# *Dendrobium* alkaloids decrease Aβ by regulating α- and β-secretases in hippocampal neurons of SD rats

**Juan Huang** [Equal first author, 1] , **Nanqu Huang** [Equal first author, 2] , **Minghui Zhang** [3] , **Nie Jing** [1] , **Yunyan Xu** [1] , **Qin Wu** [1] , **Jing-shan Shi** [Corresp. 1]

1 Key Laboratory of Basic Pharmacology and Joint International Research Laboratory of Ethnomedicine of Ministry of Education, Zunyi Medical University, Zunyi, China

2 Drug Clinical Trial Institution, The Third Affiliated Hospital of Zunyi Medical University, The First People's Hospital of Zunyi, Zunyi, China

3 Tongren People's Hospital, Tongren, China

Corresponding Author: Jing-shan Shi
Email address: shijs@zmu.edu.cn

## Background

Alzheimer's disease (AD) is the primary cause of dementia in the elderly. The imbalance between production and clearance of amyloid β (Aβ) is a very early, often initiating factor in AD. *Dendrobium nobile* Lindl. alkaloids (DNLA) extracted from a Chinese medicinal herb, which have been shown to have anti-aging effects, protected against neuronal impairment in vivo and in vitro. Moreover, we confirmed that DNLA can improve learning and memory function in elderly normal mice, indicating that DNLA has potential health benefits. However, the underlying mechanism is unclear. Therefore, we further explored the effect of DNLA on neurons, which is closely related to learning and memory, based on Aβ.

## Methods

We exposed cultured hippocampal neurons to DNLA to investigate the effect of DNLA on Aβ in vitro. Cell viability was evaluated by MTT assays. Proteins were analyzed by Western blot analysis.

## Results

The cell viability of hippocampal neurons was not changed significantly after treatment with DNLA. But DNLA reduced the protein expression of amyloid precursor protein (APP), disintegrin and metalloprotease 10 (ADAM10), β-site APP cleaving enzyme 1 (BACE1) and Aβ1-42 of hippocampal neurons in rats and increased the protein expression of ADAM17.

## Conclusions

DNLA decreases Aβ by regulating α- and β-secretase in hippocampal neurons of SD rats.

1  *Dendrobium* **alkaloids decrease Aβ by regulating α- and β-secretases**

2  **in hippocampal neurons of SD rats**

3  Juan Huang[1†], Nan-qu Huang[2†], Ming-hui Zhang[3], Jing Nie[1], Yun-yan Xu[1], Qin Wu[1] and Jing-

4  shan Shi[1]

5  [1]Key Laboratory of Basic Pharmacology and Joint International Research Laboratory of

6  Ethnomedicine of Ministry of Education, Zunyi Medical University, Zunyi, Guizhou, China

7  [2]Drug Clinical Trial Institution, The Third Affiliated Hospital of Zunyi Medical University, The

8  First People's Hospital of Zunyi, Guizhou, China

9  [3]Tongren People's Hospital, Tongren, Guizhou, China

10  Corresponding Author:

11  Jingshan Shi[1]

12  No. 6, Xuefu West Road, Xinpu New District, Zunyi, Guizhou, 563003, China

13  Email address: shijs@zmu.edu.cn

14  [†]These authors have contributed equally to this work.

15  **Abstract**

16  **Background**

17  Alzheimer's disease (AD) is the primary cause of dementia in the elderly. The imbalance between

18  production and clearance of amyloid β (Aβ) is a very early, often initiating factor in AD. *Dendrobium nobile*

19  Lindl. alkaloids (DNLA) extracted from a Chinese medicinal herb, which have been shown to have anti-

20  aging effects, protected against neuronal impairment *in vivo* and *in vitro*. Moreover, we confirmed that

21  DNLA can improve learning and memory function in elderly normal mice, indicating that DNLA has

22  potential health benefits. However, the underlying mechanism is unclear. Therefore, we further explored

23  the effect of DNLA on neurons, which is closely related to learning and memory, based on Aβ.

24

25  **Methods**

26  We exposed cultured hippocampal neurons to DNLA to investigate the effect of DNLA on Aβ *in vitro*. Cell

27  viability was evaluated by MTT assays. Proteins were analyzed by Western blot analysis.

28

29  **Results**

30  The cell viability of hippocampal neurons was not changed significantly after treatment with DNLA. But

31  DNLA reduced the protein expression of amyloid precursor protein (APP), disintegrin and metalloprotease

32  10 (ADAM10), β-site APP cleaving enzyme 1 (BACE1) and $A\beta_{1-42}$ of hippocampal neurons in rats and

33  increased the protein expression of ADAM17.

34

35    **Conclusions**

36    DNLA decreases Aβ by regulating α- and β-secretase in hippocampal neurons of SD rats.

37

38    **Keywords** *Dendrobium nobile* Lindl. alkaloids; hippocampal neurons; α-secretase; β-secretase; γ-secretase

39    # Introduction

40    Alzheimer's disease (AD) is a neurodegenerative disease of the central nervous system that causes dementia

41    in a large percentage of the aged population and for which there are only symptomatic treatments.

42    Clinically, AD is typically characterized by progressive loss of memory, declining cognitive function,

43    decreasing physical function and ultimately death. The biology of AD is characterized by two major protein

44    abnormalities in the brain of affected individuals: the extracellular accumulation of amyloid β (Aβ) plaques

45    and intraneuronal deposits of neurofibrillary tangles (NFTs) (De-Paula et al. 2012). At present, the

46    pathogenesis of AD has yet to be fully elucidated, and the main hypotheses proposed include deposition of

47    Aβ protein, loss of choline neurons, abnormal activation of inflammatory reactions, disturbance of energy

48    metabolism, genetic abnormalities and oxidative stress. (Choudhry et al. 2012; Mouton-Liger et al. 2012).

49    In addition, environmental factors, diet and diseases increase the risk of AD (Ashok et al. 2015; Banerjee

50    et al. 2014; Cansu et al. 2017; Cheng et al. 2015; Faraco et al. 2016; Yousof Ali et al. 2015). The Aβ peptide

51    cascade plays a critical role in the development of AD (Baranello et al. 2015).

52    Aβ is generated by amyloid precursor protein (APP) metabolism. APP in its mature form can be processed

53    by at least two proteolytic pathways (Kowalska 2004), the so-called "non-amyloidogenic" and the

54    "amyloidogenic" pathways. In the first pathway, α-secretases cleaves APP with the γ-secretases, thus

55    impeding the formation of the toxic Aβ peptide. In the second pathway, Aβ, which mainly consists of the

56    $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, is produced in a two-step proteolytic process initiated by β-secretase and next

57    mediated by γ-secretase. In brief, α-, β- and γ-secretases play key roles in the metabolic processing of APP.

58    Strategies to reduce the formation of toxic Aβ are an obvious approach to prevent AD. Activation of α-

59    secretase promotes the cleavage of APP, which can reduce the production of Aβ (Lichtenthaler 2012). And

60    the inhibition of β-secretase (Hilpert et al. 2013) and γ-secretase (Wolfe 2012) activity can also reduce the

61    production of Aβ. Therefore, targeting α-, β- and γ-secretases is a promising focus of AD research.

