NOVEL DIMERIC ANTIACETYLCHOLINESTERASE BIS(12)-HUPYRIDONE: NEUROPROTECTIVE AND NEURONAL DIFFERENTIATION-PROMOTING ACTIVITIES AND THEIR UNDERLYING MOLECULAR MECHANISMS

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Novel Dimeric Antiacetylcholinesterase Bis(12)-hupyridone: Neuroprotective and Neuronal Differentiation-Promoting Activities and Their Underlying Molecular Mechanisms

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Abstract

Neurodegenerative disorders have emerged as the major public health problem in the world, leading to death, disability and economic losses. The causes and mechanisms of neurodegenerative disorders are largely unknown. However, they may share some common pathways of neuronal damage due to impairment caused by oxidative stress and excitotoxicity caused by the overactivation of the N-methyl-D-aspartate (NMDA) receptor. Moreover, impairments of neurogenesis and neuronal differentiation are also observed in neurodegenerative disorders. Therefore, drugs capable of inhibiting the overactivation of the NMDA receptor, reducing neuronal impairment induced by oxidative stress, as well as promoting neuronal differentiation may be beneficial to these disorders.

Bis(12)-hupyridone (B12H) is a novel dimeric acetylcholinesterase (AChE) inhibitor derived from an ineffective fragment of huperzine A which is a natural compound isolated from the Chinese medical herb (*Huperzia serrata*). Although our previous studies have shown that B12H potentially inhibits AChE both *in vitro* and *in vivo*, its neuroprotective effects remain elusive. In this study, the neuroprotective and neuronal differentiation-promoting properties of B12H were examined, and the molecular mechanisms underlying these actions were also elucidated.

B12H prevented glutamate-induced neuronal apoptosis and moderately blocked the NMDA receptor activity at its MK-801 binding site. However, the efficacy of B12H to block the NMDA receptor activity did not match its efficacy in inhibiting glutamate-induced apoptosis, suggesting that B12H might act on multiple targets. Indeed, B12H also attenuated H_2O_2-induced neuronal apoptosis. In addition, this neuroprotection effect appeared to be independent of the inhibition of AChE, but

from reversing the inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2)/Akt/glycogen synthase kinase 3β (GSK3β) pathway. Furthermore, B12H was found to promote neuronal differentiation by activating the extracellular response kinase pathway through α7-type nicotinic acetylcholine receptor (α7nAChR).

In conclusion, B12H exerts a novel neuroprotective action by both moderately blocking the NMDA receptor and regulating the pro-survival signaling pathway. Moreover, B12H also promotes neuronal differentiation in neural stem cells. All these results might offer not only a novel and clinically relevant modality for neuroprotection by dimers, but also a rational approach for developing new drugs for the prevention and treatment of neurodegenerative disorders.

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2. **Cui W,** Li W, Zhao Y, Mak S, Gao Y, Luo J, Zhang H, Liu Y, Carlier PR, Rong J, **Han Y.** (2011) Preventing H_2O_2-induced apoptosis in cerebellar granule neurons by regulating the VEGFR-2/Akt signaling pathway using a novel dimeric antiacetylcholinesterase bis(12)-hupyridone. ***Brain Res.*** 1394:14-23.

3. **Cui W,** Li W, Han R, Mak S, Zhang H, Hu S, Rong J, **Han Y.** (2011) Phosphoinositide 3-kinase/Akt and ERK pathways activated by vascular endothelial growth factor play opposite roles in 1-methyl-4-phenylpyridinium ion-induced neuronal apoptosis. ***Neurochem Int.*** 59(6):945-53.

4. Liu Y, Wen XM, Lui EL, Friedman SL, **Cui W**, Ho NP, Li L, Ye T, Fan ST, Zhang H. (2009) Therapeutic targeting of the PDGF and TGF-beta-signaling pathways in hepatic stellate cells by PTK787/ZK222584. ***Lab Invest.*** 89(10):1152-60.

5. Fu H, Dou J, Li W, **Cui W**, Mak S, Hu Q, Luo J, Lam CS, Pang Y, Youdim MB, **Han Y.** (2009) Promising multifunctional anti-Alzheimer's dimer bis(7)-Cognitin acting as an activator of protein kinase C regulates activities of alpha-secretase and BACE-1 concurrently. ***Eur J Pharmacol.*** 623(1-3):14-21.

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7. Zhao Y, Dou J, Luo J, Li W, Chan HH, **Cui W**, Zhang H, Han R, Carlier PR, Zhang X, **Han Y**. (2011) Neuroprotection against excitotoxic and ischemic insults by bis(12)-hupyridone, a novel antiacetylcholinesterase dimer, possibly via acting on multiple targets. ***Brain Res.*** 1421:100-9.

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChRs	Acetylcholine receptors
AD	Alzheimer's disease
AIF	Apoptosis-inducing factor
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl- 4-isoxazolpropionic acid
ANOVA	Analysis of variation
APP	β-amyloid precursor protein
ATM	Ataxia telangiectasia mutant
ATP	Adenosine triphosphate
Atr	Atropine
Aβ	β-amyloid peptide
B12H	Bis(12)-hupyridone
BDNF	Brain-derived neurotrophic factor
BnH	Bis(n)-hupyridone
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
CAS	Catalytic anionic site
CDK-5	Cyclin dependent-kinase 5
CGNs	Cerebellar granule neurons
ChAT	Choline acetyltransferase
CMA	Chaperon mediated autophagy
CREB	cAMP response element-binding
Cyt c	Cytochrome c
DA	Dopaminergic
DAPI	4',6-diamidino-2-phenylindole
dbcAMP	Dibutyryl cAMP
DIV	Day *in vitro*
EGF	Epidermal growth factor
ERK	Extracellular response kinase
FDA	The U.S. Food and Drug administration
GABA	γ-aminobutyric acid
GAP-43	Growth associated protein 43
GSK3β	Glycogen synthase kinase 3β
HupA	Huperzine A
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HSF1	Heat shock factor-1
JAK	The janus kinase
JNK	c-Jun N-terminal kinase
KA	Kainate
LY	LY294002
MAPK	Mitogen-activated protein kinase
MEF2C	Myocyte enhancer factor-2C
MEK	The ERK kinase
mGluR	Receptors of metabotropic glutamate
MLA	Methyllycaconitine
MTT	3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

nAChRs	nicotinic acetylcholine receptors
NFTs	Neurofibrillary tangles
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
nNOS `	Neuronal nitric oxide synthase
NO	Nitric oxide
NSAIDS	Non-steroidal anti-inflammatory drugs
NSCs	Neural stem cells
OD	Optical density
PAS	Peripheral anionic site
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PDK-1	Phosphoinositides-dependent kinase-1
PI3-K	Phosphatiodylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PS	Presenilin
PTK	PTK787/ZK222584
RA	Retinoic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	standard error of the mean
SGL	Subgranular layer
SOD	Superoxide dismutase
SPM	Synaptic plasma membrane
STAT	Signal transducers and activators of transcription
SVZ	Subventricular zone
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
Wort	Wortmannin
α7nAChR	α7-type nicotinic acetylcholine receptor

Chapter One Introduction

Overview

Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) are characterized by a progressive and selective loss of neuronal cell populations in the neuronal systems (Lin and Beal, 2006). Despite the different exhibitions of these diseases, oxidative stress is likely to be a crucial common feature as it contributes to disease pathogenesis by inducing neuronal dysfunction or death. Oxidative stress in neurodegenerative disorders is mainly caused by the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radical, as a result of the reaction of oxygen with accumulated metal ions in the aggregates formed by neurodegenerative disorder-associated proteins. The excessive production of ROS dysregulates the level of intracellular calcium, stimulates multiple pathways and finally induces an apoptotic cascade (Bush, 2002; Curtain et al., 2001; Lee et al., 2003). Excitotoxicity induced by overactivation of the N-methyl-D-aspartate (NMDA) receptor is another major cause of neuronal loss in neurodegenerative disorders (Herrmann et al., 2011; Lipton, 2004). Overstimulation of the NMDA receptor could lead to the excessive calcium influx and the overproduction of intracellular nitric oxide (NO), and further cause neuronal death by activating pro-apoptotic pathways. Moreover, NO could also react with superoxide to produce a strong oxidant, indicating that overproduction of ROS and overstimulation of NMDA receptor may play synergistic roles in the neuronal loss in neurodegenerative disorders (Ferreira et al., 2010). Neuroprotective drugs that either directly antagonize neurotoxins or indirectly block the downstream pro-apoptotic signaling pathways may produce therapeutic benefits to prevent and treat neurodegenerative disorders (Schober, 2004). Stem cell-based therapy has been suggested as another strategy for treating neurodegenerative disorders because it may provide novel neurons to replace those lost during the progress of neurodegenerative disorders (Zhongling et al., 2009). However, this strategy is at least partially limited by the micro-environment which is unsuitable for neurogenesis and neuronal differentiation in the brains of patients. Agents capable of promoting neurogenesis and neuronal differentiation might have therapeutical usage for treating neurodegenerative disorders when used in

combination with stem cell-based therapy (Waldau and Shetty, 2008). Bis(12)-hupyridone (B12H), which is under investigation in this research project, is a synthesized dimer derived from an ineffective fragment of huperzine A (hupA) (Carlier et al., 2000; Wong et al., 2003). It has been proposed as a promising anti-AD agent based on its superior power in inhibiting acetylcholinesterase (AChE) (Li et al., 2007b) and potency in memory-enhancement (Li et al., 2007b).

In the following literature review section, AD is presented, as a representative of neurodegenerative disorders, in its etiology, pathogenesis mechanisms, and available therapeutic treatments. The relationship between AD, oxidative stress, overactivation of NMDA receptors, neuronal apoptosis, neurogenesis and neuronal differentiation will also be presented as background knowledge for this study. The pathogenic features of other major chronic neurodegenerative disorders will be briefly discussed to highlight the important roles played by neuronal apoptosis mediated by oxidative stress and/or overactivation of the NMDA receptor, as well as impaired neurogenesis and neuronal differentiation in the pathogenesis of neurodegenerative disorders. Previous research findings on B12H will also be described and analyzed.

1.1 Alzheimer's Disease

AD, first described by the German neuropathologist, Alois Alzheimer, more than 100 years ago, is the most common form of dementia among the elderly, affecting 26.6 million people throughout the world (Lahiri et al., 2004). The number of AD patients increases dramatically with 4.6 million new cases per year worldwide. In China, the number of AD patients is predicted to experience a rapid increase and will reach 26.1 million in 2040 (Ferri et al., 2005). A prevalence study shows that AD occurs in an age-dependent manner, and it affects 7-10% of people over the age of 65 and about 40% those over the age of 80 (Ferri et al., 2005). The major clinical characteristics of AD are global cognitive impairments such as memory loss, behavior changes and dysfunctional daily living activities (Pereira et al., 2005). Important neuropathological markers include extracellular senile plaques predominantly composed of β-amyloid peptide (Aβ) fibrils, and intracellular neurofibrillary tangles (NFTs) consist of abnormal hyperphosphorylated tau protein (Selkoe, 2001). The exact mechanisms of the onset of AD still remain unknown, and no effective therapeutic treatment is yet available. Four drugs have been approved by the U.S. Food and Drug Administration (FDA) and another one has been approved by China for combating AD (Doraiswamy and Xiong, 2006).

1.1.1 Risk factors

Family history and age are two major risk factors for AD. About 5-10% cases of AD are familial early-onset AD. Three genes are involved in these cases: the gene of β-amyloid precursor protein (APP) localized on chromosome 21, and the presenilin (PS) 1 and PS2 genes that translate the PS1 and PS2 proteins to form γ-secretases complex and are located on chromosome 14 and 1, respectively (Myers et al., 2005). In sporadic AD, risk increases with age after 65 (Schroecksnadel et al., 2004). Moreover, there are several environmental factors that may affect the occurrence of AD, such as education level, daily diet and sex. Epidemiological findings suggest that a relatively low education level and high fat diets may increase the risk of AD. AD is also more prone to affect women than men for yet unknown reasons (Alberca et al., 2002).

1.1.2 Molecular bases

1.1.2.1 Cholinergic system dysfunction and AChE toxicity

It was suggested very early that dysfunction of the cholinergic system may be the primary pathological event in the AD brain. In addition to the loss of cholinergic neurons, activities of choline acetyltransferase (ChAT) and AChE, and release of acetylcholine (ACh) are also abnormal in AD brains (Auld et al., 2002). However, recent studies have shown that these alterations of cholinergic markers may happen at the late stage of the disease in AD patients, indicating that they might be the result but not the cause of AD pathogenesis (Del Villar and Miller, 2004).

AChE is an enzyme assumed to be only responsible for hydrolyzing ACh. However, recent findings suggest that the role of AChE is far beyond its hydrolyzing capability. Intracellular AChE may mediate neuronal apoptosis induced by many stimuli (Zhang et al., 2002). In AD brains, although the total protein level of AChE is decreased, the activity of AChE is enhanced around the Aβ plaques (Talesa, 2001). Moreover, the interaction between AChE and Aβ could promote Aβ toxicity by increasing the aggregation of abnormal Aβ protein (Inestrosa et al., 1996). Therefore, AChE inhibitors may ameliorate the development of AD both by their cholinergic revision and by inhibiting Aβ aggregation.

1.1.2.2 The Aβ cascade hypothesis

Aβ is a 4 KD protein derived from APP. APP is cleaved sequentially by β- and γ-secretases rather than α-secretase to form Aβ (Glenner and Wong, 1984). Although the exact causes of AD are yet undefined, Aβ is thought to play an important role in its pathogenesis (Hardy and Selkoe, 2002). This hypothesis is further supported by the lines of evidence that accumulation of Aβ is the initial event that triggers AD, and the Aβ aggregate is a neurotoxin both *in vitro* and *in vivo*.

However, recent studies have shown only a weak correlation between the progression of Aβ plaques and the severity of cognitive impairment and this has led to a modified amyloid cascade hypothesis (Golde, 2003). Given the fact that 1) soluble non-fibrillar forms of Aβ are more toxic than insoluble aggregated forms of Aβ; 2) the level of Aβ oligomer is shown to have increased in affected regions of AD brains where no

amyloid plaques are found (Gong et al., 2003); and 3) the synaptic accumulation of soluble Aβ correlates better with the memory and cognitive decline observed in AD patients than the formation of amyloid plaques (Kawarabayashi et al., 2004), it is assumed that soluble oligomer of Aβ may be responsible for the pathology in AD.

1.1.2.3 Tau phosphorylation

Tau proteins participate in the regulation of neuronal morphology. However, both *in vitro* and *in vivo* studies have shown that abnormal tau proteins, which are hyperphosphorylated by both glycogen synthase kinase 3β (GSK3β) and cyclin dependent-kinase 5 (CDK-5), form the tangles which result in neurodegeneration (Ballard et al., 2011; Spittaels et al., 2000; Takahashi et al., 2003).

1.1.2.4 Glutamatergic system dysfunction

Glutamate, one of the most important excitatory neurotransmitters in the cortical and hippocampus, is involved in the cognitive impairment in AD (Bliss and Collingridge, 1993). Under physiological conditions, glutamate binds to either ionotropic glutamate receptors such as the receptors of NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) and kainate (KA), or metabotropic glutamate receptors (mGluR), and thus fulfills its physiological functions in learning and memory (Sheng and Kim, 2002). However, in pathological conditions, overactivation of the NMDA receptor leads to neuronal and synaptic damage. This NMDA receptor-related neuronal damage is largely dependent on the impairment of mitochondria caused by the overactivation of calcium-dependent neuronal nitric oxide synthase (nNOS) (Forder and Tymianski, 2009). Besides, as glutamatergic neurons are also influenced by other neurotransmitters such as ACh, γ-aminobutyric acid (GABA) and serotonin, different synaptically intercalated neurotransmitter systems can synergistically lead to the cognitive impairments in AD. This further suggests that targeting only one neurotransmitter will have only limited success (Doraiswamy, 2002).

1.1.2.5 Oxidative stress

Oxidative stress plays a significant role in the pathogenesis of AD. It occurs early in the AD brain, even before the important plaque pathology onset (Reddy, 2009). A

variety of oxidative stress markers are found in the AD brain, especially in neuritic plaques and NFTs (Butterfield et al., 2001). Increased levels of products of lipid peroxidation are found in the urine of AD patients. Antioxidant treatment may delay the progression of AD (Palacios et al., 2011).

Aβ has been proposed to be the primary source of oxidative stress in AD (Tamagno et al., 2003). Further, Aβ-induced oxidative stress can dysregulate the functions of synapses and promote Aβ deposition, up-regulate genes related to apoptosis and mitochondrial metabolism in transgenic APP mice (Reddy et al., 2004).

Abnormal homeostasis of metal ions is another important source of oxidative stress in AD. Increased concentrations of zinc, copper and iron are found in AD brains (Lovell et al., 1998). Redox-active metals such as iron and copper may cause neurotoxicity by generating toxic hydroxyl radicals from interaction of their reduced transition forms with H_2O_2. Furthermore, the inactivation of ROS scavengers may be another cause of metal ions-induced oxidative damage. For example, CuZn superoxide dismutase (SOD-1), which serves as the primary defence against ROS by removing O_2^-, could be inactivated by the co-administration of Aβ and copper (Atwood et al., 2000). Most importantly, the high affinity of metal ions and Aβ leads to hyper-metalized Aβ, which is resistant to cellular clearance. This hyper-metalized Aβ thus provides the relatively high concentrations of ROS and causes neuronal apoptosis (Fig. 1.1) (Maynard et al., 2002).

