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## Pathology of nNOS expressing GABAergic neurons in mouse model of Alzheimer's disease

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### Abstract

Alzheimer's disease (AD) is the most common form of dementia that is often accompanied by mood and emotional disturbances and seizures. There is growing body of evidence that neurons expressing  $\gamma$ -aminobutyric acid (GABA) play an important role in regulation of cognition, mood, and emotion as well as seizure susceptibility, but participation of GABAergic neuronal pathology in Alzheimer's disease (AD) is not understood well at present. Here, we report that transgenic mice expressing human amyloid precursor protein Swedish-Deutch-Iowa mutant (APP<sub>SweDI</sub>) exhibit early loss of neurons expressing GAD67, a GABA synthesizing enzyme, in advance to the loss of pyramidal neurons in hippocampal CA1 region. The loss of GAD67<sup>+</sup> neurons in APP<sub>SweDI</sub> mice accompanied with decreased spatial cognition as well as increased anxiety-like behaviors and kainic acid-induced seizure susceptibility at early phase. In the hippocampal CA1 region, GAD67<sup>+</sup> neurons expressed high basal levels of neuronal nitric oxide synthase (nNOS) and nitrosative stress (nitrotyrosine). Similarly, GAD67<sup>+</sup> neurons in primary cortical and hippocampal neuron cultures also expressed high basal levels of nNOS and degenerated in response to lower A $\beta$  concentrations due to their high basal levels of nitrosative stress. Given the role of GABAergic neurons in cognitive and neuropsychiatric functions, this study reports the role of nNOS mediated nitrosative stress in dysfunction of GABAergic neurons and its potential participation in early development of cognitive and neuropsychiatric symptoms in AD.

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#### Conflicts of interest

The authors declare no actual or potential conflicts of interest for this work.

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## Keywords

GABAergic neurons; hippocampus; nNOS; nitrosative stress; amyloid- $\beta$  ( $A\beta$ )

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## Introduction

The area of brain hippocampus plays the major role in the process of learning and memory and is the most affected area in Alzheimer's disease (AD). Neural network of hippocampus is composed of different types of neurons (e.g. pyramidal, granular, and GABAergic neurons). Among them, GABAergic neurons are the local neurons which comprise a relatively small population but distributed throughout the hippocampus to provide inhibitory inputs to virtually all excitatory units (Chamberland S and Topolnik L, 2012). AD brains are associated with GABAergic neuronal degeneration and thus disruptions in the GABAergic neurotransmission [see review (Lanctot KL et al., 2004)]. In addition, transgenic mice expressing human  $A\beta$  precursor protein (APP), tau, or apolipoprotein E4 allele (ApoE<sub>4</sub>) also develop age-dependent loss of GABAergic neurons in the hippocampus with decreased learning/memory functions (Andrews-Zwilling Y et al., 2010; Levenson J et al., 2013; Verret L et al., 2012). In hippocampus, GABAergic neurons play a crucial role in long-term potentiation (LTP) (Meredith RM et al., 2003), a form of neuroplasticity playing a critical role in memory formation. These observations, therefore, suggest a potential involvement of GABAergic neuronal pathology in development of cognitive impairment in AD.

GABAergic neurons also play an important role in regulation of mood and emotion (Shiah IS and Yatham LN, 1998) and defects in their functions have been implicated in schizophrenia, depression, and anxiety (Luscher B et al., 2011; Nuss P, 2015; Taylor SF and Tso IF, 2015). In parallel with the disruption of GABAergic system in AD brains (Lanctot KL, Herrmann N, Mazzotta P, Khan LR and Ingber N, 2004), depression is also reported as the most frequent comorbidity of AD (Modrego PJ, 2010). Moreover, similar depression-like behaviors were also observed in mouse models of AD (Romano A et al., 2015). Anxiety is another significant complication in AD (Kaiser NC et al., 2014) and anxiety-like behaviors were reported in transgenic mice expressing human mutant APP and ApoE<sub>4</sub> (España J et al., 2010; Tong LM et al., 2014). Loss of GABAergic neurons also shifts the neuronal excitation and inhibition balance towards excitation (Ben-Ari Y and Represa A, 1990) and thus increases the probability of seizure onset (Knopp A et al., 2005; Wozny C et al., 2003). In AD, seizures are highly prevalent (Born HA, 2015; Vossel KA et al., 2013), and, accordingly, animal models of AD also show increased spontaneous seizure activities as well as increased seizure susceptibility to proconvulsant agents (Born HA, 2015). These studies document the role of GABAergic degeneration in development of cognitive, neurological, and psychiatric symptoms in AD.

Nitric oxide (NO) is a key signaling molecule throughout the body. NO is produced by a family of enzymes called nitric oxide synthases (NOS). There are three NOS isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), all of which differ in physiological role and expression profile. nNOS is an enzyme responsible for the synthesis of NO in neurons but not all neurons are known to express nNOS. In hippocampus

and cortex, nNOS is primarily expressed in specific subpopulations of GABAergic neurons (Tricoire L and Vitalis T, 2012), where it regulates GABA release (Gasulla J and Calvo DJ, 2015; Getting SJ et al., 1996; Lee JJ, 2009; Maggesissi RS et al., 2009; Wall MJ, 2003; Yang Q et al., 2007), local blood flow (Cauli B and Hamel E, 2010; Cauli B et al., 2004; Perrenoud Q et al., 2012), and seizure and epileptiform activities (Demchenko IT and Piantadosi CA, 2006; Gholipour T et al., 2010; Rajasekaran K et al., 2003). nNOS is activated by calcium ( $\text{Ca}^{2+}$ )-dependent calmodulin and secondary modification of nNOS (e.g. denitrosylation and phosphorylation) (Gingerich S and Krukoff TL, 2008; Hayashi Y et al., 1999; Komeima K et al., 2000; Qu ZW et al., 2012). Under physiological conditions, the activated nNOS produces NO from L-arginine (coupled nNOS reaction). However, under oxidative stress conditions where its cofactor tetrahydro-biopterin is decreased, nNOS also produces superoxide anion ( $\text{O}_2^{\bullet-}$ ), in addition to NO, and thus leading to generation of peroxynitrite ( $\text{ONOO}^-$ ), the most reactive NO-derived oxidant (Pall ML, 2007; Xia Y et al., 1996). AD brains are reported to have decreased level of tetrahydrobiopterin (Barford PA et al., 1984) and increased level of nitrotyrosine (N-Tyr) (Sultana R et al., 2009). In addition, nNOS expressing neurons in different areas of AD brains were reported to be highly susceptible to  $\text{A}\beta$  induced neurodegeneration (Thorns V et al., 1998). These reports indicate a potential role of nNOS dysregulation in neuronal degeneration in AD, but the involvement of nNOS dysfunction in GABAergic neuronal degeneration and associated cognitive and neuropsychiatric disorders in AD is not well understood at present.

In this study, we report that transgenic mice expressing human APP Swedish-Dutch-Iowa mutant ( $\text{APP}_{\text{SweDI}}$ ) had loss of GAD67 immunoreactivity in *stratum pyramidale* of hippocampal CA1 region in advance to the loss of pyramidal neurons. The  $\text{GAD67}^+$  neurons expressed high basal levels of nNOS and nitrosative stress and exhibited higher vulnerability to  $\text{A}\beta$  induced cytotoxicity than other types of neurons. The loss of GAD67 expressing cells accompanied with increased cognitive deficits and neuropsychiatric-like symptoms as well as increased seizure susceptibility in  $\text{APP}_{\text{SweDI}}$  mice. Although pathological relationship between the observed loss of  $\text{GAD67}^+$  neurons and increased cognitive/neuropsychiatric deficits in  $\text{APP}_{\text{SweDI}}$  is not completely understood, these data suggest the potential role of nNOS dysregulation in cell death or hypo-function of GABAergic neurons and thus loss of GABAergic function for cognitive and neuropsychiatric functions in AD mouse model.

