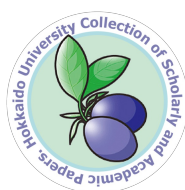




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Facilitation of brain mitochondrial activity by 5-aminolevulinic acid in a mouse model of Alzheimer's disease

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Abstract

The activities of mitochondrial enzymes, which are essential for neural function, decline with age and in age-related disease. In particular, the activity of cytochrome c oxidase (COX/complex IV) decreases in patients with Alzheimer's disease (AD). COX, a mitochondrial inner membrane protein complex that contains heme, plays an essential role in the electron transport chain that generates ATP. Heme synthesis begins with 5-aminolevulinic acid (5-ALA) in mitochondria. 5-ALA synthetase is the rate-limiting enzyme in heme synthesis, suggesting that supplementation with 5-ALA might help preserve mitochondrial activity in the aged brain. We administered a diet containing 5-ALA to triple-transgenic AD (3xTg-AD) model mice for 6 months, starting at 3 months-of-age. COX activity and protein expression, as well as mitochondrial membrane potential, were significantly higher in brains of 5-ALA-fed mice than in controls. Synaptotagmin protein levels were also significantly higher in 5-ALA-fed mice, suggesting improved preservation of synapses. Although brain A β levels tended to decrease in 5-ALA-fed mice, we observed no other significant changes in other biochemical and pathological hallmarks of AD. Nevertheless, our study suggests that daily oral administration of 5-ALA could preserve mitochondrial enzyme activities in the brains of aged individuals, thereby contributing to the preservation of neural activity.

Keywords: 5-aminolevulinic acid, cytochrome c oxidase, aging, Alzheimer's disease, amyloid- β , synaptotagmin

Introduction

In general, mitochondrial activity attenuates with age; consequently, aged individuals have less capacity to produce ATP for use in energy-dependent cellular events.^{1, 2} For example, the levels of respiratory chain complexes are reduced in liver mitochondria of aged rats.³ To perform higher-order brain functions, neurons must consume huge amounts of energy. The levels of cytochrome c oxidase (COX/complex IV) decrease in Alzheimer's disease;⁴ this may be related to the diminished cognitive ability of aged subjects. Heme-integrated proteins such as COX, hemoglobin, and myoglobin play fundamental roles in animal physiology, and heme itself serves as a cofactor for multiple enzymes. Thus, heme is a critical for multiple cellular phenomena.⁵

The heme molecule is synthesized from the precursor 5-aminolevulinic acid (5-ALA), which in animal cells is produced from glycine and succinyl-CoA via the classical Shemin pathway.⁵ 5-ALA synthetase is the rate-limiting enzyme in the heme synthesis pathway, suggesting that supplementation with 5-ALA could upregulate heme synthesis and facilitate multiple biological functions by increasing mitochondrial activity.⁶ The impairment of neural functions associated with aging may be the result of reduced mitochondrial activity in neurons, potentially contributing to neurodegenerative disorders such as Alzheimer's disease (AD), the most common form of senile dementia. In other words, preservation of mitochondrial activity may contribute to the maintenance of neural function in aged subjects who develop cognitive impairment.^{7, 8} Consistent with this, mitochondrial dysfunction has been reported in mouse models of AD.^{9, 10} These observations suggest that prevention of mitochondrial deterioration could slow neurodegeneration in the early or prodromal phase of AD. Thus, supplementation with 5-ALA could be an effective means of preserving or increasing mitochondrial activity in the brains of aged individuals. Although administration of 5-ALA to mice increases COX activity in the liver,¹¹ it remains unknown whether oral administration of 5-ALA can preserve mitochondrial function in the brain and prevent biological alterations characteristic of neurodegenerative disorders such as AD.

In this study, we analyzed the effect of orally administrated 5-ALA in the brain of triple transgenic AD (3xTg-AD) model mice¹². First, to characterize general mitochondrial activity in 3xTg-AD mice, we examined mitochondrial COX expression and activity in the hippocampus and cerebral cortex of animals undergoing daily

administration of 5-ALA. Second, to explore the effects of 5-ALA on the pathobiology of AD, we analyzed changes in synaptic proteins and proteins known to play causative roles in AD.

Materials and methods

Animals

All animal studies were conducted in compliance with the guidelines of Animal Studies Committee of Hokkaido University. Triple-transgenic Alzheimer's disease (3xTg-AD)¹² model mice, which harbor the PS1_{M146V}, APP_{swe}, and tau_{P301L} transgenes, were purchased from Jackson Laboratories. Mice were housed in a specific pathogen-free environment with Allentown MicroVent units throughout these studies. Mice were weaned at 1 month and raised on a commercial diet (PicoLab mouse diet 20, LabDiet, St. Louis, MO, USA) until the age of 3 months. Subsequently, the mice received either a control diet or a diet supplemented with 0.12% (w/w) 5-ALA (chow: Oriental Yeast Co Ltd., Tokyo, Japan; 5-ALA: SBI Pharmaceuticals Co Ltd, Tokyo, Japan). Mice were cultivated for 6 months under free-feeding conditions. During this interval, mice consumed 1.5–2 g of food per day, i.e., 1.8–2.4 mg of 5-ALA per day. At the age of 9 months, the mice were sacrificed and their brains dissected. The brain region encompassing the hippocampus and cerebral cortex was used for all examinations, except for immunohistochemical analysis.