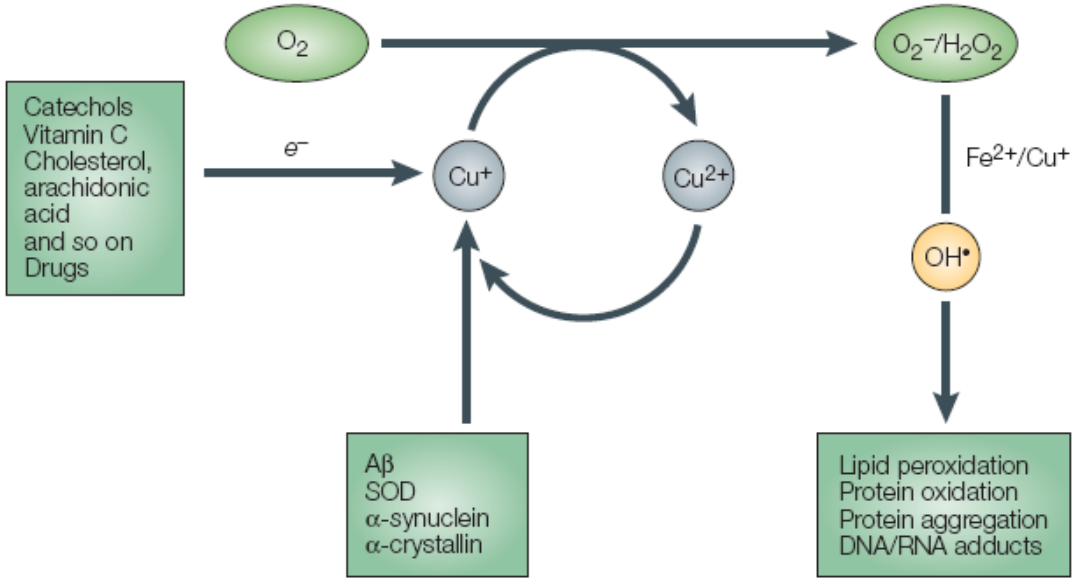

Figure 1.1 ROS generation by abnormal reaction of O_2 with protein-bound iron or copper

Redox-active metals, such as copper and iron, hypermetallate proteins. This abnormal metalation will increase the generation of ROS, leading to oxidative stress that is observed in AD (Barnham et al., 2004).

1.1.2.6 Neurotrophic factor deficiency

Neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are small proteins that maintain neuronal survival and physiological functions (Schindowski et al., 2008). Dysfunction of neurotrophic factor systems is found in AD. For example, pro-NGF, which plays a pro-apoptotic role in the activation of $p75^{NTR}$, is increased in the cortex; NGF, which mainly exerts a pro-survival function in the activation of TrkA, is decreased in the terminals of affected neurons in AD brain (Peng et al., 2005). Moreover, the expressions of BDNF, a protein that plays a key role in memory by regulating synaptic plasticity, and its receptor TrkB, are found to have decreased in the hippocampus of AD brain (Allen et al., 1999; Murer et al., 2001). Furthermore, a single-nucleotide polymorphism of BDNF is implicated in the risk of familial AD, indicating a close relationship between the dysregulation of neurotrophic factor systems and the pathology of AD (Akatsu et al., 2006).

1.1.3 Therapies for AD

1.1.3.1 AChE inhibitors

AChE inhibitors were initially approved to treat AD due to their cholinergic system-promoting properties. However, recent studies have shown that AChE inhibitors have other neuroprotective effects such as influencing the processing of APP, and reducing the production and aggregation of Aβ. There are four AChE inhibitors that have been approved by FDA, namely galantamine (Reminyl, 2001), rivastigmine (Exclon, 2000), donepezil (Aricept, 1996) and tacrine (Cognex, 1993) (Michaelis, 2003), although tacrine is now rarely used due to its hepatotoxicity. Moreover, hupA, an alkaloid compound with anti-AChE properties found in the plant *Huperzia serrata*, has been used for treating AD in China. However, a lack of Intellectual property protection of hupA has limited its usage in the western countries (Wang et al., 2009).

1.1.3.2 Therapies targeting Aβ and tau

As Aβ is formed by the sequential cleavage of APP by β- and γ-secretases, and induces neurotoxicity by oligomerization, fibrillization and aggregation, chemicals or antibodies targeting Aβ fragment may have potential clinical significance. For

example, antibodies against the β-secretase site of APP could inhibit the production of Aβ (Citron, 2004). LY450139, a γ-secretase inhibitor, has also reached the stage of clinical trials (Portelius et al., 2010).

Moreover, inhibitors of tau phosphorylation are being developed to treat AD. For example, lithium has shown benefits in an animal model, and research on its use has reached the clinical trial stage. Blockers of tau aggregation and inhibitors of tau proteolysis are also being studied (Cotman et al., 2005; Khlistunova et al., 2006).

1.1.3.3 NMDA receptor antagonist

Memantine (Namenda), approved by FDA in 2003, is an uncompetitive, moderate affinity antagonist of NMDA receptor with a fast off-rate property. It could protect neurons from glutamate-induced pathological excitotoxicity without affecting the physiological properties of the NMDA receptor. Compared with AChE inhibitors, memantine may benefit the later stages of AD by slowing the deterioration of cognitive functions (Rogawski and Wenk, 2003).

1.1.3.4 Antioxidant therapies

Although both *in vivo* and *in vitro* evidences have indicated that treatments against oxidative stress might be useful in AD, only a small number of antioxidant agents have been studied in AD patients in clinical trial (Jomova and Valko, 2011).

It has been suggested that vitamins E and C are beneficial to AD patients (Lee et al., 2010). Plant extracts have been widely used in dementia therapy. Their usages vary from culture to culture. In China, the pharmacological properties of plants with cognitive- or memory-enhancing power have been studied for thousands of years. *Gingko biloba*, which contains compounds with antioxidant and neuroprotective properties, may have anti-AD functions. EGb716, an extract from *Gingko biloba*, attenuates neuronal apoptosis induced by various stimuli *in vitro* and antagonizes age-related behavioral impairment and neuropathology of AD patients (Gertz and Kiefer, 2004).

1.1.3.5 Neurotrophic factors

As deficiency in neurotrophic factor system may contribute to the loss of cholinergic neurons in AD patients, the boosting of neurotrophic factors to fight against AD has been studied recently. For example, fibroblasts transduced with NGF-encoding vector were implanted into the forebrains of patients in the hope that this might improve both cerebral blood flow and their cognitive performance (Tuszynski et al., 2005).

1.1.3.6 Stem cell-based therapies

At present, drugs that are prescribed to treat AD have shown only modest and symptomatic effects as they could only lessen the degree of impairment without preventing or curing since they are unable to induce neurogenesis (Maggini et al., 2006). Therefore, transplantation of neural stem cells (NSCs) is considered a potential therapeutic strategy because it may provide a source of unlimited neurons for replacing those lost in the brains of AD patients, and reverse the progress of neurodegeneration (Zhongling et al., 2009). However, this approach is quite difficult as the transplanted stem cells have to be pre-differentiated into cholinergic neurons *in vitro* and then implanted in a variety of affected areas in the AD brain (Lindvall and Kokaia, 2010).

One alternative approach is to enhance the proliferation and differentiation properties of endogenous hippocampal NSCs that are disturbed in AD (Zhao et al., 2008). Compounds or antibodies that modify the microenvironment and promote neurogenesis in the AD brain could have therapeutic potential (Fig. 1.2).

1.1.3.7 Other potential therapeutic treatments

High blood cholesterol is considered to be a risk factor for AD. At high level of cholesterol, β-secretase cleavage of APP occurs preferentially to α-secretase cleavage, producing the more toxic form of o toxic Aβ (Kojro et al., 2001). Moreover, homeostasis of cholesterol is also important for the function of neurotransmitter systems (Lutjohann and von Bergmann, 2003). Therefore, agents capable of modulating cholesterol homeostasis may have therapeutic power against AD.

Estrogen has shown protective effects against AD in aging women. It could prevent the progress of the disease by reducing oxidative stress, up-regulating

neurotransmitter systems and increasing the formation of synapses (Owens, 2002). Moreover, it has been found that estrogen can also enhance memory (Henderson, 2009). Therefore, estrogen replacement therapy is prescribed to women with familial AD (Owens, 2002).

Neuroinflammation is also thought to be involved in the progress of AD. Therefore, non-steroidal anti-inflammatory drugs (NSAIDS), which suppress inflammation, might have therapeutic potential against AD (Mackenzie, 2001).

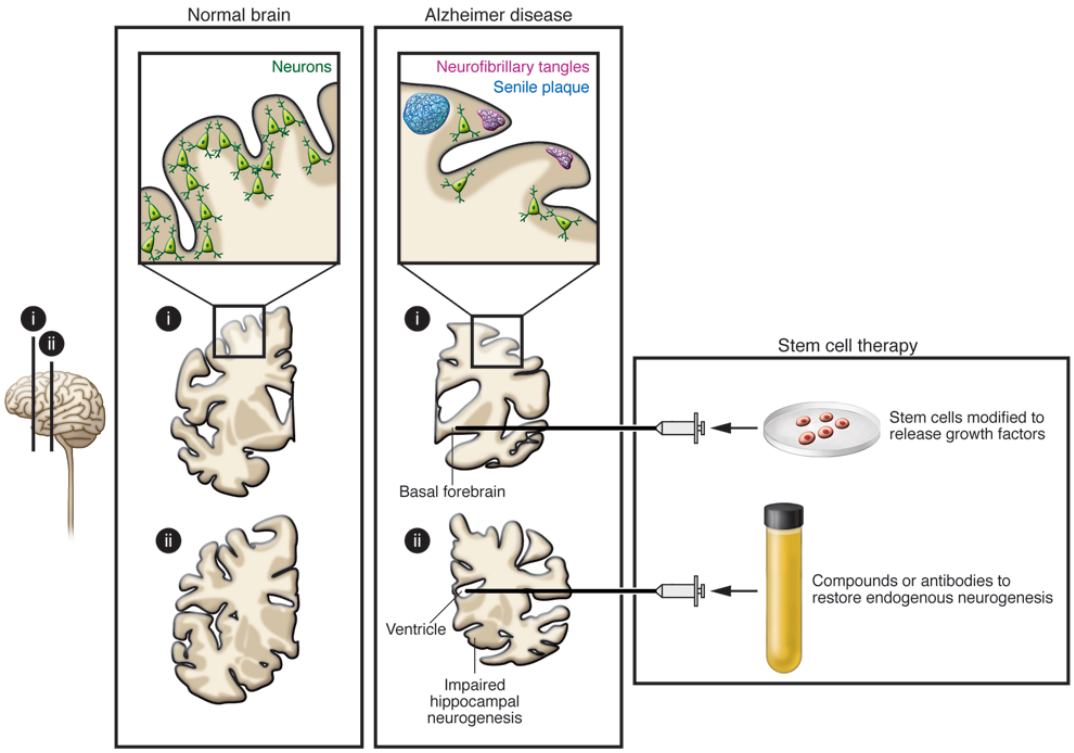

Figure 1.2 Stem cell-based therapies for AD

AD leads to the loss of neurons mainly in the cholinergic systems and the formation of NFTs and senile plaques. Stem cell-based therapy can be applied to reverse the progress of AD by stem cell transplantation, or by using compounds or antibodies that restore endogenous neurogenesis (Lindvall and Kokaia, 2010).

1.1.4 Neuronal apoptosis

1.1.4.1 Types of cell death

Necrosis, apoptosis and autophagic cell death are three main types of neuronal cell death (Gorman, 2008).

Necrosis, an acute form of cell death, is characterized by the loss of adenosine triphosphate (ATP) and the disruption of ion gradients. The cell swells and lyses during necrosis. No obvious biochemical pathway is identified in necrosis (Gorman, 2008).

Apoptosis is characterized by the shrinkage of cell bodies and budding of apoptotic bodies. Biochemically, apoptosis is regulated by certain proteins from pathways that are either intrinsic or extrinsic. In the intrinsic pathway, the release of cytochrome c from the mitochondria plays a central role, as cytochrome c interacts with Apaf-1 and thus activates caspase-9 (Li et al., 1997). Activated capase-9 passes the death signal by activating downstream effectors such as caspase-3. In the extrinsic pathway, a multi-protein complex is formed after the activation of death receptors and leads to downstream apoptosis. However, this procedure is not associated with activation of the caspase cascade (Schulze-Osthoff et al., 1998). Moreover, other proteins may also regulate apoptosis. For example, the Bcl-2 protein family, including both pro-apoptotic (Bax, Bak, Bad) and anti-apoptotic (Bcl-2, Bcl-$_{XL}$) members, could affect apoptosis by regulating the cytosol level of pro-apoptotic factors (Willis and Adams, 2005).

Autophagic cell death, which has been thought of as a pro-survival mechanism for the cell against starvation by providing amino acids and other blockers, has been reported to be associated with neurodegenerative diseases (Yang and Mao, 2010). The induction of autophagic cell death is dependent on the formation of autophagesomes initiated by proteins such as Beclin1 and type III phosphatiodylinositol 3-kinase (PI3-K) (Maiuri et al., 2009).

1.1.4.2 Cell death in AD

In AD, apoptosis is reported as one of the major mechanisms that lead to the loss of functional neurons (Schindowski et al., 2003). *In vivo* studies have shown that neurons may die of apoptosis with certain apoptosis markers such as increased DNA fragments, activated caspases and altered expressions of members of the Bcl-2 family in the AD brains (Shimohama, 2000). In the progress of neuronal apoptosis, neurons may still be recovered by drugs that activate pro-survival signals, which leads to the focus on the prevention of the onset or delaying the progress of neuronal apoptosis in AD therapeutics (Bachis et al., 2001).

Autophagic cell death caused by alterations of protein degradation systems such as the ubiquitin-proteasome system (UPS) and the autophagy systems has been reported to be associated with AD. The activities of proteasome are impaired in AD brains (van Leeuwen et al., 1998). Protein aggregations such as NFTs and senile plaques might result from the accumulation of ubiquitin conjugates, indicating the existence of an impaired UPS system in AD brains (Lennox et al., 1988). Chaperon mediated autophagy (CMA) is a specific type of autophagy that selectively degrades the substrate proteins containing a KFERQ-like motif. Although not established, the presence of a KFERQ-like motif in Aβ peptide indicates the involvement of CMA in AD pathology (Yang and Mao, 2010).

1.1.4.3 Signaling pathways in oxidative stress-induced apoptosis

The mediation of a number of signaling pathways, such as the PI3-K/Akt, mitogen-activated protein kinases (MAPKs), and phospholipase C (PLC) γ1/protein kinase C (PKC) pathways, in oxidative stress-induced apoptosis in AD has been reported. Generally, oxidative stress regulates signaling pathways by one of the two proposed mechanisms, that is by altering the activation states of specific pathways or by directly affecting proteins in pathways (Finkel, 1998).

The signal transductions pathways underlying oxidative stress-induced apoptosis are complex. In general, the PI3-K/Akt pathways, which can be specifically activated by growth factors, may inhibit neuronal apoptosis. The activation of the c-Jun N-terminal kinase (JNK) and the p38 pathways may promote cell death. The extracellular signal-regulated kinase (ERK) may either promote or inhibit apoptosis depending on

the differences between cell types and stresses (Fig. 1.3) (Martindale and Holbrook, 2002; Yao and Cooper, 1995).

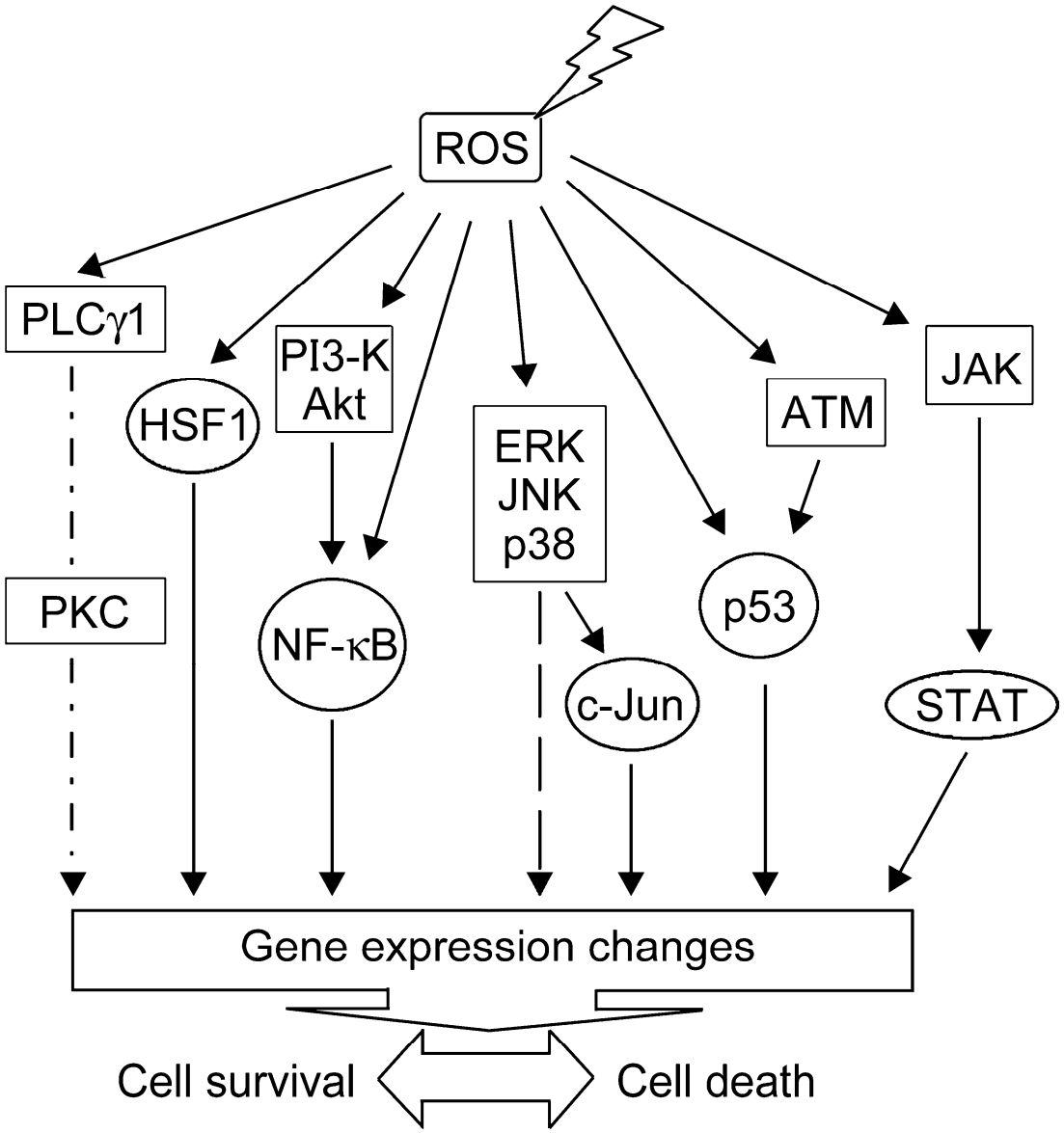

Figure 1.3 Putative pathways mediated by oxidative stress

ROS activate a variety of signaling pathways. Solid lines represent already characterized pathways, whereas dashed lines represent hypothesized pathways. Some signaling pathways, namely the p38, JNK, p53, ataxia telangiectasia mutant (ATM) and the janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathways, are proposed to be involved in promoting cell death. Other cascades, such as the Heat shock factor-1 (HSF1) and PI3-K/Akt pathways, have demonstrated a protective role against oxidative stress. Whether the nature of the ERK and (nuclear factor kappa-light-chain-enhancer of activated B cells) NF-κB pathways is pro- or anti-apoptosis has been widely debated (Taylor and Crack, 2004).