## Experimental Procedure

### Animals

All animal procedures were in accordance with the animal experiment guidelines of the Medical University of South Carolina and National Institute of Health. Wild-type (C57Bl/6J; The Jackson Laboratories, Bar Harbor, ME; <https://www.jax.org/strain/000664>) and  $\text{APP}_{\text{SweDI}}$  mice [C57BL/6-Tg(Thy1-APP<sub>SweDutIowa</sub>)B<sub>W</sub>evn/M<sub>m</sub>jax; The Jackson Laboratories; <https://www.jax.org/strain/007027>] were housed in cages under controlled temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity ( $55 \pm 10\%$ ), with a 12-hr light/12-hr dark cycle. Equal number of male and female WT and  $\text{APP}_{\text{SweDI}}$  mice (6 and 14 months old) were used.

## Histology and immuno-fluorescent staining

Cryosections (40  $\mu\text{m}$  thick) obtained from 4% paraformaldehyde-fixed brain tissues or paraffin-embedded sections obtained from formalin-fixed brain tissues were used for Nissl staining (Nissl stain kit; IHCWORLD, Woodstock, MD) or immunofluorescent staining for A $\beta$  (BC05; Wako, Osaka, Japan, Cat# 010-26903), GAD67 (Millipore, Billerica, MA, Cat# MAB5406, RRID: AB\_2278725), Neurogranin (Millipore, Cat# AB5620, RRID: AB\_91937), nNOS (Santa Cruz, Dallas, TX, Cat# sc-5302, RRID: AB\_626757) and N-Tyr (Millipore, Cat# 487923, RRID: AB\_212231). For immunofluorescent staining of neuron culture, the neurons cultured on poly-D-lysine (Sigma-Aldrich, Saint Louis, MO) coated chamber slides (Nunc Lab-Tek, Rochester, NY) were fixed in formaldehyde and incubated with MAP2 (Santa Cruz, Cat# sc-20172, RRID: AB\_2250101), GAD67, nNOS, or N-Tyr. DAPI (4',6-diamidino-2-phenylindole; Thermo-Fisher Scientific, Houston, TX) was used for staining of nuclei. BX60 Olympus fluorescent/light microscope equipped with DP-70 camera (Olympus, Tokyo, Japan) was used for histological and immunofluorescent imaging. The number of stained cells were manually counted in three randomly selected microscope fields.

## Western immunoblot analysis

For Western immunoblot of hippocampal tissue, hippocampi of mice were dissected and homogenized in SDS sample loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) by sonication and 20  $\mu\text{g}$  of protein/well was resolved in SDS-PAGE gel (4–20%, Life Science Research, Hercules, CA, USA) and then transferred to polyvinylidene fluoride membranes. The membrane was blocked with 5% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20) and incubated with antibodies against GAD67, EMX1 (Santa Cruz, Cat# sc-28220, RRID: AB\_2262220), A $\beta$ /6E10, nNOS, p35/p25 (Cell Signaling Technology, Danvers, MA, Cat# 2680S, RRID: AB\_1078214), phospho-Ser<sup>9</sup>-GSK-3 $\beta$  (p-GSK-3 $\beta$  S9; Cell Signaling Technology, Cat# 9336, RRID: AB\_331405), Cleaved Caspase 3 (Cell Signaling Technology, Cat# 9664, RRID: AB\_2070042), or  $\beta$ -actin (Santa Cruz, Cat# sc-47778, RRID: AB\_626632), then with appropriate secondary antibody-conjugated with horseradish peroxidase (HRP). The signal of HRP was detected by ECL-plus Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

## Morris water maze test

Morris water maze was employed to assess spatial learning and memory according to previously published methods with modification (Won JS et al., 2013). The test was performed in a circular pool (124 cm in diameter/60 cm in depth) filled with water clouded by nontoxic white paint. The circular pool consisted of four equal virtual quadrants. A circular area (radius 20 cm from the center of the platform) was defined as the target zone, equivalent to 4.9% of the total water maze area. All other experimental conditions are identical with our previous report (Won JS, Kim J, Annamalai B, Shunmugavel A, Singh I and Singh AK, 2013).

### Open field test for anxiety-like and exploratory behaviors

Open field test was performed to assess anxiety-like and exploratory behaviors as described previously with modification (Miyakawa T et al., 2001). Briefly, the mice were placed in the center of an open arena (length 1 m; width 1 m; side walls 20 cm height) for 5 min and explorative behavior was recorded by a video-tracking system (Accuscan, Ohio). Total distance traveled and the relative time spent in the center of the arena were recorded and quantified. In addition, number and time spent in freezing (immobility longer than 3 seconds), grooming, rearing, and defecation were measured.

### Kainic Acid (KA) treatment

The mice were treated with repeated low dose (RLD) of KA (5mg/kg/i.p. per every 30 min, up to a maximum of 6 times) based on previous report (Tse K et al., 2014). Following the first KA injection, the time to reach status epilepticus (stage 5 in Racine scale; behaviors such as generalized tonic-clonic convulsions with lateral recumbence or jumping and wild running followed by generalized convulsions (Racine RJ, 1972)) were analyzed. Following the last KA injection, the number of mice that reached stage 5 seizure and survival rate were analyzed. At 3 days after KA injection, the survived mice were sacrificed and the brains were extracted for histological analysis.

### Primary neuronal culture

Primary neuron cultures were prepared from the cerebral cortex and hippocampus of embryos of Sprague Dawley rats at embryonic day 17 (E17) as described in our previous report (Won JS, Kim J, Annamalai B, Shunmugavel A, Singh I and Singh AK, 2013). The cultured neurons were maintained in Neurobasal media (Invitrogen, Carlsbad, CA) supplemented with 2% B27 supplement (Invitrogen), 0.5 mM glutamine, 25  $\mu$ M glutamate, 50 units/ml penicillin, 50  $\mu$ g/ml streptomycin under humidified atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub>, at 37 °C. All experiments presented in this work were performed on neuronal cells at 7 days in vitro.

### Preparation of A $\beta$

Synthetic human A $\beta$ <sub>25-35</sub> and A $\beta$ <sub>1-42</sub> peptides were purchased from Anaspec (Fremont, CA). Soluble, oligomeric, and fibrillary forms of A $\beta$ <sub>1-42</sub> peptides was generated as previously described (Stine WB et al., 2011). Briefly, A $\beta$ <sub>1-42</sub> peptide was solved in hexafluoro-2-propanol (HFIP), lyophilized in vacuum centrifugation, and reconstituted in dimethylsulfoxide (monomeric A $\beta$ <sub>1-42</sub>). Then the monomeric A $\beta$ <sub>1-42</sub> peptide was further incubated in phenol-free F-12 cell culture media for 24h at 4°C for preparation of oligomeric A $\beta$ <sub>1-42</sub> or resuspended in 10 mM HCl for 24h at 37°C for preparation of fibrillary A $\beta$ <sub>1-42</sub>. Aggregation of A $\beta$ <sub>1-42</sub> was confirmed by Western blot analysis.

### Neuronal death/viability assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed for analysis of apoptotic neuronal death by using commercial kit (Roche, Indianapolis, IN). For analysis of cell specific apoptosis, the TUNEL assay was combined with immunofluorescent staining using antibody specific to MAP2 (pan neuronal marker;

Santa Cruz) or GAD67 (GABAergic neuronal marker; Millipore). For neuronal viability analysis, cell counting kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan) was used. Briefly, primary cultured neurons ( $4 \times 10^3$  cells/well in 96-well plates) were incubated with A $\beta$  peptides in the presence or absence of N $\omega$ -Propyl-L-arginine hydrochloride (NPLA, Tocris, Bristol, UK) or 3-morpholininosydnonimine (SIN-1) (Tocris) for 24 hours, then CCK-8 was added to each well and further incubated for 3 hours at 3 $^{\circ}$  C. The neuronal viability was detected by spectrometrically at 450 nm using SpectraMax 190 microplate reader (Molecular devices, Sunnyvale, CA).