62    *Dendrobium* (*D.*) *nobile* Lindl. is a traditional Chinese herbal medicine and medicinal material of Guizhou

63    Province. The chemical constituents of *D. nobile* Lindl. include alkaloids, sesquiterpene, bibenzyl,

64    fluorenone, phenolic acid, phenylpropanoid, phenanthrene, polysaccharides, and lignans. An ancient

65    medical book notes that this treatment can strengthen the body and extend life. Most compounds had good

66    physiological activity, could significantly improve memory loss and cerebral ischemia, and showed anti-

67    fatigue, antioxidative, blood sugar lowering, antitumor and anti-inflammatory effects. As a

68    pharmacologically active ingredient of *D. nobile* Lindl., *D. nobile* Lindl. alkaloids (DNLA) was originally

69    extracted from *D. nobile* Lindl and has significant protective effects in the nervous system. The chemical

70  structures of DNLA are shown in the Figure 1, and the chromatograms of the sample solutions are shown
71  in Fig. 1B (Nie et al. 2016). Previous studies indicated that DNLA can improve the neuronal disruption
72  caused by lipopolysaccharide (Y. Li et al. 2011), oxygen-glucose deprivation (Wang et al. 2010) and
73  reperfusion and decrease neuronal apoptosis, hyperphosphorylation of tau protein, and Aβ deposition in the
74  rat brain (Nie et al. 2016). Furthermore, we observed amelioration of the spatial learning performance in
75  AD model rats induced by $Aβ_{25-35}$, and this effect may be related to decreases in the generation of $Aβ_{1-42}$
76  (Zhang Minghui et al. 2016) and alleviation of $Aβ_{25-35}$-induced axonal injury by improving autophagic flux
77  in neurons *in vitro* (Li et al. 2017; Zhang et al. 2017). Notably, DNLA improved learning and memory
78  function in elderly normal mice (Linshan. et al. 2016). This caught our attentions and we really want to
79  know why there is such an improvement.
80  Therefore, based on the background of AD and our previous works, we designed the following experiment,
81  that aims to investigate the effect of DNLA on Aβ and related secretases in the normal hippocampal neurons
82  of rats and to further explore the mechanism underlying the regulation of the APP metabolic pathway.
83

## Materials & Methods

84  **Materials**

85  Dendrobium was purchased from Xintian Traditional Chinese Medicine Industry Development Co., Ltd.,
86  of Guizhou Province. DNLA was isolated from the extracts and analyzed by LC MS/MS. Alkaloids
87  accounted for 79.8% of the DNLA and were comprised of 92.6% dendrobine ($C_{16}H_{25}O_2N$), 3.3%
88  dendrobine-N-oxide ($C_{16}H_{25}O_3N$), 2.0% nobilonine ($C_{17}H_{27}O_3N$), 0.9% dendroxine ($C_{17}H_{25}O_3N$), 0.32% 6-
89  hydroxy-nobilonine ($C_{17}H_{27}O_4N$), and 0.07% 13-hydroxy-14-oxodendrobine ($C_{16}H_{23}O_4N$) (Nie et al. 2016).
90  Trypsin (SH30042.01) and DMEM/F12 medium (SH3002301B) were purchased from HyClone (America).
91  FBS (S9030) was purchased from Solarbio (China). DES, Neurobasal A (A2477501), and B27 (17504044)
92  were purchased from Gibco (America). The goat anti-mouse IgG H&L (Alexa Fluor® 488) was purchased
93  from Life Technologies (America). Anti-beta III tubulin antibody (ab14545), goat anti-rabbit IgG H&L
94  (ab150113), anti- disintegrin and metalloprotease (ADAM)10 (ab1997), anti-β-site APP cleaving enzyme
95  1(BACE1) (ab2007), anti-presenilin 1(PS1) (ab76083), and anti-$Aβ_{1-42}$ (ab201060) were purchased from
96  Abcam (America). Anti-APP (D260097) and anti-ADAM17 (D151531) were purchased from Sangon
97  Biotech (China).
98

99  **Animals**

100  Sprague-Dawley (SD) rats (approximately 200 ~250 g) were purchased from the Laboratory Animal
101  Center, Chongqing, China [grade: specific pathogen-free (SPF), certificate no. SCXK 2012-0005] and
102  housed at 22 ~23°C with a 12-hour light/dark cycle. Five SD rats (male: female = 1:4) were housed in each
103  cage to propagate the newborn SD rats and had free access to food and water. All procedures were

105 undertaken with the approval of the Animal Experimental Ethical Committee of Zunyi Medical University

106 [No. (2019)2-995, 11 Mar 2019].

107

**Pretreatment of DNLA**

109 DNLA was soluble in dimethyl sulfoxide (DMSO) and was stored at -20°C. When used, DNLA was diluted

110 to different concentrations with Neurobasal A supplemented with 2% B27. The final concentration of

111 DMSO was 0.01% (v/v) in our experimental system.

112

**Culture of hippocampal primary neurons and identification**

114 Hippocampal tissues from newborn SD rats born within 24 ~ 48 hours were separated on ice and then

115 incubated in 0.125% trypsin at 37°C with 5% $CO_2$ for 15 minutes. Hippocampal tissues were triturated by

116 passing repeatedly through a 1-mL pipette tip and filtered through a sieve with 200, 300, and 400 mesh.

117 Then, the cells were collected by centrifugation at 179 g for 8 minutes. Cells were seeded on poly-L-lysine-

118 coated (4 mg/mL) 6-well, 24-well plates or 96-well plates and cultured in DMEM/F12 medium

119 supplemented with 10% FBS, 10% DES, 100 U/mL penicillin and streptomycin at 37°C with 5% $CO_2$.

120 After 4 hours, DMEM/F12 medium was replaced with Neurobasal A supplemented with 2% B27 for the

121 duration of the experiments. Half of the liquid was exchanged every two or three days. On the 3rd day,

122 neurons were treated with cytarabine. On the 8th day, neurons in good condition were used for the following

123 experiments. The animal procedures were approved by the Animal Experimentation Ethics Committee of

124 Zunyi Medical University. The profile of neurons was visualized by immunofluorescence staining using

125 mouse monoclonal anti-beta III tubulin antibody (1:1000) and goat anti-mouse IgG H&L (1:1000). DAPI

126 (1:20) was used to mark the cell nucleus.

127

**Assessment of cell viability by MTT assays**

129 Neurons were seeded into 96-well plates and treated with DNLA for a desired time period at the indicated

130 concentrations. Five replicates were made for each treatment. After treatment, cell viability was evaluated

131 by MTT assays as previously described. The cell viability is expressed as a percentage of the OD of cells

132 with the indicated treatments to that in cells treated with the DMSO control.

133

**Western blot assay**

135 Total protein was extracted from cultured neurons using a total protein extraction kit and quantified by a

136 BCA protein assay kit. Equal amounts of protein (20 μg) per lane were separated by SDS-PAGE gels and

137 then transferred to a PVDF (0.45 μm) membrane. The membranes were incubated with the following

138 primary antibodies: anti-APP (1:500), anti-ADAM10 (1:1000), anti-ADAM17 (1:1000), anti-BACE1

139 (1:1000), anti-PS1 (1:1000), anti-A$\beta_{1-42}$ (1:1000), anti-β-actin (1:2000), and anti-GAPDH (1:2000) at 4°C

140 overnight, followed by incubation with secondary antibody at 4°C for 1 hour. The membranes were

141     visualized using the chemiluminescence reagent ECL Plus (E003-100). The image was scanned, and band

142     densities were quantified using Quantity One 1D analysis software v4.52 (Bio-Rad). GAPDH or β-actin

143     was used to normalize protein loading.

