Abstract. Synaptophysin is a synaptic vesicle glycoprotein involved in the regulation process for neurotransmitter release, which is distributed throughout neuroendocrine cells and all neurons in the brain and spinal cord. In an effort to determine whether amyloid β (Aβ)-42 peptides could influence the quantity and biochemical properties of synaptophysin, alterations in the levels of the synaptophysin protein in various soluble fractions were detected in the brains of four genotypes of transgenic mice (Tg) including Non-Tg, neuron-specific enolase (NSE)-hPS2m, NSE-hAPPsw and hAPPsw/hPS2m double Tg mice. Among the four genotypes of Tg mice, the highest levels of Aβ-42 peptides were noted in hAPPsw/hPS2m, followed by NSE-hAPPsw, NSE-hPS2m and Non-Tg mice. In the brains of these mice displaying different levels of Aβ-42 peptides, the levels of soluble synaptophysin were reduced significantly only in the hAPPsw/hPS2m double Tg mice compared to the Non-Tg mice. However, immuno-histochemical analysis revealed no differences in the levels of total synaptophysin protein between the neocortex and hippocampus of the four different genotypes of mice. Western blot analysis using four-step fractions with differing solubility revealed a marked decrease in synaptophysin levels in the Tris-buffer saline fraction of hAPPsw/hPS2m double Tg mice and a significant increase in the formic acid fraction, relative to the Non-Tg mice. The results obtained from our in vivo experiments in mice are identical to the results observed in SK-N-MC cells treated with 100 nM Aβ-42 peptides. Therefore, our experiments collectively suggest that Aβ-42 peptides may alter the solubility without changing the total amount of synaptophysin.

Introduction

Synaptophysin is a 38-kDa calcium-binding glycoprotein which is distributed throughout the neuronal synaptic vesicles and certain endocrine cells (1-3). Synaptophysin is a valuable marker protein for synaptic vesicles and terminals at the microscopic level. Functionally, it appears to play a crucial role in exocytosis, facilitating the fusion of synaptic vesicles to the presynaptic membrane during neurotransmitter release (4,5). Levels of synaptophysin protein are enhanced during synaptogenesis (6,7), and are thus recognized as markers for synaptic re-organization following denervation (8,9). In particular, in the auditory system, synaptophysin is present in the synaptic terminals of the efferent fibers projecting to the cochlea and sensory axons, and their putative termination in the central nervous system (8,10).

Synaptophysin immunoreactivity (SYN-IR) has been previously reported in Tg mice with Alzheimer's disease (AD). Reduced, increased, or unchanged immunoreactivity has been described, depending on the mouse strain, transgene type, and age. An early study reported that 8-month-old platelet-derived growth factor promoter expressing amyloid precursor protein (PDAPP) Tg mice harboring a human β-amyloid precursor protein (hAPP) V717F gene under the control of the platelet-derived growth factor B promoter displayed significantly reduced SYN-IR within the dentate gyrus molecular layer (11). In another study, APPind H6 and wild-type hAPP Tg mice also displayed reduced SYN-IR in the dentate gyrus, despite displaying greater brain Aβ deposition (12). However,
a subsequent study conducted with PDAPP heterozygous Tg mice aged 3-4, 6-7, and 10-12 months reported elevated SYN-IR within the cerebral cortex (13). Other studies using AD Tg mice displaying Aβ deposition and neuritic plaques have detected no alterations in SYN-IR. Irizarry et al (14) observed that SYN-IR expression remained unaltered in 18-month PDAPP Tg mice. A study conducted with 16-month-old Tg2576 mice also showed that SYN-IR was unaltered in the dentate gyrus, CA1, cingulate cortex, entorhinal cortex, and thalamus (15). Twelve-month-old hAPPsw-human Presenilin 1 (hPS1) Tg mice displayed no loss of SYN-IR in the frontal neocortex or dentate gyrus, although the hAPPsw+hPS1 double Tg mice exhibited a greater amyloid deposition than the single APPsw or hPS1 Tg mice (16).

Therefore, resolution of this disparity requires investigations of the changes in the biochemical properties of synaptophysin induced by Aβ-42 peptides. In this study, the synaptophysin levels were determined in four different soluble fractions collected from the brains of AD mouse models, using a specific antibody. The results are consistent with the notion that the deposition of Aβ-42 peptide may be strongly correlated with the solubility of synaptophysin in the cortex and hippocampus in the AD-affected brain.