### Assay of nitro tyrosine

Brain levels of nitro tyrosine (N-Tyr) was performed using OxiSelect Nitrotyrosine ELISA Kit (Cell Biolabs, Inc., San Diego, CA). Briefly, brain tissues (n=3) were homogenized (dounce homogenizer) in assay diluent provided with the kit and equal amounts (500  $\mu$ g) of brain lysates and nitrated BSA standards were added to an N-Tyr coated enzyme Immunoassay (EIA) plate and followed by incubation with anti-nitro tyrosine antibody. Following washing, the plates were incubated HRP conjugated secondary antibody and the levels of N-Tyr were measured by incubation with 3,3',5,5'-tetramethylbenzidine (TMB) solution and colorimetric analysis at 450 nm.

### Statistical analysis

Statistical analysis was performed using Graphpad prism. An unpaired student t test was used for comparison between two groups. One-way ANOVA with Tukey's multiple comparison test were used for comparison of multiple groups. Criterion for significance was  $p < 0.05$ .

## Results

### Loss of GABAergic neurons in *stratum pyramidale* of hippocampal CA1 of APP<sub>SweDI</sub> mice

Pyramidal neurons in CA1 region of hippocampus (*stratum pyramidale*) play critical roles in learning and memory (Kesner RP et al., 2011) as well as mood and emotions (Freeman-Daniels E et al., 2011; Modol L et al., 2011). In this study, we investigated A $\beta$ <sub>42</sub> deposition and associated neuronal loss in CA1 *stratum pyramidale* of APP<sub>SweDI</sub> mice. For detection of human A $\beta$  deposition, the brain sections from 2, 6, and 14 months old APP<sub>SweDI</sub> mice and their age-matched wild type (WT) controls (C57BL/6 mice) were immunostained for A $\beta$ <sub>42</sub> using the antibody specific to A $\beta$ <sub>42</sub> peptide (BC05). Figure 1A shows no significant deposition of human A $\beta$ <sub>42</sub> in *stratum pyramidale* of hippocampal CA1 of WT mice at all ages as well as APP<sub>SweDI</sub> mice at 2 months of age, increased deposition of A $\beta$ <sub>42</sub> in the same brain region of 6 months old APP<sub>SweDI</sub> mice, and the even greater deposition of A $\beta$ <sub>42</sub> in 14 months old APP<sub>SweDI</sub> mice.

Next, sections from the mice were immunostained with antibody specific to GAD67 for detection of GABAergic neurons or neurogranin (NRGN) for detection of pyramidal neurons (Xiong K et al., 2008). We observed no loss of GABAergic and pyramidal neurons at 2 months of age (Fig. 1B). However, increased A $\beta$  deposition paralleled with reduced number of GAD6<sup>+</sup> neurons in *stratum pyramidale* of hippocampal CA1 of APP<sub>SweDI</sub> mice



as compared to that of WT at 6 months of age (Fig. 1B). Moreover, almost complete loss of GAD67<sup>+</sup> neurons was observed in 14 months old APP<sub>SweDI</sub> mice (Fig. 1B). Accordingly, Western analysis of hippocampal lysates shows decreased levels of GAD67 protein in APP<sub>SweDI</sub> mice as compared to WT mice at 6 months of age (Fig. 1C). On the other hand, APP<sub>SweDI</sub> mice did not show any obvious changes in the number of NRG1<sup>+</sup> neurons (pyramidal neurons) at 6 months of age as compared to WT but these neurons were lost in advanced disease at 14 months of age. Studies have shown that GABAergic neurons have capacity to downregulate GAD67 expression yielding GABAergic hypo-function without neuronal loss (Zamberletti E et al., 2014). Therefore, the decreased GAD67 immunoreactivity and thus GABAergic hypo-function in *stratum pyramidale* of hippocampal CA1 of APP<sub>SweDI</sub> mice may not be only due to the loss of GABAergic neurons, but also due to the decreased gene expression of GAD67. Interestingly, we observed predominant deposition of human A $\beta$  in *stratum oriens* and *stratum radiatum* in APP<sub>SweDI</sub> mice (Fig. 1A). However, we observed relatively less deposition of A $\beta$  in *stratum pyramidale*, where nNOS<sup>+</sup> GAD67<sup>+</sup> and NRG1<sup>+</sup> neurons were undergoing degeneration (Fig. 1B). Overall, these data indicate occurrence of GABAergic hypo-function (decreased GAD67 expression or degeneration of GABAergic neurons) at early stage of disease in advance to the loss of pyramidal neurons in APP<sub>SweDI</sub> mice.

#### Alterations of cognitive function and neuropsychiatric behaviors of APP<sub>SweDI</sub> mice

Since GABAergic neurons play important role in memory and learning processes (Andrews-Zwilling Y et al., 2012; Meredith RM, Floyer-Lea AM and Paulsen O, 2003; Shinoda Y et al., 2011) as well as regulation of mood and emotion (Luscher B, Shen Q and Sahir N, 2011; Nuss P, 2015; Shiah IS and Yatham LN, 1998; Taylor SF and Tso IF, 2015), we next investigated relationship between the GABAergic hypo-function and developments of cognitive deficits and neuropsychiatric-like symptoms. For analysis of cognitive function, 6 months old APP<sub>SweDI</sub> mice and WT mice were subjected to Morris water maze test. Fig. 2A shows that APP<sub>SweDI</sub> mice had longer escape latency with shorter time spent in target quadrant than WT mice, indicating that the APP<sub>SweDI</sub> mice develop defective spatial learning and memory dysfunction at 6 months of age.

Next, to analyze neuropsychiatric-like symptoms in APP<sub>SweDI</sub> mice, 6 months old APP<sub>SweDI</sub> mice and WT mice were subjected to open field test. Fig. 2B-i shows that APP<sub>SweDI</sub> mice did not show any decreases in distance and time traveled in center, a typical behavioral measure for anxiety-like behavior (Walsh RN and Cummins RA, 1976). However, the mice developed increased sympathetic reactivity (defecation and urination) (Fig. 2B-ii) with increased frequency of freezing (Fig. 2B-iii), another typical indicators of high anxiety-like behavior (Gray JA, 1971). APP<sub>SweDI</sub> mice did not show any differences in grooming behavior (Fig. 2B-iv), a displacement behavior (Espejo EF, 1997), but developed decreased locomotor and exploratory activities as observed by decreased total distance traveled and number of rearing compared to WT (Fig. 2B-v). These data suggest possible involvement of early loss of GABAergic neurons in increased anxiety-like behaviors and decreased locomotor/exploration activities in APP<sub>SweDI</sub> mice.

### Altered seizure susceptibility of APP<sub>SweDI</sub> mice

GABAergic neurons in hippocampus are known to play a critical role in modulation of seizure activity (Esclapez M et al., 1997). Therefore, we next assessed the susceptibility of APP<sub>SweDI</sub> mice to kainic acid (KA)-induced seizure activity. KA is an epileptogenic glutamate analogue and a reliable tool to study temporal lobe epilepsy, the most common type of epilepsy. Since treatment with repeated low dose (RLD) of KA treatment develops a robust and reliable mouse model of status epilepticus (Tse K, Puttachary S, Beamer E, Sills GJ and Thippeswamy T, 2014), 6 months old APP<sub>SweDI</sub> and WT mice were treated with RLD of KA and seizure activities, during and after treatments, were analyzed based on Racine scale (see Materials and Method section) (Racine RJ, 1972).