Brain fractionation

The hippocampus and cerebral cortex were homogenized in 10 volumes of extraction buffer (10 mM HEPES [pH 7.5], 200 mM mannitol, 70 mM sucrose, 1 mM EGTA) containing 2 mg/ml bovine serum albumin (BSA) with 25 strokes of a Dounce homogenizer. After the lysate was centrifuged at $600 \times g$ for 10 min, the post-nuclear supernatant (PNS) was further centrifuged at $100,000 \times g$ for 1 h. The precipitated membrane fraction (P100) was used for Western blotting to detect membrane proteins.

PNS was centrifuged at $11,000 \times g$ for 10 min, the supernatant removed, and the precipitate suspended in extraction buffer. The resultant suspension was centrifuged at $600 \times g$ for 5 min and the supernatant further centrifuged at $11,000 \times g$ for 10 min.

The precipitated fraction was suspended in storage buffer (10 mM HEPES [pH 7.4], 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K₂HPO₄, 1 mM dithiothreitol) and used as the mitochondrial fraction. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotech, Rockford, IL, USA).

Mitochondrial enzyme and membrane potential assays

Cytochrome c oxidase in the mitochondrial fraction from the brain (hippocampus plus cerebral cortex) was assayed using the Cytochrome c Oxidase Assay Kit (CYTOCOX1, Sigma-Aldrich, St. Louise). In this study, 1 µg mitochondria protein was assayed in ferrocytochrome c substrate solution; absorbance at 550 nm was measured in an Infinite 200 multimode microplate reader (TECAN, Mannedorf, Switzerland) after a 30-sec incubation.

Mitochondrial membrane potential was measured in an tetraethylbenzimidazolycarbocyanine iodide (JC-1) uptake assay (JC-1 Mitochondrial membrane potential Assay Kit, Abcam, Cambridge UK). Fluorescence was measured on an Infinite 200 multimode microplate reader at an excitation wavelength of 490 nm and emission wavelength of 590 nm.

Antibodies and Western blot analysis

Anti-synaptotagmin, anti-PSD95, and anti-flotillin-1 antibodies were purchased from BD Bioscience (Franklin Lakes, NJ, USA). Anti-actin (Chemicon/Millipore, Billerica, MA, USA), anti-COX IV (Cell Signaling, Danvers, MA, USA), anti- α -tubulin (Wako Pure Chemical Industries, Osaka, Japan), and anti-VDAC1 (Abcam, Cambridge, UK) antibodies were purchased from the indicated suppliers. Anti-APP cytoplasmic antibody (G369, from Dr. Gandy) to detect APP FL and CTF,¹³ anti-BACE1 (3D5, from Dr. Vassar),¹⁴ and anti-PS1 CTF (G1L3, from Dr. Tomita)¹⁵ were obtained as gifts from the indicated sources. Mouse monoclonal anti-A β antibodies 6E10 (raised against human A β 1-16) and 4G8 (raised against human A β 17-24) were purchased from BioLegend (San Diego, CA, USA), and anti-A β antibody 82E1 (raised against human A β 1-16) was purchased from IBL (Fujioka, Gunma, Japan). Anti-GluR1 (Merck Millipore, Darmstadt, Germany), anti-NR2A (Thermo Fisher Scientific, Waltham, MA,

USA), and anti-NR2B (BD Biosciences) antibodies were purchased from the indicated suppliers.

Quantitation of A β 40 and A β 42

A β quantification was performed as described previously.¹⁶ Briefly, samples of hippocampus plus cerebral cortex from each hemisphere were homogenized in four volumes of Tris-buffered saline (20 mM Tris [pH 7.6], 137 mM NaCl) with 30 strokes of a Dounce homogenizer and then centrifuged at $200,000 \times g$ for 20 min at 4°C. The precipitate was further homogenized with 30 strokes in nine volumes of TBS and then centrifuged at $100,000 \times g$ for 20 min at 4°C. After addition of one volume of 6 M guanidine chloride, the precipitate was sonicated twice for 10 sec each and allowed to stand for 1 h at room temperature. The samples were then centrifuged at $130,000 \times g$ for 20 min at 4°C. The supernatant was diluted with enzyme immunoassay buffer (PBS containing 1% BSA and 0.05% Tween 20) and centrifuged at $14,000 \times g$ for 15 min. The resultant supernatant was assayed using appropriate ELISA kits (IBL 27714 for human A β 40 and IBL 27712 for human A β 42).

Immunohistochemistry

3xTg-AD mouse brain tissue sections (25 μ m thick) were prepared and incubated overnight with the indicated mouse monoclonal antibody, as previously described.¹⁶ Immunostained sections were washed in PBS, incubated with a biotin-conjugated horse anti-mouse IgG antibody, and then incubated with ABC complex (Vector Laboratories, Burlingame, CA USA). Peroxidase activity was revealed using diaminobenzidine (DAB) as the chromogen. Images were acquired under a BZ-X710 microscope (KEYENCE, Osaka, Japan).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA). The threshold for significance is indicated with p value (*, $p < 0.05$).

