Pen-2 overexpression induces Aβ-42 production, memory defect, motor activity enhancement and feeding behavior dysfunction in NSE/Pen-2 transgenic mice

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Received June 8, 2011; Accepted July 21, 2011

DOI: 10.3892/ijmm.2011.767

Abstract. Pen-2 is a key regulator of the γ-secretase complex, which is involved in the production of the amyloid β (Aβ)−42 peptides, which ultimately lead to Alzheimer’s disease (AD). While Pen-2 has been studied in vitro, Pen-2 function in vivo in the brains of transgenic (Tg) mice overexpressing human Pen-2 (hPen-2) protein has not been studied. This study aimed to determine whether Pen-2 overexpression could regulate the AD-like phenotypes in Tg mice. NSE/hPen-2 Tg mice were produced by the microinjection of the NSE/hPen-2 gene into the pronucleus of fertilized eggs. The expression of the hPen-2 gene under the control of the NSE promoter was successfully detected only in the brain and kidney tissue of NSE/hPen-2 Tg mice. Also, 12-month-old NSE/hPen-2 Tg mice displayed behavioral dysfunction in the water maze test, motor activity and feeding behavior dysfunction in food intake/water intake/motor activity monitoring system. In addition, tissue samples displayed dense staining with antibody to the Aβ-42 peptide. Furthermore, NSE/hPen-2 Tg mice exhibiting feeding behavior dysfunction were significantly more apt to display symptoms related to diabetes and obesity. These results suggest that Pen-2 overexpression in NSE/hPen-2 Tg mice may induce all the AD-like phenotypes, including behavioral deficits, motor activity and feeding behavior dysfunction, Aβ-42 peptide deposition and chronic disease induction.

Introduction

Pen-2 is a component of γ-secretase, which also consists of a heterodimeric form of presenilins (PSs), a glycosylated mature form of nicastrin (NCT) and APH-1. Pen-2 is responsible for the intramembranous cleavage of β-amyloid precursor protein (APP) in Alzheimer's disease (AD) (1). Pen-2 was first identified in Caenorhabditis elegans through genetic screening for modifiers of the PS homologues SEL-12 and HOP-1 (2). In humans, Pen-2 is designated PSENEN, and encodes a 101 amino acid polypeptide with a hairpin-like topology, in which both the N- and C-termini are exposed to the lumen (3,4). Also, Pen-2 is incorporated into the PS-NCT-APH-1 trimeric intermediate during the assembly of the γ-secretase complex and is responsible for intramembrane proteolysis of a variety of type I membrane proteins including APP, Notch and p75 (5-7). Both the length of the exposed C-terminus and the sequence of a highly-conserved DYLSF Pen-2 motif contribute to the interaction of Pen-2 to other PS complex components in the function of the γ-secretase during NCT maturation, PS endoproteolysis and the production of amyloid β (Aβ) peptides (8).

Many in vitro studies have reported on the functional role of Pen-2 in the formation and activity of γ-secretase, although a definitive role has yet to be established. Basically, in most of these studies, Pen-2 overexpression could induce the increase of Aβ-42 production, while the loss of Pen-2 activity resulted in a reduction of PS levels (2,9,10), PS endoproteolysis (5,6,11) and a loss of γ-secretase activity, which generates Aβ-42 peptides, S3-site cleavages and the Notch intracellular domain (NICD) (2). Moreover, cells overexpressing one component of the γ-secretase complex display a marginal elevation of γ-secretase activity levels (5). When overexpressed by transient transfection,
Pen-2 can be incorporated into the endogenous complex with a low degree of efficiency (1). Also, the co-expression of APPsw and Pen-2 can induce minor increases in PS1 fragment levels and a corresponding reduction in the levels of full-length PS1 (5). NCT overexpression induces the increase in γ-secretase activity without a change in PS component levels (12). However, Pen-2 suppression with transfection of small interfering RNA (siRNA) fragment induces the loss of fragmented forms of PS as well as an accumulation of the PS holoprotein (6), while APH-1 or NCT siRNA do not induce the same result (2,13).