Figs. 3A-i and ii show the percent of mice that reached stage 5 and the time to reach stage 5 seizure in Racine scale (most severe behaviors including generalized tonic-clonic seizure with lateral recumbence or jumping and wild running). APP<sub>SweDI</sub> mice reached stage 5 with lesser doses of KA and in shorter time than WT mice (Figs. 3A-i and ii). APP<sub>SweDI</sub> mice also exhibited higher lethality than WT (Fig. 3A-iii). Three days after the trials, the brain sections from surviving mice were subjected to Nissl staining for assay of neuronal morbidity. Figure 3B shows that the number of pyramidal cells in CA1 and CA3 of KA-treated APP<sub>SweDI</sub> mice were reduced to greater degree than KA-treated WT mice. Moreover, greater empty spaces in pyramidal layer (blue arrow head) and higher number of dark Nissl bodies (blue arrow head) in KA-treated APP<sub>SweDI</sub> mice indicate higher degree of active neurodegeneration in these mice. Previous studies have reported that CA3 damage occurs more rapidly than CA1 (within 48hr) (Ben-Ari Y et al., 1979; Charriat-Marlangue C et al., 1996; Friedman LK, 1998; Hartley Z and Dubinsky JM, 1993). Accordingly, we observed more post-epileptic neurodegeneration in CA3 compared to CA1 (Fig. 3B). In this study, we observed that some of pyramidal neurons in CA3 hippocampus of APP<sub>SweDI</sub> mice also underwent neurodegeneration without KA treatment (Fig. 3B-ii), suggesting that pyramidal neurons in CA3 may be more susceptible to A $\beta$  toxicity than those in CA1. Overall, these data suggest the possible involvement of early loss of GABAergic neurons in increased vulnerability of APP<sub>SweDI</sub> mice to KA induced seizure and post-seizure neurodegeneration.

### GABAergic neurons have high vulnerability to A $\beta$ -induced cytotoxicity

Since APP<sub>SweDI</sub> mice had loss of GAD67<sup>+</sup> immunoreactivity in CA1 hippocampus prior to the loss of pyramidal neurons at early stages (6 months old) of disease (Fig. 1), we next investigated the vulnerability of GABAergic neurons to A $\beta$ -induced cytotoxicity using primary rat neuron culture. For identification of GABAergic neurons in culture, the primary cultured cortical and hippocampal neurons were immunostained for MAP2 (pan neuronal marker) and GAD67 (GABAergic neuronal marker). Fig. 4A shows that both cortical and hippocampal neuron cultures contained 15~20% of GAD67<sup>+</sup>/MAP<sup>+</sup> GABAergic neurons (Fig. 4A). Next, the cultured cortical and hippocampal neurons were treated with soluble (*s*-), oligomeric (*o*-), and fibrillary (*f*-) forms of A $\beta$ <sub>42</sub>. The different A $\beta$ <sub>42</sub> assemblies of A $\beta$ <sub>42</sub> (*s*-, *o*-, and *f*- forms) were generated as described previously (Stine WB, Jungbauer L, Yu C and LaDu MJ, 2011) and characterized by Western analysis (Fig. 4B-i). As reported previously (Tomic JL et al., 2009), *o*-A $\beta$ <sub>42</sub> was most toxic to the cultured neurons than *s*-A $\beta$ <sub>42</sub> and *f*-A $\beta$ <sub>42</sub> as shown by MTT cell viability assay (Fig. 4B-ii). Next, we examined the



effect of *o*-A $\beta$ <sub>42</sub> treatment on the expression of GAD67 (marker for GABAergic neurons) and EMX-1 (marker for glutamatergic neurons) (Chan CH et al., 2001) in the cortical neuron culture. Fig. 4B-iii shows that treatment of the cultured neurons with lower concentration of *o*-A $\beta$ <sub>42</sub> (5  $\mu$ M) selectively decreased the expression of GAD67 without affecting EMX-1 expression, while higher concentration of *o*-A $\beta$ <sub>42</sub> (10  $\mu$ M) reduced expressions of both GAD67 and EMX-1. *o*-A $\beta$ <sub>42</sub> at lower concentration (5  $\mu$ M) selectively induced apoptosis of GAD67<sup>+</sup> neurons while higher concentration of *o*-A $\beta$ <sub>42</sub> (10  $\mu$ M) also induced apoptosis of other types of neurons (MAP2<sup>+</sup>/GAD67<sup>-</sup>) in both cortical and hippocampal neuron cultures (Fig. 4C). Similar to the effects of *o*-A $\beta$ <sub>42</sub>, A $\beta$ <sub>23-35</sub> peptide, a main toxic part of the full-length A $\beta$  peptides (Mattson MP et al., 1992), also decreased the viability of cultured cortical neurons in a dose dependent manner (data not shown). A $\beta$ <sub>23-35</sub> peptide at low concentration (10  $\mu$ M) selectively decreased GAD67 expression, but without affecting EMX-1 expression, while A $\beta$ <sub>23-35</sub> peptide at higher concentration (50–100  $\mu$ M) reduced expressions of both GAD67 and EMX-1. Accordingly, lower concentration of A $\beta$ <sub>23-35</sub> (10  $\mu$ M) selectively induced apoptosis of GAD6<sup>+</sup> neurons, while higher concentration of A $\beta$ <sub>23-35</sub> (50  $\mu$ M) increased apoptosis of other MAP2<sup>+</sup> neurons in addition to GAD67<sup>+</sup> neurons. These data indicate that GABAergic neurons are relatively more vulnerable to lower concentrations of A $\beta$  than other types of neurons.

### GABAergic neurons in CA1 area of hippocampus express high basal levels of nNOS and nitrosative stress

In AD brains, neurons expressing nNOS are highly susceptible to NFTS and A $\beta$  induced neurodegeneration (Thorns V, Hansen L and Masliah E, 1998). Since nNOS is known to be expressed primarily in specific subtypes of GABAergic neurons (Tricoire L and Vitalis T, 2012), we examined whether GAD67<sup>+</sup> neurons in hippocampal CA1 express nNOS. Figure 5A-i shows that GAD67<sup>+</sup> neurons in *stratum pyramidale* of hippocampal CA1 region in WT mice (6 months old) express high levels of nNOS. In APP<sub>SweDI</sub> mice of the same age (6 months), GAD67<sup>+</sup> neurons also expressed nNOS but the number of GAD67 and nNOS double positive neurons was decreased as the number of GAD67<sup>+</sup> neurons was decreased (Fig. 1B-i). However, APP<sub>SweDI</sub> mice showed increased nNOS expression as shown by Western analysis of hippocampal tissue lysates (Fig. 5A-ii), specifically in pyramidal neurons in hippocampal CA1 (Fig. 5A-i). Similar aberrant expression of nNOS in pyramidal neurons in hippocampus was also reported previously in AD brains (Luth HJ et al., 2000; Tang Z et al., 2013) but underlying mechanism is not understood at present. Interestingly, the nNOS and GAD67 double positive neurons in WT mice and nNOS positive pyramidal neurons in APP<sub>SweDI</sub> mice expressed high N-Tyr immuno-reactivity (Fig. 5A-iii and iv), indicating the basal (GABAergic neurons) or induced (pyramidal neurons) nitrosative stress in those neurons.

Next, we examined whether nNOS<sup>+</sup> GABAergic neurons in culture also express high basal levels of nitrosative stress. Figure 5B-i shows that ~30% of MAP2<sup>+</sup> neurons in culture-expressed nNOS. In addition, figure 5B-ii shows that ~40% of nNOS<sup>+</sup> neurons expressed GAD67<sup>+</sup>. These observations indicate that 12% of MAP2<sup>+</sup> neurons express both nNOS and GAD67 (Fig. 5B-ii). Similar to the observation in *stratum pyramidale* of hippocampal CA1 of WT mice, the nNOS expressing GABAergic neurons in culture also expressed high basal

levels of N-Tyr immunoreactivity (Fig. 5B-iii), thus suggesting that nNOS expressing GABAergic neurons express higher basal levels of nitrosative stress compared to other neurons even under normal conditions.

