pLG72 levels increase in early phase of Alzheimer’s disease but decrease in late phase

Chieh-Hsin Lin1,2,3, Chih-Chiang Chiu4,5, Chiung-Hsien Huang6, Hui-Ting Yang7 & Hsien-Yuan Lane2,8,9

pLG72, named as D-amino acid oxidase activator (although it is not an activator of D-amino acid oxidase demonstrated by later studies), in mitochondria has been regarded as an important modulator of D-amino acid oxidase that can regulate the N-methyl-D-aspartate receptor (NMDAR). Both oxidative stress in mitochondria and NMDAR neurotransmission play essential roles in the process of neurodegenerative dementia. The aim of the study was to investigate whether pLG72 levels changed with the severity of neurodegenerative dementia. We enrolled 376 individuals as the overall cohort, consisting of five groups: healthy elderly, amnestic mild cognitive impairment [MCI], mild Alzheimer’s disease [AD], moderate AD, and severe AD. pLG72 levels in plasma were measured using Western blotting. The severity of cognitive deficit was principally evaluated by Clinical Dementia Rating Scale. A gender- and age- matched cohort was selected to elucidate the effects of gender and age. pLG72 levels increased in the MCI and mild AD groups when compared to the healthy group. However, pLG72 levels in the moderate and severe AD groups were lower than those in the mild AD group. D-serine level and D- to total serine ratio were significantly different among the five groups. L-serine levels were correlated with the pLG72 levels. The results in the gender- and age- matched cohort were similar to those of the overall cohort. The finding supports the hypothesis of NMDAR hypofunction in early-phase dementia and NMDAR hyperfunction in late-phase dementia. Further studies are warranted to test whether pLG72 could reflect the function of NMDAR.
pLG72 has been proposed to interact with D-amino acid oxidase (DAAO)\textsuperscript{12}. DAAO is capable of degrading D-amino acids including D-serine and D-alanine, which are co-agonists of the NMDAR\textsuperscript{13}. DAAO concentration in peripheral blood has been found to reflect cognitive aging\textsuperscript{1}. A DAAO inhibitor, sodium benzoate, showed beneficial effect for the cognitive and global function in patients with early-phase dementia\textsuperscript{14,15}. G72 is a susceptibility gene for schizophrenia\textsuperscript{24}. Sodium benzoate also showed efficacy for schizophrenia patients\textsuperscript{16,17}. In fact, there are some similarities between schizophrenia and AD: both reveal cognitive and functional deficits\textsuperscript{18–20}, behavioral problems\textsuperscript{21}, implication with NMDAR\textsuperscript{22,23} and response to the DAAO inhibitor. Previous study found that pLG72 concentration in the peripheral blood was higher in patients with schizophrenia than in controls\textsuperscript{21}. The aim of this study is to investigate whether pLG72 protein levels display a linear or nonlinear pattern in patients with neurodegenerative dementia.

**Results**

Totally 376 participants were enrolled: 108 healthy elders (controls), 81 amnestic MCI patients, 124 mild AD patients, 35 moderate AD patients, and 28 severe AD patients.

**Unmatched cohort.** There were more females in the controls than the other four AD groups (p = 0.015). The age distribution, education and MMSE scores were significantly different among the five groups (p < 0.001). The percentages of patients taking anti-dementia drugs (including memantine and AChEI) were different significantly among the four groups with cognitive deficits (p < 0.001). In the amino acids measured, the inter-groups differences were significant for D-serine level and D- to total serine ratio (p = 0.001, 0.018, respectively). The clinical and demographic characteristics are shown in Table 1.

**Matched cohort.** The five groups were matched further for age and gender. The gender and age distributions were not significantly different among the five groups in the matched cohort (p = 0.126, 0.109, respectively). The education level of the controls was significantly higher than the four groups with cognitive deficits (p < 0.001). The percentages of patients taking anti-dementia drugs were significantly different among the four groups with cognitive deficits (p = 0.018). For the five amino acids in the matched cohort, the inter-groups difference was significant for D-serine level (p = 0.015). The clinical and demographic characteristics are shown in Table 2 (matched cohort).

**pLG72 levels were highest in mild AD patients.** The pLG72 levels of the healthy elders, amnestic MCI, mild AD, moderate AD, and severe AD were 1.4 ± 0.7 ng/mL, 2.3 ± 1.1 ng/mL, 2.9 ± 1.6 ng/mL, 2.7 ± 1.4 ng/mL and 2.0 ± 1.3 ng/mL, respectively (p < 0.001) (Table 1 and Fig. 1). Bonferroni method was used for post-hoc analysis. The result revealed that the pLG72 levels in control group were lower than those in amnestic MCI, mild AD, and moderate AD (p < 0.001, <0.001, <0.001, respectively). The pLG72 levels in mild AD were significantly higher than those in amnestic MCI and in severe AD (p = 0.021, 0.006, respectively). The inter-groups differences of pLG72 levels among other groups were not significant (p > 0.05). The pLG72 levels in participants with or without anti-dementia agents were not significantly different (Table 1).