Several reports have described the correlation between Pen-2 and PS with respect to the regulation of gene expression. At the level of transcriptional regulation, Pen-2 mRNA and its promoter transcription were reduced by the depletion of PS. Especially, in fibroblasts this regulation depends on the p53 tumor suppressor (14). Also, at the translational level, PS selectively enhances the stability of Pen-2 and protects it from proteosomal degradation (15). However, there are no studies on the overexpression of human Pen-2 (hPen-2) gene in transgenic (Tg) mice, despite the importance of this approach in investigating the function of Pen-2 and for the screening of AD therapeutic drugs.

The present study addressed this need by developing and characterizing a novel NSE/hPen-2 Tg mouse system. Experiments using these Tg mice implicates Pen-2 in the onset of AD through the regulation of Aβ production, behavioral defects and feeding behavior dysfunction.

Materials and methods

Gene constructions. The pNSE/hPen-2 plasmid, which harbors hPen-2 under the control of the NSE promoter, was previously constructed (16). Briefly, the hPen-2 gene (GenBank accession no. NM-172341) was amplified by polymerase chain reaction (PCR) using a full-length RNA isolated from SK-N-MC cells. The primers used for the amplification were the sense primer, 5'-ggatccATGgaaca aaaac ttatt tctga agatc tg AACCT GGAGC GAGTG TCCAA TG-3' (italics, BamHI site; ATG, start codon; small letters, c-Myc tag; capital letters, p53 tumor suppressor (14). Also, at the translational level, PS selectively enhances the stability of Pen-2 and protects it from proteosomal degradation (15). However, there are no studies on the overexpression of human Pen-2 (hPen-2) gene in transgenic (Tg) mice, despite the importance of this approach in investigating the function of Pen-2 and for the screening of AD therapeutic drugs.

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Production of NSE/hPen-2 Tg mice. The pNSE/hPen-2 plasmid was digested with BglII/PvuII in order to remove the prokaryotic sequence and electrophoresed on an agarose gel. The linear gene fragment of the pNSE/hPen-2 fragment (2,254-bp) was excised and extracted from the agarose gel. The pNSE/hPen-2 fragment was purified by electrophoresis, diluted to 4 ng/µl and microinjected into the male pronucleus of fertilized embryos, which were obtained by the crossing a female with a male BDF1 mouse originated from a mating between female C57BL/6 and male DBA/2 mice (17). The injected egg was then transferred into the oviducts of a pseudopregnant ICR recipient female on Day 1. The founder mice, into which the NSE/hPen-2 transgene had been inserted, were identified by DNA-PCR of the tail-derived DNA. For DNA-PCR, 10 pmole of the sense, 5'-GCT ATG AAC CTG GAG CGA GTG-3' and antisense, 5'-GAA GGA GAG GTA GTC CCC AAG G-3' primers were added into genomic DNA template mixture, and the reaction mixtures were subjected to 25 cycles of amplification. Amplification was conducted in a thermal cycler (Perkin-Elmer, Waltham, MA, USA), under the following conditions: 30 sec at 94˚C, 30 sec at 62˚C and 45 sec at 72˚C. The amplified PCR products were separated on a 1% agarose gel and the bands were detected using the Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY, USA). The Tg founder mice were then crossed onto the parental strain of the C57/BL6 background to establish the transgenic lines. Subsequently, all pedigrees were hemizygous for their transgene.

Reverse transcription (RT)-PCR analysis. For the preparation of total-RNA, tissues frozen in liquid nitrogen were chopped with scissors and homogenized in RNAzol B solution (Tet-Test, Austin, TX, USA). The isolated RNA was then quantified using an Ultraspec 1000 system (Amersham Pharmacia Biotech, Buckinghamshire, UK). To characterize the expression of transgenes, RT-PCR was conducted using 5 µg of total-RNA from each of the tissue samples. Oligo(dT) primers of 500 ng (Gibco-BRL, Grand Island, NY, USA) were annealed for 10 min at 70˚C. Complementary DNA, which was utilized as a template for further amplification, was synthesized via the addition of dATP, dCTP, dGTP and dTTP, as well as 200 units of reverse transcriptase. Thereafter, 10 pmole of the sense and antisense primers were added, and the reaction mixtures were subjected to 28 cycles of amplification. Amplification was conducted in the afore-mentioned thermal cycler under the afore-mentioned conditions. In each case, minus-RT controls were included to distinguish between the DNA and RNA products. This experiment was repeated three times, and the relative differences in RNA quantity were also reproducibly observed in the three experiments. Finally, the levels of the Pen-2 RT-PCR product were quantified using the afore-mentioned electrophoresis documentation and analysis system on a 1% agarose gel.