### Involvement of nitrosative stress in high vulnerability of nNOS expressing GABAergic neurons to A $\beta$ cytotoxicity

We next investigated the involvement of nitrosative stress in A $\beta$ -induced neurotoxicity of nNOS and GAD67 double positive neurons. Fig. 6A-i show that treatment of cultured neurons with selective nNOS inhibitor *N* $\omega$ -propyl-L-arginine (NPLA; 50  $\mu$ M) significantly protected against neuronal death induced by lower concentrations (5 – 7.5  $\mu$ M) of *o*-A $\beta$ <sub>42</sub>. However, NPLA was unable to block loss of neuronal viability induced by higher concentration of *o*-A $\beta$ <sub>42</sub> (10  $\mu$ M) (Fig. 6A-i). These data indicate that nNOS activity participates in neuronal degeneration under conditions of lower load of A $\beta$ . However, inability of nNOS inhibitor in the protection of neurons at higher concentration of *o*-A $\beta$ <sub>42</sub> (10  $\mu$ M) suggests the participation of additional cell death mechanisms at higher A $\beta$  load. Next, the effect of NPLA (50  $\mu$ M) on A $\beta$ -induced GABAergic neuronal death was examined by double-immunofluorescent staining of neuron culture for GAD67 and TUNEL. Figure. 6B-i and ii show that NPLA treatment almost completely inhibited *o*-A $\beta$ <sub>42</sub> (5  $\mu$ M) induced apoptosis of GAD67<sup>+</sup> neurons. As observed by Western analysis (Fig. 6C), NPLA treatment also restored the GAD67 expression decreased by low concentration of *o*-A $\beta$ <sub>42</sub> (5  $\mu$ M). However, NPLA was not able to restore the expressions of GAD67 and EMX1 decreased by higher concentration of *o*-A $\beta$ <sub>42</sub> (10  $\mu$ M) (Fig. 6C). These data document that *o*-A $\beta$ <sub>42</sub> induces apoptosis of GABAergic neurons at lower load of A $\beta$  is mediated via nNOS-dependent pathway. However, additional mechanisms of cell death pathways may participate at high load of A $\beta$  during the late stages of AD.

Since nNOS expressing GABAergic neurons also express high levels of cellular N-Tyr (Fig. 5B-iii) and are highly susceptible to A $\beta$ -induced neurotoxicity (Fig. 4C), we next assessed whether ONOO<sup>-</sup> and A $\beta$  cooperatively induce neuronal death. For it, the cultured rat neurons were treated with subtoxic concentrations of *o*-A $\beta$ <sub>42</sub> and 3-morpholiniosydnonimine (SIN-1). SIN-1 is known to generate stoichiometric amounts of NO and O<sub>2</sub><sup>-</sup> and thus ONOO<sup>-</sup> (Hogg N et al., 1992). Figure 6D-ii shows that combination of subtoxic concentrations of SIN-1 (200  $\mu$ M; Fig. 6D-i) and *o*-A $\beta$ <sub>42</sub> (1  $\mu$ M; Fig. 4B-ii), which did not induce cell death individually, synergistically reduced cell viability (Fig. 6B-ii). Consistent with this data, combination of subtoxic concentrations of SIN-1 and *o*-A $\beta$ <sub>42</sub> also induced p35 cleavage to p25, a critical event in aberrant Cdk5 activation, and activations of GSK3 $\beta$  (dephosphorylation of Ser<sup>9</sup>) and caspase 3 (cleaved caspase 3) which are involved in neuronal degeneration under AD conditions (Fig. 5D-iii) (Camins A et al., 2006; Pei JJ et al., 2008; Snigdha S et al., 2012). These data document that nNOS expressing GABAergic neurons are highly vulnerable to A $\beta$ -induced toxicity due to nitrosative stress induced mechanisms.

## Discussion

GABAergic neurons play critical roles in learning and memory (Andrews-Zwilling Y, Gillespie AK, Kravitz AV, Nelson AB, Devidze N, Lo I, Yoon SY, Bien-Ly N, Ring K, Zwilling D, Potter GB, Rubenstein JL, Kreitzer AC and Huang Y, 2012; Meredith RM, Floyer-Lea AM and Paulsen O, 2003; Shinoda Y, Sadakata T, Nakao K, Katoh-Semba R, Kinameri E, Furuya A, Yanagawa Y, Hirase H and Furuichi T, 2011), neuropsychiatric behaviors (mood and anxiety states) (Luscher B, Shen Q and Sahir N, 2011; Nuss P, 2015; Shiah IS and Yatham LN, 1998; Taylor SF and Tso IF, 2015), and suppression of seizure activity (Cohen I et al., 2002; Knopp A, Kivi A, Wozny C, Heinemann U and Behr J, 2005; Wozny C, Kivi A, Lehmann TN, Dehnicke C, Heinemann U and Behr J, 2003). Patients with AD frequently exhibit GABAergic dysfunction (Lanctot KL, Herrmann N, Mazzotta P, Khan LR and Ingber N, 2004) along with neuropsychiatric symptoms (Egashira N et al., 2005; Kaiser NC, Liang LJ, Melrose RJ, Wilkins SS, Sultzer DL and Mendez MF, 2014; Modrego PJ, 2010; Romano A, Pace L, Tempesta B, Lavecchia AM, Macheda T, Bedse G, Petrella A, Cifani C, Serviddio G, Vendemiale G, Gaetani S and Cassano T, 2015) as well as late onset seizures before and after the onset of cognitive deficits (Born HA, 2015; Mendez M and Lim G, 2003; Romanelli MF et al., 1990; Vossel KA, Beagle AJ, Rabinovici GD, Shu H, Lee SE, Naasan G, Hegde M, Cornes SB, Henry ML, Nelson AB, Seeley WW, Geschwind MD, Gorno-Tempini ML, Shih T, Kirsch HE, Garcia PA, Miller BL and Mucke L, 2013). However, answers to when, where, and how GABAergic neurons degenerate under AD conditions and what is the impact of GABAergic neuronal loss on cognitive deficits and neuropsychiatric symptoms in AD are still elusive.

In this study, we report that number of neurons expressing GAD67 in CA1 hippocampus of APP<sub>SweDI</sub> mice decreases at early stages of disease with relatively lower A $\beta$  load in advance to the loss of pyramidal neurons (Fig. 1). In the CNS, GAD67 is known to provide more than 90% of GABA (Asada H et al., 1997), thus suggesting that the loss of GAD67 immunoreactivity and expression may directly induce GABAergic hypo-function in the CA1 of hippocampus. GAD67<sup>+</sup> neuronal loss accompanies with the loss of spatial learning and memory as well as increased anxiety-like behaviors (Fig. 2) and increased vulnerability to KA-induced seizures in APP<sub>SweDI</sub> mice (Fig. 3). In primary cultured neurons, GAD67<sup>+</sup> neurons are highly vulnerable to A $\beta$ -induced cytotoxicity than other types of neurons (Fig. 4). GAD67<sup>+</sup> neurons in primary neuron cultures as well as in hippocampal CA1 region express high basal levels of nNOS and nitrosative stress (Fig. 5) and treatment of those neurons with pharmacological nNOS inhibitor protects against A $\beta$ -induced apoptosis of GAD67<sup>+</sup> neurons, while ONOO<sup>-</sup> donor treatment enhances A $\beta$ -induced apoptosis of other types of neurons (Fig. 6). These observations suggest the potential role of nNOS-mediated nitrosative stress in early development of GABAergic hypo-function (e.g. decrease in GAD67 expression and/or degeneration of GAD67<sup>+</sup> neurons) under early AD condition.