Results

Orally administered 5-ALA activates mitochondria in the hippocampus and cerebral cortex

A previous study shows that oral administration of 5-ALA increases COX activity in the liver of 6-week-old female wild-type C57BL6/N mice¹¹. Therefore, we investigated the effect of 5-ALA on COX activity in the mitochondrial fraction of the brain region encompassing the hippocampus and cerebral cortex (**Fig. 1A**). COX activity was slightly but significantly elevated in the mitochondrial fractions of 5-ALA-fed mouse brains relative to those of controls. Therefore, we performed Western blotting to examine the COX IV level in the mitochondrial fraction from hippocampus and cerebral cortex (**Fig. 2**, lower rows); COX levels were normalized against the level of voltage-dependent anion-selective channel protein 1 (VDAC1) in the same sample (**Fig. 3A**). The COX IV protein level in the mitochondrial fraction increased significantly in mice that received 5-ALA. By contrast, levels of VDAC1, which is localized largely on the outer mitochondrial membrane, were not altered by the administration of 5-ALA (**Fig. 3A**). The change in the COX IV level was relatively modest, but had a measurable physiological effect: mitochondrial membrane potential was significantly higher in 5-ALA-fed mice than in control mice (**Fig. 1B**). These data indicate that prolonged daily oral administration of 5-ALA can activate mitochondrial function in the brain, at least in the region encompassing the hippocampus and cerebral cortex.

Changes in AD-related and causative proteins in the brain upon 5-ALA administration

The 3xTg-AD model mouse exhibits AD pathologies associated with extracellular A β deposits by the age of 6 months.¹² Accordingly, we performed Western blotting to analyze changes in protein levels, including accumulation of A β , in 9-month-old mice that had consumed a diet with or without 5-ALA for 6 months. In this study, we used PNS/cytoplasmic and crude membrane fractions from the brain region encompassing the hippocampus and cerebral cortex.

Because 3xTg-AD mice exhibit cognitive impairment,¹⁷ we first examined the synaptic proteins of 5-ALA-fed and control mice. Specifically, we measured the levels of presynaptic synaptotagmin and postsynaptic PSD95 (**Fig. 2**). The synaptotagmin

level increased significantly when normalized against actin, but not when normalized against α -tubulin (**Fig. 3B**). The level of postsynaptic PSD95 did not change in 5-ALA-fed mice relative to controls, irrespective of the choice of normalization control (**Figs. 3B**). We also examined the levels of other postsynaptic proteins, including the NR2A and NR2B subunits of the NMDA receptor and the GluR1 subunit of the AMPA receptor (**Fig. 2**); these proteins did not change in 5-ALA-fed mice when compared to controls (**Fig. 3C**). Thus, the effect of 5-ALA on synaptic proteins of 9-month-old mice, if any, may be limited. It is thought that in AD neurotoxic A β oligomers impair synapses primarily, and that the A β accumulation starts prior to change of the levels of synaptic components.¹⁸

Therefore, we next examined A β precursor and the enzymes responsible for A β generation. To this end, we analyzed brain membrane fractions by Western blotting to measure the levels of APP full-length (APP FL), the APP carboxyl-terminal fragment generated by β -secretase/BACE1 or α -secretase cleavage of APP FL (APP CTF β and CTF α , respectively),¹⁹ BACE1²⁰, and PS1 carboxyl-terminal fragment (PS1 CTF), a component of active γ -secretase complex²¹ (**Figs. 2**). Contrary to the findings of the previous original report,¹² the levels of CTF β , APP, and BACE1 tended to be higher in females than in males. Production of APP CTF β tended to be higher in the brains of 5-ALA-fed mice, but the difference was not significant (**Fig. 3D**). This trend may have been due to the different numbers of females in the 5-ALA-fed and control cohorts rather than to an effect 5-ALA. In the 3xTg-AD mouse, the APP transgene carrying the Swedish mutation expresses two APP species, mature and immature APP, and generates much more CTF β than CTF α (**Fig. 2**). Identification and characterization of the APP FL and CTFs forms has been described elsewhere.²² Overall, 5-ALA administration did not exert a quantitative effect on the levels of APP, APP CTFs, BACE1, and PS1 CTF.

Third, we examined brain A β levels in the brain region encompassing the hippocampus and cerebral cortex. Because the level of CTF β , the precursor of A β 40 and A β 42, differed slightly between males and females, we measured A β 40 and A β 42 separately in male and female mice (**Fig. 4**). In both males (panel A) and females (panel B), A β 40 (left) tended to decrease in 5-ALA-fed mice compared to controls; however, we did not perform statistical analysis because of the small number of mice examined (two 5-ALA-fed males and two control-fed females) (**Fig. 4A left and B left**). The A β 42 level (right) also decreased in males, but not in females (**Fig. 4A right and B**

right). Importantly, the A β levels differed between female and male 3xTg mice (4–5-fold for A β 40 and ~20-fold for A β 42) regardless of 5-ALA administration. Therefore, to determine the statistical significance of differences in A β levels, we compared relative brain A β levels in all mice examined; for this comparison, the A β level of control mice brain was set at 1.0 (**Fig. 4C**). The A β 40 level decreased significantly in 5-ALA-fed brain (**Fig. 4C left**), but no significant difference was observed for A β 42.

It may not be possible to explain the higher accumulation of brain A β in female mice relative to male mice (4–5-folds for A β 40, ~ 20-fold for A β 42) based solely on the slightly higher levels of CTF β in females (~1.5 fold) (**Figs. 2 and 3D**). Instead, the levels of the transgene products may also be influenced by sex-specific differences in APP metabolism.