144

145     **Statistical analysis**

146     All data are expressed as the mean ± SD and were analyzed statistically by SPSS 17.0 software. The

147     normally distributed data were first analyzed statistically via one-way analysis of variance (ANOVA). $P <$

148     0.05 was considered to be statistically significant.

149     # Results

150     **The purity of hippocampal neurons in SD rats**

151     First, we examined the purity of neurons by immunofluorescence staining to ensure the accuracy and

152     feasibility of the experiment. The neuronal purity was at least 92.5% (Figure 2).

153

154     **Effects of DNLA on the cell viability of hippocampal neurons in SD rats**

155     Second, to determine whether DNLA improved the cell viability of hippocampal neurons from SD rats, we

156     treated primary hippocampal neurons with DNLA for 48 hours and detected the viability of neurons in each

157     group by MTT assays. The results showed that DNLA did not significantly change the cell vitality of

158     hippocampal neurons (Figure 3). We concluded that DNLA does not alter the overall state of the cells in

159     premature aging hippocampal neurons. However, did the internal state of the neurons change? We further

160     examined this question based on the "Aβ cascade hypothesis".

161

162     **DNLA decreased the accumulation of Aβ by decreasing APP**

163

164     As mentioned above, dementia is attributed to synaptic dysfunction and neuronal loss in the hippocampus

165     and its associated cortex, which are caused by the accumulation of Aβ oligomers. Thus, we detected the

166     protein expression of $A\beta_{1-42}$, and found that DNLA decreased the protein expression of $A\beta_{1-42}$ (Figure 4 B).

167     To determine whether the effects of DNLA on the protein expression of $A\beta_{1-42}$ in hippocampal neurons of

168     SD rats were related to APP, which is the source of Aβ and is cleaved to $A\beta_{1-42}$ and the other Aβ fragment,

169     we examined the protein expression of APP in hippocampal primary neurons by Western blot analysis.

170     Furthermore, PS1 (γ-secretase) participates in both the "non-amyloidogenic" and the "amyloidogenic"

171     pathways. Therefore, we also examined the protein expression of APP by Western blot analysis. As shown

172     in Figure 4, DNLA decreased the protein expression of APP in hippocampal primary neurons of rats but

173     did not change the protein expression of PS1. We found that DNLA decreased the accumulation of Aβ by

174     decreasing APP.

175

176 **DNLA decreased the accumulation of Aβ by regulating α-secretase**

177 As a protective factor for the progression of AD, α-secretase plays a significant role in the "non-
178 amyloidogenic" pathway. Moreover, ADAM10 and ADAM17 are considered to be the most important α-
179 secretases involved in the physiological processing of APP in the brain. We further probed the effect of
180 DNLA on α-secretases (ADAM10 and ADAM17). And we examined the protein expression of ADAM10
181 and ADAM17 by Western blot analyses. Our results indicated that DNLA increased the protein expression
182 of ADAM17 but decreased the protein expression of ADAM10 (Figure 5). Thus, DNLA decreased the
183 accumulation of Aβ by regulating α-secretase

184

185 **DNLA decreased the accumulation of Aβ by inducing BACE1 (β-secretase)**

186 In addition to γ-secretase, β-secretase, the crucial factor in the "amyloidogenic" pathways, is related to APP
187 metabolism, which is the source of Aβ. BACE1 was shown to have important physiological roles in Aβ
188 production. Inhibiting the activity of BACE1 can reduce the accumulation of Aβ, which triggers the
189 exacerbation of AD. We confirmed that DNLA could decrease the production of Aβ. Moreover, the
190 reduction of Aβ involved APP and α-secretase. Thus, we examined whether this effect was related to
191 BACE1. Accordingly, we detected the protein expression of BACE1 by Western blots analyses. As
192 indicated in Figure 6, DNLA could decrease the accumulation of Aβ by reducing the protein expression of
193 BACE1 in hippocampal neurons from SD rats.

194

195 # Discussion

196 DNLA was extracted from *D. nobile* Lindl, which is a traditional Chinese medicinal material and is
197 produced in Chishui, Guizhou. As a pharmacologically active ingredient of *D. nobile*, DNLA has a
198 protective effect on the nervous system. Previous studies showed that APP, β-secretase and Aβ protein in
199 the hippocampus of Tg2576 transgenic mice were reduced. Additionally, DNLA substantially improved
200 the learning and memory of middle-aged APP/PS1 transgenic mice and wild-type mice, suggesting that
201 DNLA has potential health benefits (Linshan. et al. 2016; Nie et al. 2018). In this experiment, neurons in
202 the normal growth state were treated with different concentrations of DNLA, and the effects of DNLA on
203 cell viability, APP, Aβ and its related secretases (α-, β- and γ- secretases) in normal conditions were
204 preliminarily explored.

205 The senile plaque formed by extracellular deposition of Aβ is one of the main pathological characteristics
206 of AD. Moreover, with increasing age, Aβ deposition increases (Niedowicz et al. 2014). Aβ mainly includes
207 $Aβ_{1-40}$ and $Aβ_{1-42}$. $Aβ_{1-42}$ has stronger neurotoxicity and hydrophobicity than $Aβ_{1-40}$, and it has a strong
208 tendency to oligomerize than $Aβ_{1-40}$ and is thus more easily polymerized in the brain. $Aβ_{1-42}$ accumulates to
209 a high level, forming "senile plaques", which produce neurotoxic effects that injure the neurons and cause
210 damage. Thus, inhibiting the production of $Aβ_{1-42}$ can delay the pathogenesis of AD (Zhu et al. 2011). In

211  this study, the protein expression of $A\beta_{1-42}$ in hippocampal neurons of SD rats was detected. The results
212  showed that DNLA can reduce the protein expression of $A\beta_{1-42}$ in hippocampal neurons.

213  APP is the precursor protein of $A\beta$ and is a member of a family of related proteins, including amyloid
214  precursor-like proteins (APLP1 and APLP2) in mammals and amyloid precursor protein-like (APPL) in
215  *Drosophila*, all with large extracellular structures. These proteins have a one-way transmembrane domain,
216  but only APP produces amyloidosis fragments (O'Brien & Wong 2011). In theory, reductions in APP, as
217  the precursor protein of $A\beta$, could decrease the production of $A\beta$ from the source. In this study, DNLA
218  decreased the protein expression of APP.