In this study, we observed early loss of GAD67<sup>+</sup> and nNOS<sup>+</sup> neurons, in advance to the loss of pyramidal neurons, in *stratum pyramidale* of CA1 hippocampus of 6 months old APP<sub>SweDI</sub> mice (Fig. 1B). In addition, the loss of GAD67<sup>+</sup> and nNOS<sup>+</sup> neurons accompanies with the increased cognitive deficits and neuropsychiatric-like symptoms (Figs. 2 and 3). The role of GAD67<sup>+</sup> and nNOS<sup>+</sup> neurons in regulation of cognitive function and

neuropsychiatric behaviors under conditions of AD is not well understood at present; therefore, it is not clear whether the degeneration of these neurons causes the observed cognitive deficits and neuropsychiatric-like deficits APP<sub>SweDI</sub> mice. However, the reported efficacies of GABAergic agonists, such as lorazepam and carbamazepine, against behavioral and psychiatric symptoms of dementia (Anton RF et al., 1986; Gleason RP and Schneider LS, 1990; Lanctot KL, Herrmann N, Mazzotta P, Khan LR and Ingber N, 2004; Lemke MR, 1995; Olin JT et al., 2001; Tariot PN et al., 1994; Tariot PN et al., 1999) support the importance of GABAergic neuronal protection for controlling cognitive, neurological, and psychiatric symptoms in early AD conditions.

Hippocampal GABAergic neurons play important roles for modulation of seizure activities (Cohen I, Navarro V, Clemenceau S, Baulac M and Miles R, 2002; Knopp A, Kivi A, Wozny C, Heinemann U and Behr J, 2005; Orban-Kis K et al., 2015; Wozny C, Kivi A, Lehmann TN, Dehnicke C, Heinemann U and Behr J, 2003). Therefore, the loss of hippocampal GABAergic transmission by decreased GAD67 expression or GABAergic neuronal degeneration may increase incidence of seizures in AD patients. AD patients are reported to have increased risk for late-onset seizures and neuronal network abnormalities (Born HA, 2015; Mendez M and Lim G, 2003; Romanelli MF, Morris JC, Ashkin K and Coben LA, 1990; Vossel KA, Beagle AJ, Rabinovici GD, Shu H, Lee SE, Naasan G, Hegde M, Cornes SB, Henry ML, Nelson AB, Seeley WW, Geschwind MD, Gorno-Tempini ML, Shih T, Kirsch HE, Garcia PA, Miller BL and Mucke L, 2013). Accordingly, this study also documents that increased seizure susceptibility of APP<sub>SweDI</sub> mice to KA treatment at 6-month age accompanies with decreased number of GAD67<sup>+</sup> neurons in hippocampal CA1 region as well as decreased expression of GAD67 protein in CA1 hippocampus (Fig. 3A). In addition to the loss of GAD67<sup>+</sup> cells, we also observed increased expression of nNOS and nitrosative stress in pyramidal neurons in 6-month-old APP<sub>SweDI</sub> mice (Fig. 5A). At present, the role of increased nNOS expression and nitrosative stress in pyramidal neurons in KA-induced seizure activity is not understood. Previous studies reported that NO produced by nNOS promotes initiation of seizure-like events in hippocampus and entorhinal cortex slice cultures (Kovacs R et al., 2009). Therefore, loss of GABAergic neurons as well as increased nNOS expression and nitrosative stress in pyramidal neurons may participate in increased seizure susceptibility of APP<sub>SweDI</sub> mice. Interestingly, APP<sub>SweDI</sub> mice exhibited more post-seizure damage in the hippocampus than WT mice (Fig. 3B). Recent studies reported that an estimated 10 to 22 percent of AD patients develop late-onset seizures and those who develop seizures typically have more severe symptoms and rapid disease progression (Vossel KA, Beagle AJ, Rabinovici GD, Shu H, Lee SE, Naasan G, Hegde M, Cornes SB, Henry ML, Nelson AB, Seeley WW, Geschwind MD, Gorno-Tempini ML, Shih T, Kirsch HE, Garcia PA, Miller BL and Mucke L, 2013). Taken together, these studies document that the loss of hippocampal GABAergic neurons and thus increased seizure susceptibility may influence disease course and thus severity of AD. Therefore, protection of hippocampal GABAergic neurons may be important in modulation of seizure-associated worse outcome in AD.

Under physiological conditions, nNOS catalyzes the oxidation of L-arginine resulting in the formation of L-citrulline and NO (coupled nNOS reaction). However, when the levels of its cofactor (tetrahydrobiopterin) and substrate (L-arginine) are low, nNOS produces relatively higher amounts of O<sub>2</sub><sup>-</sup> (uncoupled nNOS reaction) (Pall ML, 2007; Xia Y, Dawson VL,

Dawson TM, Snyder SH and Zweier JL, 1996). Under such conditions, the nNOS produced  $O_2^-$  readily reacts with NO to form ONOO<sup>-</sup>, which in turn causes cellular and tissue nitrosative damage (Koppenol WH, 2001; Pacher P et al., 2007). In this study, we observed that nNOS expressing GAD67<sup>+</sup> neurons have higher intrinsic nitrosative stress compared to other types of cells in CA1 of hippocampus as well as in neuron culture (Fig. 5) and the number of these neurons was greatly reduced in APP<sub>SweDI</sub> mice as well as in neuron culture treated with A $\beta$ . In addition, the observed inhibitory effect of nNOS inhibitor on A $\beta$ -induced GABAergic neuronal death as compared to synergistic effect of ONOO<sup>-</sup> donor on A $\beta$ -induced death of other types of neurons suggests that nitrosative stress, especially in nNOS expressing GABAergic neurons, may participate in increased susceptibility to A $\beta$ -induced neuronal death. These data further postulate that nNOS expressing GABAergic neurons in the hippocampus degenerate at earlier stages of AD with relatively lower A $\beta$  load due to their high intrinsic nitrosative stress.

In summary, the present study documents that APP<sub>SweDI</sub> mice exhibit selective loss of nNOS expressing GABAergic neurons in CA1 *stratum pyramidale* in advance to the loss of pyramidal neurons during early disease development. The nNOS expressing GABAergic neurons were highly susceptible to A $\beta$  probably due to their intrinsic high basal levels of nitrosative stress. The loss of GABAergic neurons is accompanied with development of cognitive deficits and neuropsychiatric-like symptoms as well as increased seizure susceptibility in APP<sub>SweDI</sub> mice. GABAergic neurons are known to play critical roles in learning and memory, neuropsychiatric behaviors (mood and anxiety states), and suppression of seizure activity. Therefore, protection of nNOS expressing GABAergic neurons by drugs targeting these early stages of the disease may potentially alleviate cognitive, neurological, and psychiatric symptoms at the early stages of disease.

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## Abbreviations

<b>A<math>\beta</math></b>	amyloid- $\beta$
<b>AD</b>	Alzheimer's disease
<b>APP<sub>SweDI</sub></b>	human amyloid precursor protein Swedish-Deutch-Iowa mutant
<b>GABA</b>	$\gamma$ -aminobutyric acid
<b>KA</b>	kainic acid
<b>N-Tyr</b>	nitrotyrosine
<b>nNOS</b>	neuronal nitric oxide synthase
<b>NO</b>	nitric oxide
<b>NPLA</b>	N $\omega$ -Propyl-L-arginine hydrochloride

<b>NRGN</b>	neurogranin
<b>O<sub>2</sub><sup>•-</sup></b>	superoxide anion
<b>ONOO<sup>-</sup></b>	peroxynitrite
<b>RLD</b>	repeated low dose
<b>SIN-1</b>	3-morpholinostyridone
<b>WT</b>	wild type