The 3xTg-AD model mice exhibited higher levels of A β accumulation in the female brain, and the A β 40 level was high in control mice relative to 5-ALA-fed mice (**Fig. 4B and C**). As noted above, 3xTg-AD mice exhibit A β deposition at 6 months of age¹² and they accumulate pTau in the hippocampus with age.²³ Therefore, we examined the effect of 5-ALA on AD pathology (specifically, amyloid plaque formation and accumulation of pTau) by immunostaining female brain sections with anti-A β (Panel A, 82E1; Panel B, 4G8; Panel C, 6E10) and anti-pTau (Panel D, AT8) antibodies (**Fig. 5**). Contrary to expectations, we did not detect obvious amyloid plaques in either control or 5-ALA fed mice using any of the three antibodies, although we observed strong A β signals within the neurons of the cortex, hippocampus, and amygdala (**Fig. 5A–C**; typical staining is indicated by the arrow in panel B), as previously reported.²⁴ According to expectation, no pTau staining was observed using the AT8 antibody (**Fig. 5D**), consistent with a previous report showing that AT8 detects pTau in the hippocampus of 17-month-old mice.²³ We observed no marked differences between 5-ALA-fed mice and control animals (at an age of 9 months) with respect to A β and pTau immunostaining, although 5-ALA-fed mice did exhibit reduced A β accumulation in the brain (**Fig. 4C**).

Discussion

Cytochrome c/COX activity decreases with age,^{1, 25} as well as in patients with Alzheimer's disease (AD).^{26, 27} Because heme deficiency directly results in a decrease in the level of COX proteins,²⁸ facilitation of heme production is expected to increase the activity of mitochondrial enzymes, including COX. This could conceivably slow aging and prevent the development of neurodegenerative disorders such as AD. Although 5-ALA increases COX activity in the liver¹¹, this is the first study to examine the effect of orally administered 5-ALA on the brain.

Using the 3xTg-AD mouse model, we showed that oral administration of 5-ALA increased both the activity and protein levels of COX in the brain. In addition, 5-ALA increased mitochondrial activity, as reflected by the membrane potential. These results were obtained in 9-month-old mice that received a 5-ALA-containing diet for 6 months. Although the increase in mitochondrial activity was small, our results demonstrate that 5-ALA had a physiological effect on brain function, at least in the region encompassing the hippocampus and cerebral cortex. It is possible that orally administered 5-ALA is quickly adsorbed by body tissues prior to transportation into brain neurons, potentially explaining why such a small effect on mitochondrial activity was observed in the brains of 5-ALA-fed mice. Nevertheless, because AD is a chronic disease of aged individuals, long-term administration may be preferable to administration of higher doses over shorter periods. This study was a first trial of the effect of 5-ALA in an AD mouse model. Further studies with other AD mouse models or longer-term administration of 5-ALA may reveal clearer effects on the levels of mitochondrial proteins and activities. A recent report described synaptosomal mitochondrial dysfunction in the 5xFAD mouse model, overexpressing human APP-bearing mutations (Swedish, Florida and London) and PS1 mutations (M146L and L286V).¹⁰ This dysfunction was remarkable in that it was specific to mitochondria in synaptosomes, rather than all mitochondria in the brain.

We sought to determine whether oral administration of 5-ALA is effective in suppressing AD-related pathobiology, such as amyloidogenic processing of APP, accumulation of A β and pTau in the brain, and the reduction in synapses or synaptic proteins.^{29, 30} We observed that APP CTF β and A β levels were clearly higher in female mice than in males; however, levels of APP FL expression and CTF α generation were

comparable between male and female mice. Therefore, we analyzed male and female samples separately. When compared relative brain A β levels in all mice examined, the A β 40 level decreased significantly in 5-ALA-fed mice. Contrary to expectation, we did not detect any AD pathology, including A β plaques, in control or 5-ALA-fed mice of either sex at the age of 9 months. Thus, the effect of 5-ALA to AD pathology could not be examined at this age. To analyze AD pathobiology, we may need to conduct longer-term experiments in older mice.

Recent studies report that synaptic impairment occurs prior to the onset of AD pathology, and that this phenomenon is induced by A β oligomers.^{18, 29} We observed significantly higher levels of synaptotagmin in 5-ALA-fed mice than in controls. However, levels of the synaptic protein PSD95 and various subunits of the NMDA and AMPA receptors did not change significantly following 5-ALA administration. The slight effect on synaptotagmin suggests that 5-ALA exerts a slight preservative effect on synapses prior to the onset of AD pathology; however, we need to perform further studies involving alternative aged AD mouse models and longer-term administration of 5-ALA to confirm this effect. Furthermore, as suggested by a recent report,¹⁰ analysis of the synaptosomal fraction may reveal clearer effects of 5-ALA on the preservation of synaptic proteins.

Finally, we wish to propose a possible mechanism by which preservation of mitochondrial function could slow A β -induced neuronal impairment in AD. Neurons have long axons and highly branched dendrites, which are supported functionally and architecturally by a well-organized membrane, cytoskeletal components such as microtubules, and molecular motor proteins carrying membrane cargos and mitochondria.³¹ Dysfunction of intraneuronal transport causes neurodegeneration.³²⁻³⁴ Interestingly, APP, the precursor of neurotoxic A β , plays an important role as a cargo of kinesin-1, which is composed of two kinesin light chains and two kinesin heavy chains (KHCs).^{33, 35} Mitochondria are also transported by KIF5, which is composed of two KHCs.³⁶ Suppression of the kinesin-dependent transport system in neurons increases generation of A β ,^{32, 33} and the A β oligomer impairs both mitochondria (intracellularly) and synapses (extracellularly).^{12, 17, 18} Intracellular transport by kinesin is an energy-consuming process; consequently, mitochondrial dysfunction affects this transport system and induces additional A β generation, which in turn further impairs mitochondria and synapses. This feedback loop between A β generation and

mitochondrial dysfunction could facilitate neurodegeneration, ultimately resulting in AD. Therefore, it is reasonable to postulate that preservation of mitochondrial function, including ATP production, in aged individuals could suppress both amyloidogenic processing of APP and generation of A β . If 5-ALA can significantly preserve mitochondrial function in brain neurons of aged individuals, in whom mitochondrial activity is reduced, it could slow the progression of A β -dependent neuronal impairment by suppressing the downward spiral of A β generation and mitochondrial dysfunction.