Materials and methods

Animal care and use. All animal experimental procedures employed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea FDA and Pusan National University. All mice were supplied by the breeding center of a Korea FDA facility and were handled in a Korea FDA accredited animal facility in accordance with the AAALAC International Animal Care policy (Accredited Unit, Korea Food and Drug Administration: Unit no. 000996) and PNU-Laboratory Animal Resources Center. The mice were housed in cages under a strict light cycle (lights on at 06:00 h and off at 18:00 h) at 23±1˚C and a relative humidity of 50%. Additionally, all mice were given a standard irradiated chow diet (Purina Mills Inc., USA) ad libitum and maintained in a specified pathogen-free state.

Production and identification of hAPPsw/hPS2m double Tg mice. hAPPsw/hPS2m double Tg mice were generated from male NSE-hAPPsw and female NSE-hPS2m Tg mice. The NSE-hAPPsw Tg mice were developed via the insertion of the hAPPsw gene under the control of an NSE promoter. These Tg mice display the neuropathological phenotype typical of AD, which includes behavioral dysfunction, Aβ-42 deposition, elevated Tau phosphorylation and Cox-2 induction at 12 months of age (17). The NSE-hPS2m Tg mice also express human mutant Presenilin 2 (hPS2, N141I), under the control of the NSE promoter (18). These Tg mice exhibit behavioral dysfunction, Aβ-42 deposition, and caspase-3 and Cox-2 induction at 12 months of age.

Three weeks after birth, offspring with four different genotypes, hAPPsw/hPS2m double, single NSE/hAPPsw, NSE/hPS2m and Non-Tg, were identified via polymerase chain reaction (PCR) analysis using APP and PS2-specific primers. The primer sequences were as follows: APP-specific primers, sense, 5'-CACGATGACGCTGATCATGATG-3'; and PS2-specific primers, sense, 5'-TCTAGATGCCTGATTCGTG-3' and antisense, 5'-GAGGAAAGATGATGATGAG-3'. The PCR consisted of 25 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 62˚C, and extension for 45 sec at 72˚C. The amplified PCR products were loaded on a 1.0% agarose gel in a buffer containing 30% glycerol, 0.25% bromophenol blue (BBP), and 0.25% xylene cyanol (XC), and electrophoresed. The resolved bands were visualized with ethidium bromide (EtBr).

Preparation of total soluble protein from mouse brain. Frozen brain tissues were chopped with scissors and homogenized in lysis solution [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% sodium dodecyl sulfate (SDS), 1% Igepal-630, 0.5% sodium fluoride, 0.5% sodium deoxycholate, 0.5 mM EDTA, 0.1 mM EGTA and proteinase inhibitor] using a glass homogenizer. The soluble homogenate was separated from the total lysate mixture via 15 min of centrifugation at 15,000 rpm at 4˚C. The supernatant was subsequently collected and employed for Western blot analysis as described below.

Preparation of solubility-based fractionation from mouse brains. The frozen brain tissues were sequentially extracted as previously described (19). At each step, sonication [40 sec at an amplitude of 20 using a Vibra Cell, VC130; Sonics & Materials, USA] in an appropriate buffer was followed by 1 h of centrifugation at 100,000 x g at 4˚C. The supernatant was removed and the pellet was sonicated in the next solution in the sequential extraction process. For the four-step extraction, the sonication of frozen brain tissue samples (100 mg) commenced in TBS (20 mM Tris and 137 mM NaCl, pH 7.6) containing protease inhibitors (protease inhibitor cocktail; Sigma-Aldrich, USA) (extraction step 1). The next three sequential extraction steps used 1% Triton X-100 in TBS with protease inhibitors (extraction step 2), 2% aqueous SDS with the same protease inhibitors (extraction step 3), and 70% formic acid in water (extraction step 4). Each fraction was then stored at -80˚C until further analysis.

Western and slot blot analyses. For Western blot analysis, protein prepared from the brain was separated via gradient (4-20%) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h. The resolved proteins were then transferred to a nitrocellulose membrane for 2 h at 40 V. Each membrane was separately incubated with a 1:1,000 dilution of primary antibody to Aβ-42 (Chemicon, USA), synaptophysin (Sigma-Aldrich), or actin (Sigma-Aldrich) overnight at 4˚C. The membranes were then washed in washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4 and 0.05% Tween-20) and incubated with a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Zymed, USA) at room temperature for 2 h. Blots were developed with a Chemiluminescence Reagent Plus kit (Amersham Pharmacia Biotech, USA). For the slot blot analysis, proteins prepared from the mouse brain samples were transferred to a nitrocellulose membrane using a Slot Blot kit (Amersham Pharmacia Biotech). Anti-Aβ-42 was applied in accordance with the manufacturer's recommendations to specifically detect Aβ-42 on a slot blot. Finally, each blot was developed
using Nitro Blue tetrazolium chloride (NBT) with 5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt (BCIP) as the substrate.