Western blotting and slot blotting. For slot blotting, protein prepared from cells transfected with NSE/SV40 and NSE/hPen-2 plasmids were transferred to a nitrocellulose membrane using a Slot Blot kit (Amersham Pharmacia Biotech). The membrane was incubated separately with primary rabbit polyclonal anti-Aβ-42 unconjugated at 2 µg in blocking buffer at room temperature for 3 h, and were washed in washing buffer and then incubated with secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:1,000 for 1 h at room temperature. The slot band for the Aβ-42-specific peptide was detected by an NBT/BCIP substrate. For Western blotting, 10 µg of protein were separated by electrophoresis on a 10% polyacrylamide gel for 3 h and the resolved species were transferred to a nitrocellulose membrane by electroblotting for 2 h. The membrane was incubated with the following primary antibodies: anti-human Pen-2 (Calbiochem, San Diego, CA; 1:1,000 dilution), anti-Glut-1 (Abcam, Cambridge, UK; 1:1,000 dilution), anti-Glut-3 (Abcam; 1:1,000 dilution) or anti-actin (Sigma-Aldrich; 1:1,000 dilution) overnight at 4˚C. Each membrane was washed with buffer (137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, and 0.05% Tween-20) and incubated with a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at room temperature for 2 h. The membrane blots were developed using the Enhanced Chemiluminescence Reagent Plus kit (Amersham Biosciences).

Perfusion and immunohistochemical analysis. Brain perfusion and immunohistochemical analysis was performed as previously described (18). Briefly, mice were anesthetised with Zoletil 50 (Virbac, Carros cedex, France) and transcardially perfused with 1X PBS followed by buffered 4% formaldehyde to effectively remove the blood and fix the brain tissue. After perfusion, each mouse brain was isolated from the skull and fixed overnight in formaldehyde. Each brain was dehydrated and embedded in paraffin. A series of brain sections (10 µm) were cut from paraffin-embedded tissue using a Leica microtome (Leica Microsystems, Bannockbrun, IL, USA). For immunohistochemical analysis, these sections were de-paraffinized with xylene, rehydrated and pretreated for 30 min at room temperature with PBS blocking buffer containing 10% goat serum. Next, the sections were incubated with mouse anti-Pen-2 antibody and anti-Aβ-42 antibody (Invitrogen), both at a dilution of 1:100 in PBS blocking buffer. The antigen-antibody complexes were visualized with biotinylated secondary antibody (goat anti-rabbit)-conjugated HRP streptavidin (Histostain-Plus kit; Zymed, South San Francisco, CA, USA), at a dilution of 1:1,500 in PBS blocking buffer. Pen-2 protein and Aβ-42 peptide were detected using stable 3,3'-diaminobenzidine (DAB; Invitrogen) and observed using the B XSOF-3 optical microscope (Olympus, Tokyo, Japan).