In summary, we showed here that oral administration of 5-ALA can activate mitochondrial enzymes in the brain. Further studies using multiple synaptic markers within synaptosomes should seek to confirm these effects in the early stage of AD and/or in aged individuals.

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Figure legends

Figure 1. 5-ALA administration increases COX activity and mitochondrial membrane potential. (A) COX activity and (B) membrane potential in mitochondrial fractions prepared from the brains of 5-ALA-fed (+) and control (-) 3xTg-AD mice. Brain samples included the regions encompassing the hippocampus and cerebral cortex. Statistical analysis was performed using Student's t test. Values represent the mean $\pm$ S.E. Statistical significance is indicated by an asterisk (n=7; *, p<0.05).

Figure 2. Changes in protein levels in 5-ALA-fed and control 3xTg-AD mouse brain. Lysates of 5-ALA-fed and control mouse brain (hippocampus plus cerebral cortex) were fractionated into post-nuclear supernatant (upper six rows), crude membrane (middle eight rows), and mitochondrial (lower four rows) fractions. Respective protein samples (30 μ g for post-nuclear fraction; 10 μ g for crude membrane and mitochondrial fractions) were analyzed by Western blotting with antibodies against synaptotagmin, PSD95, NR2A, NR2B, GluR1, α -tubulin, APP/CTF, BACE1, PS1 CTF, flotillin, actin, COX IV, and VDAC1. In APP FL, arrowheads indicate mature APP (N- and O-glycosylated form) and arrow indicates immature APP (N-glycosylated form). A longer exposure of the blot for APP CTF α is also shown. Male (M) and female (F) mice fed with (+) or without (-) 5-ALA were analyzed at 9 months-of-age.

Figure 3. Quantitation of protein levels of 5-ALA-fed and control 3xTg-AD mouse brain. Protein levels shown in Figure 2 were quantitated on a LAS-4000 mini (Fujifilm). Levels were normalized against the corresponding levels of α -tubulin and actin (for cytoplasmic proteins), flotillin-1 (for membrane proteins), or VDAC1 (for mitochondrial proteins). Levels of COX IV and VDAC1 (A), synaptotagmin and PSD95 (B), NR2A, NR2B, and GluR1 (C) and APP FL, APP CTF β , BACE1, and PS1 CTF (D) are shown. Statistical analysis was performed using Student's t test. Values represent the mean $\pm$ S.E. Statistical significance is indicated by an asterisk (n=7; *, p<0.05).

Figure 4. Human A β levels in 3xTg-AD mouse brains fed with or without 5-ALA. Quantitation of A β 40 (left panels) and A β 42 (right panels) levels in brain samples

(hippocampus plus cerebral cortex) from male (A) and female (B) mice fed with (+) or without (-) 5-ALA. Concentrations were normalized to the amount of total protein. Relative ratios of A β 40 and A β 42 accumulation in all animals are shown (C). Levels of A β 40 (left) and A β 42 (right) in 5-ALA-fed mice (+) are indicated as relative ratios (the level in control mice not subjected to 5-ALA administration (-) was defined as 1.0). Data were analyzed by Student's t test. Values represent the mean $\pm$ S.E. Statistical significance is indicated by an asterisk [n=7 for 5-ALA (-) and n=6 for 5-ALA (+); *, p<0.05].

Figure 5. Immunohistochemical analysis of 3xTg-AD mice with anti-A β and anti-pTau antibodies. Immunostaining of coronal hemisphere sections of brain regions including the cerebral cortex, hippocampus, and amygdala (indicated with arrows) of 9-month-old 3xTg-AD female fed with (ALA) or without (control) 5-ALA. Brain sections were stained with antibodies against A β [82E1 (A), 4G8 (B), and 6E10 (C)] and pTau (D). Bar (shown in panel A “Control”), 300 μ m.