**Immunohistochemistry.** Sixteen 8-month-old mice were used for synaptophysin immunostaining analysis: four hAPPsw+hPS2m, four NSE-hAPPsw, four NSE-hPS2m, and four Non-Tg mice. These mice were perfused as previously described (20). After perfusion, the brain tissue was fixed for 12 h in 5% formalin at 4°C, and then sequentially transferred to sucrose solutions of 10, 20 and 30%. Sections (20 µm) were prepared and pretreated for 1 h at room temperature with PBS-blocking buffer containing 10% goat serum. Each section was separately incubated with a 1:100 dilution of primary rabbit polyclonal anti-synaptophysin antibody (Sigma-Aldrich) in blocking buffer overnight at 4°C. The sections were washed in washing buffer, then incubated with a 1:2,500 dilution of the secondary antibody (HRP-conjugated goat anti-rabbit IgG; Zymed) for 2 h at room temperature. Synaptophysin protein in the brain tissue was detected by the development of color using stable 3,3'-diaminobenzidine (DAB) (Invitrogen, USA).

**Cell culture and Aβ-42 peptide treatment.** SK-N-MC human neuroepithelial cells were maintained for 24-36 h in Dulbecco’s modified Eagle’s medium (Gibco-BRL, USA) containing 10% fetal bovine serum, 1% non-essential amino acids, 2 ml L-glutamine, 100 IU/ml penicillin and 100 µg/ml of streptomycin. Firstly, the Aβ-42 peptide (Sigma-Aldrich) was prepared by dissolving lyophilized Aβ-42 in dimethyl-sulfoxide at a concentration of 100 µM and these peptides in solution were aggregated for 24 h at 37°C. In order to treat the Aβ-42 peptides into cells, SK-N-MC cells were seeded at a density of 2x10⁴ cells per 100-mm-diameter dish. After reaching 80-90% confluence, aggregated Aβ-42 peptide was added at 10 or 100 nM, and the cells were incubated for an additional 24 h at 37°C (21). Protein samples were prepared from these cells using the afore-mentioned method for protein extraction from the mouse brains.

**Statistical analysis.** One-way ANOVA was used to determine whether significant differences existed between the Non-Tg and Tg groups (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). Additionally, differences in the responses of single Tg (NSE-hAPPsw and NSE-hPS2m) and hAPPsw+hPS2m double Tg mice were evaluated via a post-hoc test (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL) of the variance and significance levels. A total of seven founder mice were identified as Tg mice; three hAPPsw+hPS2m single Tg (#2789, #2790, and #2792), an NSE-hAPPsw single Tg (Fig. 1A), two NSE-hPS2m single Tg mice (#2787 and 2788), and a Non-Tg mouse (#2793) (Fig. 1A). These results reflect the successful acquisition of the four different genotypes, and demonstrate that the NSE-hAPPsw and NSE-hPS2m Tg mice successfully transferred the transgene into their progenies in a Mendelian inheritance manner.

**Results**

**Identification of Tg mice.** The Aβ-42 peptide level in the brains of different mouse AD models can differ depending on the inserted gene and the physiological condition of the mice (17,18). Prior to investigating whether the Aβ-42 peptide could induce alterations of synaptophysin under in vivo conditions, appropriate animal models harboring different levels of Aβ-42 peptide in the brain were obtained via mating. For this mating, two AD models including NSE-hAPPsw Tg mice and NSE-hPS2m Tg mice were selected from five AD models previously developed in our laboratory (17,18). In order to identify the four different genotypes in the offspring produced from the mating between the male NSE-hAPPsw Tg mice and female NSE-hPS2m Tg mice, DNA-PCR analysis using the genomic DNA isolated from the tail of the 4-week founder mice was performed. Following electrophoresis of the PCR products, the hPS2m- and hAPPsw-specific PCR products were detected on agarose gels as bands of 442 and 509-bp, respectively. A total of seven founder mice were identified as Tg mice; three hAPPsw+hPS2m double Tg mice (#2789, #2790, and #2792), an NSE-hAPPsw single Tg (#2791) mouse, two NSE-hPS2m single Tg mice (#2787 and 2788), and a Non-Tg mouse (#2793) (Fig. 1A). These results reflect the successful acquisition of the four different genotypes, and demonstrate that the NSE-hAPPsw and NSE-hPS2m Tg mice successfully transferred the transgene into their progenies in a Mendelian inheritance manner.