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase activated in response to PI3-K. Activation of Akt upon exposure to oxidative stress appears to be mediated by growth factor/growth factor receptor systems. Growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), may inhibit oxidative stress-induced apoptosis via activating Akt through stimulation of individual factors (Klotz et al., 2000; Wang et al., 2000). Activated Akt inactivates some pro-apoptotic proteins such as Bad, caspase-9 and GSK3β by phosphorylation and in turn further inhibits apoptosis (Grimes and Jope, 2001; Tulasne and Foveau, 2008). For example, inhibiting the activity of GSK3β by the PI3-K/Akt pathway prevents neuronal loss by suppressing pro-apoptotic proteins such as p53 and caspase-3, as well as by inhibiting mitochondrial failure (King et al., 2001).

The ERK cascade (Raf, the ERK kinase (MEK) and ERK) plays important roles in growth, stress and differentiation by either phosphorylating target proteins in the cytosol or phosphorylating transcription factors in nucleus (Kolch, 2000). Activation of the ERK pathway has been proposed to be a survival factor following oxidant injury because 1) oxidative stress reduces the activation of ERK and 2) pharmacological agents that cause the activation of ERK enhance survival in cells treated with oxidant (Guyton et al., 1996). However, the activation of the ERK pathway can also contribute to apoptosis in certain cell types under certain stimuli (Bhat and Zhang, 1999; Brand et al., 2001).

The JNK and p38 pathways and the members of MAPK pathways are activated by a wide range of oxidative stresses. p53 may be the potential target of pro-apoptotic signaling by both the JNK and p38 pathways. Both pathways have been shown to be capable of phosphorylating p53 and regulating p53 expression level under oxidative stress (Bulavin et al., 1999; Fuchs et al., 1998).

1.1.4.4 Glutamate-induced apoptosis

Overactivation of the NMDA receptor leads to neuronal apoptosis by producing a large amount of free radicals and activating pro-apoptotic signaling pathways (Figure 1.4). In the AD brain, the excessive release of glutamate is usually accompanied by

the disruption of energy homeostasis. The depolarized state of the membrane is found in neurons that lack energy, as neurons cannot maintain ion homeostasis without an adequate energy supply. This depolarized state further leads Mg^{2+}, an ion that normally blocks the NMDA receptor, to dissociate. Overactivation of the NMDA receptor then occurs. The prolonged overactivation of the NMDA receptor caused by excessive glutamate further leads to the loss of neurons and failure of synaptic plasticity (Molinuevo et al., 2005). Progressive impairment results in overload of Ca^{2+} in mitochondria, leading to the formation of ROS and activation of the caspases-induced apoptosis pathway, the activation of Ca^{2+}-dependent nNOS, resulting in the formation of reactive nitrogen species, and to the stimulation of the p38 signaling pathway, which further influences neurotoxicity by activating transcription factors (Dawson et al., 1993; Tenneti et al., 1998; Yun et al., 1998).

1.1.5 Neurogenesis and neuronal differentiation

1.1.5.1 Neurogenesis and learning and memory

Adult human brain contains NSCs which could proliferate and further differentiate into neurons and glia (Sharpless and DePinho, 2007). Recent studies have shown two areas of neurogenesis: the subventricular zone (SVZ), where NSCs could differentiate into neurons and migrate to the olfactory bulb, and the subgranular layer (SGL), where NSCs could differentiate into granule neurons (Lazarov et al., 2010). Newly formed neurons in the adult brain play an important role in learning and memory. Stress stimuli, as well as anti-mitotic agents, decrease neurogenesis and further impair hippocampal-dependent memory (Deng et al., 2010). Neurogenesis in the SVZ could regulate synaptic plasticity in the olfactory bulb (Doetsch and Hen, 2005). Focal irradiation of SVZ impairs neurogenesis, and inhibits long-term olfactory memory (Lazarini et al., 2009).

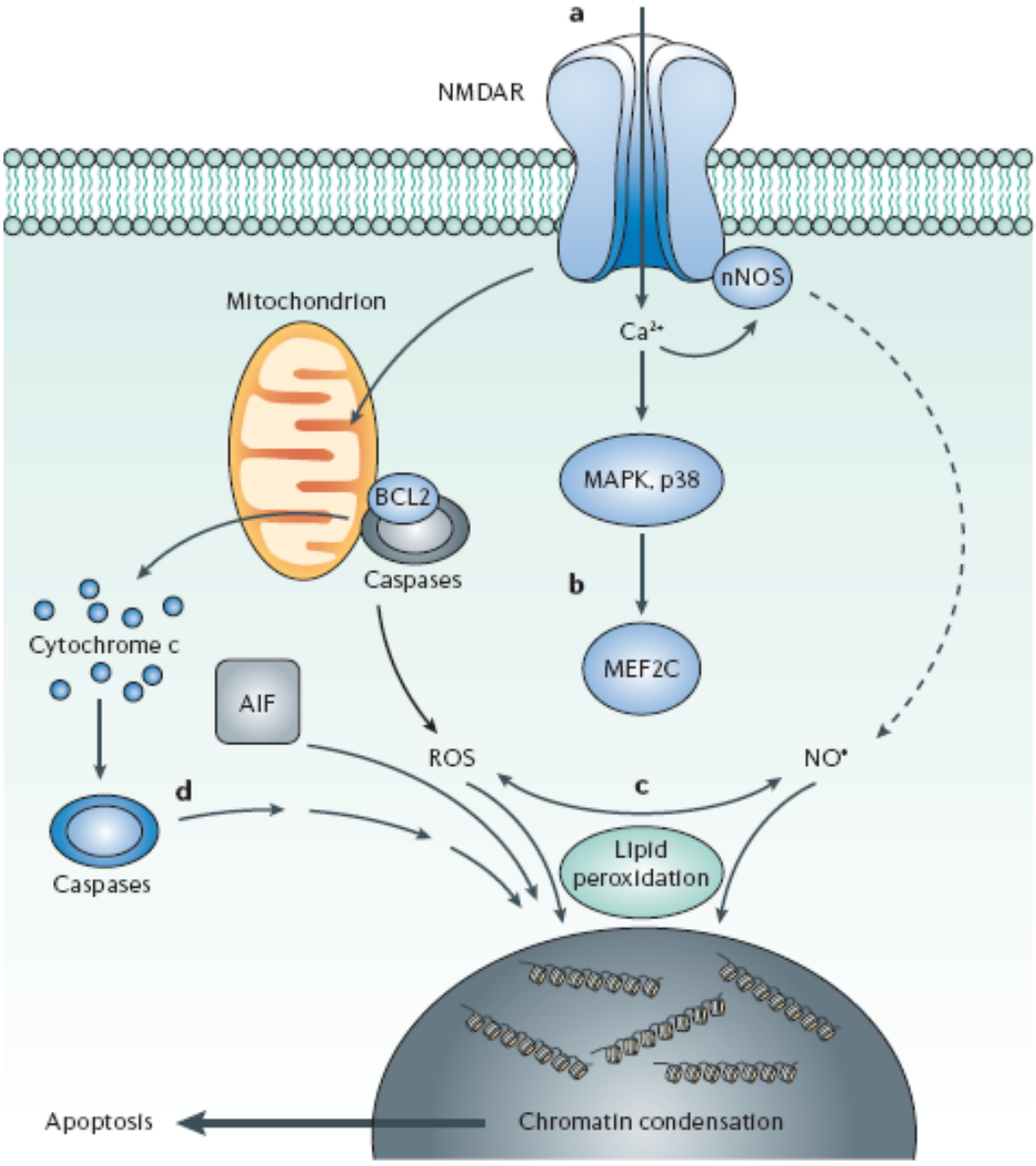

Figure 1.4 Scheme of the pro-apoptotic pathways triggered by excessive NMDA receptor activity

The cascade includes (a) NMDA receptor overactivation; (b) activation of the p38-myocyte enhancer factor-2C (MEF2C) pathway; (c) toxic effects of free radicals such as NO and ROS; and (d) activation of apoptosis-inducing enzymes, including caspases and apoptosis inducing factors. AIF, apoptosis-inducing factor; Cyt c, cytochrome c (Lipton, 2006).

1.1.5.2 Neurogenesis and neuronal differentiation impairments in AD

Impairment of neurogenesis is observed in the AD brain. The neurogenesis areas of SGL and SVZ are influenced by senile plaques and NFTs during the progress of AD. Moreover, APP and PS1 mutant mice have shown impaired neurogenesis that begins prior to the formation of Aβ plaques and the deterioration of memory (Demars et al., 2010). Furthermore, impairment of neurogenesis in AD could also result in a deficient of neuronal progenitor cells which could differentiate into neurons. As progressive decline in memory and learning is considered as the primary characteristic of AD, it is hypothesized that impaired neurogenesis might exacerbate the impairment of memory and learning in AD by affecting hippocampal and olfactory neural circuits (Lazarov and Marr, 2010). Therefore, drugs capable of promoting neuronal differentiation and neurogenesis might have therapeutic potential in the treatment of AD.

1.2 Other Neurodegenerative Disorders

Other neurodegenerative disorders, such as PD, ALS and HD, also share some common pathological effects with AD (Crunkhorn, 2011). The following section will introduce two other major types of neurodegenerative disorders: PD and ALS, to illustrate the molecular pathological events involved in these complex disorders.

1.2.1 Parkinson's disease

PD is primarily characterized by a progressive loss of dopaminergic (DA) neurons in the *substantia nigra*. The major pathological event of PD is thought to be oxidative stress. Dopamine, an essential neurotransmitter, could yield $H_2O_{2\ by}$ interacting with metal ions such as Fe^{3+} and Cu^{2+} (Double et al., 2003). Neuromelanin, a pigment that accumulates metal ions in the substantia nigra, is also found to be pro-oxidant after binding high levels of metal ions (Ben-Shachar et al., 1991). As the NMDA receptor is expressed in the DA neurons of the substantia nigra and the glutamatergic input is transmitted from the cerebra cortex and subthalamic nucleus to the substantia nigra, excitotoxicity caused by the overactivation of NMDA receptor might aggravate the damage induced by oxidative stress in PD. Antagonists of the NMDA receptor could thus protect DA neurons against neurotoxicity (Barnham et al., 2004). Moreover, decreased neurogenesis and neuronal differentiation in the SVZ and SGL and a reduced number of new neurons in the olfactory bulb have been observed in the early

stage of PD, indicating that impairment of neurogenesis and neuronal differentiation is also associated with the mechanisms of PD (Simuni and Sethi, 2008).

1.2.2 Amyotrophic lateral sclerosis

ALS is characterized by a loss of motor neurons in the spinal cord and cerebral cortex. It has been reported that the aggregation of a misfolded mutant of copper/zinc SOD in neurons occurs in ALS (Bruijn et al., 1998). The SOD mutants cause the gain of toxicity function, leading to pro-oxidant proteins that could produce ROS. For example, Zn-deficient SOD could elevate endogenous ROS by increasing O^{2-}, which would further produce $ONOO^-$ by reacting with NO (Goto et al., 2000). In addition, a significant reduction in the NMDA receptor and an elevation of glutamate levels, have been observed in the brains of ALS patients, indicating that glutamate-induced excitotoxicity might also be involved in the pathogenesis of ALS (Canton et al., 1998). Moreover, a reduced ability of NSCs to differentiate into neurons is also observed in ALS brains (Liu and Martin, 2006), showing the connection between neuronal differentiation and ALS.

Different neurodegenerative disorders may share some common pathways that cause neuronal damage, and oxidative stress and glutamate-induced excitotoxicity are two major pathways involved. Therefore, antioxidants capable of antagonizing NMDA receptors may provide potential therapeutic approaches to these diseases. Moreover, impairment of neurogenesis and neuronal differentiation is also observed in neurodegenerative disorders, indicating that drugs with the property of promoting neuronal differentiation may also be used in the treatment of neurodegenerative disorders.

1.3 Bis(12)-hupyridone, A Novel Dimeric AChE Inhibitor

B12H ((5S)-5-[(12-dodecyl) amino]-1,2,5,6,7,8-hexahydroquinolin-2-one) is a novel dimeric AChE inhibitor derived from the bivalent linkage of the pharmacophores of an ineffective fragment of hupA (Figs. 1.5A and B). It is a white solid poorly soluble in water, which dissolves easily in organic solvent. It potently and selectively inhibits AChE, and also displays a variety of other activities as found by others (Li, et al, 2007b, Yu et al, 2008) and deciphered in this thesis.

1.3.1 Bivalent ligand strategy

The bivalent ligand strategy is widely used in the synthesis of novel drugs in which identical or different pharmacophores are linked via a suitable linker (Haviv et al., 2005). The advantage of this strategy is the chelate effect, which creates a bifunctional ligand with enhanced affinity for its target. The occurrence in the molecular structure of AChE of one active site within the gorge (catalytic anionic site, CAS) and another at the extremity (peripheral anionic site, PAS), makes AChE a particularly attractive target for applying this strategy. To use this strategy, a series of novel bis(n)-hupyridone (BnH) dimers, derived from the hupyridone pharmacophore have been developed. Among them, a dimer with a 12-carbon linker, bis(12)-hupyridone or B12H, displays the strongest potency to inhibit AChE, by simultaneously binding at both the CAS and the PAS (Fig. 1.5C). Since butyrylcholinesterase (BuChE) lacks a PAS, AChE is inhibited selectively by B12H (Carlier et al., 2000).

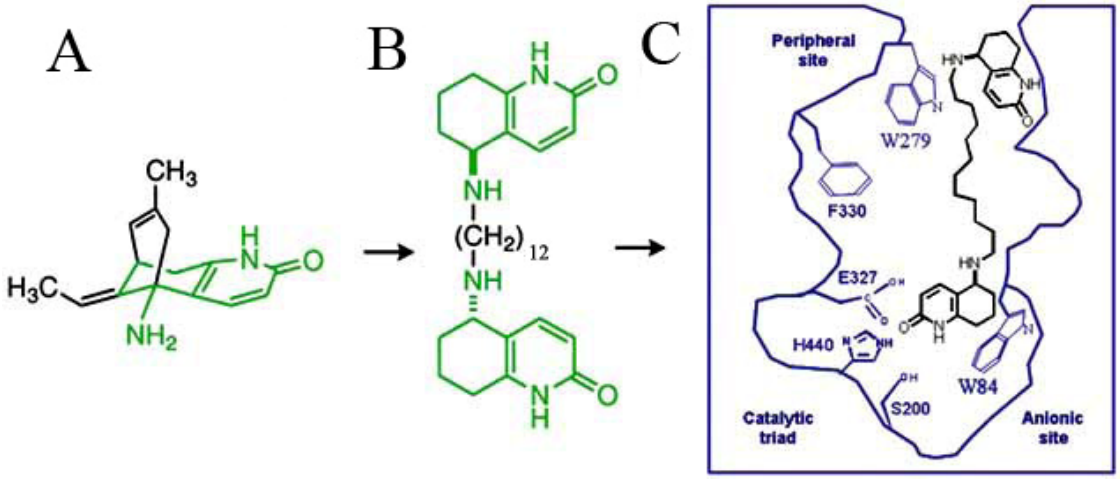

Figure 1.5 Structure-based rational drug designs for B12H

(A) HupA; (B) B12H; (C) The fitting of B12H into the AChE gorge by binding to both the CAS and PAS (Li et al., 2007b).

1.3.2 Potentially advantageous features

B12H exhibits high potency as an AChE inhibitor. Its IC_{50} for AChE is 52 nM, which is 185 times lower than that towards BuChE *in vitro* (Carlier et al., 2000). It is about twice as potent (52 nM *versus* 114 nM) but with a relatively lower selectivity for AChE (185 *versus* 1170) when compared to hupA. Significant inhibition of the AChE activity has also been found in various brain regions (cortex, hippocampus, and striatum) of rats in a concentration-dependent manner following the administration of B12H. For example, the maximum enzymatic inhibition of AChE was found in the rat brain cortex (17%), hippocampus (14%), striatum (16%) and serum (26%) after 30 min *i.p*. administration of B12H at 45 μmol/kg *in vivo*. And this enzymatic inhibition could last for 120 min and return to the normal level after 240 min (Li et al., 2007b). Moreover, B12H could rescue the scopolamine-induced learning and memory impairments in rats (Li et al., 2007b). In addition, B12H can be absorbed and permeated into brain tissue and raise the level of acetylcholine quickly after an intraperitoneal injection into mice, indicating that it easily crosses the blood brain barrier (Yu et al., 2008). All these findings indicate the potential use of B12H as a therapeutic agent for AD patients.

1.4 The aims of the study

Neurodegenerative disorders are caused by different initiators but may share some common pathways producing neuronal damage such as oxidative stress-induced neuronal apoptosis and overactivation of NMDA receptor-induced excitotoxicity (Santos et al., 2011). Moreover, impairments in neurogenesis and neuronal differentiation are also observed in neurodegenerative disorders (Winner et al., 2011). Drugs capable of 1) preventing neuronal apoptosis by reducing oxidative stress-induced impairment and/or antagonizing NMDA receptor; and/or 2) promoting neurogenesis and neuronal differentiation, may have potential therapeutic ability in the treatment of neurodegenerative disorders. B12H, which was chemically designed in this laboratory, has primarily performed as a powerful AChE inhibitor and has shown promise in AD animal studies (Li et al., 2007b). However, more studies need to be done to confirm these therapeutic advantages, and to understand the molecular mechanisms underlying the properties of B12H. Therefore, in my research, I would like to achieve the following specific aims:

- To elucidate the molecular mechanisms by which B12H protects against neuronal apoptosis induced by glutamate
- To elucidate the molecular mechanisms by which B12H protects against neuronal apoptosis induced by H_2O_2
- To elucidate the molecular mechanisms by which B12H promotes neuronal differentiation

Chapter Two Materials and Methods

2.1 Primary Cerebellar Granule Neurons Culture

Cerebellar granule neurons (CGNs) were prepared from 8-day-old Sprague–Dawley rats as described in our previous publication (Luo et al., 2010). Briefly, neurons were seeded at a density of 2.7×10^5 cells/cm^2 in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. Granule cells were identified by a combination of several criteria, including their size, shape and relative proportion to the total cell population as determined by phase contrast microscopy (Messer, 1977). With the use of this protocol, more than 95% of the cultured cells were granule neurons.