In the gender- and age-matched cohort, the pLG72 levels in the controls, amnestic MCI, mild AD, moderate AD and severe AD were 1.4 ± 0.6 ng/mL, 2.3 ± 1.0 ng/mL, 2.9 ± 1.5 ng/mL, 2.4 ± 1.3 ng/mL and 2.1 ± 1.3 ng/mL, respectively (p < 0.001) (Table 2 and Fig. 2). Post-hoc analysis (Bonferroni method) revealed that the pLG72 levels in controls were lower than those in amnestic MCI, mild AD and moderate AD (p = 0.001, <0.001, 0.010, respectively). The inter-groups differences of pLG72 levels among other groups were not significant (p > 0.05). The pLG72 levels in participants with and without anti-dementia agents were also not significantly different in the matched cohort (Table 2).

**The relationship between pLG72 level and L-serine levels.** Multiple linear regression analyses were used to test the relationship between pLG72 and amino acids. Age and sex were adjusted in the regression models for the overall cohort. Because the co-linearity was high between D- to L-form ratios and amino acids levels, amino acids levels only but not ratios were included in the models. There were significant associations between pLG72 levels and age and L-serine levels in the overall cohort (adjusted R\textsuperscript{2} = 0.114) (Table 3). For the matched cohort, age and sex were not adjusted in the regression models. There was also significant association between pLG72 levels and L-serine levels in the matched cohort (adjusted R\textsuperscript{2} = 0.035) (Table 3).

**Discussion**

The results of this study in the elderly population showed that pLG72 protein levels reveal a non-linear association with the severity of cognitive decline. In healthy elders and patients with early-phase dementia (including MCI and mild AD), the pLG72 levels increased when the cognitive deficits were worsened, with the highest level in mild AD. However, the pLG72 levels decreased in patients with moderate or severe AD when compared to those in early-phase dementia. There was a weak association between pLG72 levels and L-serine levels in peripheral blood. Furthermore, the results were similar in both the unmatched cohort and the age- and gender-matched cohort. This novel finding suggests that pLG72 may play a role in the process of neurodegenerative dementia possibly related to the NMDAR modulation.

The function of pLG72 protein in brain and its disorders is still an enigma\textsuperscript{24}. pLG72 protein is detectable in many brain regions such as cerebellum, striatum and frontal cortex\textsuperscript{4}. However, it is not yet clear which cells in the brain contain pLG72 protein. Further study is warranted to find out specific cells containing pLG72 protein. Considering its exclusive existence in human being and three other primates, pLG72 protein may be important for advanced cognitive functions. pLG72 plays a pivotal role in the modulation of NMDAR via DAAO activation\textsuperscript{25}. Accumulating evidence suggests that the function of NMDAR decreases in patients with cognitive decline. The density of NMDARs decreases during the normal process of aging\textsuperscript{26}. In patients with AD,
Table 1. Demographic characteristics of the overall cohort (n = 376). NA, not associated; *Chi-square test; **ANOVA test; *Kruskal-Wallis test; Comparison among MCI, mild, moderate and severe AD groups. Abbreviations: CDR, Clinical Dementia Rating; MMSE, Mini Mental Status Examination; pLG72, D-amino acid oxidase activator; T-serine, total serine; T-alanine, total alanine; D/T-serine ratio, D-serine/total serine ratio; D/T-alanine ratio, D-alanine/total alanine ratio.

|                         | Healthy elderly (n = 108) | MCI (CDR = 0, n = 81) | Mild AD (CDR = 1, n = 124) | Moderate AD (CDR = 2, n = 35) | Severe AD (CDR = 3, n = 28) | p Value |
|-------------------------|---------------------------|-----------------------|-----------------------------|------------------------------|----------------------------|---------|
| Gender, female, n (%)   | 47 (43.5)                 | 47 (58.0)             | 79 (63.7)                   | 24 (68.6)                    | 15 (53.6)                  | 0.015*  |
| Age, mean (SD)          | 67.2 (9.8)                | 68.2 (7.5)            | 73.6 (7.9)                  | 79.5 (8.2)                   | 77.8 (9.1)                 | <0.001* |
| Education, year, mean (SD) | 11.2 (4.1)            | 6.7 (5.0)             | 5.0 (4.2)                   | 5.1 (5.2)                    | 5.8 (4.9)                  | <0.001* |
| MMSE, mean (SD)         | 28.1 (1.5)                | 23.3 (3.1)            | 18.9 (4.4)                  | 11.5 (3.7)                   | 8.1 (4.2)                  | <0.001* |