**Deposition of Aβ-42 peptides in the brains of four different mouse genotypes.** To compare the deposition of Aβ-42
peptides in the brains of four different mouse genotypes, the levels of brain Aβ-42 peptides were quantified at 8 months of age via Western blot and slot blot analyses. As demonstrated in Fig. 2B, the levels of Aβ-42 peptides were significantly greater in the brains of the single NSE-hAPPsw and NSE-hPS2m Tg mice relative to those of the Non-Tg mice. Additionally, the levels of Aβ-42 peptides in the brains of the hAPPsw+hPS2m double Tg mice were 2- to 3-fold greater than those detected in the brains of the single NSE-hAPPsw and NSE-hPS2m Tg mice. However, no differences in the Aβ-42 peptide levels were observed between the brains of the single NSE-hAPPsw and NSE-hPS2m Tg mice (Fig. 1B). These observations in the four mouse genotypes may reflect different levels of Aβ-42 peptides in their brains. In particular, the hAPPsw+hPS2m double Tg mice displayed the highest levels of Aβ-42 peptides among the four mouse genotypes, respectively.

Effects of Aβ-42 peptides on the level of soluble and total synaptophysin. In order to determine whether the Aβ-42 peptides, which are known to induce cortical and hippocampal disorganization, would affect synaptophysin level, the soluble protein and total protein levels of synaptophysin were measured via Western blot and immunohistochemical analyses in order to detect the soluble form of the specific protein, as well as the localization and distribution of the total protein, respectively. The level of the soluble form of synaptophysin was significantly lower in hAPPsw+hPS2m double Tg mice relative to the single and Non-Tg littermate mice. By way of contrast, no significant changes were noted in the levels of synaptophysin expression between the brains of the single and Non-Tg mice, although the level in the single Tg mice was increased slightly relative to the Non-Tg mice (Fig. 2A). However, in immunohistochemical analysis, the immunoreactivity of total synaptophysin within the cortex and hippocampus regions of the brains between the double hAPPsw+hPS2m and control Tg mice (NSE-hAPPsw, NSE-hPS2m, and Non-Tg mice) did not differ (Fig. 2B). Therefore, these results suggest that the biochemical properties, such as the solubility of synaptophysin, may be altered in the brains of double hAPPsw+hPS2m Tg mice with high levels of deposited Aβ-42 peptides, although the amount of total synaptophysin proteins remains uniform.

Effects of Aβ-42 peptides on the solubility of synaptophysin. The next experiment addressed the hypothesis that Aβ-42 deposition in the brains of mouse models of AD induces alterations in the biochemical properties of synaptophysin without affecting its quantity. To test this hypothesis, the four soluble synaptophysin fractions were extracted from the brains of the progeny of the four mouse genotypes, and their levels were ascertained via Western blot analysis. In the TBS fraction harboring highly soluble proteins, synaptophysin levels in the hAPPsw+hPS2m double Tg mice was markedly reduced as compared with the single NSE-hAPPsw, NSE-hPS2m, and Non-Tg mice (Fig. 3A). However, the level of synaptophysin in the 70% formic acid fraction, which harbored poorly soluble proteins, was higher in the brains of the hAPPsw+hPS2m double Tg mice than in the single NSE-hAPPsw, NSE-hPS2m, and Non-Tg mice (Fig. 3A). By way of contrast, we detected no significant differences between the hAPPsw+hPS2m double Tg mice and the control mice with regard to synaptophysin levels from the Triton X-100- and SDS-soluble fractions containing proteins of intermediate solubility (Fig. 3). These results are consistent with the suggestion that copious levels of Aβ-42 peptides may induce a significant reduction in synaptophysin solubility. These changes were only observed in the TBS and formic acid fractions in the brains of hAPPsw+hPS2m double Tg mice.