2.2 Primary Hippocampal Neuron Culture

Primary hippocampal neurons were obtained from 18-day-old Sprague-Dawley rat embryos as described earlier (Li et al., 2005). Briefly, the hippocampal neurons were plated at a density of 4×10^5/ml cells. Neurons were maintained in Neuro-basal/B27 medium containing 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Half-changes of culture media were done twice weekly.

2.3 Measurement of Neurotoxicity

Neurotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the neurons were cultured in 96-well plates, 10 μl of 5 mg/ml MTT labeling reagent was added to each well in 100 μl of medium, and the plate was incubated for 4 h in a humidified incubator at 37°C. After the incubation, 100 μl of the solvating solution (0.01 N HCl in 10% sodium dodecyl sulfate (SDS) solution) was added to each well for 16-20 h. The absorbance of the samples was measured at a wavelength of 570 nm with 655 nm as a reference wavelength.

2.4 Analysis of Chromatin Condensation

Chromatin condensation was detected by nucleus staining with Hoechst 33342 as previously described (Li et al., 2005). CGNs grown in a 6-well plate were washed with ice-cold PBS and fixed with 4% formaldehyde. Cells were then stained with Hoechst 33342 (5 μg/ml) for 5 min at 4^{o}C. The nuclei were visualized using a fluorescence microscope at ×400 magnification.

2.5 Fluorescein Diacetate/Propidium Iodide Double Staining Assay

Viable granule neurons were stained with fluorescein derived from fluorescein diacetate, which is de-esterified only by living cells (Li et al., 2005). Propidium iodide can penetrate cell membranes of dead cells to intercalate into double-stranded nucleic acids. Briefly, neurons were washed twice with PBS. After incubation with 10 μg/ml fluorescein diacetate and 5 μg/ml propidium iodide for 15 min, the neurons were examined and photographed using a fluorescence microscope.

2.6 DNA Fragmentation Assay

DNA fragmentation was assessed using a soluble DNA preparation (Li et al., 2005). The DNA fragments were electrophoresed in 1.5% agarose gel in TBE buffer. The DNA bands were then imaged by ethidium bromide staining and photographed.

2.7 PC12 Cell Culture

PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium containing 6% fetal bovine serum, 6% horse serum, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 μg/ml). The medium was renewed every 2–3 days.

2.8 Measurement of Neurite Outgrowth

PC12 cells were plated in growth medium at 5×10^4 cells per well in poly-L-lysine-coated 6-well plates and allowed to attach overnight. Cells were washed and incubated in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, 1% horse serum and various concentrations of experimental compounds the next day. When cells had differentiated for several days, experimental compounds were added every two days. Quantification of neurite outgrowth was done

as previously described (Shi et al., 2006). Briefly, cells were examined under a microscope and only processes longer than 20 μm were considered as neurites. Neurite outgrowth was quantified as the percentage of cells bearing neurite length of at least the length of one cell diameter.

2.9 Adult Rat Hippocampus Neural Stem Cells Culture

Adult rat hippocampus neural stem cells, obtained from Millipore (Billerica, MA, USA), were maintained in the rat neural stem cell expansion medium containing 20 ng/ml basic fibroblast growth factor.

2.10 Immunocytochemical Staining

Cells were fixed in 4% formaldehyde, membrane-permeabilized in Triton X-100 and blocked in bovine serum albumin (BSA). Cells were then exposed to a primary antibody overnight at 4^{o}C followed by incubation at room temperature with an Alexa Fluor 488-conjugated secondary antibody. Immunofluorescence images were acquired by fluorescence microscopy.

2.11 DAPI Staining

Neural stem cells were fixed in 4% formaldehyde, membrane-permeabilized in Triton X-100 and then exposed to 4,6-diamidino-2-phenylindole (DAPI) overnight at 4^{o}C. Fluorescent images were acquired by fluorescence microscopy.

2.12 Western Blot Assay

Western blot analysis was performed as previously described (Li et al., 2001). In brief, neurons were harvested in a cell lysis buffer. The protein (30 μg) was separated on a 10% SDS–polyacrylamide gel. Proteins were detected using primary antibodies. After incubation overnight at 4^{o}C, signals were obtained using a secondary antibody. Blots were developed using an ECL plus kit (Amersham Bioscience, Aylesbury, UK), exposed to Kodak autoradiographic films and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). All data from three independent experiments were expressed as the ratio to optical density (OD) values of the corresponding controls for the statistical analyses (Tukey's test).

2.13 Whole-Cell Electrophysiological Analysis

Cultured hippocampal neurons were used for whole cell patch clamp recording 7-15 days after plating (Luo et al., 2007). 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to 340 mOsm with sucrose. A low concentration of Ca^{2+} was used to minimize the calcium-dependent desensitization of NMDA-activated current. Pipettes pulled from borosilicate glass had resistances of 2–4 MΩ when filled with a pipette solution containing (in mM): 140 CsCl, 10 EGTA, 10 HEPES, and 5 MgATP with pH 7.3 and 315 mOsm in osmolarity. The holding potential was set at −60 mV, except when indicated otherwise. Data were acquired using pClamp 9.0 software (Axon Instruments). Currents were filtered at 2 kHz and digitized at 5 kHz.

2.14 Receptor-Ligand Binding Assay

The binding assay was performed as previously described (Li et al., 2007a). Briefly, a synaptic plasma membrane (SPM) was prepared from the rat brain using discontinuous sucrose density gradients. SPM proteins (150–200 g) were incubated with 4 nM [^{3}H] MK-801 and different concentrations of testing compounds for measurements of receptor-ligand binding. Non-specific binding was determined by incorporating an excess of the unlabeled MK-801. The samples on Whatman GF/B filters were collected by rapid filtration with a MD-24 Sample Harvester, and then soaked into scintillation cocktails overnight and measured in a scintillation counter. Specific [^{3}H] ligand binding to receptors was determined by subtraction of the counts for nonspecific binding, which was defined by use of 0.1 mM unlabeled MK-801. The K_i value was derived from the IC_{50} values by correcting for receptor occupancy by [^{3}H] ligand. The binding parameters were obtained with the ligand binding module in Sigmaplot 9.0 program.

2.15 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Dunnett's test, Duncan's multiple range test or

Tukey's test was used for statistical comparisons. Levels of $p < 0.05$ were considered to be of statistical significance.

Chapter Three Results

3.1 Protection against Glutamate-Induced Neuronal Apoptosis by Blocking NMDA Receptors

3.1.1 B12H prevents glutamate-induced apoptosis in CGNs

Cell death caused by glutamate at low concentrations is mainly through apoptosis (Li et al., 2005). Using our established model published, the effects of B12H on glutamate-induced excitotoxicity in CGNs were observed (Li et al., 2005). At 8 day *in vitro* (DIV), CGNs were pretreated with 0.1 nM to 1 μM B12H for 2 h， and then exposed to glutamate at 75 μM for 24 h. The MTT assay showed that B12H prevented glutamate-induced neuronal loss in a concentration-dependent manner (Fig. 3.1.1A). Treatments with B12H alone at the same concentrations (0.1 nM – 1 μM) for 26 h showed no cell proliferative or cytotoxic effects (Fig. 3.1.1A). B12H reached its maximal protection at 1 μM. A time-dependent potency profile of B12H protecting against excitotoxicity caused by glutamate was determined. CGNs were treated with B12H at 1 μM for 2 h before the challenge of glutamate at 75 μM (-2 h), at the same time as glutamate (0), or 0.5 and 1 h after glutamate (0.5 and 1). At 24 h after the glutamate challenge, cell viability was measured by the MTT assay. B12H at 1 μM retained its neuroprotective effects even when it was added at 0.5 h after the glutamate challenge, whereas memantine at this time point completely lost its neuroprotective capability. However, the neuroprotective effects of B12H significantly lost their potency with gradually reduced treatment duration, and it had no effect at all when added at 1 h after the glutamate insult (Fig. 3.1.1B).

Fluorescein diacetate/propidium iodide double staining, Hoechst 33324 staining and DNA fragmentation gel assays were concurrently used to determine the effects of B12H on glutamate-induced neuronal apoptosis. CGNs were pretreated with 1 μM B12H and exposed to 75 μM glutamate 2 h later, Fluorescein diacetate/propidium iodide double staining assay showed that B12H significantly blocked the loss of neurons and reversed the morphological alterations induced by glutamate, including the presence of unhealthy cell bodies and a broken extensive neuritic network (Fig.

3.1.2A). The Hoechst 33342 staining assay showed that B12H significantly reversed the glutamate-involved nuclear condensation (Fig. 3.1.2A), and blocked the apoptotic bodies from 47% in the glutamate group to 9% (Fig. 3.1.2B). The DNA fragmentation assay showed that B12H substantially blocked the DNA fragmentation induced by glutamate in CGNs (Fig. 3.1.2C).

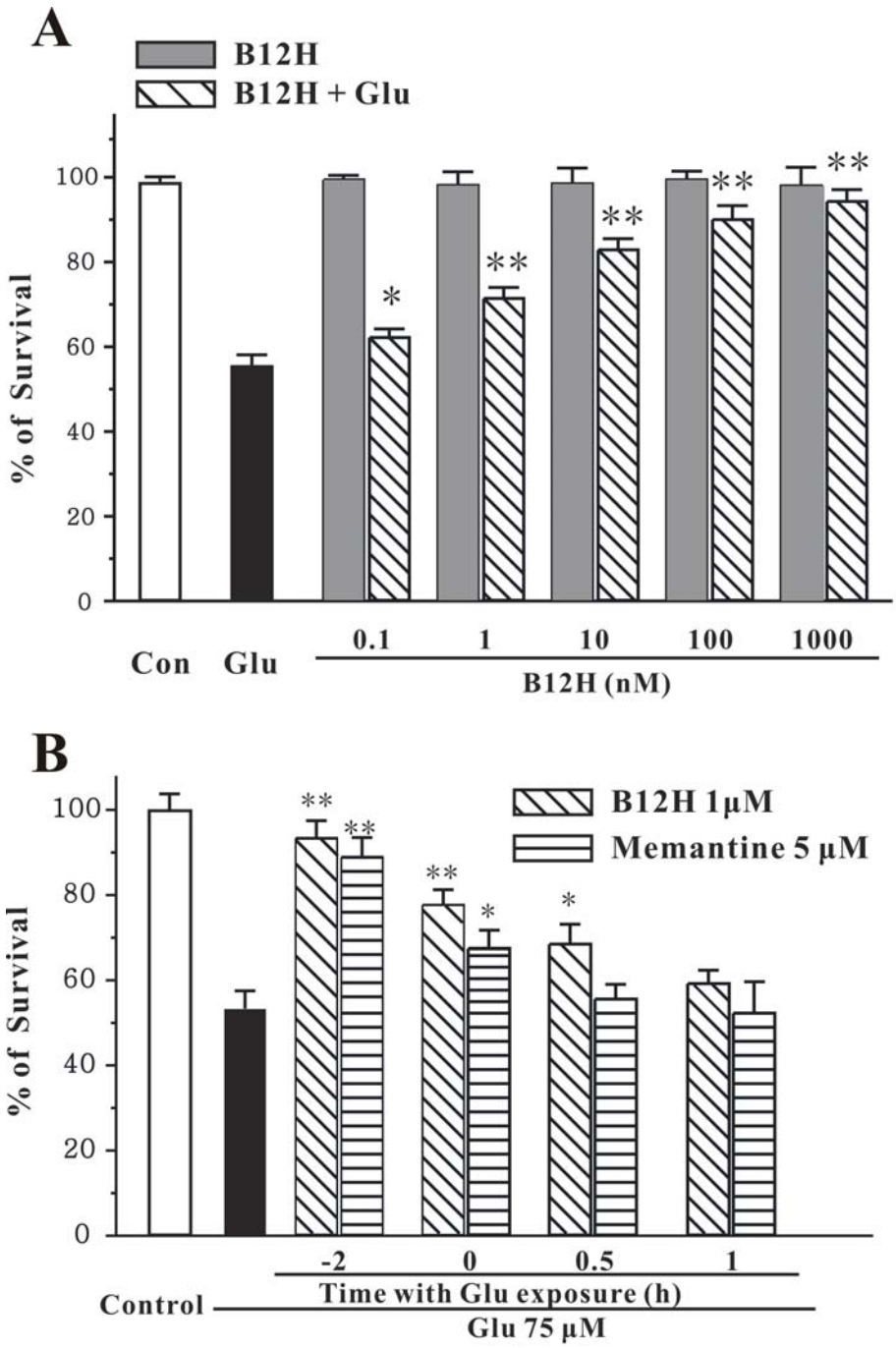

Figure 3.1.1 B12H prevents neuronal death induced by glutamate in CGNs

At 8 DIV, CGNs were pre-incubated with B12H at various concentrations as indicated and exposed to 75 μM glutamate 2 h later. (B) CGNs were exposed to 75 μM glutamate with 2-h pretreatment (2 h), co-treatment (0 h), 0.5 h post-treatment (0.5 h) or 1 h post-treatment (1 h) of 1 μM B12H and 5 μM memantine. Cell viability was measured by the MTT assay 24 h after glutamate challenge. All data, expressed as percentage of control, were the mean ± SEM of three separate experiments, $*p < 0.05$ and $**p < 0.01$ versus glutamate group.

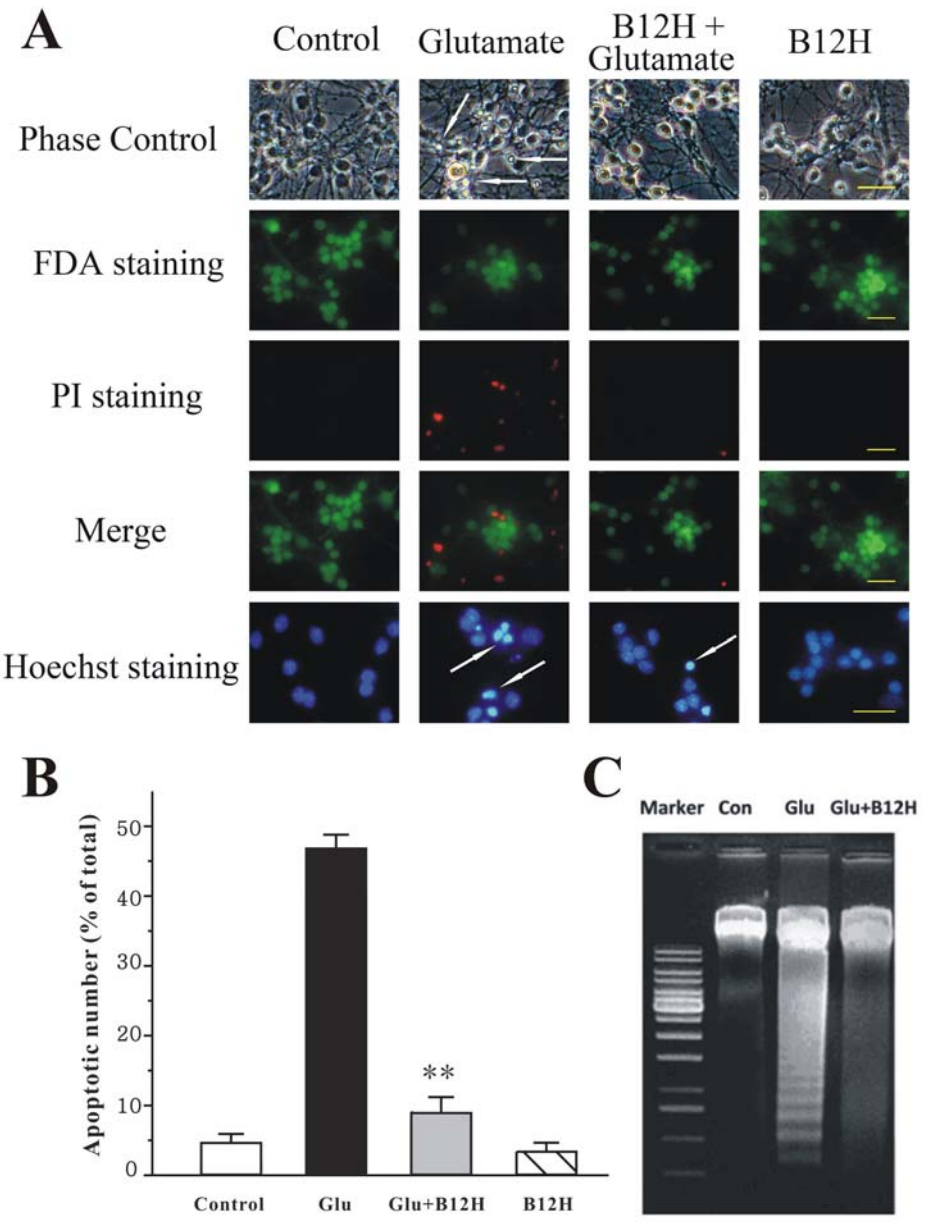

Figure.3.1.2 B12H blocks apoptosis induced by glutamate in CGNs

(A) At 8 DIV, CGNs were pre-incubated with or without 1 μM B12H, and exposed to 75 μM glutamate 2 h later. At 24 h after the glutamate challenge, CGNs were assayed with a phase contrast microscope, fluorescein diacetate/propidium iodide double staining and the Hoechst 33324 staining. (B) The counts of apoptotic bodies by Hoechst staining assay in (A). Values, expressed as percentage of total number of nuclei counted, were the mean ± SEM of three separate experiments, $^{**}p < 0.01$ versus glutamate group. (C) Under the same treatment conditions as (A), DNA fragments were accessed 24 h after the challenge, and agarose gel electrophoresis and ethidium bromide staining were used to visualize the extracted DNA.