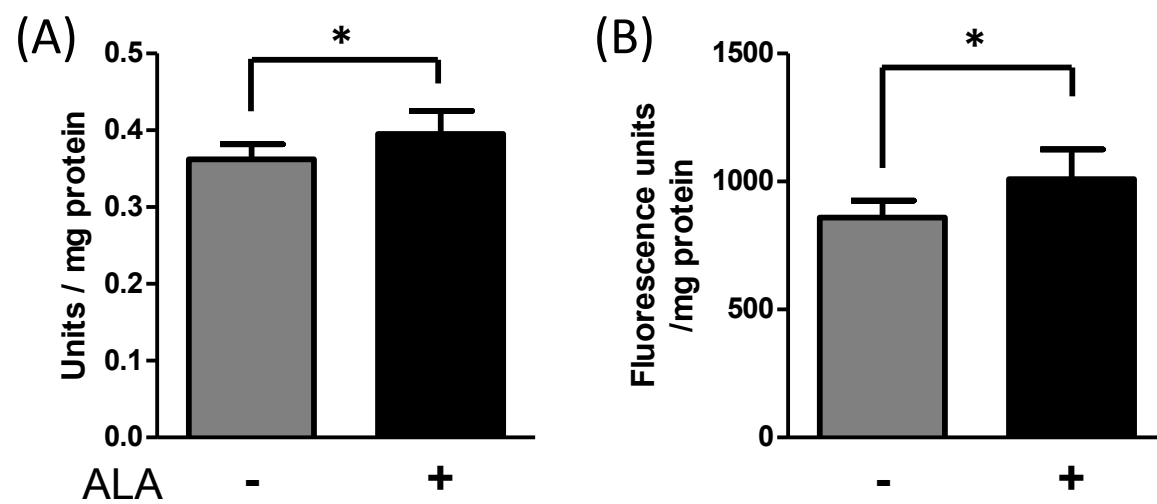


Figure 1

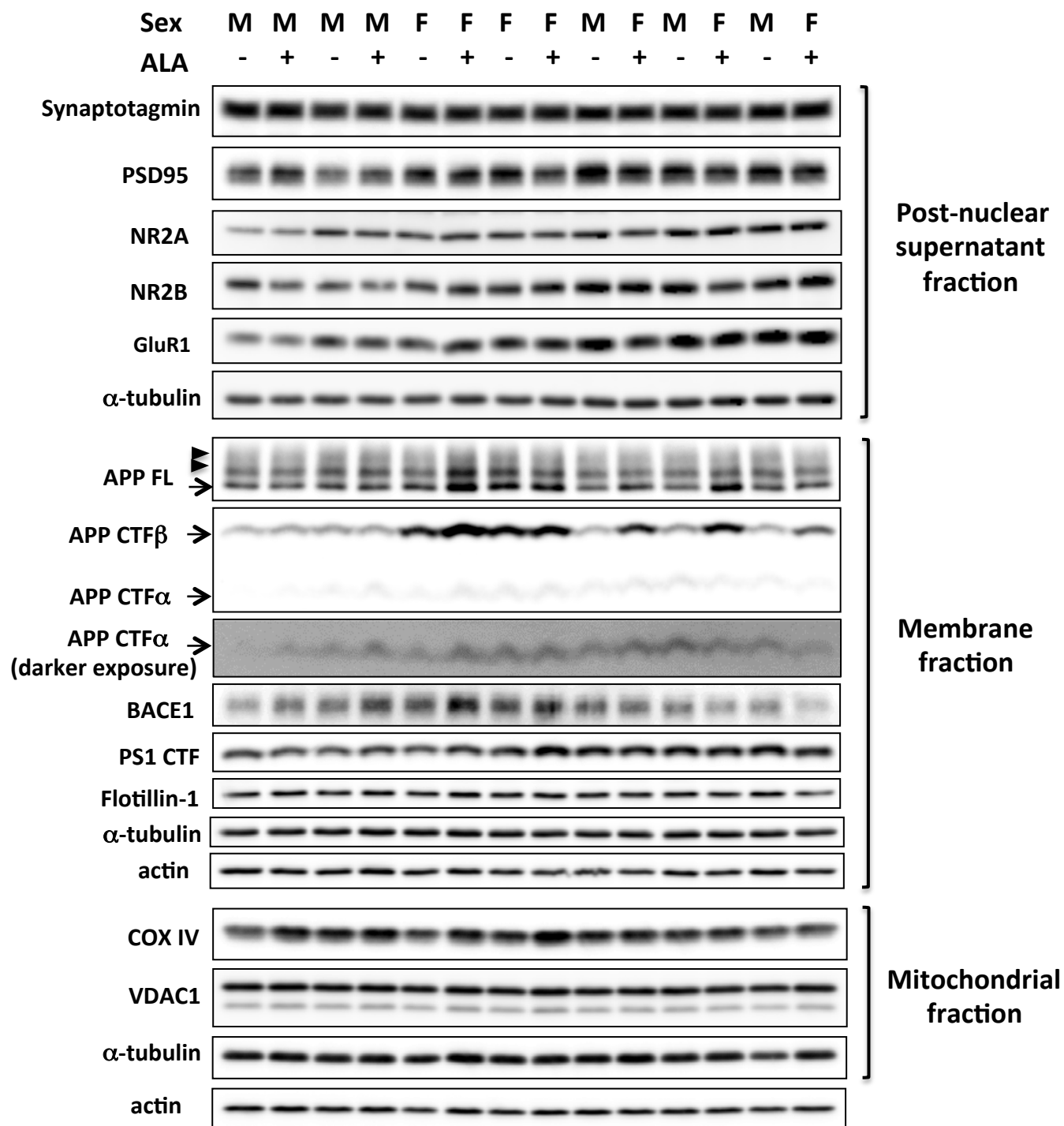


