62**, 468-477 (2013).
151. Otori, T.*, et al.* Long-term measurement of cerebral blood flow and metabolism in a rat chronic hypoperfusion model. *Clinical and experimental pharmacology & physiology* **30**, 266-272 (2003).
152. Shim, Y.S., Yang, D.W., Kim, B.S., Shon, Y.M. & Chung, Y.A. Comparison of regional cerebral blood flow in two subsets of subcortical ischemic vascular dementia: statistical parametric mapping analysis of SPECT. *J Neurol Sci* **250**, 85-91 (2006).
153. Chen, H.*, et al.* Tacrine(10)-Hupyridone prevents post-operative cognitive dysfunction via the activation of BDNF pathway and the inhibition of AChE in aged mice. *Front Cell Neurosci* **12**, 396 (2018).
154. Contestabile, A. Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. *Cerebellum* **1**, 41-55 (2002).
155. Ciani, E., Virgili, M. & Contestabile, A. Akt pathway mediates a cGMP-dependent survival role of nitric oxide in cerebellar granule neurones. *J Neurochem* **81**, 218-228 (2002).
156. Chen, H.*, et al.* A novel tetramethylpyrazine derivative protects against glutamate-induced cytotoxicity through PGC1alpha/Nrf2 and PI3K/Akt signaling pathways. *Front Neurosci* **12**, 567 (2018).
157. Barbero-Camps, E., Fernandez, A., Martinez, L., Fernandez-Checa, J.C. & Colell, A. APP/PS1 mice overexpressing SREBP-2 exhibit combined Abeta accumulation and tau pathology underlying Alzheimer's disease. *Hum Mol Genet* **22**, 3460-3476 (2013).
158. Sattler, R. & Tymianski, M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol* **24**, 107-129 (2001).

159. Zhang, H.*, et al.* Tacrine(2)-ferulic acid, a novel multifunctional dimer, attenuates 6-hydroxydopamine-induced apoptosis in PC12 cells by activating Akt pathway. *Neurochem Int* **59**, 981-988 (2011).
160. Beurel, E., Grieco, S.F. & Jope, R.S. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* **148**, 114-131 (2015).
161. Frame, S., Cohen, P. & Biondi, R.M. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol Cell* **7**, 1321-1327 (2001).
162. Ma, R.*, et al.* Erythropoietin protects PC12 cells from beta-amyloid(25-35)-induced apoptosis via PI3K/Akt signaling pathway. *Neuropharmacology* **56**, 1027-1034 (2009).
163. Cui, W.*, et al.* Bis(12)-hupyridone, a novel acetylcholinesterase inhibitor, protects against glutamate-induced neuronal excitotoxicity via activating alpha7 nicotinic acetylcholine receptor/phosphoinositide 3-kinase/Akt cascade. *Chem Biol Interact* **203**, 365-370 (2013).
164. Bajda, M., Guzior, N., Ignasik, M. & Malawska, B. Multi-target-directed ligands in Alzheimer's disease treatment. *Curr Med Chem* **18**, 4949-4975 (2011).
165. Hardingham, G.E. Coupling of the NMDA receptor to neuroprotective and neurodestructive events. *Biochem Soc Trans* **37**, 1147-1160 (2009).
166. O'Collins, V., Howells, D. & Markus, R. Neurotoxicity and Stroke. 1483-1509 (2014).
167. Bading, H. Therapeutic targeting of the pathological triad of extrasynaptic NMDA receptor signaling in neurodegenerations. *J Exp Med* **214**, 569-578 (2017).
168. Paul, S. & Connor, J.A. NR2B-NMDA receptor-mediated increases in intracellular Ca2+ concentration regulate the tyrosine phosphatase, STEP, and ERK MAP kinase signaling. *J Neurochem* **114**, 1107-1118 (2010).
169. Anegawa, N.J., Lynch, D.R., Verdoorn, T.A. & Pritchett, D.B. Transfection of N-methyl-D-aspartate receptors in a nonneuronal cell line leads to cell death. *J Neurochem* **64**, 2004-2012 (1995).
170. H., M.W. The dementias and progressive diseases of the basal ganglia, in Neuropathology. Vol. 3 rd Edn (ed. G, G.J.) 475–521 (E. Arnold, London, 1961).
171. Aarts, M.M. & Tymianski, M. TRPMs and neuronal cell death. *Pflugers Archiv : European journal of physiology* **451**, 243-249 (2005).
172. Rothman, S.M. & Olney, J.W. Glutamate and the pathophysiology of hypoxic--ischemic brain damage. *Annals of neurology* **19**, 105-111 (1986).
173. Dirnagl, U., Iadecola, C. & Moskowitz, M.A. Pathobiology of ischaemic stroke: an integrated view. *Trends in neurosciences* **22**, 391-397 (1999).
174. Atochin, D.N.*, et al.* A novel dual NO-donating oxime and c-Jun N-terminal kinase inhibitor protects against cerebral ischemia-reperfusion injury in mice. *Neurosci Lett* **618**, 45-49 (2016).
175. Pluta, R.M.*, et al.* Effects of nitric oxide on reactive oxygen species production and infarction size after brain reperfusion injury. *Neurosurgery* **48**, 884-892; discussion 892-883 (2001).
176. Khan, M.*, et al.* Promoting endothelial function by S-nitrosoglutathione through the HIF-1alpha/VEGF pathway stimulates neurorepair and functional recovery following experimental stroke in rats. *Drug design, development and therapy* **9**, 2233-2247 (2015).