No. of subjects using anti-dementia drugs

| Total number (%)       | NA | 9 (11.1) | 43 (34.7) | 4 (11.4) | 6 (21.4) | <0.001* |
| Donepezil (dose, mean ± SD) | NA | 8 (6.9 ± 2.6) | 28 (9.1 ± 2.0) | 1 (10.0 ± 0.0) | 4 (10.0 ± 0.0) | 0.011* |
| Rivastigmine (dose, mean ± SD) | NA | 1 (9.0) | 6 (6.8 ± 2.5) | 0 | 2 (5.5 ± 0.7) | 0.226* |
| Galantamine (dose, mean ± SD) | NA | 0 | 9 (15.1 ± 2.7) | 1 (16.0) | 0 | 0.035* |
| Memantine (dose, mean ± SD) | NA | 0 | 0 | 2 (20.0 ± 0.0) | 0 | 0.004* |
| pLG72 level (ng/mL), mean (SD) | 1.4 (0.7) | 2.3 (1.1) | 2.9 (1.6) | 2.7 (1.4) | 2.0 (1.3) | <0.001* |
| pLG72 with anti-dementia drugs | NA | 2.3 (1.1) | 2.7 (1.9) | 2.3 (1.0) | 2.7 (1.8) | 0.999* |
| pLG72 without anti-dementia drugs | 1.4 (0.7) | 2.3 (1.1) | 3.0 (1.4) | 2.7 (1.4) | 1.8 (1.1) | 0.001* |

the number of glutamate terminals reduces in the hippocampus. While some study found that the levels of D-serine (the primary co-agonist of NMDAR) slightly fell and L-serine slightly rose in the serum, other studies noted that D-serine levels increased in the brain, CSE and serum in AD patients. The peripheral DAAO levels were found to be higher in the patients with MCI or AD than the healthy elderly individuals. "Glutamate excitotoxicity theory" is one of the possible pathogeneses of AD, particularly in the later phase. Memantine, a partial NMDAR antagonist that blocks NMDAR overactivation, has been approved for use as a medication for moderate-severe AD. We hypothesize that pLG72 may play a pivotal role on the modulation of NMDAR function in neurodegenerative dementia, yet its function in different phases of dementia has not yet been clear. Further studies are warranted for verification.

In addition to its role in modulating DAAO, pLG72 is also a mitochondrial protein that regulates the production of reactive oxygen species. A cell line study using systemic approaches indicated that pLG72 might be involved in the induction of ROS. Another study found that pLG72 interacted with a mitochondrial protein (methionine-R-sulfoxide reductase B2) that functioned in oxidative stress defense. Oxidative stress has been regarded to contribute to aging with the assumption that free radicals damage cell constituents and connective tissues. Increased oxidative stress might play an important role underlying processes of aging or neurodegenerative diseases. Age-related cognitive decline is correlated with a decrease in brain and plasma antioxidants, for example, glutathione (GSH). The role of pLG72 in the regulation of oxidative stress that occurs in the process of neurodegenerative dementia deserves further elucidation.

D-serine level and D- to total serine ratio were significantly different among the five groups of participants. Results from the present study revealed a significant but weak association between pLG72 level and L-serine level (the adjusted R squares was low) but not D-serine (Table 3). The relationship between pLG72 and L-serine needs to be confirmed in further studies with larger sample sizes. Previous study showed that patients with congenital defects in the L-serine synthesizing enzymes manifest severe neurological abnormalities. The role of L-serine in neurodegenerative dementia has not yet been clear. D-serine is a major endogenous co-agonist of the NMDAR. pLG72 has been thought to be able to modulate the D-serine metabolism. Of note, D-serine is also regulated by many other mechanisms such as serine racemase that synthesizes D-serine from L-serine. Serine racemase has also been thought to play a role in the aging process. Other modulatory mechanisms should be also considered when the relationship between pLG72 and D-serine and L-serine is explored. In addition, the relationship between pLG72 level and severity of cognitive deficits was non-linear in this study. The non-linear dynamics has been observed in many research fields including neuroscience and cognitive psychology. The non-linearity phenomenon may reflect the dynamic complexity of brain fine-tuning function. Analyses that are appropriate for approaching biological non-linearity will be helpful in exploring the interactions among pLG72 and amino acids in future longitudinal studies.

This study has several limitations. Firstly, the finding from this study is limited by its cross-sectional design. Secondly, the peripheral blood-CNS relationship of pLG72 requires investigation in patients with...
| Study Details | Healthy elderly (CDR = 0, n = 56) | MCI (CDR = 0.5, n = 45) | Mild AD (CDR = 1, n = 99) | Moderate AD (CDR = 2, n = 20) | Severe AD (CDR = 3, n = 22) | p Value |
|----------------|----------------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|---------|
| Gender, female, n (%) | 23 (41.1) | 28 (62.2) | 61 (61.6) | 12 (60.0) | 12 (54.5) | 0.126* |
| Age, mean (SD) | 72.9 (9.9) | 72.2 (6.6) | 73.2 (7.1) | 77.2 (4.8) | 75.4 (7.2) | 0.189* |
| Education, mean (SD) | 9.6 (4.2) | 5.4 (3.9) | 5.6 (4.5) | 4.9 (5.2) | 5.2 (5.1) | <0.001* |
| MMSE, mean (SD) | 27.6 (1.6) | 23.1 (3.2) | 19.3 (4.2) | 11.9 (4.4) | 7.6 (4.3) | <0.001* |