219  Regardless, $\gamma$-secretase participates in the "non-amyloidogenic" and the "amyloidogenic" pathways and
220  plays an indispensable role in the progression of APP metabolism to generate $A\beta$. Notably, DNLA reduced
221  APP in a dose-dependent manner, but the effects were only significant at a dose of 350 ng/mL. One possible
222  reason is that the data for these statistics are not sufficient to produce significant differences at other doses.
223  Another possible reason is that the other dosages did not result in a significant change in this indicator.
224  Simultaneously, some studies have suggested that the inhibition of $\gamma$-secretase activity could reduce the
225  production of $A\beta$. Moreover, $\gamma$-secretase contains PS (including PS1 and PS2), NCT, Pen-2 and Aph-1 and
226  is also the rate-limiting enzyme of APP production of $A\beta$ (T. Li et al. 2011). Many studies have shown that
227  inhibition of $\gamma$-secretase activity helps reduce $A\beta$ production (Wolfe 2012). As the core catalytic subunit of
228  $\gamma$-secretase, PS1 is the main active component (Garcia-Ayllon et al. 2013) of this enzyme and has an
229  independent $\gamma$-secretase role (Woodruff et al. 2013). PS1 overexpression may be a risk factor for late-onset
230  SAD (T. Li et al. 2011). Conversely, inhibition of PS1 expression reduced $A\beta$ production (Futai et al. 2016).
231  In our study, the protein expression of PS1 showed no significant changes. These results indicated that
232  DNLA did not affect PS1. $A\beta_{1-42}$ produced by $\gamma$-secretase did not increase and was reduced. Therefore, we
233  concluded that DNLA affects APP metabolism to $A\beta$ in other ways. We further explored this phenomenon.
234  Next, to explore the effect of DNLA on the non-amyloidogenic pathway, we detected the $\alpha$-secretases,
235  mainly the ADAM10 and ADAM17 proteins. ADAM17 is an important $\alpha$-secretase involved in the non-
236  amyloidogenic pathway of APP. Enhancing the activity of ADAM17 increased the secretion of a soluble
237  sAPP$\alpha$ fragment with neuroprotective activity and decreased $A\beta$ production. Therefore, ADAM17 is
238  considered a potential therapeutic target for AD (Qian et al. 2016). The results of this experiment showed
239  that DNLA could increase the protein expression of ADAM17 and reduce the production of $A\beta$.
240  Additionally, ADAM10 also degrades APP by the non-amyloidogenic pathway. On one hand, it cleaves
241  APP to produce sAPP$\alpha$ and avoids the production of $A\beta$. On the other hand, studies have also found an age-
242  dependent increase in ADAM10 levels (Schuck et al. 2016). However, experiments by B. Shackleton et al.
243  (Shackleton et al. 2016) suggested that inhibition of ADAM10 promoted the clearance of $A\beta_{1-40}$ and $A\beta_{1-42}$
244  in the brains of mice with AD, thereby reducing the level of $A\beta$. This result may be because the other $\alpha$-
245  secretase process APP when ADAM10 is reduced or absent. Other studies have reported that other members
246  of the $\alpha$-secretase family, such as ADAM9 and ADAM17, can compensate for the reduction in ADAM10

247     activity (Asai et al. 2003; Hartmann et al. 2002). When ADAM10 was absent or reduced, sAPPα production

248     was shown to be reduced or Aβ was significantly increased, which may be due to the lack of compensation

249     for α-secretase (Kuhn et al. 2010; Postina et al. 2004; Suh et al. 2013). In addition, inhibition of BACE1

250     increased ADAM10 cleavage of APP, but decreasing ADAM10 activity increased the risk of AD by

251     increasing β-secretase cleavage of APP (Colombo et al. 2013). In this experiment, ADAM10 protein

252     expression in hippocampal neurons treated with DNLA was decreased. However, interestingly, ADAM17

253     protein expression was increased, and the Aβ protein level was decreased. These findings suggested that

254     DNLA can reduce the expression of ADAM10 protein, which promotes the clearance of Aβ and reduces

255     the Aβ level in hippocampal neurons of SD rats.

256     In the amyloidogenic pathway, APP finally produces Aβ by β- and γ-secretase cleavage. β-secretase,

257     including BACE1, cathepsin S (Cat S), cathepsin L (Cat L) (Schechter & Ziv 2011), cathepsin D (Cat D)

258     and cathepsin B (Cat B) (Zhou et al. 2012), is the rate-limiting enzyme of APP production of Aβ. BACE2

259     was previously suggested to belong to the β-secretase family, but it was later proven to be a newly

260     discovered η-secretase (Willem et al. 2015). Although Cat S, Cat L and Cat B may be further developed as

261     targets for the treatment of AD (Schechter & Ziv 2011), the current main target for β-secretase remains

262     BACE1. Numerous studies have shown that inhibition of BACE1 can reduce the production of Aβ (Adwan

263     et al. 2014; Yan & Vassar 2014; Yun et al. 2013; Zhu et al. 2012). The results of this experiment showed

264     that BACE1 protein expression was decreased after treatment with DNLA, suggesting that DNLA can

265     reduce the protein expression of BACE1 in neurons. This change likely caused the reduced protein

266     expression of $A\beta_{1\text{-}42}$, which is crucial for the development of AD.

267     Under the experimental conditions, there are some drawbacks in our study. We need further direct evidence

268     showing that DNLA reduces Aβ by affecting α- and β-secretase. For further analysis of the effects of DNLA

269     on α-, β- and γ-secretase, the products of APP, such as sAPPα, P3, sAPPβ and CTF-γ, which were lysed by

270     the corresponding secretases, should be further tested to explain the effect of DNLA on related secretases

271     and its functions. In addition, the α-, β-, and γ-secretase activity should be directly detected for further

272     confirmation of our results. Moreover, Aβ was reduced by decreasing its source and promoting its

273     clearance. In this experiment, $A\beta_{1\text{-}42}$ protein expression in hippocampal neurons of SD rats was reduced,

274     which was the result of various mechanisms. Therefore, the exact mechanism of $A\beta_{1\text{-}42}$ reduction requires

275     further elucidation.

276     In summary, DNLA decreases Aβ accumulation by regulating α- and β-secretases in hippocampal neurons

277     from SD rats. We verified that DNLA has potential health benefits and provided a theoretical basis for the

278     anti-aging effect of DNLA.

279

## Conclusions

281     The results showed that DNLA can decrease $A\beta_{1\text{-}42}$ in hippocampal neurons of rats by regulating α- and β-

282    secretase.

283

284    **References**

285    Adwan L, Subaiea GM, and Zawia NH. 2014. Tolfenamic acid downregulates BACE1 and protects against
286         lead-induced upregulation of Alzheimer's disease related biomarkers. Neuropharmacology 79:596-
287         602. 10.1016/j.neuropharm.2014.01.009

288    Asai M, Hattori C, Szabo B, Sasagawa N, Maruyama K, Tanuma S, and Ishiura S. 2003. Putative function
289         of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. Biochemical and Biophysical
290         Research Communications 301:231-235. 10.1016/S0006-291X(02)02999-6

291    Ashok A, Rai NK, Tripathi S, and Bandyopadhyay S. 2015. Exposure to As-, Cd-, and Pb-mixture induces
292         Abeta, amyloidogenic APP processing and cognitive impairments via oxidative stress-dependent
293         neuroinflammation in young rats. Toxicological Sciences 143:64-80. 10.1093/toxsci/kfu208

294    Banerjee P, Sahoo A, Anand S, Ganguly A, Righi G, Bovicelli P, Saso L, and Chakrabarti S. 2014. Multiple
295         mechanisms of iron-induced amyloid beta-peptide accumulation in SHSY5Y cells: protective
296         action of negletein. Neuromolecular Medicine 16:787-798. 10.1007/s12017-014-8328-4

297    Baranello RJ, Bharani KL, Padmaraju V, Chopra N, Lahiri DK, Greig NH, Pappolla MA, and Sambamurti
298         K. 2015. Amyloid-beta protein clearance and degradation (ABCD) pathways and their role in
299         Alzheimer's disease. Curr Alzheimer Res 12:32-46. 10.2174/1567205012666141218140953

300    Cansu GB, Atilgan S, Balci MK, Sari R, Ozdem S, and Altunbas HA. 2017. Which type 2 diabetes mellitus
301         patients should be screened for subclinical Cushing's syndrome? Hormones (Athens) 16:22-32.
302         10.14310/horm.2002.1716