3.1.2 B12H moderately blocks NMDA receptors at the MK-801 site

It has been reported that glutamate-induced neuronal apoptosis is mainly mediated by the overstimulation of NMDA receptors (Pi et al., 2004). To further dissect the possible interaction between B12H and NMDA receptors, the patch clamp technique was used on cultured hippocampal neurons. The NMDA receptor-activated currents were elicited using fast application of NMDA (30 μM, i.e., EC_{50} for NMDA-activated currents) and measured at −60 mV. The currents from neurons treated with 30 μM NMDA in the presence of B12H (0.1 – 1000 μM) were plotted by using SigmaPlot 9.0. Fig. 3.1.3A shows that B12H inhibited NMDA (30 μM)-activated currents with an IC_{50} value of 22 μM. Furthermore, it has been demonstrated that MK-801 prevented glutamate (75 μM)-induced cell death of CGNs with an EC_{50} of 0.023 μM (Li et al., 2007a). The receptor-ligand binding assay showed that B12H moderately competed with [^{3}H] MK-801 with a K_i of 7.7 μM in the cerebellar cortex membrane (Fig. 3.1.3B). (Part of the work in this section was done by Dr. Zhao Yuming)

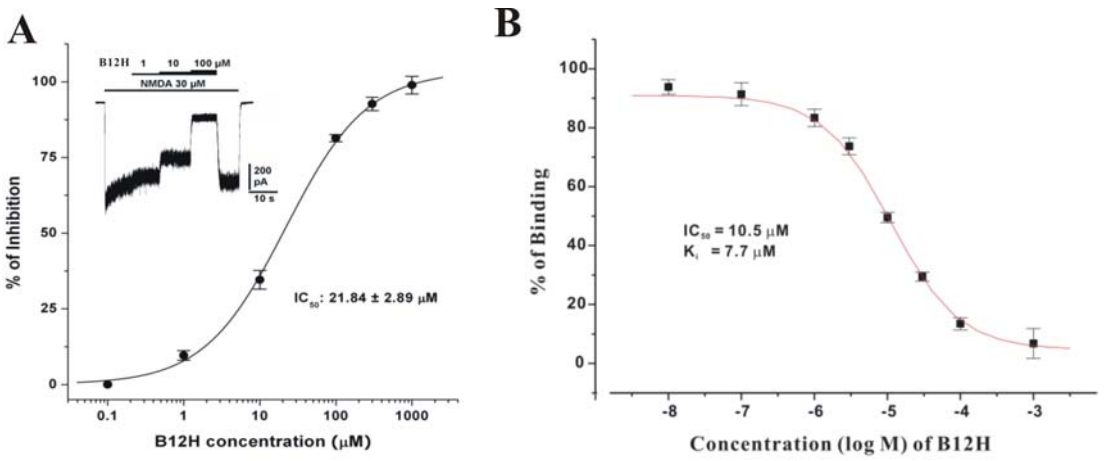

Figure 3.1.3 B12H inhibits NMDA-activated whole-cell currents in primary cultured hippocampal neurons

(A) Whole cell currents recorded from hippocampal neurons treated with NMDA (30 μM) alone or with NMDA plus various concentrations of B12H. Upper panel: representative current traces taken from the same neuron showing 1, 10 and 100 μM B12H inhibition of NMDA-evoked currents. Lower panel: the concentration-response curve for the blockade of NMDA-evoked currents was fitted by the equation $Y = E_{max} / [1+ (IC_{50}/X)^{n}]$ (the slope factor = 0.83 ± 0.083). The data are expressed as the mean ± SEM of $(1 - I/I_0) \times 100\%$ where I_0 is the current with NMDA alone and I is the current with NMDA and the tested drug at a certain concentration. (B) Inhibition of [^{3}H] MK-801 binding to rat cerebellar cortex membranes by B12H. The membranes from rat cerebellar cortex were incubated with [^{3}H] MK-801 (4 nM) and B12H at gradually increasing concentrations as described under Materials and Methods. The data, expressed as percentages of control, were the means of three experiments, and the graphs were plotted using Sigmaplot software.

3.2 Prevention of H_2O_2-Induced Apoptosis via Regulating the VEGFR-2/Akt Signaling Pathway

3.2.1 B12H attenuates H_2O_2-induced apoptosis in CGNs

To evaluate the effect of H_2O_2 on neuron viability, at 8 days *in vitro* (DIV), CGNs were treated with various concentrations of H2O2 (0: non-treated group, 10, 20, 30, 40, 50 μM) for 6 h or 30 μM H_2O_2 for various times. Cell viability was measured using the MTT assay. As shown in Figs. 3.2.1A and B, cell viability was significantly reduced in a concentration- and time-dependent manner. Based on these results, 30 μM H_2O_2 for 6 h was selected as an optimal treatment for subsequent experiments (Fig. 3.2.1A).

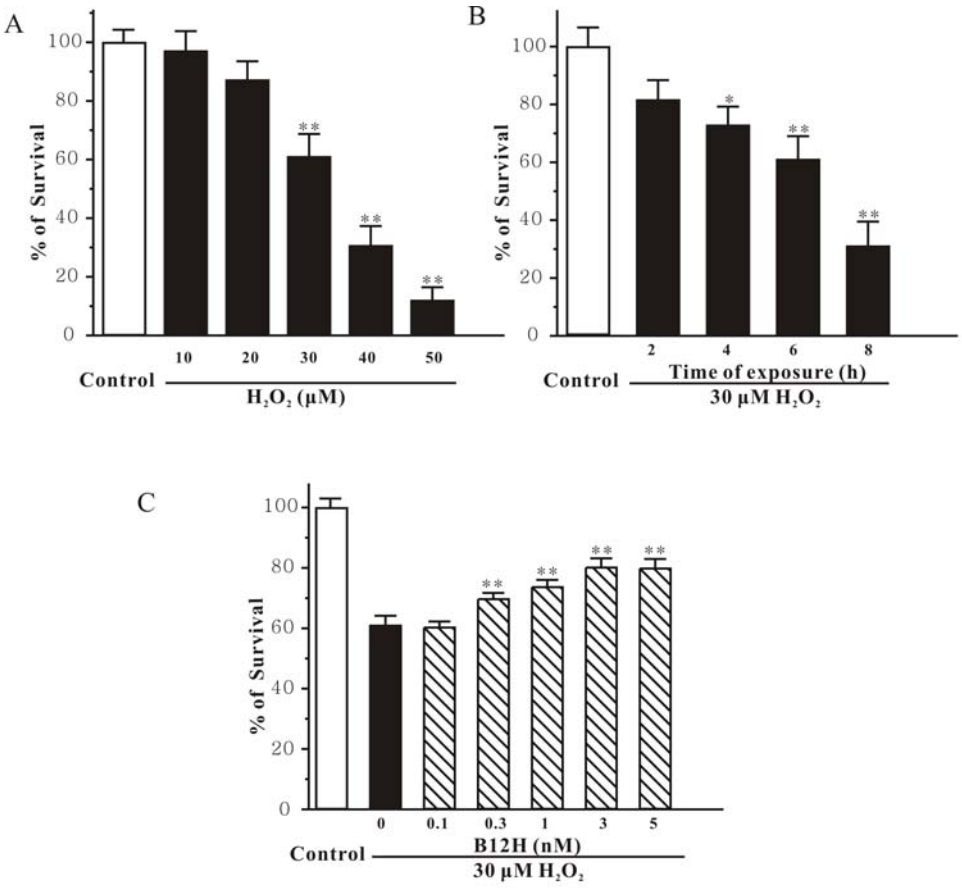

Figure 3.2.1 B12H inhibits H_2O_2-induced cell death in a concentration-dependent manner

H_2O_2 induces concentration-dependent cytotoxicity. CGNs were exposed to 10, 20, 30, 40 or 50 μM H_2O_2 for 6 h, and cell viability was then measured by the MTT assay. (B) H_2O_2 induces a time-dependent cytotoxicity. CGNs were exposed to 30 μM H_2O_2 for various time points as indicated, and cell viability was then measured by the MTT assay. (C) B12H prevents H_2O_2-induced cell death in a concentration-dependent manner. CGNs were treated with B12H at 0.1, 0.3, 1, 3 or 5 nM for 2 h, and then exposed to 30 μM H_2O_2. Cell viability was measured by the MTT assay at 6 h after the H_2O_2 challenge. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; *$p < 0.05$ and **$p < 0.01$ *versus* control in (A) and (B) or *versus* H_2O_2 in (C) (ANOVA and Dunnett's test).

To investigate the effects of B12H on H_2O_2-induced neurotoxicity, CGNs were pre-treated with various concentrations of B12H (0.1, 0.3, 1, 3 or 5 nM) for 2 h and then treated with 30 μM H_2O_2 for 6 h. Cell viability was measured using the MTT assay. Compared with 30 μM H_2O_2-treated cells, treatment with 0.3, 1, 3 or 5 nM B12H increased cell viability in a concentration-dependent manner (69.9 ± 1.9%, $p <$ 0.01; 73.8 ± 2.2%, $p < 0.01$; 80.3 ± 2.8%, $p < 0.01$; and 80.0 ± 3.0%, $p < 0.01$, respectively) (Fig. 3.2.1C). However, CGNs treated with B12H alone at the same concentrations (0.1 – 5 nM) for 8 h showed no cell proliferative or cytotoxic effects (data not shown).

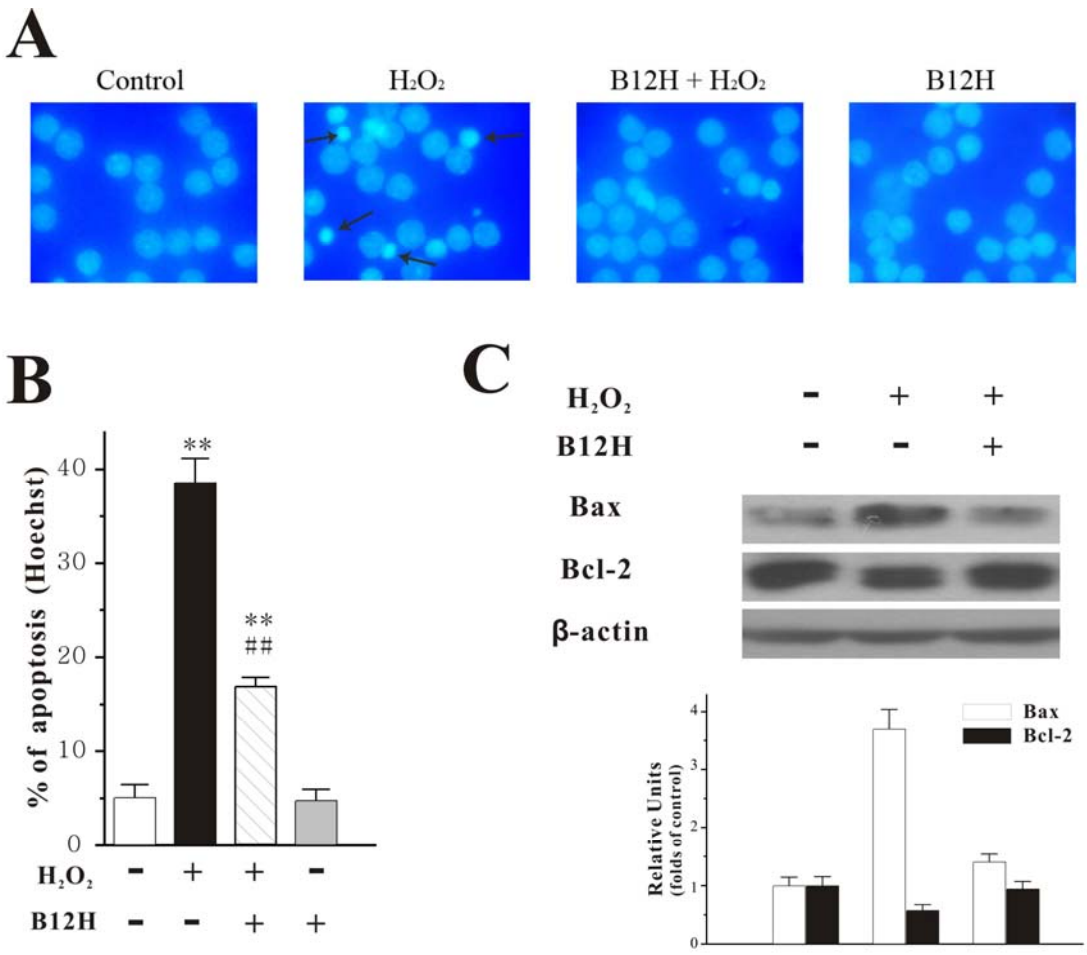

Figure 3.2.2 B12H blocks H_2O_2-induced neuronal apoptosis

(A) CGNs were exposed to 30 μM H_2O_2 for 6 h with or without pre-treatment with 3 nM B12H for 2 h. They were then assessed by the Hoechst 33342 staining. Apoptotic nuclei with condensed chromatin were marked by black arrows. (B) The number of apoptotic nuclei with condensed chromatin was estimated using representative photomicrographs, and was presented as a percentage of the total number of nuclei counted. Data were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ *versus* control and $^{\#\#}p < 0.01$ *versus* H_2O_2 group (ANOVA and Duncan's multiple range test). (C) CGNs were pre-treated with 3 nM B12H for 2 h and then exposed to 30 μM H_2O_2 for 6 h. Total protein was collected from the neurons for western blot assay with antibodies specific for Bax, Bcl-2 and β-actin.

In CGNs, H_2O_2 causes necrosis and/or apoptosis depending on the experimental conditions. The results demonstrated that 30 μM H2O2 induced apoptosis and B12H could prevent H_2O_2-induced apoptosis (Figs. 3.2.2A and B). At 8 DIV, CGNs were pre-treated with 3 nM B12H, and then exposed to 30 μM H_2O_2 2 h. The Hoechst 33342 staining assay showed that B12H significantly reversed the induced nuclear condensation. The counts of apoptotic nuclei stained by Hoechst 33342 showed that B12H significantly blocked H_2O_2-induced apoptotic bodies by 53% (Fig. 3.2.2B). However, treatment of 3 nM B12H alone did not affect apoptosis compared with the control group (Figs. 3.2.2A and B). To further confirm that the neuronal apoptosis was caused by H_2O_2, a Western blot assay was conducted on the above-mentioned cultures. The results showed that H_2O_2 decreased the expression level of Bcl-2 and increased the expression level of Bax. Moreover, pre-treatment with B12H reversed the alteration of the expression levels of these proteins induced by H_2O_2 (Fig. 3.2.2C).

3.2.2 Neuroprotective effects of B12H are independent of AChE enzymatic activity inhibition

Neostigmine and tacrine, two specific AChE inhibitors, were used to investigate whether they may prevent H_2O_2-induced apoptosis in CGNs. The MTT assay showed that neither neostigmine (3-30 μM) nor tacrine (0.3-3 μM) could block neuronal death induced by 30 μM H_2O_2 (Fig. 3.2.3).

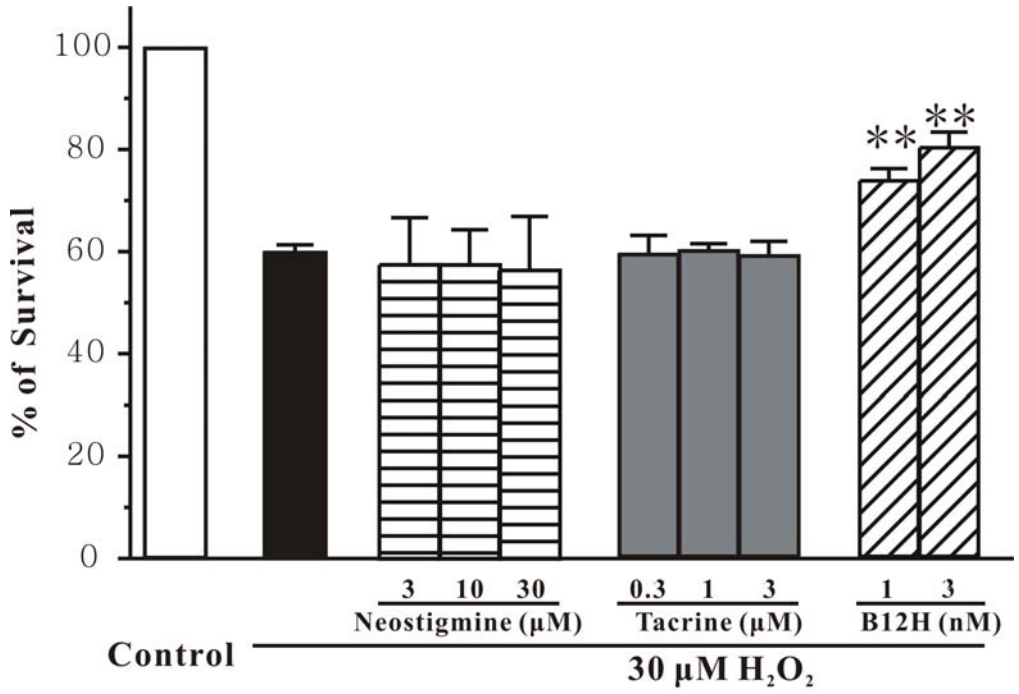

Figure 3.2.3 Neuroprotective effects of B12H against H_2O_2 are independent of AChE inhibition

At 8 DIV, CGNs were pre-incubated with 3, 10 or 30 μM neostigmine, 0.3, 1 or 3 μM tacrine, 1 or 3 nM B12H, and then exposed to 30 μM H_2O_2 2 h later. Cell viability was measured at 6 h after the H_2O_2 challenge by the MTT assay. Data, expressed as percentage of the control, were the mean ± SEM of three separate experiments, $^{**}p < 0.01$ *versus* control (ANOVA and Dunnett's test).