Table 2. Demographic characteristics of the gender- and age- matched cohort (n = 242). NA, not associated; *Chi-square test; **ANOVA test; *Mann-Whitney U test; *Comparison among MCI, mild, moderate and severe AD groups. Abbreviations: CDR, Clinical Dementia Rating; MMSE, Mini Mental Status Examination; pLG72, D-amino acid oxidase activator; T-serine, total serine; T-alanine, total alanine; D/T-serine ratio, D-serine/total serine ratio; D/T-alanine ratio, D-alanine/total alanine ratio.

neurodegenerative dementia. Thirdly, only Han Chinese populations were recruited in this study. The findings need to be tested in other populations. Fourthly, the sample sizes in the moderate AD and the severe AD groups were relatively small that might hinder us to draw definite conclusion. Fifthly, the education levels between the control group and the cognitively impaired group were different. Although the grouping of participants was based on CDR score which is not influenced by education, further study on participants with matched education levels is warranted for further elucidating the education effect on pLG72 levels. Lastly, although we had performed a thorough physical and mental work-up to exclude patients with comorbid mental or organic disorders, it was not possible to exclude with certainty all other neurodegenerative diseases (e.g. amyotrophic lateral sclerosis) that may alter pLG72 levels.

In summary, this study suggests that the pLG72 levels in the peripheral blood increase in patients with early-phase dementia with the peak at mild AD, but decrease with the severity of cognitive deficits in later phase of AD. The findings which need to be confirmed and explored for underlying mechanisms by further studies indirectly supports the hypo-NMDAR hypothesis in early-phase AD and glutamate excitotoxicity hypothesis in late-phase AD. In the future, combining pLG72 level with other potential biomarkers such as DAAO and β-amyloid levels for assisting the diagnosis of AD particularly in its early phase may be favorable. For example, molecules that bind the DAAO-pLG72 complex have been discovered. The fluctuation of pLG72 concentration in the progression from the early phase of AD to its later phase needs to be confirmed in prospective longitudinal, larger-scale studies. It is worthy to test whether pLG72 could serve as a biomarker reflecting the function of NMDAR. Finally, it is also interesting to explore whether pLG72 blockers can be developed as a treatment for AD in its early phase and whether pLG72 enhancers, as a treatment for AD in its late phase.

Materials and Methods

Participants. All participants were recruited and evaluated from Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan, China Medical University Hospital, Taichung, Taiwan, and Taipei City Municipal Hospital, Taipei, Taiwan. This study was carried out in accordance with the recommendations of Good Clinical Practice (GCP), Institutional Review Board of Kaohsiung Chang Gung Memorial Hospital, Institutional Review Board of China Medical University Hospital, and Institutional Review Board of Taipei City Municipal Hospital, Taiwan. The protocol was officially approved by the institutional review boards of these hospitals. All subjects provided written informed consent according to the Declaration of Helsinki.

All participants were 50–100-year-old Han Chinese who were physically healthy with normal blood routine and biochemical tests. All participants were evaluated by research physicians thoroughly. Participants were recruited if they [1] had adequate education for effective communication, [2] were able to complete the evaluations in this study, and [3] provided written informed consent to join this study. The exclusion criteria included major medical, neurological, or psychiatric disorders other than AD; delirium symptoms; substance dependence or abuse (including alcohol); Hachinski Ischemic Score >4; history of significant cerebrovascular disease; severe
hearing or visual impairment; and being unable to follow protocol. The healthy volunteers were free from any psychiatric disorder. Similarly, all healthy participants had no substance abuse or dependence (including alcohol) diagnosed by DSM-IV.

- Patients with MCI satisfied amnestic MCI criteria, in which a probable degeneration course including subjective memory decline with a Clinical Dementia Rating (CDR) score 0.5 is noted, but the impairments in cognitive and global function are insufficient to meet the NINCDS-ADRDA criteria (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association).
- Patients with mild AD satisfied NINCDS-ADRDA criteria for probable AD with a CDR score 1.
- Patients with moderate AD satisfied NINCDS-ADRDA criteria for probable AD with a CDR score 2.

Table 3. Multiple linear regression analyses of independent factors associated with pLG72 level in overall and matched cohorts (stepwise). The regression model was adjusted with age and sex for the overall cohort. The variables were L-serine level, D-serine level, glycine level, L-alanine level, D-alanine level, D/T-serine ratio and D/T-alanine ratio. Significant variables are shown in the Table (p < 0.05).
• Patients with severe AD satisfied NINCDS-ADRDA criteria for probable AD with a CDR score 3.
• Healthy individuals had a CDR score 0.

All patients with AD were enrolled at the departments of outpatient clinic of the aforementioned hospitals. The healthy individuals were enrolled in the communities of southern, central and northern Taiwan.