303    Cheng XJ, Gao Y, Zhao YW, and Cheng XD. 2015. Sodium Chloride Increases Abeta Levels by
304         Suppressing    Abeta    Clearance    in    Cultured    Cells.    PloS    One    10:e0130432.
305         10.1371/journal.pone.0130432

306    Choudhry F, Howlett DR, Richardson JC, Francis PT, and Williams RJ. 2012. Pro-oxidant diet enhances
307         beta/gamma secretase-mediated APP processing in APP/PS1 transgenic mice. Neurobiology of
308         Aging 33:960-968. 10.1016/j.neurobiolaging.2010.07.008

309    Colombo A, Wang H, Kuhn PH, Page R, Kremmer E, Dempsey PJ, Crawford HC, and Lichtenthaler SF.
310         2013. Constitutive alpha- and beta-secretase cleavages of the amyloid precursor protein are
311         partially coupled in neurons, but not in frequently used cell lines. Neurobiology of Disease 49:137-
312         147. 10.1016/j.nbd.2012.08.011

313    De-Paula VJ, Radanovic M, Diniz BS, and Forlenza OV. 2012. Alzheimer's disease. Sub-Cellular
314         Biochemistry 65:329-352. 10.1007/978-94-007-5416-4_14

315    Faraco G, Park L, Zhou P, Luo W, Paul SM, Anrather J, and Iadecola C. 2016. Hypertension enhances
316         Abeta-induced neurovascular dysfunction, promotes beta-secretase activity, and leads to
317         amyloidogenic processing of APP. Journal of Cerebral Blood Flow and Metabolism 36:241-252.
318         10.1038/jcbfm.2015.79

319    Futai E, Osawa S, Cai T, Fujisawa T, Ishiura S, and Tomita T. 2016. Suppressor Mutations for Presenilin
320         1 Familial Alzheimer Disease Mutants Modulate gamma-Secretase Activities. Journal of
321         Biological Chemistry 291:435-446. 10.1074/jbc.M114.629287

322   Garcia-Ayllon MS, Campanari ML, Brinkmalm G, Rabano A, Alom J, Saura CA, Andreasen N, Blennow
323        K, and Saez-Valero J. 2013. CSF Presenilin-1 complexes are increased in Alzheimer's disease. Acta
324        Neuropathol Commun 1:46. 10.1186/2051-5960-1-46
325   Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lubke T, Lena
326        Illert A, von Figura K, and Saftig P. 2002. The disintegrin/metalloprotease ADAM 10 is essential
327        for Notch signalling but not for alpha-secretase activity in fibroblasts. Human Molecular Genetics
328        11:2615-2624.
329   Hilpert H, Guba W, Woltering TJ, Wostl W, Pinard E, Mauser H, Mayweg AV, Rogers-Evans M, Humm
330        R, Krummenacher D, Muser T, Schnider C, Jacobsen H, Ozmen L, Bergadano A, Banner DW,
331        Hochstrasser R, Kuglstatter A, David-Pierson P, Fischer H, Polara A, and Narquizian R. 2013. β-
332        Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in
333        Alzheimer's Disease. Journal of Medicinal Chemistry 56:3980-3995. 10.1021/jm400225m
334   Kowalska A. 2004. [The beta-amyloid cascade hypothesis: a sequence of events leading to
335        neurodegeneration in Alzheimer's disease]. Neurologia i Neurochirurgia Polska 38:405-411.
336   Kuhn PH, Wang H, Dislich B, Colombo A, Zeitschel U, Ellwart JW, Kremmer E, Rossner S, and
337        Lichtenthaler SF. 2010. ADAM10 is the physiologically relevant, constitutive alpha-secretase of
338        the amyloid precursor protein in primary neurons. EMBO Journal 29:3020-3032.
339        10.1038/emboj.2010.167
340   Li LS, Lu YL, Nie J, Xu YY, Zhang W, Yang WJ, Gong QH, Lu YF, Lu Y, and Shi JS. 2017. Dendrobium
341        nobile Lindl alkaloid, a novel autophagy inducer, protects against axonal degeneration induced by
342        Abeta25-35 in hippocampus neurons in vitro. CNS Neuroscience & Therapeutics 23:329-340.
343        10.1111/cns.12678
344   Li T, Li YM, Ahn K, Price DL, Sisodia SS, and Wong PC. 2011. Increased expression of PS1 is sufficient
345        to elevate the level and activity of gamma-secretase in vivo. PloS One 6:e28179.
346        10.1371/journal.pone.0028179
347   Li Y, Li F, Gong Q, Wu Q, and Shi J. 2011. Inhibitory effects of Dendrobium alkaloids on memory
348        impairment induced by lipopolysaccharide in rats. Planta Medica 77:117-121. 10.1055/s-0030-
349        1250235
350   Lichtenthaler SF. 2012. Alpha-secretase cleavage of the amyloid precursor protein: proteolysis regulated
351        by signaling pathways and protein trafficking. Curr Alzheimer Res 9:165-177.
352        10.2174/156720512799361655
353   Linshan. J, Fei. L, Jing. N, Qin. W, and Jingshan. S. 2016. [Effect of Dendrobium nobile Lindl.alkaloids
354        on the learning and memory function of APP/PS1 transgenic mice]. Journal of Zunyi Medical
355        University 3:246-249. 10.14169/j.cnki.zunyixuebao.2016.0057
356   Mouton-Liger F, Paquet C, Dumurgier J, Bouras C, Pradier L, Gray F, and Hugon J. 2012. Oxidative stress
357        increases BACE1 protein levels through activation of the PKR-eIF2alpha pathway. Biochimica et
358        Biophysica Acta 1822:885-896. 10.1016/j.bbadis.2012.01.009
359   Nie J, Jiang LS, Zhang Y, Tian Y, Li LS, Lu YL, Yang WJ, and Shi JS. 2018. Dendrobium nobile Lindl.
360        Alkaloids Decreases the Level of Intracellular β-Amyloid by Improving Impaired Autolysosomal
361        Proteolysis in APP/PS1 Mice. Frontiers in Pharmacology 9:1479. 10.3389/fphar.2018.01479
362   Nie J, Tian Y, Zhang Y, Lu YL, Li LS, and Shi JS. 2016. Dendrobium alkaloids prevent Abeta25-35-

363    induced neuronal and synaptic loss via promoting neurotrophic factors expression in mice. PeerJ
364    4:e2739. 10.7717/peerj.2739

365  Niedowicz DM, Reeves VL, Platt TL, Kohler K, Beckett TL, Powell DK, Lee TL, Sexton TR, Song ES,
366    Brewer LD, Latimer CS, Kraner SD, Larson KL, Ozcan S, Norris CM, Hersh LB, Porter NM,
367    Wilcock DM, and Murphy MP. 2014. Obesity and diabetes cause cognitive dysfunction in the
368    absence of accelerated β-amyloid deposition in a novel murine model of mixed or vascular
369    dementia. Acta Neuropathol Commun 2:64. 10.1186/2051-5960-2-64

370  O'Brien RJ, and Wong PC. 2011. Amyloid precursor protein processing and Alzheimer's disease. Annual
371    Review of Neuroscience 34:185-204. 10.1146/annurev-neuro-061010-113613

372  Postina R, Schroeder A, Dewachter I, Bohl J, Schmitt U, Kojro E, Prinzen C, Endres K, Hiemke C, Blessing
373    M, Flamez P, Dequenne A, Godaux E, van Leuven F, and Fahrenholz F. 2004. A disintegrin-
374    metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer
375    disease mouse model. Journal of Clinical Investigation 113:1456-1464. 10.1172/jci20864