Water maze test. Twelve-month-old mice were subjected to water maze tests following a previously-described procedure (18). Briefly, the tests were performed using the SMART-CS (Panlab, Barcelona, Spain) program, which was placed in an experimental room with a window, air-conditioning and tables. This experiment was conducted in a plastic, 1.5 m-diameter circular pool filled with water maintained at 20-22°C. The visual field of the water was obstructed by the addition of powdered milk. Mice were pre-trained by allowing them to swim to a round platform (diameter 12 cm) submerged 1 cm beneath the surface. The escape latencies, escape distances, swimming speeds and swimming patterns of the mice were monitored by the SMART-LD computer program, which was connected to a camera mounted to the ceiling directly above the pool. Prior to the experiment, a 60 sec habituation tria
was performed to verify that the mice could swim. The mice were then given five training trials in which their ability to find the hidden platform was measured for a maximum of 60 sec. If the mice failed to find the platform within the maximum time, they were physically placed on it. The training schedule consisted of two trials per day over 5 test days, and each trial was assessed based on the ability of the mouse to reach the platform within 60 sec. The second trial was conducted at least 5 min after the first one. However, the two trials were started from identical locations and the platform location was kept constant during the training period. After each trial, the mice were allowed to remain on the platform for 30 sec. On the sixth day, the mice were subjected to three probe trials, during which they swam for 60 sec with no platform in the pool. In this test, each of the two training trials and three actual trials were initially started from the right side of the water pool, and then from the opposite side. The patterns of searching, the number of times the mice swam to the former location of the platform (escape latency), the distances the mice swam (escape distance) and the swimming speeds (velocity) to the former location of the platform were recorded. All trials were recorded and stored on a videotape for subsequent analysis.

Feeding behavior and motor activity analysis. Feeding behavior and motor activity of 12-month-old NSE/hPen-2 Tg mice were detected by a food intake/water intake/motor activity monitoring system MDF-100 (Shinfactory, Tokyo, Japan). One NSE/hPen-2 Tg mouse and one non-Tg mouse that had been housed in groups (4-6 mice/cage) until 12-months-of-age and had originated from same parent were taken and separated into individual chambers of the monitoring system. Food intake, water intake and motor activity were monitored for at least 2 weeks. The data of three parameters were collected from 3-5 mice/group using ACTIMO-S Vista-compliant data acquisition software (Shinfactory, Tokyo, Japan).

Serum biochemical analysis. Following the final application of feeding behavior and motor activity, the mice were fasted for 24 h and whole blood was collected from their abdominal vein. Serum was obtained by centrifuging the blood (15,000 rpm, 4°C, 10 min), followed by incubation for 30 min at room temperature. The serum was then stored at -80°C until analysis. The glucose, total serum cholesterol and triglyceride levels in the brain of NSE/hPen-2 Tg mice were measured to

Aβ-42 enzyme-linked immunosorbent assay (ELISA). Aβ-42 levels in the brain of NSE/hPen-2 Tg mice were measured as previously described (20). Firstly, the cortex region was collected from the brain of non-Tg and NSE/hPen-2 Tg mice, and homogenized in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6). The supernatant containing soluble Aβ-42 was prepared from the homogenized mixture using centrifugation at 100,000 x g for 1 h. The levels of Aβ-42 peptides were analyzed using Signal Select Human β Amyloid (1-42) colorimetric sandwich ELISA kits (Signal Select™, BioSource, Camarillo, CA, USA) following the manufacturer’s instructions. The standard, control and brain samples were added into Aβ-42 antibody-coated wells of 96-well plates, and 50 µl of detection antibody solution were immediately added. After the incubation for 3 h, the unbound proteins were removed with washing steps and 100 µl of anti-rabbit IgG-HRP working solution was immediately added to the plate. Finally, the level of Aβ-42 peptides were measured to read the absorbance of color formed by the reaction between the stabilized chromogen and the stop solution at 450 nm.

Insulin ELISA. The insulin levels in serum obtained from non-Tg and NSE/hPen-2 Tg mice were detected using the ultra-sensitive assay procedure and reagents in the insulin ELISA kit (Mercodia, Uppsala, Sweden). Briefly, serum and standards were incubated in a plate shaker at 100-150 rpm at room temperature for 2 h on antibody-coated plates. The wells were then washed six times with an automated plate washer (Hoefer, Holliston, MA, USA), after which horseradish peroxidase (HRP) conjugate was added to each of the wells. The plates were then incubated in a shaker for 30 min at room temperature. The reaction was terminated by the addition of 50 µl of stop solution (0.5 M H₂SO₄), after which the plates were analyzed by evaluating the absorbance at 450 nm using a Molecular Devices Vmax Plate reader (Sunnyvale, CA, USA).