AD patients with and without anti-dementia drugs were both recruited. AD patients without anti-dementia drugs were free from those medications for three months or longer. For patients with anti-dementia drugs treatment, those medications had been maintained for three months or longer with unchanged doses. Medication history was determined by reviewing medical records, confirming with providers of health care, and history talking with the participants and their caregivers or family. Healthy individuals were free from anti-dementia drugs.

### The assessments of cognitive function.

The cognitive functions of the participants were evaluated by Mini-Mental State Examination (MMSE) and CDR. MMSE is common for screening dementia and measuring cognition. Nevertheless, MMSE is easily influenced by education and age, and has low sensitivity for mild cognitive deficits, therefore limiting its use.

In contrast, CDR has good discrimination power for dementia with slight impairment. Further, CDR has shown good reliability and validity for the assessment and staging of dementia with favorable inter-rater reliability. Thus, the grouping of participants was mainly based upon CDR that represented both the cognitive and global impairment of the participants. Of note, CDR score is not influenced by age, gender and education.

### Laboratory assessments.

#### \( \text{pLG72 protein level measurement.} \)

Well-trained personnel collected subjects’ peripheral blood (10 ml) into EDTA-containing blood collection tubes. The specimens were immediately centrifuged at 500 g. Plasma was dissected quickly and stored immediately at \(-80^\circ\)C after centrifugation until Western blotting.

The plasma pLG72 protein levels were examined by Western blotting. At first, 100 \( \mu \)l plasma was depleted using ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma). The low-abundant protein fractions were collected to 100 \( \mu \)l. Then, 10 \( \mu \)l of the fractions were mixed with 4X sample buffer (500 mM Tris-HCl (pH 6.8), 16% SDS, 80% glycerol, 400 mM DTT, and 0.08% bromophenol blue) and separated on 12% SDS-PAGE. Proteins in the gels were transferred to 0.45 \( \mu \)m polyvinylidene difluoride (PVDF) membrane (Millipore), which was placed in 5% nonfat dry milk in TBST (20 mM Tris-HCl pH 7.6, 500 mM sodium chloride, 0.1% Tween 20) for 1 hour at room temperature, then incubated with goat anti-pLG72 antibody (pLG72 (N15):sc-46118, Santa Cruz Biotechnology) diluted by 1:1000 in TBST overnight at 4°C. The membrane was washed thrice in TBST and incubated for 2 hours with an HRP-linked anti-goat IgG secondary antibody (sc-2030, Santa Cruz Biotechnology) diluted by 1:5000 in TBST. After 3 washes in TBST, the blots were visualized with an ECL Advance Western Blotting Detection Kit (RPN2135, GE Healthcare). The stained membranes were photographed on ImageQuant LAS 4000 mini (GE Healthcare) and quantified using ImageQuant™ TL 7.0 software (GE Healthcare) by measuring the relative intensity from each band and normalized to the pLG72 recombinant protein (20 ng) signals. The commercial pLG72 antibodies were able to specifically recognize LG72 recombinant proteins. A standard curve was generated by serial dilutions of the pLG72 protein (50, 20, 10, 5, 2.5, 1.25, and 0.625 ng), and its detection limit was as low as 0.625 ng (Supplementary Fig. 1). The Western blotting was repeated by two experienced technicians separately for quality control. The results of the Western blotting were very similar between the two technicians. The R² of the linearity between the Western blotting signals and the amounts of the pLG72 proteins was 0.988. In the Western blotting, the molecular weight of the pLG72 protein band was approximately 18 kDa. The molecular weight of the standard recombinant pLG72 protein (as control) which had a tagged protein on it was marginally higher than that of the plasma pLG72 protein (Supplementary Fig. 2). The noise-signal ratios around the points of the Western blotting were between 0.04 and 0.13. All Western blot analyses were repeated twice.

#### Amino acids levels measurement.

Serum was firstly extracted by methanol (1:3, by volume), then filtered after 15 min centrifugation (1500 \( \times \) g) with nylon membranes (0.45 mM, Minisart SRP4, Sartorius, Germany). The filtrate was diluted with proper amount of 20% methanol then derivatized with N-isobutyl-L-cysteine (IBC) and O-phthaldialdehyde (OPA) mixture for 5 minutes then injected into high performance liquid chromatography (HPLC, L-7100 Pump, L7250 Autosampler, L-7250, with L7480 fluorescence Detector, Hitachi, Japan) for analysis. Analytical column (Grom-Sil OPA-2, 5\( \mu \)m, 250 mm * 4 mm, Part No: GSOP 2051252504, SAP No: 5113679, Grace, US) with guard column (Grom-Sil OPA-2, 5\( \mu \)m, 10 mm * 4 mm, Part No: GSOP20512v0104V, Grace, US) were used for the determination. Isocratic elution of mobile phase A (23 mM sodium acetate, pH 6.0) and B (50 mM acetonitrile in 600 mL methanol) were performed under fluorescence detection (excitation 260 nm, emission 355 nm), respectively. Retention time of each amino acid was L-serine, 33.6 min; D-serine, 35.8 min; glycine, 41.5 min; L-alanine, 47.2 min; D-alanine, 50.3 min, respectively. All amino acids levels were double-checked by performing HPLC analyses for two times in order to confirm that the peaks were not artifact (Supplementary Fig. 3).