Figure 2

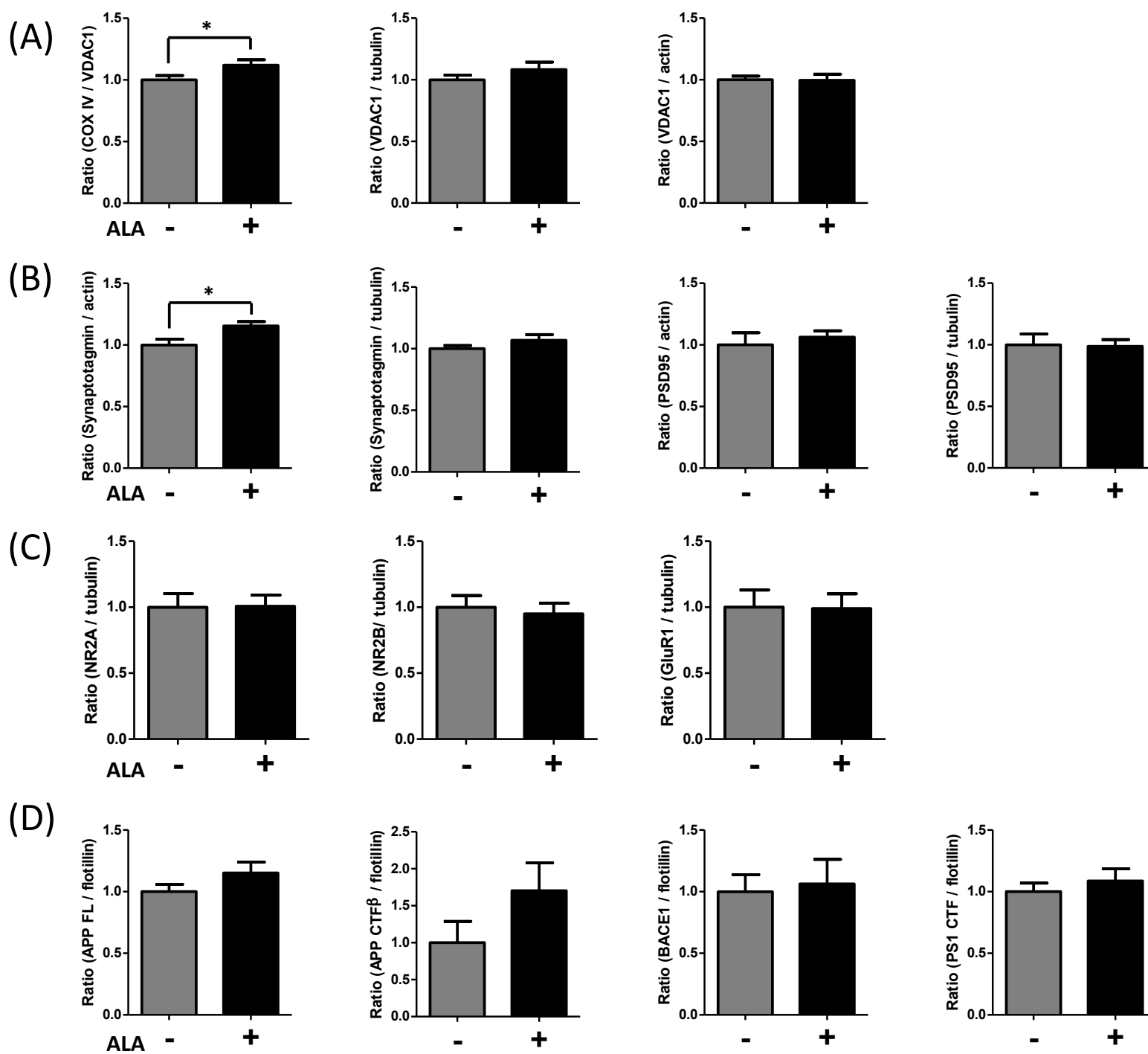


Figure 3

(A) (B)