177. Willmot, M.R. & Bath, P.M. The potential of nitric oxide therapeutics in stroke. *Expert opinion on investigational drugs* **12**, 455-470 (2003).
178. Garthwaite, J. NO as a multimodal transmitter in the brain: discovery and current status. *Br J Pharmacol* **176**, 197-211 (2019).
179. Garry, P.S., Ezra, M., Rowland, M.J., Westbrook, J. & Pattinson, K.T. The role of the nitric oxide pathway in brain injury and its treatment--from bench to bedside. *Exp Neurol* **263**, 235-243 (2015).
180. Balez, R. & Ooi, L. Getting to NO Alzheimer's Disease: Neuroprotection versus Neurotoxicity Mediated by Nitric Oxide. *Oxid Med Cell Longev* **2016**, 3806157 (2016).
181. Lee, R.H*., et al.* Memantine inhibits alpha3beta2-nAChRs-mediated nitrergic neurogenic vasodilation in porcine basilar arteries. *PloS one* **7**, e40326 (2012).
182. Kilic, U., Yilmaz, B., Reiter, R.J., Yuksel, A. & Kilic, E. Effects of memantine and melatonin on signal transduction pathways vascular leakage and brain injury after focal cerebral ischemia in mice. *Neuroscience* **237**, 268-276 (2013).
183. Liu, Y., Huang, Y., Xu, Y., Qu, P. & Wang, M. Memantine protects against ischemia/reperfusion-induced brain endothelial permeability. *IUBMB life* **70**, 336-343 (2018).
184. Zhang, N*., et al.* Neovascularization and Synaptic Function Regulation with Memantine and Rosuvastatin in a Rat Model of Chronic Cerebral Hypoperfusion. *J Mol Neurosci* **63**, 223-232 (2017).
185. Thomas, T., Miners, S. & Love, S. Post-mortem assessment of hypoperfusion of cerebral cortex in Alzheimer's disease and vascular dementia. *Brain* **138**, 1059-1069 (2015).
186. Chen, Z.Q., Mou, R.T., Feng, D.X., Wang, Z. & Chen, G. The role of nitric oxide in stroke. *Med Gas Res* **7**, 194-203 (2017).
187. Ota, H., Ogawa, S., Ouchi, Y. & Akishita, M. Protective effects of NMDA receptor antagonist, memantine, against senescence of PC12 cells: A possible role of nNOS and combined effects with donepezil. *Experimental gerontology* **72**, 109-116 (2015).
188. Tanaka, A*., et al.* Effects of Memantine on Nitric Oxide Production and Hydroxyl Radical Metabolism during Cerebral Ischemia and Reperfusion in Mice. *J Stroke Cerebrovasc Dis* **27**, 1609-1615 (2018).
189. Liu, H*., et al.* Nitric oxide synthase in hypoxic or ischemic brain injury. *Reviews in the neurosciences* **26**, 105-117 (2015).
190. Wang, C.C., Wee, H.Y., Hu, C.Y., Chio, C.C. & Kuo, J.R. The Effects of Memantine on Glutamic Receptor-Associated Nitrosative Stress in a Traumatic Brain Injury Rat Model. *World neurosurgery* **112**, e719-e731 (2018).
191. Kida, K*., et al.* Beneficial effects of nitric oxide on outcomes after cardiac arrest and cardiopulmonary resuscitation in hypothermia-treated mice. *Anesthesiology* **120**, 880-889 (2014).
192. Huang, W*., et al.* Searching for the Multi-Target-Directed Ligands against Alzheimer's disease: discovery of quinoxaline-based hybrid compounds with AChE, H(3)R and BACE 1 inhibitory activities. *Bioorg Med Chem* **19**, 7158-7167 (2011).
193. Spilovska, K*., et al.* Multitarget tacrine hybrids with neuroprotective properties to confront Alzheimer's disease. *Curr Top Med Chem* **17**, 1006-1026 (2017).

194. Grossberg, G.T., Tong, G., Burke, A.D. & Tariot, P.N. Present algorithms and future treatments for Alzheimer's disease. *J Alzheimers Dis* **67**, 1157-1171 (2019).
195. Fereidoonnezhad, M., Mostoufi, A., Eskandari, M., Zali, S. & Aliyan, F. Multitarget drug design, molecular docking and PLIF studies of novel tacrine-coumarin hybrids for the treatment of Alzheimer's disease. *Iran J Pharm Res* **17**, 1217-1228 (2018).
196. Najafi, Z.*, et al.* Novel tacrine-coumarin hybrids linked to 1,2,3-triazole as anti-Alzheimer's compounds: In vitro and in vivo biological evaluation and docking study. *Bioorg Chem* **83**, 303-316 (2019).
197. Unzeta, M.*, et al.* Multi-target directed donepezil-like ligands for Alzheimer's disease. *Front Neurosci* **10**, 205 (2016).