We also used 10 \( \mu l \) D-amino acid oxidase (DAAO) mixed with 40 \( \mu l \) serum sample for the verification of D-amino acids levels. After serial gradient heating, 150 mL methanol was added and centrifuged under 15000 \( \times \) g at 4°C for 15 minutes. The filtrate was injected into the HPLC when reacting with OPA. D-serine and D-alanine levels were markedly decreased after DAAO addition. The levels of L-form amino acids and D-glutamate were slightly decreased, representing the diluting effect in the test (Supplementary Table 1).

### Statistical analysis.

All participants’ demographic, clinical characteristics and laboratory measures are shown as number (percentage) or mean \( \pm \) SD. All percentages between groups were compared by \( \chi^2 \) test, and
mean values among groups were compared by one-way ANOVA. For three or more groups, Kruskal-Wallis test was used. Multiple linear regression analysis was used to generate correlation models for pLG72 levels and amino acids levels. Statistical significance was defined as a p value ≤ 0.05. IBM SPSS Statistics (version 22.0, SPSS inc.) was applied to conduct all statistical analyses.

**Role of the sponsor.** The sponsors were not involved in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

**References**

1. Levey, A., Lah, J., Goldstein, F., Steenland, K. & Blywise, D. Mild cognitive impairment: an opportunity to identify patients at high risk for progression to Alzheimer's disease. *Clin. Ther. 28*, 991–1001 (2006).
2. Lin, C. H., Huang, Y. J., Lin, C. J., Lane, H. Y. & Tsai, G. E. NMDA neurotransmission dysfunction in mild cognitive impairment and Alzheimer's disease. *Current pharmaceutical design* 20, 5169–5179 (2014).
3. McDonald, J. W. & Johnston, M. V. Physiological and pathophysiologic roles of excitatory amino acids during central nervous system development. *Brain Res Brain Res Rev* 15, 41–70 (1990).
4. Lin, C. H., Yang, H. T., Chiu, C. C. & Lane, H. Y. Blood levels of D-amino acid oxidase vs. D-amino acids in reflecting cognitive aging. *Scientific reports* 7, 14849 (2017).
5. Butterfield, D. A. & Boyd-Kimball, D. Redox Proteomics and Amyloid beta-Peptide: Insights into Alzheimer Disease. *Journal of neurochemistry*, https://doi.org/10.1111/jnc.14589 (2018).
6. Grimm, A. & Eckert, A. Brain aging and neurodegeneration: from a mitochondrial point of view. *Journal of neurochemistry* 143, 418–431 (2017).
7. Kvajo, M., Dhalla, A., Swor, D. E., Karayiorgou, M. & Gogos, J. A. Evidence implicating the candidate schizophrenia/bipolar disorder susceptibility gene G72 in mitochondrial function. *Molecular psychiatry* 13, 685–698 (2008).
8. Chumakov, I. et al. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America* 99, 13675–13680 (2002).
9. Jagannath, V., Marinova, Z., Monoranu, C. M., Walitza, S. & Grunblatt, E. Expression of D-Amino Acid Oxidase (DAO/DAO) and D-Amino Acid Oxidase Activator (DAOA/G72) during Development and Aging in the Human Post-mortem Brain. *Frontiers in neuroanatomy* 11, 31 (2017).
10. Velez, J. I. et al. A Mutation in DAOA Modifies the Age of Onset in PSEN1 E280A Alzheimer's Disease. *Neural plasticity* 2016, 9760314 (2016).
11. Dr Maria, E. et al. Genetic variation in the G72/G30 gene locus (DAOA) influences the occurrence of psychotic symptoms in patients with Alzheimer's disease. *Journal of Alzheimer's disease: JAD* 18, 953–960 (2009).
12. Jagannath, V., Bestańska, Z. E., Parrinello, M., Walitza, S. & Grunblatt, E. Controversial Effects of D-Amino Acid Oxidase Activator (DAOA/G72) on D-Amino Acid Oxidase (DAO) Activity in Human Neuronal, Astrocyte and Kidney Cell Lines: The N-methyl D-aspartate (NMDA) Receptor Hypofunction Point of View. *Front Mol Neurosci* 10, 342 (2017).
13. Schwickert, S. et al. pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase: effect on schizophrenia susceptibility. *The journal of biological chemistry* 283, 22244–22256 (2008).
14. Lin, C. H. et al. Benzoate, a D-amino acid oxidase inhibitor, for the treatment of early-phase Alzheimer disease: a randomized, double-blind, placebo-controlled trial. *Biological psychiatry* 75, 678–685 (2014).
15. Ma, J. et al. Further evidence for the association between G72/G30 genes and schizophrenia in two ethnically distinct populations. *Molecular psychiatry* 11, 479–487 (2006).
16. Lin, C. H. et al. Sodium Benzoate, a D-Amino Acid Oxidase Inhibitor, Added to Clozapine for the Treatment of Schizophrenia: A Randomized, Double-Blind, Placebo-Controlled Trial. *Biological psychiatry* 84, 422–432 (2018).