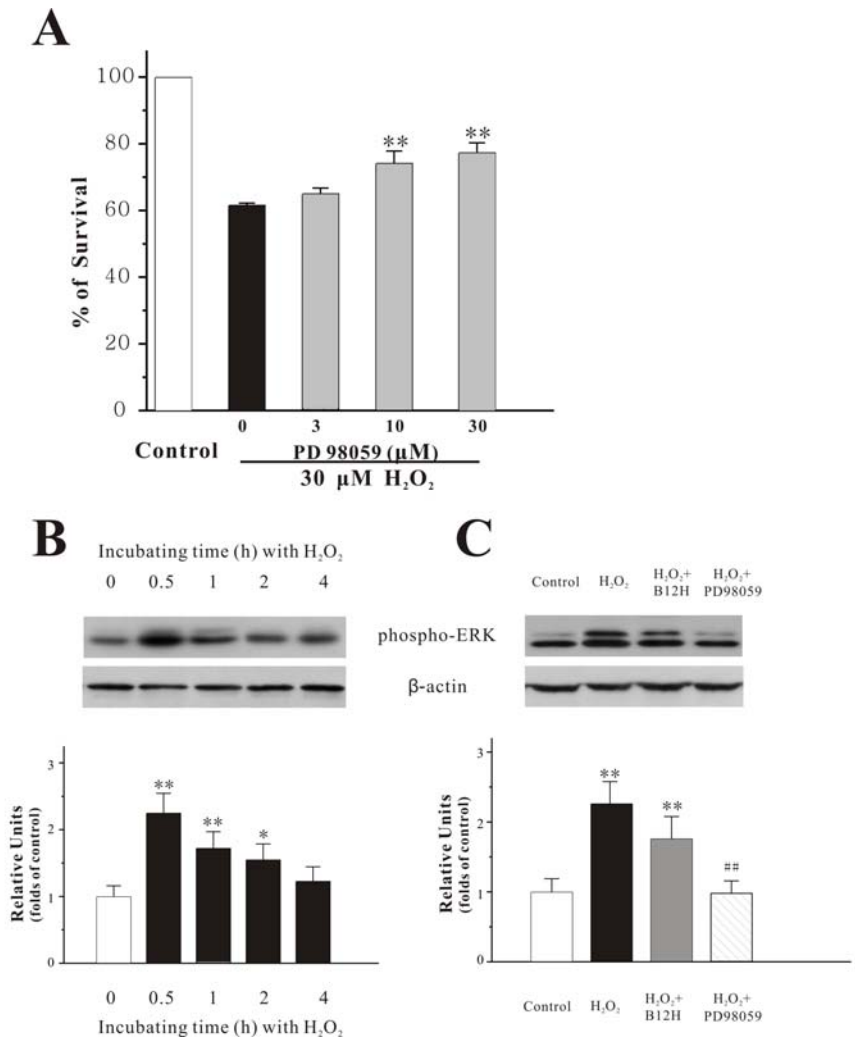

Figure 3.2.4 B12H does not significantly decreased the activation of ERK caused by H_2O_2

(A) PD98059 prevents H_2O_2-induced cell death in a concentration-dependent manner. CGNs were exposed to 30 μM H_2O_2 at 2 h after pre-treatment with PD98059 at various concentrations as indicated. Cell viability was measured at 6 h after H_2O_2 challenge by the MTT assay. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ *versus* H_2O_2 alone group (ANOVA and Dunnett's test). (B) H_2O_2 time-dependently increases the levels of phospho-ERK. CGNs were incubated with 30 μM H_2O_2 at the indicated time points, and the proteins were detected by using specific antibodies. (C) B12H could not reverse the increase of phospho-ERK caused by H_2O_2. CGNs were pre-treated with 3 nM B12H or 30 μM PD98059 for 2 h and then exposed to 30 μM H_2O_2 for 0.5 h. Proteins were detected using specific antibodies. Data were expressed as the ratio to OD values of the corresponding controls; $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* control in (B) and (C) and $^{\#\#}p < 0.01$ *versus* H_2O_2 group in (C) (Tukey's test).

3.2.3 B12H does not affect the activation of ERK caused by H_2O_2

To determine whether the ERK pathway was involved in H_2O_2-induced apoptosis in cultured CGNs, PD98059, a specific inhibitor of MEK, was used to pre-treat the CGNs for 2 h before the H_2O_2 challenge. It was observed that PD98059 at 10-30 μM attenuated H_2O_2-induced apoptosis in CGNs in a concentration-dependent manner (Fig. 3.2.4A). To examine whether B12H protected neurons through inhibition of the ERK pathway, the level of phospho-ERK was determined by Western blotting. As shown in Fig. 3.2.4B, the level of phospho-ERK peaked at 0.5 h after 30 μM H_2O_2 challenge. However, pre-treatment of B12H at 3 nM for 2 h failed to significantly reverse the increase in phospho-ERK caused by H_2O_2, indicating that B12H does not affect the activation of ERK caused by H_2O_2 in CGNs (Fig. 3.2.4C).

3.2.4 B12H inhibits the activation of GSK3β caused by H_2O_2

The activation of GSK3β is also associated with H_2O_2-induced apoptosis in neurons. To determine whether it was involved in H_2O_2-induced apoptosis in cultured CGNs, SB415286, a specific inhibitor of GSK3β, was used to pre-treat the CGNs for 2 h before the H_2O_2 challenge. It was observed that SB415286 at 10-100 μM partially prevented H_2O_2-induced apoptosis in a concentration-dependent manner (Fig. 3.2.5A). To further examine whether B12H protected neurons through the inhibition of GSK3β activity, the level of phospho-GSK3β (Ser-9) was determined by Western blotting. As shown in Fig. 3.2.5C, the level of pSer9-GSK3β peaked at 1 h and returned to its normal level 4 h after the H_2O_2 challenge. B12H at 3 nM restored the phosphorylation level of ser9 on GSK3β that has been depleted by H_2O_2 at 1 h (Fig. 3.2.5D).

3.2.5 B12H reverses the suppression of the PI3-K/Akt pathway caused by H_2O_2

To further investigate whether B12H prevented H_2O_2-induced apoptosis by activating the PI3-K/Akt pathway which decreased the activity of GSK3β by phosphorylating Ser9, the level of pSer473-Akt was also determined by Western blotting. It was found that 30 μM H_2O_2 caused a decrease in pSer473-Akt that peaked at 1 h, and that 3 nM B12H reversed the decrease in pSer473-Akt caused by H_2O_2 (Figs. 3.2.5C and

3.2.5D). In addition, wortmannin and LY294002, two PI3-K specific inhibitors, were used to investigate the neuroprotective effects of B12H. The inhibition of PI3-K by either 50 nM wortmannin or 10 μM LY294002 completely blocked the neuroprotective effects of B12H against H_2O_2-induced apoptosis (Fig. 3.2.5B).

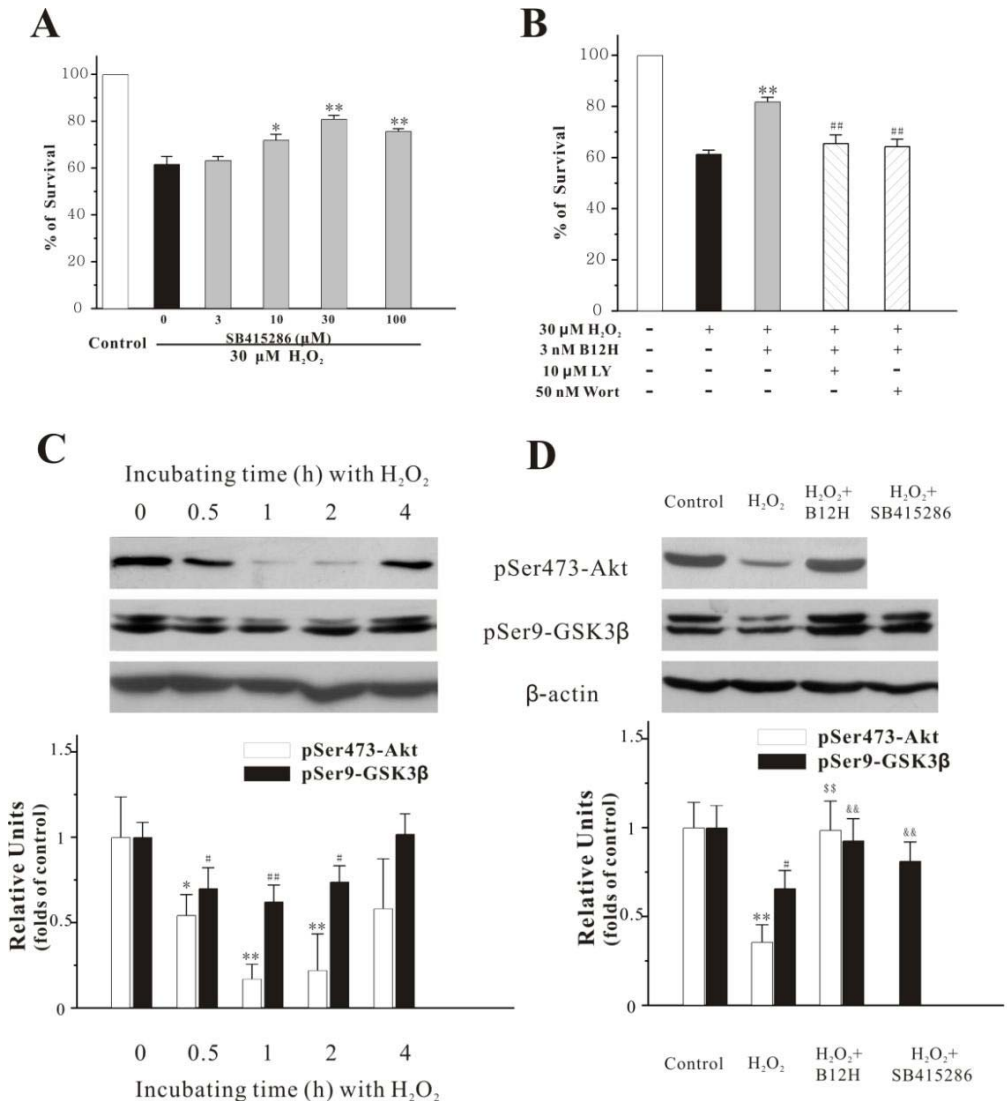

Figure 3.2.5 B12H reverses the suppression of the PI3-K/Akt/GSK3β pathway caused by H_2O_2

(A) SB415286 prevents H_2O_2-induced cell death in a concentration-dependent manner. CGNs were exposed to 30 μM H_2O_2 at 2 h after pre-treatment with SB415286 at various concentrations. Cell viability was measured at 6 h after H_2O_2 challenge by the MTT assay. (B) PI3-K-specific inhibitors abrogate the neuroprotective effects of B12H on H_2O_2-induced apoptosis. CGNs were incubated with 50 nM wortmannin (Wort) or 10 μM LY294002 (LY) for 30 min, and supplemented with 3 nM B12H for 2 h before the exposure to 30 μM H_2O_2. At 6 h after the H_2O_2 challenge, cell viability was measured by the MTT assay. Data expressed as percentage of the control, were the mean ± SEM of three separate experiments, $*p < 0.05$, $**p < 0.01$ *versus* H_2O_2 alone group in (A) and (B), $^{\#\#}p < 0.01$ *versus* B12H plus H_2O_2 group in (B) (Tukey's test). (C) H_2O_2 time-dependently decreases the levels of pSer473-Akt and

pSer9-GSK3β. CGNs were incubated with 30 μM H_2O_2 at the indicated time points, and proteins were detected using specific antibodies. (D) B12H reverses H_2O_2-induced decrease of pSer473-Akt and pSer9-GSK3β. CGNs were pre-treated with 3 nM B12H or 30 μM SB415286 for 2 h, and then exposed to 30 μM H_2O_2 for 1 h. Proteins were detected using specific antibodies. Data were expressed as the ratio to OD values of the corresponding controls; $^{*}p < 0.05$ and $^{**}p < 0.01$ or $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* control for pSer473-Akt or pSer9-GSK3β, respectively in (C) and (D); and $^{\$\$}p < 0.01$ or $^{\&\&}p < 0.01$ *versus* H_2O_2 group for pSer473-Akt or pSer9-GSK3β, respectively, in (D) (Tukey's test).

3.2.6 B12H reverses the inhibition of VEGF/VEGFR-2 system caused by H_2O_2

Recent studies have provided evidence that VEGF, assumed to specifically affect the survival of endothelial cells, is a survival factor for neurons mainly via activating VEGF receptor-2 (VEGFR-2) (Matsuzaki et al., 2001). To determine whether the VEGF/VEGFR-2 system mediated the protective effects against H_2O_2, recombinant VEGF and the VEGFR-2-specific inhibitor PTK787/ZK222584 were used in the experiments. Interestingly, VEGF at 30-100 ng/ml attenuated H_2O_2-induced apoptosis (Fig. 3.2.6A). Pre-treatment of 10 μM PTK787/ZK222584 blocked the neuroprotective effects of VEGF against H_2O_2-induced apoptosis (Fig. 3.2.6A). Furthermore, the activation of VEGFR-2 was examined in the system by determining the level of pTyr1054-VEGFR-2. It was found that 30 μM H_2O_2 caused a decease in pTyr1054-VEGFR-2, and pre-treatment of 3 nM B12H or 100 ng/ml VEGF for 2h reversed the decrease of pTyr1054-VEGFR-2 induced by H_2O_2 (Figs. 3.2.6B and C). Moreover, pre-treatment of 10 μM PTK787/ZK222584 completely abolished the neuroprotective effects of B12H (Fig. 3.2.6A).

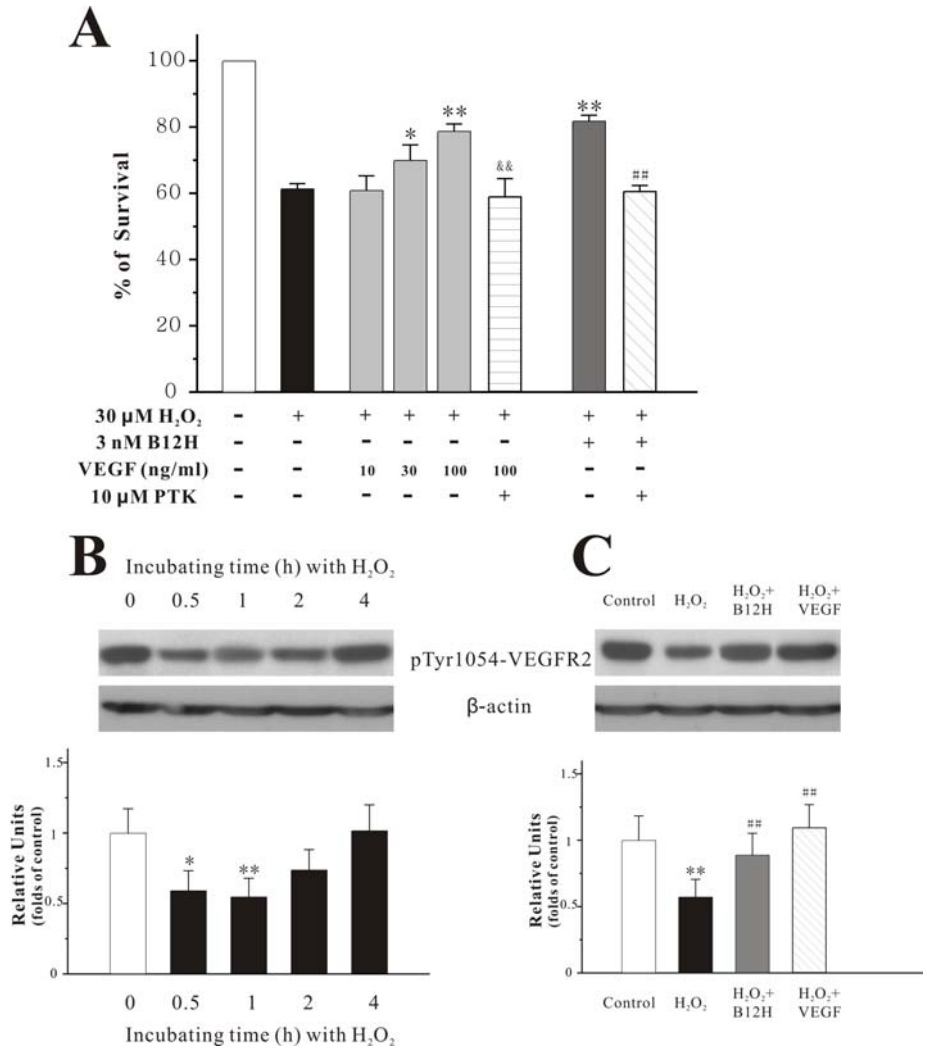

Figure 3.2.6 B12H reverses the activation of VEGF/VEGFR-2 system inhibited by H_2O_2

(A) VEGFR-2-specific inhibitors abrogate the neuroprotective effects of B12H and VEGF. CGNs were incubated with 10 μM PTK787/ZK222584 (PTK) for 30 min, supplemented with 3 nM B12H or VEGF at the concentrations indicated for 2 h before the exposure to 30 μM H_2O_2. At 6 h after the H_2O_2 challenge, cell viability was measured by the MTT assay. Data, expressed as percentage of the control, were the mean ± SEM of three separate experiments, $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* H_2O_2 alone group; $^{\#\#}p < 0.01$ *versus* B12H plus H_2O_2; $^{\&\&}p < 0.01$ *versus* VEGF at the same concentration plus H_2O_2 (Tukey's test). (B) H_2O_2 time-dependently decreases the levels of pTyr1054-VEGFR-2. CGNs were incubated with 30 μM H_2O_2 at the indicated time points, and proteins were detected using specific antibodies. (C) B12H and VEGF reverse the H_2O_2-induced decrease of pTyr1054-VEGFR-2. CGNs were pre-treated with 3 nM B12H or 100 ng/ml VEGF for 2 h and then exposed to 30 μM

H_2O_2 for 1 h. Proteins were then detected using specific antibodies. Data were expressed as the ratio to OD values of the corresponding controls; $*p < 0.05$ and $**p < 0.01$ *versus* control in (C) and (D) and $^{\#\#}p < 0.01$ *versus* H_2O_2 group in (D) (Tukey's test).

3.3 Promotion of Neuronal Differentiation Possibly through Activating α7 nAChR

3.3.1 B12H promotes neurite outgrowth in PC12 cells

NGF and dibutyryl cAMP (dbcAMP) were examined as positive controls in the PC12 neuronal cell model. Both agents triggered the outgrowth of neurites after treatment for 7 days (Fig. 3.3.1A). PC12 cells were then exposed to B12H and its monomeric analogue, hupA. It was found that the efficacy of B12H in promoting neurite outgrowth was three times higher than that of hupA (Figs. 3.3.1A and B). Morphological changes and expression of growth associated protein 43 (GAP-43) in cells treated with various agents are shown in Fig. 3.4.1C. The neurites induced by B12H appeared to have an axon-like morphology, which differs from the dendrite-like neurites induced by NGF.