Verification of the effects of Aβ-42 peptides on synaptophysin solubility using SK-N-MC cells. Finally, in an effort to confirm that the effects of the Aβ-42 peptide observed in the genotypically different mice could be detected in vitro, the synaptophysin levels were determined in the four soluble fractions collected from the SK-N-MC cells after 24 h of
treatment with Aβ-42 peptides. As shown in Fig. 4A, the high concentration (100 nM) of Aβ-42 peptide induced neuronal cell death, whereas the low dose (10 nM) did not induce any detectable changes. Additionally, Western blot analysis rarely detected synaptophysin in the TBS fraction, and the synaptophysin levels in the Triton X-100 fraction were slightly reduced in the Aβ-42-treated relative to the vehicle-treated cells (Fig. 4B). In the formic acid soluble fraction, the highest synaptophysin levels were observed in cells treated with 100 nM, followed by 10 nM and vehicle treatment. By way of contrast, we noted no differences between the Aβ-42-treated and vehicle-treated cells with regard to synaptophysin levels in the SDS fraction (Fig. 4B). The results support the suggestion that treatment with Aβ-42 peptides significantly enhances the insolubility of synaptophysin in the neuroblastoma cells, which is similar to the pattern observed in the brains of hAPPsw+hPS2m double Tg mice.

Discussion

AD is a neurodegenerative disorder that features deposits of amyloid plaques and neurofibrillary tangles within the cortex and hippocampus of the brain (22,23). The formation of the neurofibrillary tangles, the elevation of phosphorylated Tau, and the accumulation of Aβ-42 peptides in the neuronal cells of AD brains significantly inhibit intracellular transport, cellular geometry, and neuronal viability in the neuronal cells (4,5,24-26). Ultimately, these events result in apoptotic death of neuronal cells, and prevent the communication of neuronal cells (27).

In the process of communication between neuronal cells, signals from the presynaptic neuron can be transmitted into the postsynaptic neuron by the opening of an ion-channel that is bound to the neurotransmitter-receptor in the membrane of the postsynaptic neuron. Synaptophysin is crucial to exocytosis during the process of neurotransmitter release, facilitating the fusion of the synaptic vesicles to the presynaptic membrane (4,5). In AD and Parkinson's disease (PD), the release of neurotransmitters from the neuronal cells is significantly reduced or impaired relative to that observed in normal neuronal cells (28). In particular, these alterations of neurotransmitter regulation are correlated strongly with mutations in genes encoding for Presenilin in AD, and Parkin, DJ-1, PARK1, and LRRK2 in PD (28,29). However, no studies
have yet been conducted regarding the correlation between the Aβ-42 peptides increased by overexpression of the hPS2m or hAPPsw gene and the biochemical properties of synaptophysin. Therefore, it was necessary to determine whether or not the biophysical properties of synaptophysin can be altered by Aβ-42 peptides in an AD animal model harboring damaged neuronal cells.

The present study was conducted with hAPPsw+hPS2m Tg, NSE-hAPPsw Tg, NSE-hPS2m Tg and Non-Tg mice to evaluate the changes in the levels of synaptophysin in the brains of the genotypically different AD models. Significant behavioral dysfunctions in the water maze test, and the brain levels of Aβ-42, caspase-3 and Cox-2 proteins have been demonstrated in single NSE-hAPPsw or NSE-hPS2m Tg 12-month-old mice (17). Furthermore, the behavioral deficits in the hAPPsw+hPS2m double Tg mice developed much earlier than in their single and Non-Tg littermate progenies at 8 months of age (data not shown). These double Tg mice harbored 40-50% more Aβ-42 peptides in their brains (Fig. 1B). Also, previous studies have reported that the hAPPsw+PS1 double Tg mice produced from a cross between line Tg2576 and a mutant PS1M146L Tg line developed fibrillar Aβ deposits within the cortex and hippocampus more rapidly and exuberantly compared to their single Tg Tg2576 littermates (30,31). The current results obtained with the hAPPsw+hPS2m double Tg mice are consistent with these results.

Also, in our study, the levels of synaptophysin among the hAPPsw+hPS2m double, single NSE-hAPPsw and NSE-hPS2m Tg, and Non-Tg AD mice were examined to discern if there were any differences, because of the controversy of this issue. Specifically, SY-IR data from the various AD models are inconsistent (32). In a study using one of the most well-known models, SY-IR in the dentate gyrus of the brains of Tg mice expressing APP695 and APP751 of Korea (NRF) grant funded by the Korean government (MEST) (MRC, 2010-0029480).

Acknowledgements

This study was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (MRC, 2010-0029480).

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