17. Lane, H. Y. et al. Add-on treatment of benzoate for schizophrenia: a randomized, double-blind, placebo-controlled trial of D-amino acid oxidase inhibitor. *JAMA psychiatry* 70, 1267–1275 (2013).
18. Lin, C. H. et al. Clinical symptoms, mainly negative symptoms, mediate the influence of neurocognition and social cognition on functional outcome of schizophrenia. *Schizophrenia research* 146, 231–237 (2013).
19. Hsu, W. Y., Lane, H. Y. & Lin, C. H. Medications Used for Cognitive Enhancement in Patients With Schizophrenia, Bipolar Disorder, Alzheimer's Disease, and Parkinson's Disease. *Frontiers in psychiatry* 9, 91 (2018).
20. McKhann, G. et al. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34, 939–944 (1984).
21. Huang, Y. J., Lin, C. H., Lane, H. Y. & Tsai, G. E. NMDA Neurotransmission Dysfunction in Behavioral and Psychological Symptoms of Alzheimer's Disease. *Current neuropharmacology* 10, 272–285 (2012).
22. Lin, C. H., Lane, H. Y. & Tsai, G. E. Glutamate signaling in the pathophysiology and therapy of schizophrenia. *Pharmacology, biochemistry, and behavior* 100, 665–677 (2012).
23. Lin, C. H. et al. Distinctively higher plasma G72 protein levels in patients with schizophrenia than in healthy individuals. *Molecular psychiatry* 19, 636–637 (2014).
24. Saedi, S., Binelli, G. & Pollegioni, L. G72 primate-specific gene: a still enigmatic element in psychiatric disorders. *Cellular and molecular life sciences: CMLS* 73, 2029–2039 (2016).
25. Detera-Wadleigh, S. D. & McMahon, F. J. G72/G30 in schizophrenia and bipolar disorder: review and meta-analysis. *Biological psychiatry* 60, 106–114 (2006).
26. Segovia, G., Porras, A., Del Arco, A. & Mora, F. Glutamatergic neurotransmission in aging: a critical perspective. *Mec Ageing Dev* 122, 1–29 (2001).
27. Cowburn, R., Hardy, J., Roberts, P. & Briggs, R. Regional distribution of pre- and postsynaptic glutamatergic function in Alzheimer's disease. *Brain Res* 452, 403–407 (1988).
28. Hashimoto, K. et al. Possible role of D-serine in the pathophysiology of Alzheimer's disease. *Progress in neuro-psychopharmacology & biological psychiatry* 28, 385–388 (2004).
29. Madeira, C. et al. d-serine levels in Alzheimer's disease: implications for novel biomarker development. *Translational psychiatry* 5, e561 (2015).
30. Scarpini, E., Scheltens, P. & Feldman, H. Treatment of Alzheimer's disease: current status and new perspectives. *Lancet neurology* 2, 539–547 (2003).
31. Gardoni, F. et al. Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. *The journal of neuroscience: the official journal of the Society for Neuroscience* 29, 669–677 (2009).
32. Pallas, M. & Camins, A. Molecular and Biochemical Features in Alzheimer disease. *Curr. Pharm. Des.* 12 (2006).
33. Reisberg, B. et al. Memantine in Moderate-to-Severe Alzheimer Disease. *N. Engl. J. Med.* 348 (2003).
34. Drews, E., Otto, D. M. & Zimmer, A. Involvement of the primate specific gene G72 in schizophrenia: From genetic studies to pathomechanisms. *Neuroscience and biobehavioral reviews* 37, 2410–2417 (2013).
35. Wang, M. et al. Identification of pLG72-Induced Oxidative Stress Using Systemic Approaches. *BioMed research international* 2015, 429253 (2015).
36. Otte, D. M. et al. Identification of the mitochondrial MSRB2 as a binding partner of LG72. Cellular and molecular neurobiology 34, 1123–1130 (2014).
37. Harman, D. Aging: a theory based on free radical and radiation chemistry. Journal of gerontology 11, 298–300 (1956).
38. Gallagher, M. et al. Hippocampal neurodegeneration in aging. Science 274, 484–485 (1996).
39. Serrano, F. & Klann, E. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing research reviews 3, 431–443 (2004).
40. Perrig, W. J., Perrig, P. & Shahelin, H. B. The relation between antioxidants and memory performance in the old and very old. Journal of the American Geriatrics Society 45, 718–724 (1997).
41. Perkins, A. J. et al. Association of antioxidants with memory in a multiethnic elderly sample using the Third National Health and Nutrition Examination Survey. American journal of epidemiology 150, 37–44 (1999).
42. Rinaldi, P. et al. Plasma antioxidants are similarly depleted in mild cognitive impairment and in Alzheimer's disease. Neurobiology of aging 24, 915–919 (2003).
43. Tabatabaie, L., Klomp, L. W., Berger, R. & de Koning, T. J. L-serine synthesis in the central nervous system: a review on serine deficiency disorders. Molecular genetics and metabolism 99, 256–262 (2010).