376  Qian M, Shen X, and Wang H. 2016. The Distinct Role of ADAM17 in APP Proteolysis and Microglial
377    Activation Related to Alzheimer's Disease. Cellular and Molecular Neurobiology 36:471-482.
378    10.1007/s10571-015-0232-4

379  Schechter I, and Ziv E. 2011. Cathepsins S, B and L with aminopeptidases display beta-secretase activity
380    associated with the pathogenesis of Alzheimer's disease. Biological Chemistry 392:555-569.
381    10.1515/bc.2011.054

382  Schuck F, Wolf D, Fellgiebel A, and Endres K. 2016. Increase of alpha-Secretase ADAM10 in Platelets
383    Along Cognitively Healthy Aging. Journal of Alzheimer's Disease 50:817-826. 10.3233/jad-
384    150737

385  Shackleton B, Crawford F, and Bachmeier C. 2016. Inhibition of ADAM10 promotes the clearance of Abeta
386    across the BBB by reducing LRP1 ectodomain shedding. Fluids Barriers CNS 13:14.
387    10.1186/s12987-016-0038-x

388  Suh J, Choi SH, Romano DM, Gannon MA, Lesinski AN, Kim DY, and Tanzi RE. 2013. ADAM10
389    missense mutations potentiate beta-amyloid accumulation by impairing prodomain chaperone
390    function. Neuron 80:385-401. 10.1016/j.neuron.2013.08.035

391  Wang Q, Gong Q, Wu Q, and Shi J. 2010. Neuroprotective effects of Dendrobium alkaloids on rat cortical
392    neurons injured by oxygen-glucose deprivation and reperfusion. Phytomedicine 17:108-115.
393    10.1016/j.phymed.2009.05.010

394  Willem M, Tahirovic S, Busche MA, Ovsepian SV, Chafai M, Kootar S, Hornburg D, Evans LD, Moore
395    S, Daria A, Hampel H, Muller V, Giudici C, Nuscher B, Wenninger-Weinzierl A, Kremmer E,
396    Heneka MT, Thal DR, Giedraitis V, Lannfelt L, Muller U, Livesey FJ, Meissner F, Herms J,
397    Konnerth A, Marie H, and Haass C. 2015. eta-Secretase processing of APP inhibits neuronal
398    activity in the hippocampus. Nature 526:443-447. 10.1038/nature14864

399  Wolfe MS. 2012. gamma-Secretase inhibitors and modulators for Alzheimer's disease. Journal of
400    Neurochemistry 120 Suppl 1:89-98. 10.1111/j.1471-4159.2011.07501.x

401  Woodruff G, Young JE, Martinez FJ, Buen F, Gore A, Kinaga J, Li Z, Yuan SH, Zhang K, and Goldstein
402    LS. 2013. The presenilin-1 DeltaE9 mutation results in reduced gamma-secretase activity, but not
403    total loss of PS1 function, in isogenic human stem cells. Cell Reports 5:974-985.

404          10.1016/j.celrep.2013.10.018

405    Yan R, and Vassar R. 2014. Targeting the beta secretase BACE1 for Alzheimer's disease therapy. Lancet
406          Neurology 13:319-329. 10.1016/s1474-4422(13)70276-x

407    Yousof Ali M, Jung HA, and Choi JS. 2015. Anti-diabetic and anti-Alzheimer's disease activities of
408          Angelica decursiva. Archives of Pharmacal Research 38:2216-2227. 10.1007/s12272-015-0629-0

409    Yun SM, Cho SJ, Song JC, Song SY, Jo SA, Jo C, Yoon K, Tanzi RE, Choi EJ, and Koh YH. 2013. SUMO1
410          modulates Abeta generation via BACE1 accumulation. Neurobiology of Aging 34:650-662.
411          10.1016/j.neurobiolaging.2012.08.005

412    Zhang Minghui, Li Fei, Zhang Wei, Wu Qin, and Jingshan S. 2016. [Effect of Dendrobium nobil Lindl.
413          Alkaloids on the product of Aβ in hippocampus of rats induced by Aβ25-35]. Journal of Zunyi
414          Medical University 39:18-21. 10.14169/j.cnki.zunyixuebao.2016.0005

415    Zhang W, Wu Q, Lu YL, Gong QH, Zhang F, and Shi JS. 2017. Protective effects of Dendrobium nobile
416          Lindl. alkaloids on amyloid beta (25-35)-induced neuronal injury. Neural Regen Res 12:1131-
417          1136. 10.4103/1673-5374.211193

418    Zhou JW, Cheng XR, Cheng JP, Zhou WX, and Zhang YX. 2012. The activity and mRNA expression of
419          beta-secretase, cathepsin D, and cathepsin B in the brain of senescence-accelerated mouse. Journal
420          of Alzheimer's Disease 28:471-480. 10.3233/jad-2011-111469

421    Zhu F, Wu F, Ma Y, Liu G, Li Z, Sun Y, and Pei Z. 2011. Decrease in the production of beta-amyloid by
422          berberine inhibition of the expression of beta-secretase in HEK293 cells. BMC Neuroscience
423          12:125. 10.1186/1471-2202-12-125

424    Zhu HC, Wang LM, Wang M, Song B, Tan S, Teng JF, and Duan DX. 2012. MicroRNA-195 downregulates
425          Alzheimer's disease amyloid-beta production by targeting BACE1. Brain Research Bulletin
426          88:596-601. 10.1016/j.brainresbull.2012.05.018

427

# Figure 1

Fig. 1. Chemical structures of *Dendrobium nobile* Lindl alkaloids.

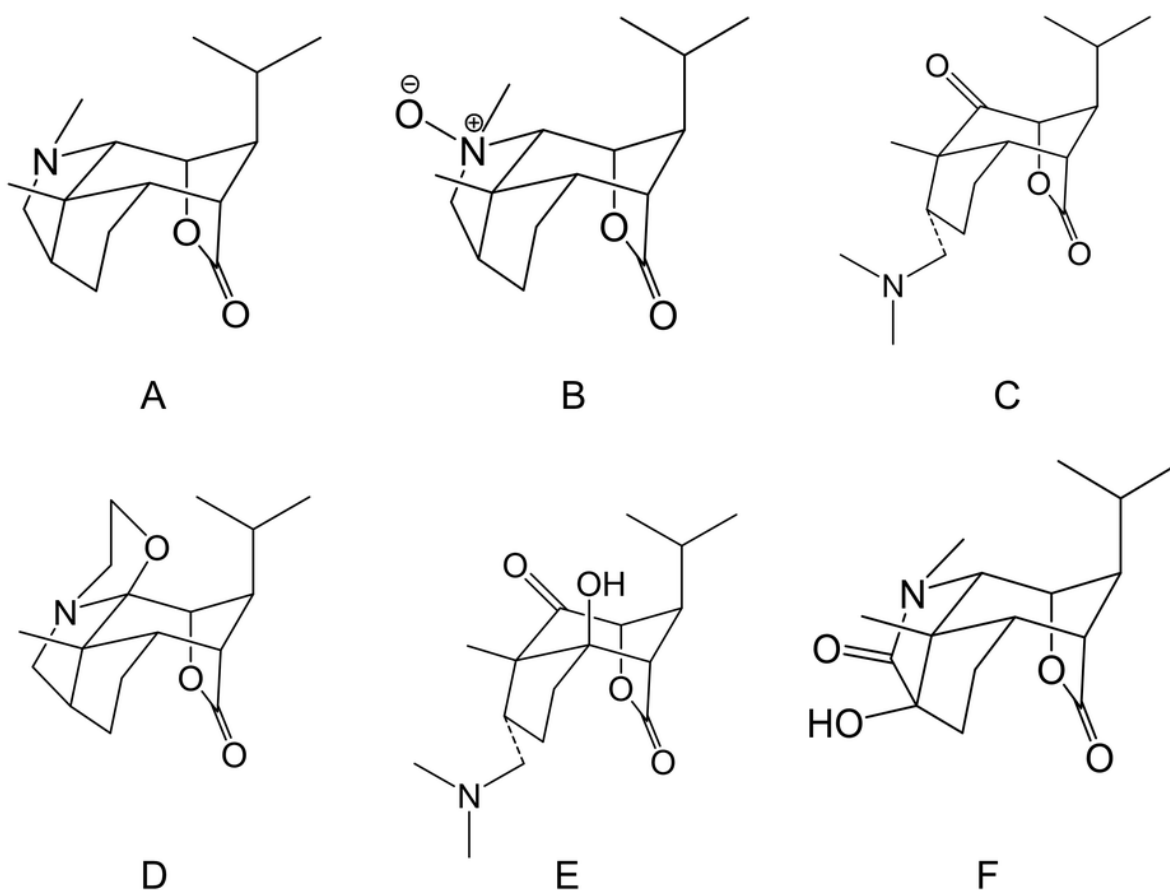
(A) Dendrobine, (B) Dendrobine-N-oxide, (C) Nobilonine, (D) Dendroxine, (E) 6-Hydroxy-nobilonine, and (F) 13-Hydroxy-14-oxodendrobine.