3.3.2 B12H induces differentiation in adult rat hippocampus neural stem cells

The effects of B12H and hupA on the differentiation of neural stem cells were evaluated by microscopy for the percentage of βIII-tubulin positively stained neurons. It was observed that B12H promoted neuronal differentiation in a concentration-dependent manner, and that the potency of 10 μM B12H was similar to that of 0.5 μM retinoic acid (RA). HupA at 2.5 or 5 μM did not however induce any differentiation (Figs 3.3.2A and B).

3.3.3 Stimulation of ERK but not the p38 signaling pathway is involved in B12H-induced neurite outgrowth in PC12 cells

To determine whether the ERK and p38 signaling pathways are involved in the model, U0126 and PD98059, specific inhibitors of MEK, and SB203580, a specific inhibitor of p38, were selected to treat cells before B12H was added to the cell culture. It was observed that U0126 and PD98059, but not SB203580, partially attenuated the outgrowth of neurites induced by B12H (Fig. 3.3.3A).

To further examine whether B12H induced neurite outgrowth through activation of the ERK pathway, the levels of phospho-ERK, phospho-Elk-1 and phospho-cAMP response element-binding (phospho-CREB) were detected by Western blotting. Fig. 3.4.3B shows that B12H caused an increase in phospho-ERK, an increase that peaked at 30 min and lasted for 6 h after treatment. It was observed that B12H at the same concentration also caused increases in phospho-Elk-1 and phospho-CREB (Fig. 3.3.3B).

3.3.4 Inhibition of α7nAChR abolishes B12H-induced neurite outgrowth in PC12 cells

To investigate whether B12H promoted neurite outgrowth by the TrkA-NGF complexed, K252a, a specific inhibitor of TrkA, was employed. It was observed that K252a did not abolish neurite outgrowth induced by B12H (Figs. 3.3.4A and 3.3.4D).

To determine whether acetylcholine receptors (AChRs) were also involved in the neurite outgrowth induced by B12H, atropine, a specific antagonist of muscarinic AChRs, and mecamylamine and tubocurarine, antagonists of nAChRs, were applied to the cells before administration of B12H. It was observed that 10 μM tubocurarine and 10 μM mecamylamine, but not 10 μM atropine, partially abolished the neurite outgrowth induced by B12H (Fig. 3.3.4B). Furthermore, methyllycaconitine (MLA), a specific inhibitor of α7-type nAChR (α7nAChR), significantly attenuated the neurite outgrowth induced by B12H (Figs. 3.3.4B and D). In addition, pre-treatment of MLA also abolished the elevated level of phospho-ERK (Fig. 3.3.4C).

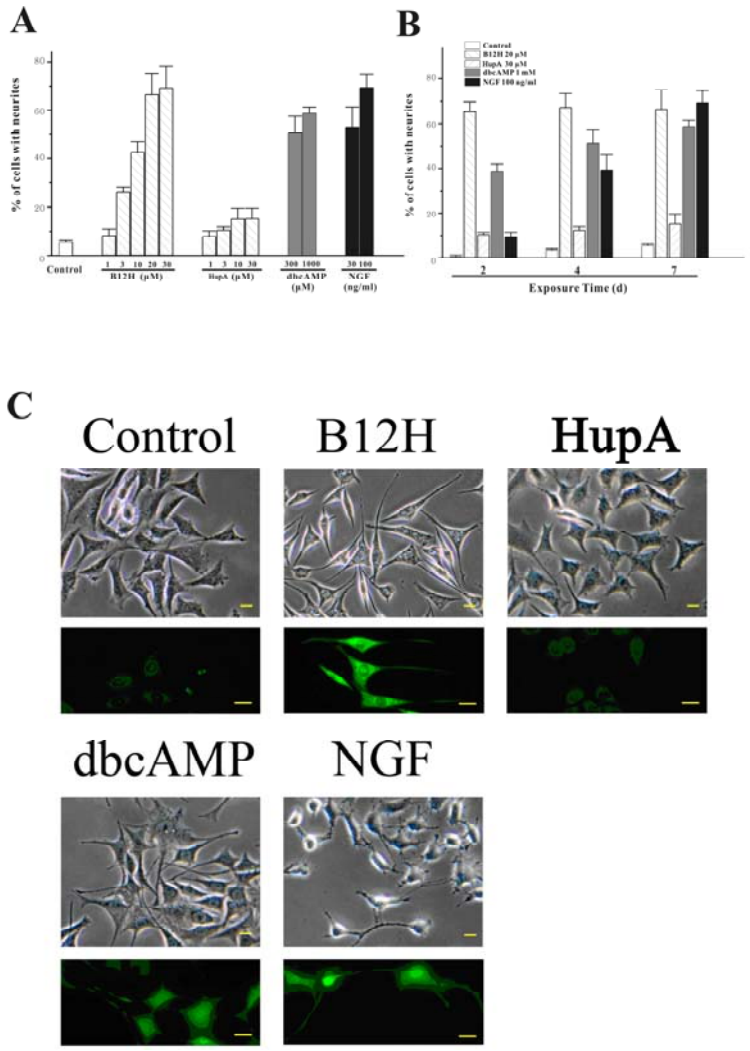

Figure 3.3.1 B12H induces neurite outgrowth in PC12 cells

(A) Induction of neurite outgrowth by B12H is concentration-dependent. PC12 cells were exposed to B12H, hupA, NGF or dbcAMP for 7 days, and the percentage of cells with neurites was determined. (B) Induction of neurite outgrowth by B12H is time-dependent. PC12 cells were exposed to 20 μM B12H, 30 μM hupA, 3 mM dbcAMP or 100 ng/ml NGF for various times, and the percentage of cells with neurites was assessed. The data, expressed as percentage of control, are the mean ± SEM of three separate experiments. (C) The effects of B12H on promoting neurite outgrowth were assessed by the morphological changes seen and the expression of GAP-43. PC12 cells were exposed to 20 μM B12H, 30 μM hupA, 3 mM dbcAMP or 100 ng/ml NGF for 7 days. The morphological changes were examined by light microscopy, and expression of GAP-43 was assessed by fluorescence microscopy. Scale bar, 5 μm.

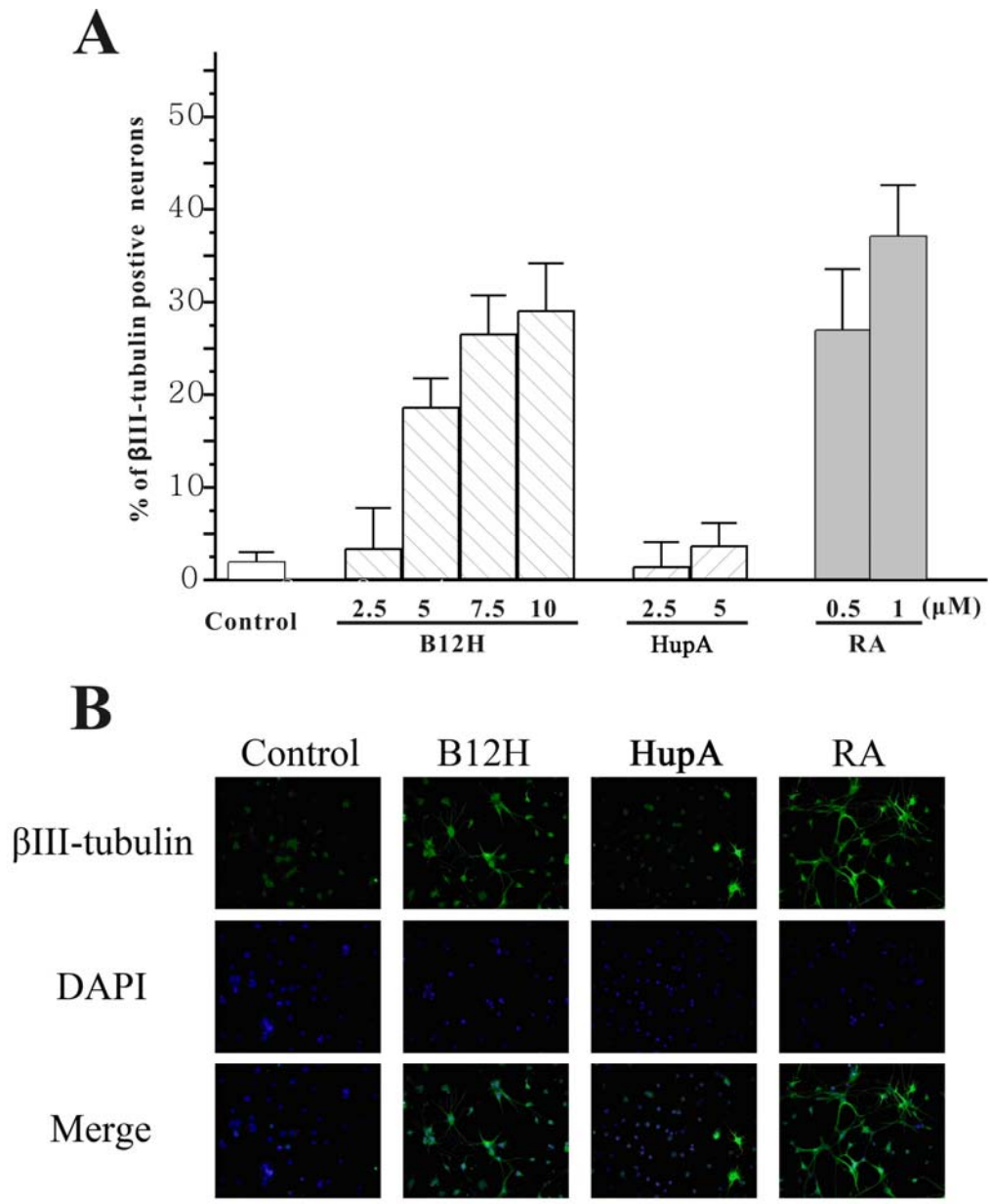

Figure 3.3.2 B12H induces neuronal differentiation in adult rat hippocampus neural stem cells

(A) The increase in the percentage of βIII-tubulin positive neurons caused by B12H is concentration-dependent. Neural stem cells were exposed to B12H, hupA or RA for 48 h, and the percentage of βIII-tubulin positive neurons was determined. (B) The expression of βIII-tubulin in the induced neural stem cells was assessed by fluorescence microscopy. Neural stem cells were exposed to 10 μM B12H, 5 μM hupA or 1 μM RA for 48 h. The cells were then subjected to βIII-tubulin immunostaining and to DAPI staining.

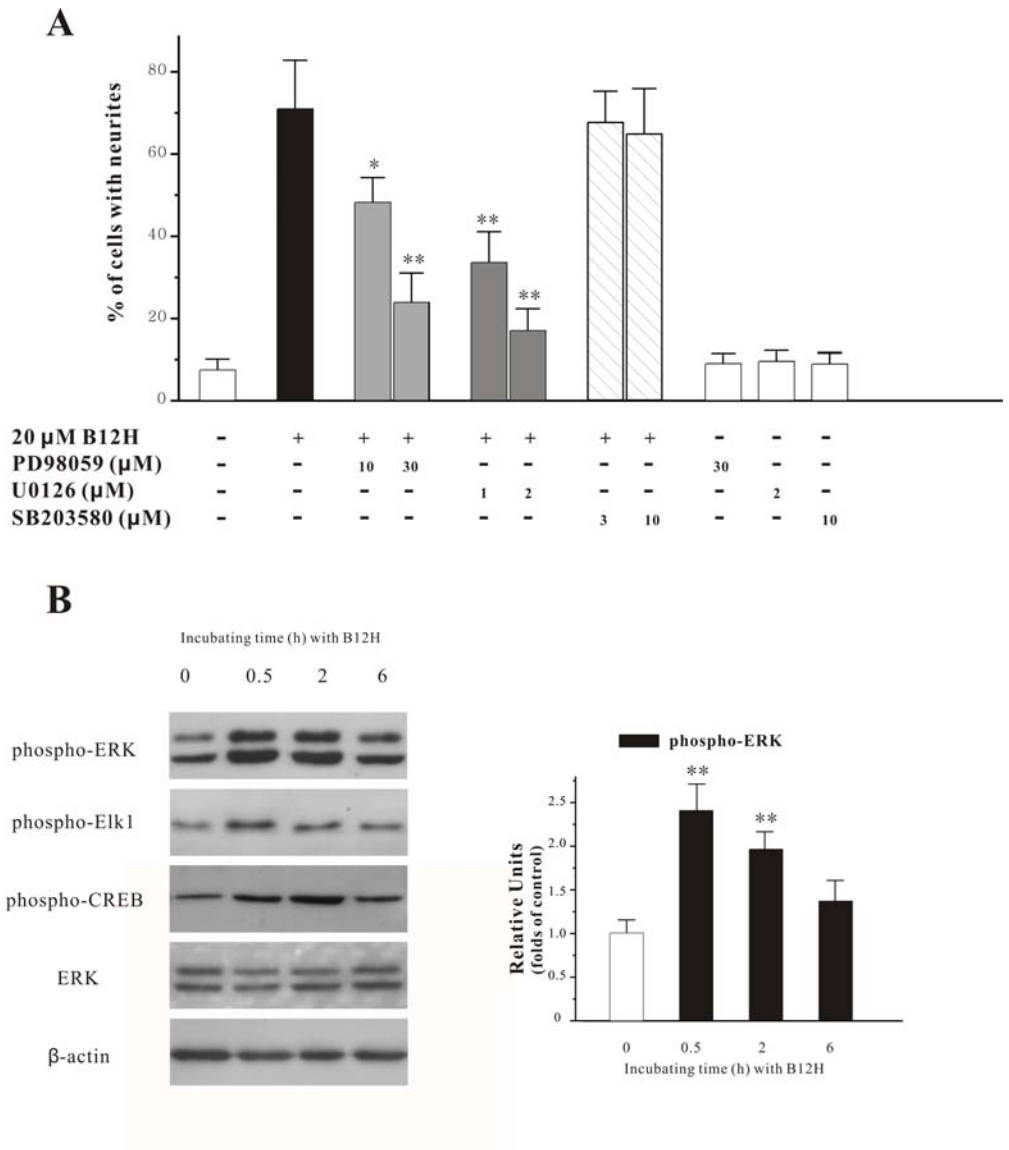

Figure 3.3.3 B12H induces neurite outgrowth by activating the ERK pathway in PC12 cells

(A) MEK-specific inhibitors, but not the p38 specific inhibitors, abolish the neurite outgrowth induced by B12H in PC12 cells. PC12 cells were incubated with various concentrations of PD98059, U0216 or SB203580 for 2 h, and then treated with 20 μM B12H. The percentage of cells with neurite outgrowth was measured 7 days after treatment with B12H. The data, expressed as percentage of control, are the mean ± SEM of three separate experiments, with $*p < 0.05$ and $**p < 0.01$ *versus* the B12H group (ANOVA and Dunnett's test). (B) B12H activates the ERK pathway in PC12 cells. PC12 cells were treated with 20 μM B12H for various times, and the total proteins were extracted for Western blot analysis with specific antibodies. Data were expressed as the ratio to OD values of the corresponding controls; $**p < 0.01$ *versus* control group (ANOVA and Dunnett's test).

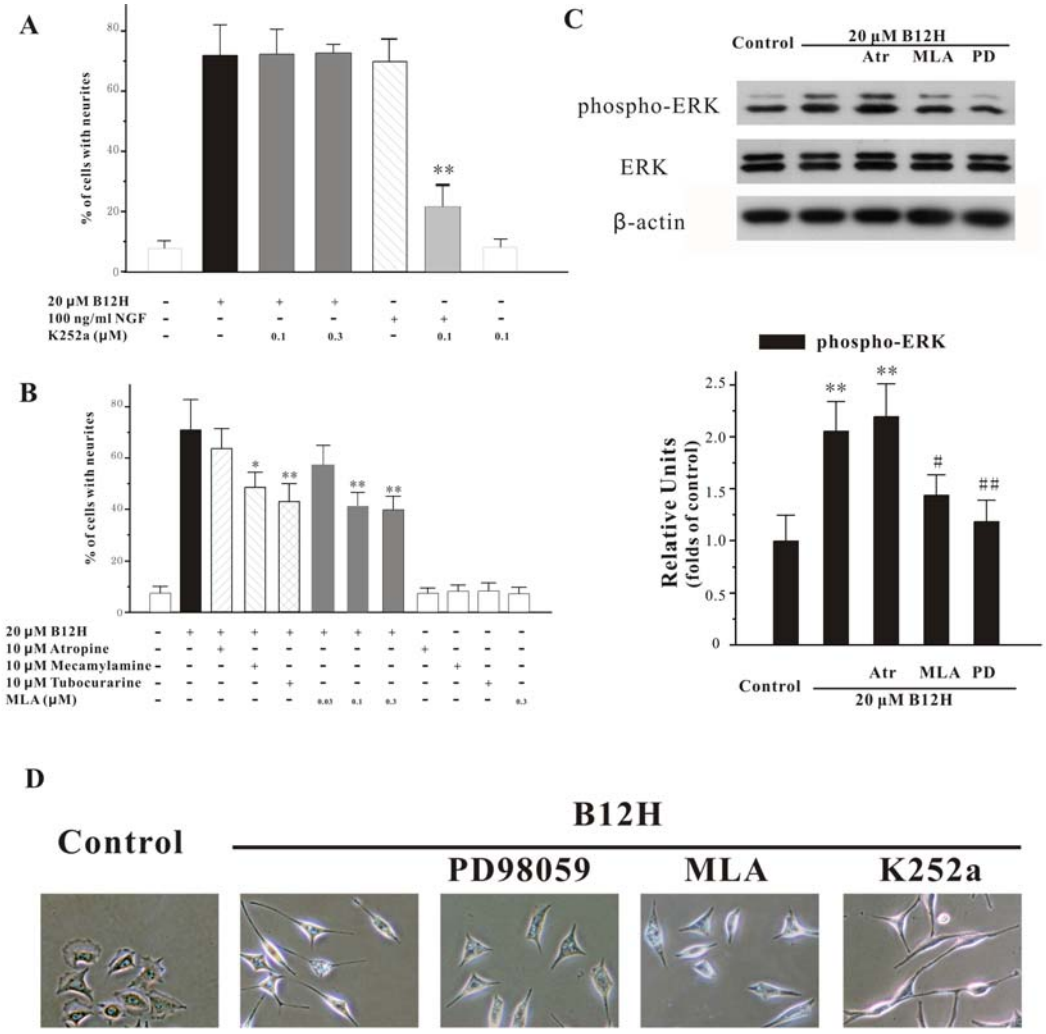

Figure 3.3.4 Inhibition of α7nAChR abolishes the B12H-induced neurite outgrowth in PC12 cells

(A) TrkA-specific inhibitor does not abolish the neurite outgrowth induced by B12H. PC12 cells were incubated with K252a for 2 h, and then treated with 20 μM B12H or 100 ng/ml NGF. The percentage of cells with neurites was measured 7 days after treatment. (B) The α7nAChR antagonist attenuates the neurite outgrowth induced by B12H. PC12 cells were incubated with tubocurarine, mecamylamine, MLA, or atropine for 2 h and treated with 20 μM B12H. The percentage of cells with neurites was measured 7 days after treatment with B12H. The data, expressed as percentage of control, are the mean ± SEM of three separate experiments, with $^{**}p < 0.01$ *versus* the NGF group in (A) and the B12H group in (B) employing ANOVA and Dunnett's test. (C) The α7nAChR antagonist attenuates the activation of ERK induced by B12H. PC12 cells were treated with 0.3 μM MLA, 10 μM atropine (Atr), or 30 μM PD98059 (PD) for 30 min before the administration of 20 μM B12H. The total proteins were

extracted 30 min after the addition of B12H for Western blot analysis with specific antibodies. Data were expressed as the ratio to OD values of the corresponding controls; $^{**}p < 0.01$ *versus* control and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* B12H group (Tukey's test). (D) PC12 cells were pre-incubated with 10 μM PD98059, 0.3 μM MLA or 0.3 μM K252a for 2 h, and then exposed to 20 μM B12H for 7 days. Morphological changes of neurites were examined by light microscopy.