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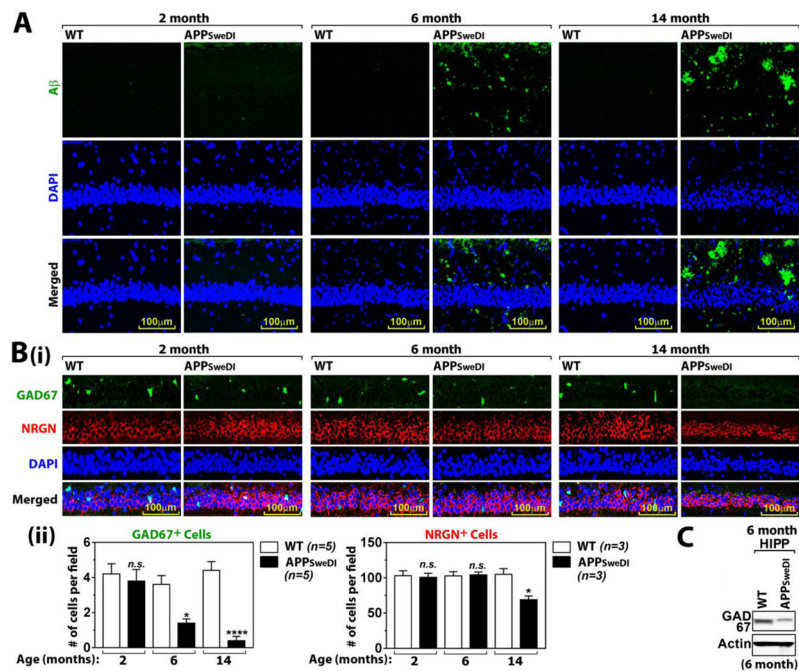
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### Highlights

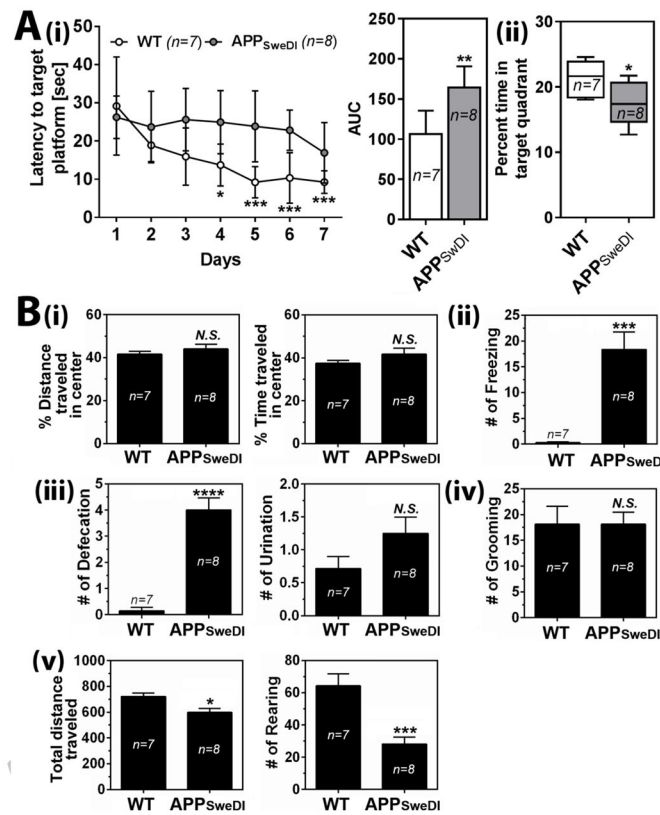
- APP<sub>SwDI</sub> transgenic mice develop early loss of GAD67 expression in CA1 of hippocampus.
- GAD67<sup>+</sup> neurons exhibit high basal expression of nNOS and nitrosative stress.
- These neurons are highly vulnerable to A $\beta$  due to the high intrinsic nitrosative stress.
- The loss of GAD67<sup>+</sup> neurons correlates with cognitive and neuropsychiatric deficits.
- These suggest a role of nitrosative stress in of Alzheimer's GABAergic pathology.



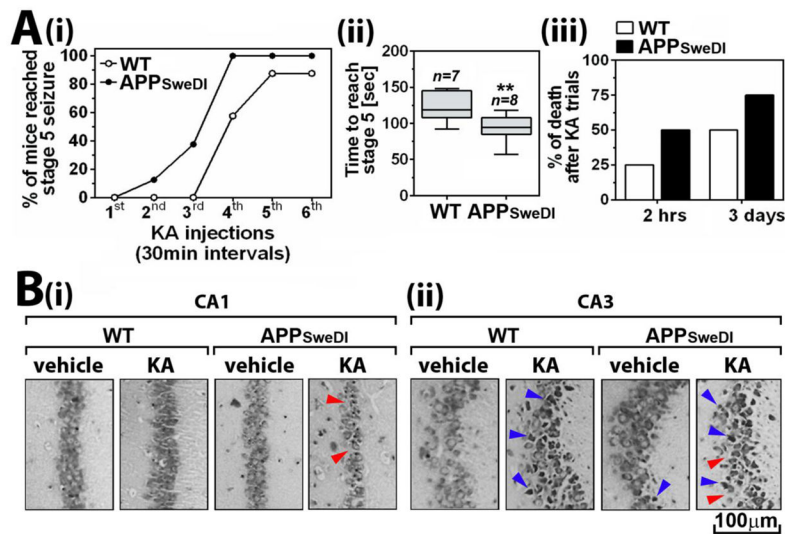
**Fig. 1. Age dependent A $\beta$  accumulation and degeneration of GABAergic neurons in hippocampus**

**A.** A $\beta_{42}$  accumulation in CA1 of Hippocampus of WT and APP<sub>SweDI</sub> mice (2, 6, and 14 months old) were analyzed by immunofluorescent staining with BC05 antibody. DAPI was used for staining for nucleus. **B.** Age dependent loss of GABAergic neurons (GAD67<sup>+</sup> cells) or pyramidal neurons (neurogranin/NRGN) were investigated (**i**). The graph shows the # of GABAergic or pyramidal neurons (**ii**). **C.** The expression of GAD67 in hippocampus (HIPP) was analyzed by Western blot. All columns are means of individual data and T-bars are standard error mean: n.s. P > 0.05. \*, P 0.05. \*\*\*\*. P 0.0001 as compared to WT mice.



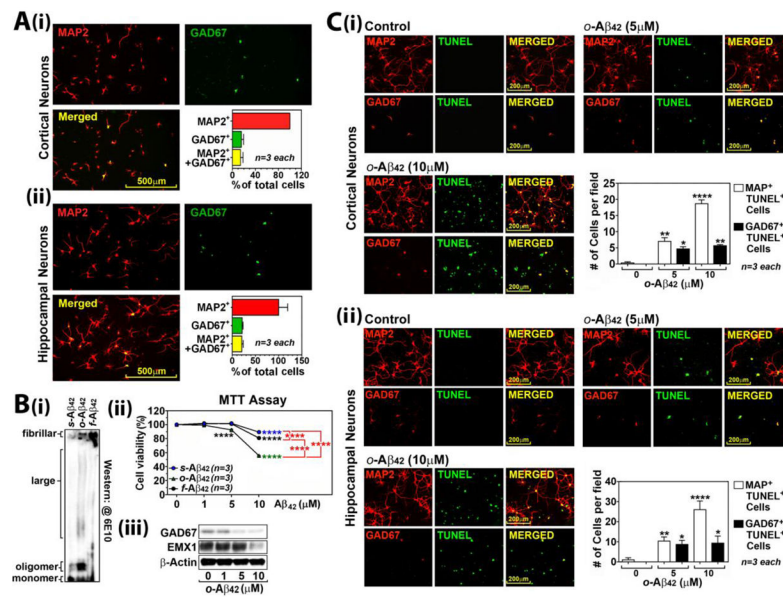


**Fig. 2. Cognitive deficits and neuropsychiatric-like symptoms in APP<sub>SweDI</sub> mice (6 months old)**  
**A.** Spatial learning (latency to invisible target platform) and memory (% time to spent in target quadrant without platform) functions were analyzed by Morris water maze test. In left panel of **A-i**, open (WT mice) and solid (APP<sub>SweDI</sub> mice) circles represent mean and T-bars represent standard error means. In right panel of **A-i**, the area under the curve (AUC) of the overall learning was calculated and represented by bar graph. In panel **A-ii**, columns show 75% of distribution; horizontal bar in each column is median; and vertical T-bars are minimum and maximum values of the data. **B.** Open field test was test for anxiety-like behaviors, such as distance/time travelled in the center area (**i**), number of freezing (**ii**), number of defecation and urination (**iii**), number of grooming (**iv**), and total travelled distance and number of rearing (**v**). All columns are means of individual data and T-bars are standard error mean: n.s. P > 0.05. \* P 0.05. \*\* P 0.01. \*\*\* P 0.001. \*\*\*\* P 0.0001 as compared to WT mice.