# Figure 2

Fig. 2. The purity of hippocampal neurons from SD rats.

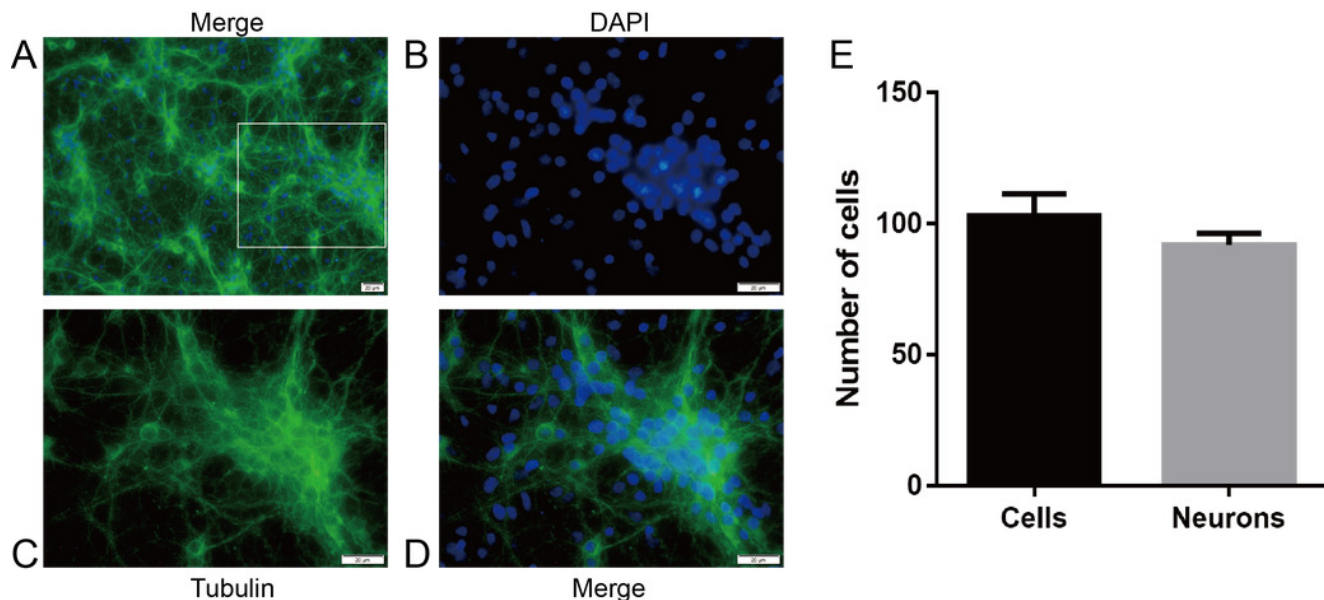
Primary cultured hippocampal neurons were cultured for 8 days before DNLA treatment. (A) hippocampal neurons 1000×. (B) DAPI 2000×. (C)Tubulin 2000×. (D) hippocampal neurons 2000×. (E) Neuron purity was more than 92.5% (n = 3). White squares represent enlarged areas.

# Figure 3

Fig. 3. Effects of DNLA on the cell viability of hippocampal neurons from SD rats.

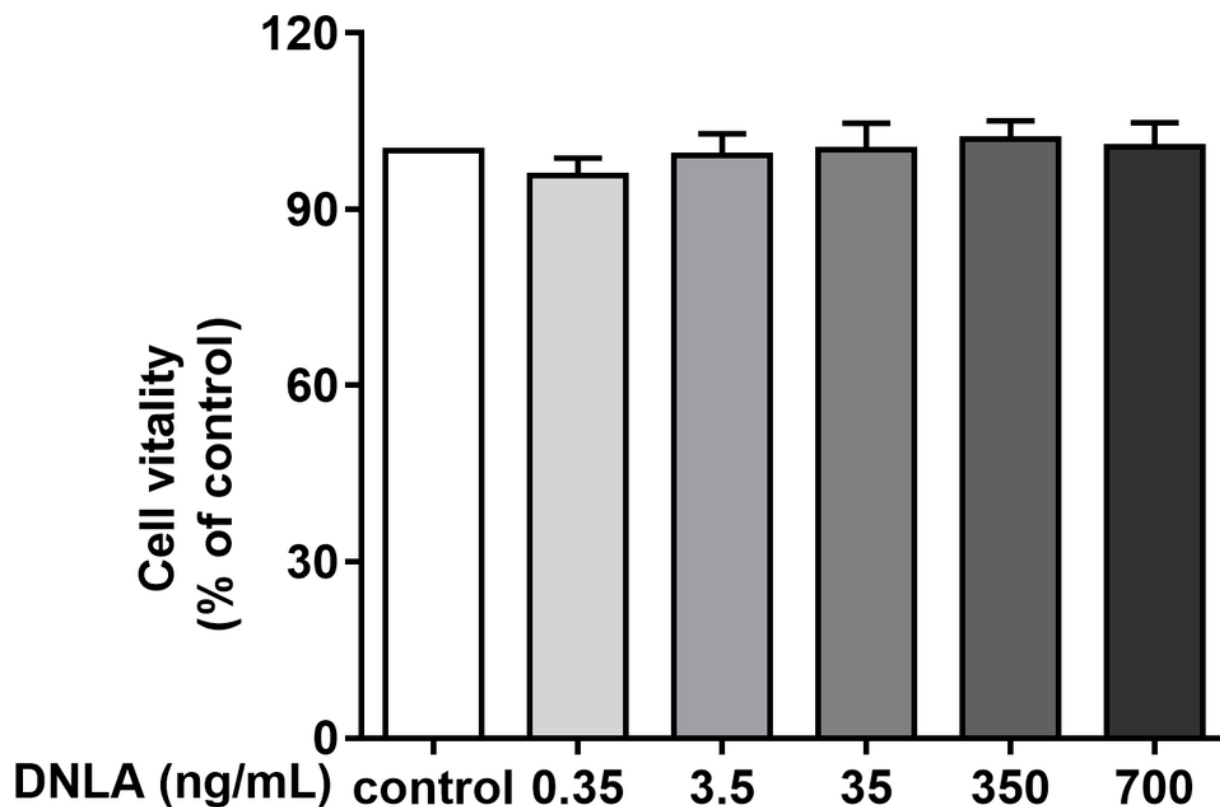
DNLA did not significantly change the cell vitality of hippocampal neurons (n = 3).