Chapter Four Discussion

4.1 B12H prevents glutamate-induced neuronal apoptosis

Excessive glutamate could induce apoptosis via over-activating NMDA receptors in CGNs (Li et al., 2005). Using a glutamate-induced apoptosis model, it was found that B12H inhibits glutamate-induced apoptosis in a concentration-dependent manner, and that its preventive effect is significant even at 1 nM ($p < 0.01$), indicating that B12H is a powerful neuroprotectant against excitotoxicity *in vitro*. But what is(are) the mechanism(s) involved? As B12H is a selective and potent AChE inhibitor, does B12H prevent glutamate-induced apoptosis by inhibiting AChE? However, it has been demonstrated that other AChE inhibitors (tacrine and donepezil) hardly show any neuroprotective properties against neuronal apoptosis induced by glutamate in CGNs (Li et al., 2005). It is thus proposed that B12H prevents apoptosis induced by glutamate independent of AChE inhibition.

4.2 B12H is a modest affinity antagonist of the NMDA receptor at MK-801 site

It is well known that overstimulation of NMDA receptors is essential in the neuronal apoptotic cell death induced by glutamate, and that the blockade of NMDA receptors may prevent neuronal cell death induced by glutamate (Danysz and Parsons, 2003; Li et al., 2005). It is reasonable to suggest NMDA receptors as the target for the action of B12H against glutamate. With the help of the whole-cell patch clamp technique and a ligand binding assay, it was found that B12H significantly reduces NMDA-evoked currents with an IC_{50} of 21.8 μM, and moderately competes with [^{3}H] MK-801 in binding to rat cerebellar cortical membranes with a Ki of 7.7 μM.

4.3 The efficacy of B12H in blocking the NMDA receptor is not as strong as its efficacy in inhibiting glutamate-induced neuronal apoptosis

Although B12H has potency to block NMDA receptors and inhibit glutamate-induced excitotoxicity *in vitro,* its NMDA receptor-blocking efficacy is not as strong as its efficacy in inhibiting glutamate-induced neuronal apoptosis. Therefore, it is reasonable to hypothesize that B12H may act on other target(s) apart from blocking

NMDA receptors.

4.4 The neuroprotective effects of B12H on H_2O_2-induced neuronal apoptosis are independent of AChE inhibition

B12H prevents H_2O_2-induced apoptosis at a low concentration (3 nM), and such prevention might be independent of inhibition of AChE activity. The IC_{50} of B12H for AChE is 52 nM *in vitro* (Carlier et al., 2000), indicating that administration of 3 nM B12H may not affect AChE activity in the system. Furthermore, other AChE inhibitors (tacrine and neostigmine), even at concentrations greater than the IC_{50} (the IC_{50} of tacrine and neostigmine with AChE are 200 nM and 12 nM, respectively), fail to show neuroprotective effects on neuronal apoptosis induced by H_2O_2. Taken together, it is reasoned that the neuroprotective effects of B12H against H_2O_2 may be a novel activity that is not related to its AChE inhibitory property.

4.5 B12H prevents H_2O_2-induced neuronal apoptosis from reversing VEGFR-2/Akt/GSK3β signaling pathway

Since activation of the ERK pathway has been reported as one of the key pathways that mediate neuronal death (Jiang et al., 2005), it may be wondered whether the neuroprotective effects of B12H are a result of the inhibition of the ERK pathway. Consistent with other studies (Hallak et al., 2001), the level of phospho-ERK is increased by H_2O_2 in CGNs. It has been found that the MEK inhibitor partially prevents apoptosis induced by H_2O_2, indicating that the ERK pathway plays a pro-apoptotic rather than pro-survival role in the system. However, B12H does not inhibit the activation of ERK caused by H_2O_2, indicating that the neuroprotective effects of B12H may involve mechanism other than the ERK pathway.

To further delineate the signaling pathways involved, the effect of B12H on the PI3-K/Akt pathway has been investigated. Previous studies have reported that the increased activity of GSK3β mediates neuronal death, and that inhibition of GSK3β protects from neuronal apoptosis caused by H_2O_2 (Koh et al., 2003). Our results confirm these conclusions based on our findings that the activity of GSK3β increases following the application of H_2O_2, and that a GSK3β inhibitor attenuates neurotoxicity in the system. Moreover, the fact that B12H inhibits the activity of

GSK3β evoked by H_2O_2 implies that B12H exerts its neuroprotective effects via signaling molecule upstream of GSK3β.

It was reported that activated Akt directly inhibits the activity of GSK3β by phosphorylating its Ser9 residue (Grimes and Jope, 2001). Moreover, the PI3-K/Akt pathway plays a central role in neuronal survival (Cantrell, 2001). These facts led us to hypothesize that B12H exerts its neuroprotective effects by regulating the PI3-K/Akt pathway. B12H reverses the suppression of increased phosphorylated Akt caused by H_2O_2. Furthermore, the neuroprotective effects of B12H are completely blocked by PI3-K specific inhibitors. This evidence suggests that B12H is likely to inhibit H_2O_2-induced apoptosis via the PI3-K/Akt pathway.

Neurotrophic factors, such as VEGF and BDNF, protect CGNs from various insults (Bazan-Peregrino et al., 2007; Zhong et al., 2005). The PI3-K/Akt pathway is one of the important pro-survival pathways downstream. Therefore, it is hypothesized that B12H exerts its neuroprotective effects by modulating the activities of the neurotrophic factor systems. Initially identified as an angiogenic peptide (Keck et al., 1989), VEGF has been shown to induce neuroprotective properties via activating the VEGF/VEGFR-2 system both *in vitro* and *in vivo*. It was found for the first time that VEGF attenuates H_2O_2-induced apoptosis in CGNs by activating VEGFR-2. More interestingly, B12H reverses the activity of VEGFR-2 inhibited by H_2O_2. Moreover, the neuroprotective effects of B12H are abolished by the VEGFR-2 specific inhibitor. All these results indicate that the activation of VEGFR-2 may contribute to the neuroprotective effects of B12H.

Although it is still unclear what the precise primary target is, this study indicates that B12H prevents H_2O_2-induced apoptosis via regulating the VEGFR-2/Akt/GSK3β pathway. As the activation of VEGFR-2 is mainly modulated by receptor dimerization and autophosphorylation after ligand binding (Holmes et al., 2007), it is speculated that B12H might either directly interact with VEGFR-2 as a potential agonist, or indirectly facilitate the activation of VEGFR-2, for example, by stabilizing the dimerization or by increasing the level of endogenous VEGF via elevating its translational, transcriptional or post-transcriptional modifications. Further

investigation is needed to understand the exact role that B12H plays in the activation of VEGFR-2.

4.6 B12H promotes neuronal differentiation

Using the percentage of neurite-bearing cells as a marker, it was found that the pro-differentiation potentials of NGF and dbcAMP in this PC12 neuronal model are comparable to those in the literature (Daniele et al., 2010). Moreover, it was found that B12H increased neurite-bearing PC12 cells in a concentration- and time-dependent manner, and with greater efficacy than hupA, indicating that this dimer is a powerful agent in promoting neurite outgrowth in PC12 cells. B12H-induced neuronal differentiation was also observed and characterized by the percentage increase in βIII-tubulin-positive neurons. Since the effect of B12H on the differentiation of NSCs is similar to that of RA, which is a widely used positive control agent for stem cell differentiation, B12H thus seems a promising drug candidate for promoting differentiation of NSCs.

4.7 B12H promotes neuronal differentiation possibly through α7nAChR

Several mechanisms underlying the effects of B12H on stimulating neurite outgrowth in PC12 cells have been explored. It is widely accepted that sustained activation of the ERK signaling pathway is critical in neuronal differentiation. Moreover, activation of the p38 pathway is also required in the neurite outgrowth induced by certain stimuli, such as osmotic shock and heat shock (Kano et al., 2004). Results showed that inhibitors specific for MEK, but not p38, decreased the number of neurite-bearing neurons induced by B12H, indicating that ERK, but not the p38 signaling pathway, may be involved in the B12H-induced differentiation in this model. Since the ERK pathway is implicated mainly in growth factor-induced neurotrophic effects, whereas the p38 pathway is implicated in the stress-induced apoptosis, these results suggest that B12H might exert neurotrophic rather than pro-apoptotic effects (Harper and Wilkie, 2003). It was found that induction of ERK phosphorylation by B12H, similarly to that reported for NGF, is quite rapid in action (Santos et al., 2007). However, the kinetics of ERK stimulation are different because NGF-induced ERK phosphorylation returns to its basal level 2 h after induction (Santos et al., 2007),

whereas B12H shows a more sustained stimulation that lasted for up to 6 h after treatment. This sustained activation of ERK might be due to its slow degradation kinetics, or to a positive feedback network shaping the response of ERK, as suggested by earlier studies (Santos et al., 2007). The sustained stimulation may partially explain the observed pleiotropic effects of B12H.

The different morphologies of the neurites induced by NGF and of those induced by B12H suggest that there different mechanisms may be underlying the neurite outgrowth effect. It is well accepted that NGF promotes neurite outgrowth by activating TrkA (Vaudry et al., 2002). However, the neurites induced by B12H could not be blocked by the TrkA inhibitor, K252a, indicating that the effect of B12H is mediated by a TrkA-independent pathway.

Several studies have shown that activation of α7nAChR plays an important role in the development of neurons *in vivo* and in the neuronal differentiation in PC12 cells (Resende et al., 2008). Activated α7nAChR allows the influx of Ca^{2+}, which in turn activates its downstream signal transduction pathways, including ERK and CREB (Ren et al., 2005). Findings that show that α7nAChR specific inhibitor abolishes neurite outgrowth in parallel to ERK activation induced by B12H confirm the importance of α7nAChR in differentiation, and support ERK as a predominant pro-differentiation pathway in PC12 cells. Our data give further evidence that α7nAChR is necessary for neuronal dendritic arborization, innervation and maturation in the brain (Campbell et al., 2010).

Many studies have suggested that some AChE inhibitors can also interact with the nAChRs (Roman et al., 2004). For example, galantamine, an AChE inhibitor approved for the treatment of AD, serves as an allosteric modulator to potentiate the agonist response of α7nAChR at modest concentrations (Maelicke et al., 2000). Physostigmine, another specific AChE inhibitor, also shows the same allosteric potentiation (Maelicke et al., 2000). Most interestingly, huprine X, a hybrid of tacrine and hupA, also has potentiation effects on the nAChRs (Roman et al., 2004). All these agents are lipophilic compounds containing a tertiary nitrogen that is protonated at neutral pH. These biological and physical properties are all shared by B12H (Maelicke et al., 2000).

4.8 Summary and conclusions

(1) In CGNs, B12H (0.1 nM to 1 μM) reduced glutamate-induced neuronal apoptosis in a concentration- and time-dependent manner. Using patch clamp and receptor-ligand binding techniques, B12H was found to reduce NMDA-activated currents with a moderate potency in cultured rat hippocampal neurons, and to compete with the antagonist, [^{3}H]MK-801, with a Ki value of 7.7 μM, in rat cerebellar cortex membranes.

(2) In CGNs, H_2O_2 generated apoptosis could be attenuated by pre-treatment with B12H in a concentration-dependent manner. Tacrine and neostigmine, however, failed to prevent neurotoxicity, indicating that the neuroprotection by B12H might not be due to its action on AChE activity. Furthermore, it was found that B12H's neuroprotective effects share the same signaling pathway as VEGF in preventing H_2O_2-induced neuronal loss via activation of the VEGF/VEGFR-2 system. B12H was also able to reverse the decreased activation of GSK3β, downstream of the VEGF/VEGF-2 signaling pathway, after H_2O_2 exposure. Thus, B12H prevents H_2O_2-induced neuronal apoptosis by regulating the VEGFR-2/Akt/GSK3β signaling pathway.

(3) B12H induced neurite outgrowth in PC12 cells in a concentration- and time-dependent manner when characterized by morphological changes and expression of GAP-43. Furthermore, B12H promoted neurogenesis as shown by the percentage increase in βIII-tubulin-positive neurons in NSCs. B12H potently transforms pro-neuronal cells to differentiated neurons by activating the ERK pathway, possibly via regulating α7nAChR. The activities of ERK, as well as of its downstream transcription factors, Elk-1 and CREB, were elevated in the B12H-treated PC12 cells. Concordantly, MEK inhibitors and α7nAChR antagonist blocked the neurite outgrowth and activation of ERK induced by B12H.

(4) In conclusion, B12H, a novel dimeric AChE inhibitor, was found to exert substantial and multiple neuroprotective effects by moderately blocking NMDA receptors at MK801 sites and reversing the VEGFR-2/Akt/GSK3β signaling pathway.

Moreover, B12H was also found to promote neuronal differentiation via the α7nAChR (Fig. 4.1). Therefore, we propose that B12H could benefit patients of various neurodegenerative disorders by acting on multiple pathological targets concurrently. Especially, as the synergism between anti-AChE, anti-NMDA receptors, anti-ROS, pro-neuronal differentiation might serve as the most effective therapeutic strategy to prevent and treat neurodegeneration in AD, our findings not only provide a new direction for the design of effective compounds with multiple targets for the prevention and the treatment of neurodegenerative disorders, but also offer novel insights into the molecular basis for the development of potent therapeutic strategies for AD.

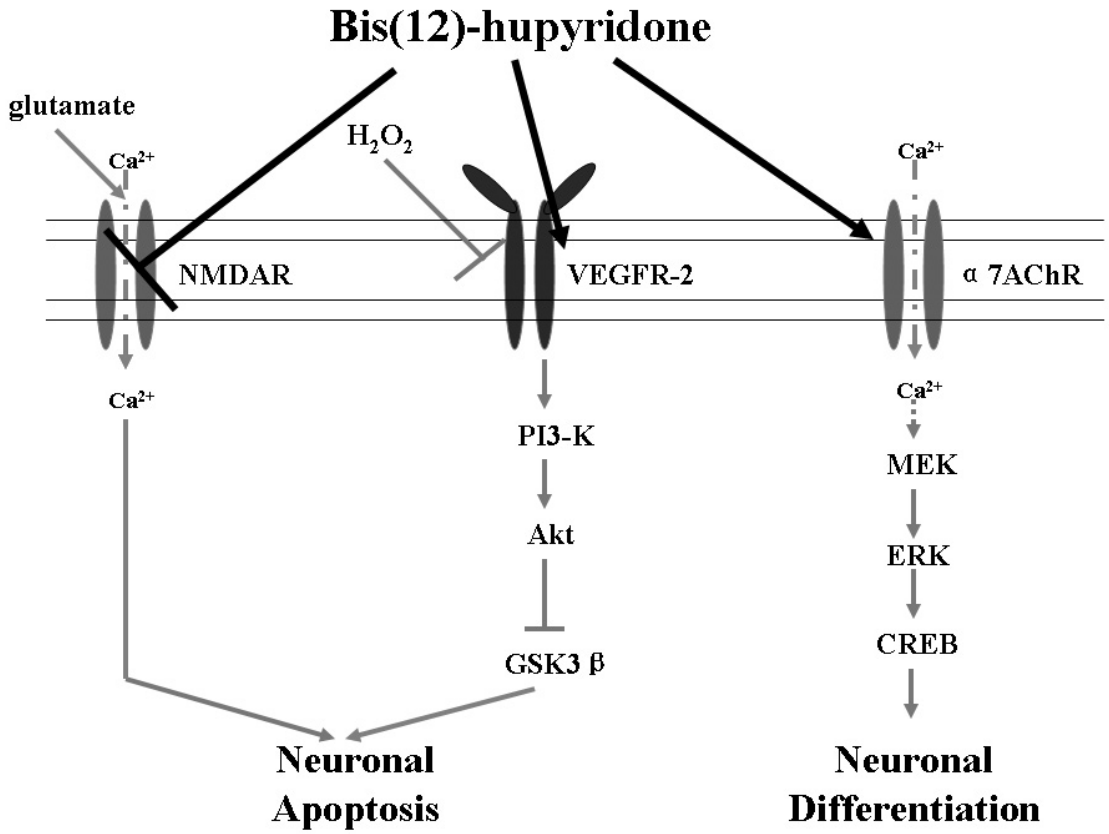

Figure 4.1 The molecular mechanisms underlying the multiple activities of B12H (The AChE inhibition mechanism is depicted in Fig. 1.5)

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