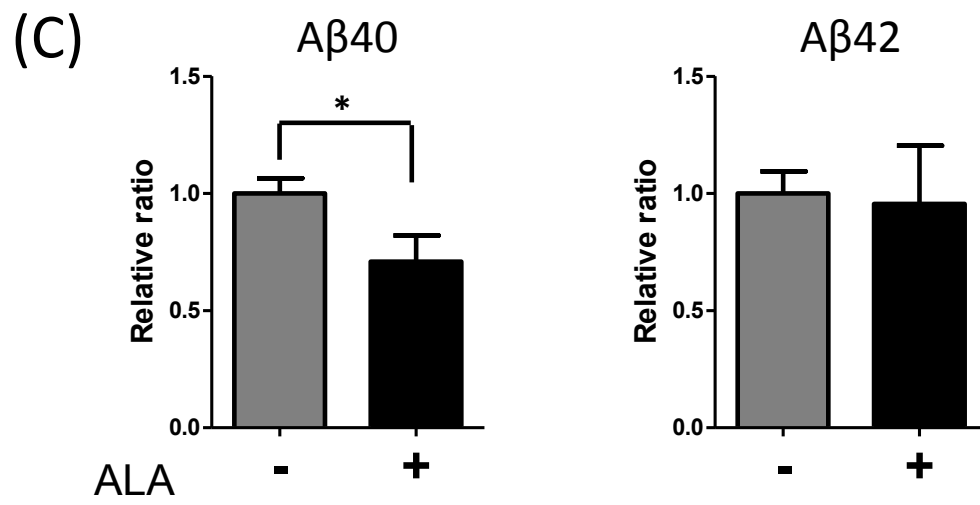
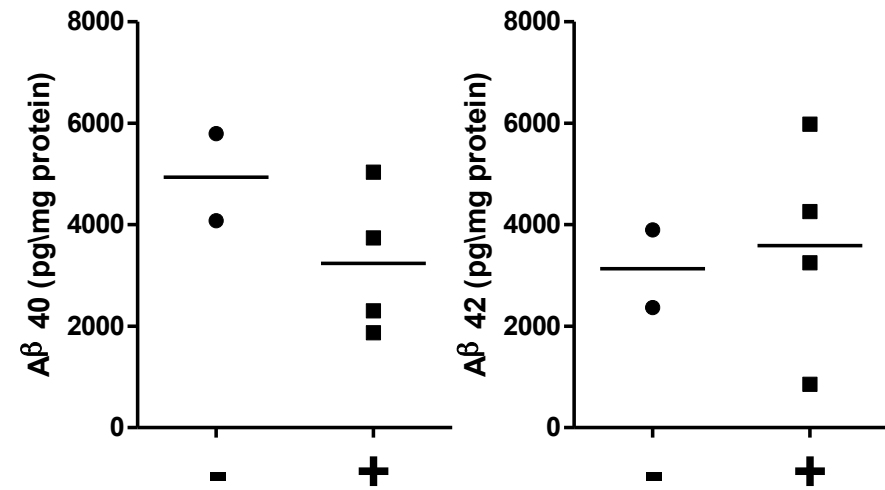
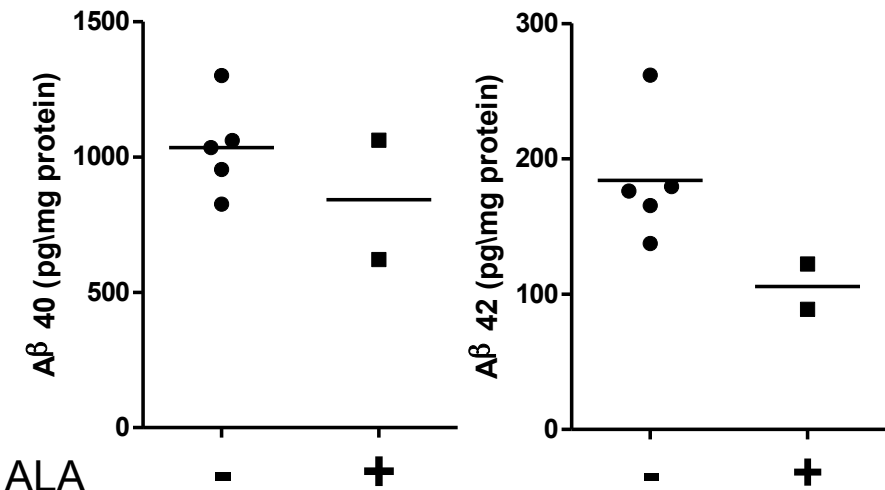


Figure 4

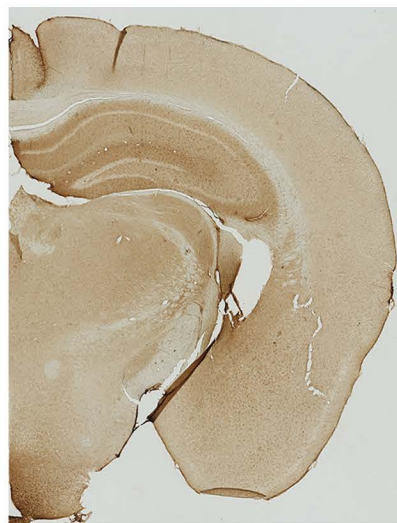
(A)



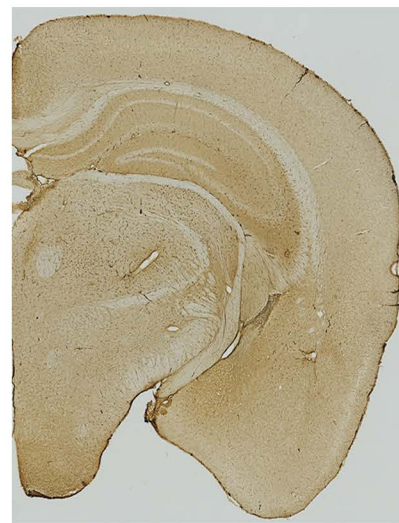
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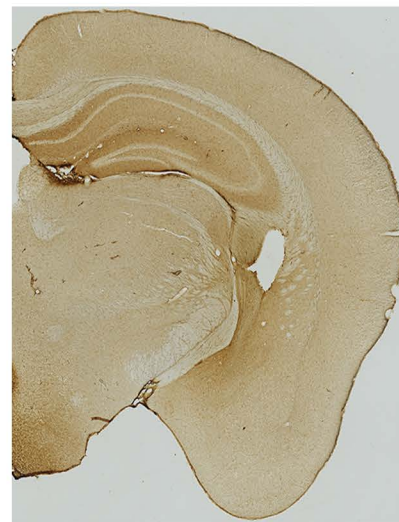
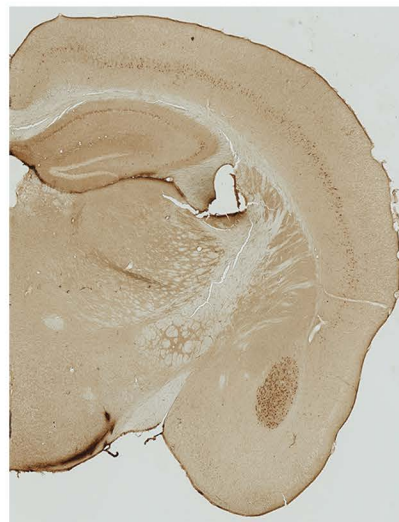
(C)



(D)



Control



ALA