# Figure 4

Fig. 4. DNLA reduced the production of Aβ by decreasing APP.

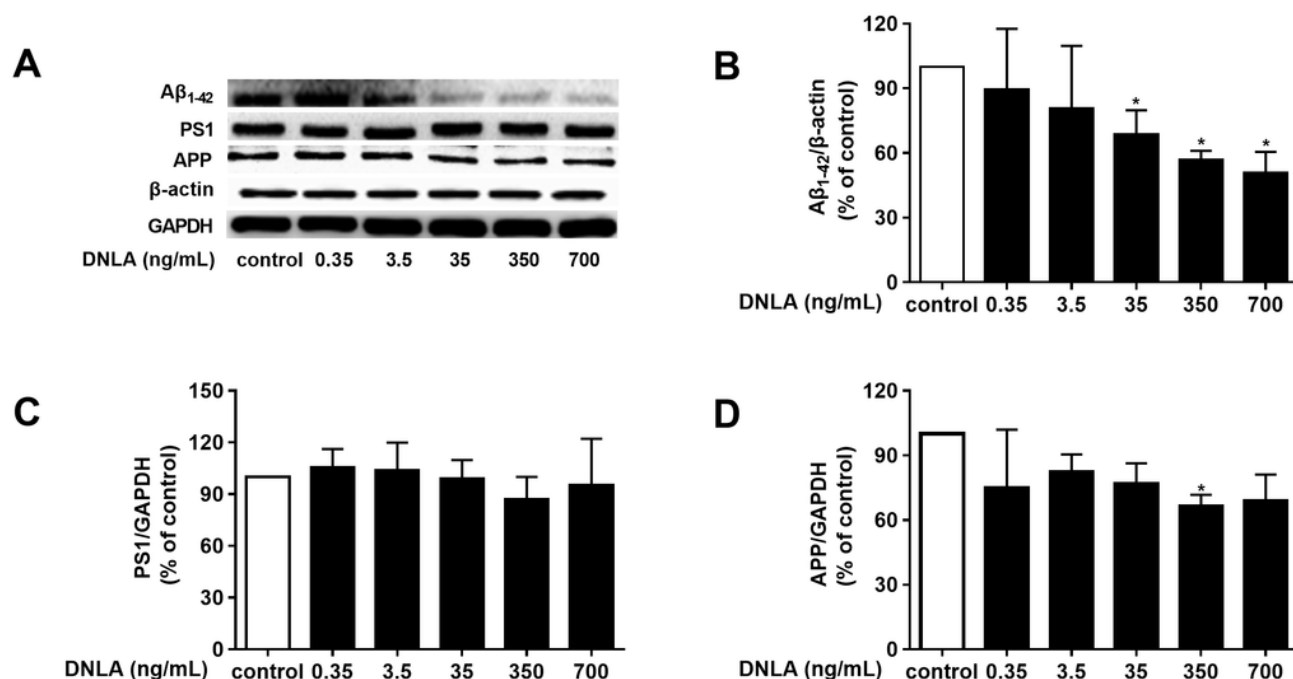
The protein expression levels of Aβ$_{1-42}$ (B), PS1 (C) and APP (D) as determined from densitometric scans of Western blots. DNLA significantly decreased the protein expression of Aβ$_{1-42}$ (35, 350, 700 ng/mL) and APP (350 ng/mL). However, DNLA did not change the protein expression of PS1. (A) Representative strips of these proteins. Data are presented as the mean ± SD (n = 4). *$P < 0.05$ versus the sham group.

# Figure 5

Fig. 5. DNLA reduced the accumulation of Aβ through non-amyloidogenic pathways.

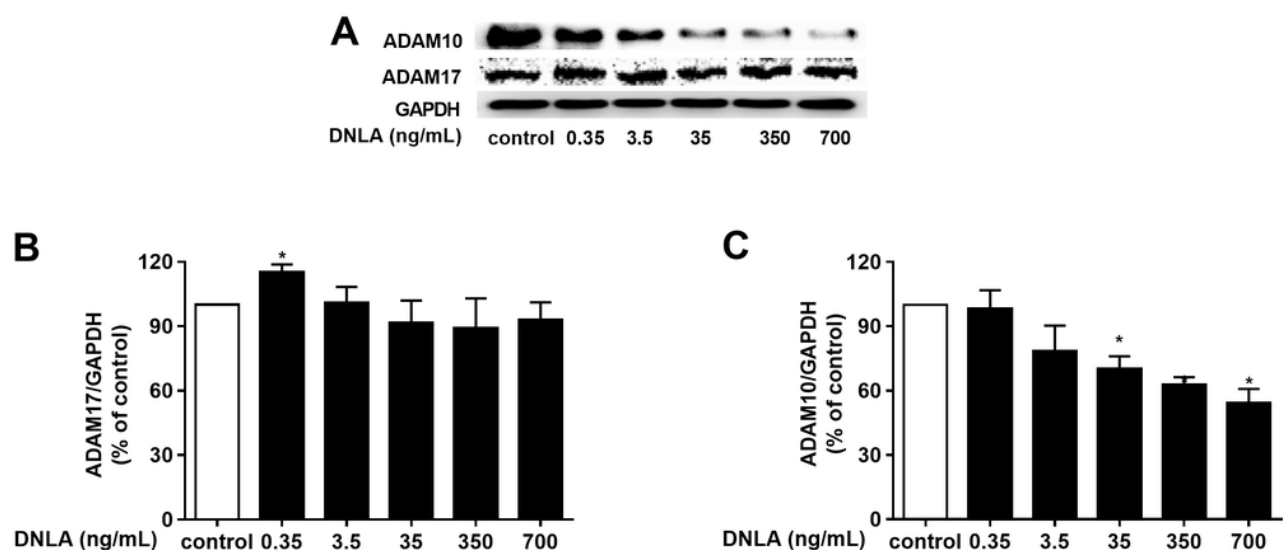
The protein expression levels of ADAM17 (B) and ADAM10 (C) as determined from densitometric scans of Western blots analyses. DNLA significantly decreased the protein expression of ADAM10 (35, 350, 700 ng/mL). However, this treatment significantly increased the protein expression of ADAM17 (0.35 ng/mL). (A) Representative strips of these proteins. Data are presented as the mean ± SD (n = 4). *$P < 0.05$ versus the sham group.

# Figure 6

Fig. 6. DNLA reduced the production of Aβ through amyloidogenic pathways.

Representative strips of these proteins (A). The protein expression level of BACE1 (B) as determined from densitometric scans of Western blots. DNLA significantly decreased the protein expression of BACE1 (35, 350, 700 ng/mL). Data are presented as the mean ± SD (n = 4). *P < 0.05 versus the sham group.