44. Pollegioni, L., Puibelli, L., Molla, G. & Rosini, E. D-Amino Acid Oxidase-pLG72 Interaction and D-Serine Modulation. Frontiers in molecular biosciences 5, 3 (2018).
45. Wolosker, H. et al. Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proceedings of the National Academy of Sciences of the United States of America 96, 721–725 (1999).
46. Billard, J. M. Changes in Serine Racemase-Dependent Modulation of NMDA Receptor: Impact on Physiological and Pathological Brain Aging. Front Mol Biosci 5, 106 (2018).
47. Mattei, T. A. Unveiling complexity: non-linear and fractal analysis in neuroscience and cognitive psychology. Frontiers in computational neuroscience 8, 17 (2014).
48. Zueva, M. V. Fracality of sensations and the brain health: the theory linking neurodegenerative disorder with distortion of spatial and temporal scale-invariance and fractal complexity of the visible world. Frontiers in aging neuroscience 7, 135 (2015).
49. Kim, J., Avants, B., Whyte, J. & Gee, J. C. Methodological considerations in longitudinal morphometry of traumatic brain injury. Frontiers in human neuroscience 7, 52 (2013).
50. Hughes, C. P., Berg, L., Danziger, W. L., Cohen, L. A. & Martin, R. L. A new clinical scale for the staging of dementia. The British journal of psychiatry: the journal of mental health science 140, 566–572 (1982).
51. Hwang, C. S., Tsai, C. H., Liu, G. T., Li, W. & Chang, H. T. Decreased level of serum autoantibody against LG72 is a biosignature of amyotrophic lateral sclerosis. Biomarkers in medicine 10, 73–79 (2016).
52. Glenner, G. G. & Wong, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochemical and biophysical research communications 120, 885–890 (1984).
53. Terry-Lorenzo, R. T. et al. High-Throughput Screening Strategy Identifies Allosteric, Covalent Human D-Amino Acid Oxidase Inhibitor. J Biomol Screen 20, 1218–1231 (2015).
54. Kato, Y. & Fukui, K. Structure models of G72, the product of a susceptibility gene to schizophrenia. Journal of biochemistry 161, 223–230 (2017).
55. Chang, S. L. et al. The C-terminal region of G72 increases D-amino acid oxidase activity. International journal of molecular sciences 15, 29–43 (2013).
56. Lu, P. H. et al. Donepezil delays progression to AD in MCI subjects with depressive symptoms. Neurology 72, 2115–2121 (2009).
57. Morris, J. C. The Clinical Dementia Rating (CDR): current version and scoring rules. Neurology 43, 2412–2414 (1993).
58. Folstein, M. F., Folstein, S. E. & McHugh, P. R. Mini-mental state”. A practical method for grading the cognitive state of patients for the clinician. Journal of psychiatric research 12, 189–198 (1975).
59. Crum, R. M., Anthony, I. C., Bassett, S. S. & Folstein, M. F. Population-based norms for the Mini-Mental State Examination by age and educational level. Jama 269, 2386–2391 (1993).
60. Tombaugh, T. N. & Mcintyre, N. J. The mini-mental state examination: a comprehensive review. Journal of the American Geriatrics Society 40, 922–935 (1992).
61. Lim, W. S., Chong, M. S. & Sahadevan, S. Utility of the clinical dementia rating in Asian populations. Clinical medicine & research 5, 61–70 (2007).
62. Rockwood, K., Strang, D., MacKnight, C., Downer, R. & Morris, J. C. Inter-rater reliability of the Clinical Dementia Rating in a multicenter trial. Journal of the American Geriatrics Society 48, 558–559 (2000).
63. Kato, S., Kito, Y., Hemmi, H. & Yoshimura, T. Simultaneous determination of D-amino acids by the coupling method of D-amino acid oxidase with high-performance liquid chromatography. J Chromatog B Analyt Technol Biomed Life Sci 789, 3190–3195 (2011).

Acknowledgements

This work was funded by Ministry of Science and Technology, Taiwan (MOST 105-2314-B-182A-059-; MOST 107-2632-B-039-001), National Health Research Institutes (NHRI-EX108-1073N1), Kaohsiung Chang Gung Memorial Hospital, Taiwan (NMRPG8F0111, BMRPC04), China Medical University Hospital, Taiwan (DMR-108-093) and Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW108-TDU-B-212-133004).

Author Contributions

C.H. Lin and H.Y. Lane involved in conception and design, literature review, data interpretation, and manuscript writing; C.H. Lin, C.C. Chiu and H.Y. Lane involved in participants enrollment; C.H. Huang performed the laboratory procedures; All authors reviewed and approved the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-49522-1.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