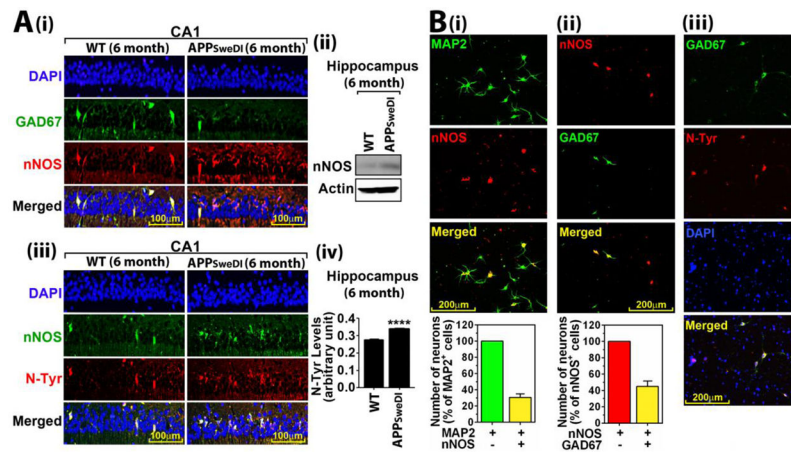
**Fig. 3. Altered seizure susceptibility of APP<sup>SweDI</sup> mice (6 months old)**

**A.** Seizure responses of mice to KA was assessed for analysis of seizure vulnerability of the mice. The mice were treated with repeated low dose of KA (5mg/kg; 30 min intervals) till each mouse reached stage 5 Racine scale. The number of KA treatment (**i**) and time to reach stage 5 seizure (**ii**) and death of mice following the KA treatment (**iii**) were analyzed. The columns in panel **A-ii** show 75% of distribution; horizontal bar in each column is median; and vertical T-bars are minimum and maximum values of the data: \*\*,  $P < 0.01$  as compared to WT mice. **B.** Three days of KA treatment, hippocampal morphology and pyramidal neuronal status in CA1 (**i**) and CA3 (**ii**) regions were assessed by Nissle staining. Red and blue arrow heads indicate neuronal loss (empty areas) and degenerating neurons (dark Nissl bodies), respectively.



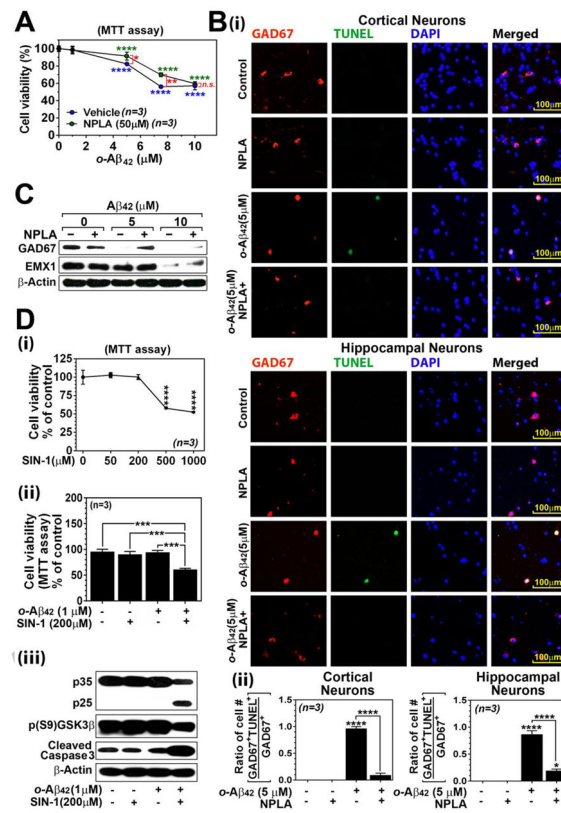
**Fig. 4. Higher vulnerability of GABAergic neurons than glutamatergic neurons to Aβ-induced cytotoxicity**

**A.** Primary rat cortical and hippocampal neurons were stained for MAP2 (pan neuronal marker) and GAD67 (GABAergic neuronal marker) (i) for quantification of GABAergic neurons in culture (ii). **B.** Soluble (*s*-), oligomeric (*o*-), and fibrillary (*f*-) forms of Aβ<sub>42</sub> were generated in vitro and characterized by Western blot (i) and their neurotoxicity was assessed in primary cultured cortical neurons by MTT assay (ii). The cortical neuron cultures were treated with *o*-Aβ<sub>42</sub> peptide and expressions of GAD67 vs. EMX1 were analyzed by Western analysis (iii). **C.** Apoptotic death of GABAergic neurons (TUNEL assay) was also analyzed by double immunostaining for MAP2 (pan neuronal marker) and TUNEL or GAD67 (GABAergic neuronal marker) and TUNEL in primary cultured cortical (i) and hippocampal neurons (ii). All columns and lines are means of individual data and T-bars are standard error mean: \* P 0.05. \*\* P 0.01. \*\*\* P 0.001. \*\*\*\* P 0.0001.



**Fig. 5. Expression of nNOS and nitrotyrosine in GABAergic neurons**

**A.** Expression of nNOS in GABAergic and pyramidal neurons (**i** and **ii**) and its co-localization with nitrotyrosine (N-Tyr) were analyzed by immuno-fluorescent staining of CA1 regions of hippocampus in 6 months old WT and APP<sup>SweDI</sup> mice (**iii**). The levels of N-Tyr were also measured in hippocampus by ELISA (**iv**). **B.** Presence of nNOS<sup>+</sup> GABAergic (GAD67<sup>+</sup>) neurons and their co-localization with N-Tyr were analyzed in primary cultured rat cortical neurons. All columns are means of individual data (n=3) and T-bars are standard error mean: \*\*\*\*, P < 0.0001.



**Fig. 6. Role of nitrosative stress in A $\beta$ -induced GABAergic neuronal loss**

Effect of nNOS inhibitor, *N* $\omega$ -propyl-L-arginine (NPLA; 20 $\mu$ M), on  $\alpha$ -A $\beta_{42}$  induced loss of neuronal viability (MTT assay) (**A**), apoptosis of GABAergic neurons (TUNEL assay) (**B**), and decreased protein levels of GAD67 and EMX-1 (**C**) were analyzed in primary rat cortical and hippocampal neuron cultures. Loss of cell viability (MTT assay) by serial concentrations of 3-morpholinosydnonimine (SIN-1, a donor of ONOO<sup>-</sup>) (**D-i**), effect of subtoxic concentrations of SIN-1 and  $\alpha$ -A $\beta_{42}$  on cell viability (**D-ii**), p35 cleavage to p25, GSK3 $\beta$  phosphorylation (Ser<sup>9</sup>), active (cleaved) caspase 3 were analyzed (**D-iii**). All columns or lines are means of individual data and T-bars are standard error mean: n.s. P > 0.05. \*. P 0.05. \*\*. P 0.01. \*\*\*. P 0.001. \*\*\*\*. P 0.0001 as compared to WT mice.