Statistical analysis. Tests for significance were conducted using one-way ANOVA analyses of variance (SPSS, release 10.01; SPSS, USA). All values were expressed as the mean ± standard deviation (SD). Significance was set as a value of P<0.05.

Results

Identification of hPen-2 protein expression and Aβ-42 production in neuroblastoma cells. Prior to testing of the effects of hPen-2 overexpression in Tg mice, the NSE/hPen-2 plasmid was applied to detect the expression of hPen-2 protein in neuroblastoma cells and quantify the Aβ-42 levels produced by γ-secretase activation. After transfection with NSE/hPen-2 or NSE/SV40 plasmid, the 12-kDa Pen-2 protein was detected by Western blotting using a Pen-2 specific antibody. The levels of Pen-2 protein were determined to be elevated only in cells transfected with NSE/hPen-2 as compared to those transfected with NSE/SV40 (Fig. 1Ba). Also, fluorescence activated cell sorting (FACS) analysis revealed that Pen-2 proteins in the cell membrane were significantly increased and successfully detected in cells transfected with NSE/hPen-2 (Fig. 1Bb). Furthermore, the level of Aβ-42 peptides was higher in NSE/hPen-2 transfectants than in NSE/SV40 transfectants (Fig. 1C). The results from transiently-transfected cells suggest that the NSE/hPen-2 plasmid may be useful for this study, as it mimics conditions of the actual in vivo abnormally enhanced Pen-2 overexpression.

Identification of NSE/hPen-2 Tg mice. To produce NSE/hPen-2 Tg mice overexpressing the hPen-2 protein in each tissue, the 2,254-bp NSE/hPen-2 fragment was microinjected into the male pronucleus of fertilized embryos obtained by crossing a female with a male BDF1 mouse. Of a total of 118 offspring, eight mice (#6, 18, 21, 46, 49, 60, 86 and 95) from the first lineage founder mice possessed the NSE/hPen-2 gene, which was identified by genomic DNA-PCR using the hPen-2 specific primer (Fig. 1D). The founder mice containing the
The NSE/hPen-2 gene were then mated with the C57BL/6 mice to produce a large number of animals. The NSE/hPen-2 gene introduced into their genomes was transmitted to all offspring with approximately 50% hemizygotes in a Mendelian fashion.

**Tissue-specific regulation of hPen-2 expression in the NSE/hPen-2 Tg mice.** To determine whether the regulation of the introduced hPen-2 gene was expressed under the control of the NSE promoter in neural tissues, the transcriptional levels of Pen-2 from various tissues, including the brain, heart, lung, liver, kidney, intestine and muscles of Tg mice were examined by RT-PCR analysis. The RT-PCR analysis showed that the highest level of hPen-2 expression was observed in the kidney, followed by the brain and heart (Fig. 2A). Furthermore, to detect the localization and distribution of the Pen-2 protein in brain tissues, Pen-2 protein immunoreactivity was analyzed in the brain using optical microscopy. The immunostaining intensity in the NSE/hPen-2 Tg mice was spread throughout the CA1-3 of the hippocampus and dentate gyrus (DG) in the brain (Fig. 2B). However, the level of intensity in the non-Tg
littermates was slightly lower than that of the NSE/hPen-2 Tg mice (Fig. 2B). Therefore, all of the above observations indicate that the regulatory sequence of the NSE promoter could be successfully attributed to the synthesis of the hPen-2 transcripts and protein in the NSE/hPen-2 Tg mice.

**Behavioral deficits of NSE/hPen-2 Tg mice.** To assay for early behavioral defects, the escape latency and distance, and swimming patterns and speeds were measured in 12-month-old non-Tg and NSE/hPen-2 Tg mice by water maze tests. After the last training (Day 5), all the mice were given three prove trials on Day 6, in which they swam in the pool for 60 sec with the platform removed. NSE/hPen-2 Tg mice appeared to be slightly impaired, about 30% than the non-Tg mice, in both the escape latencies (Fig. 3A) and distances (Fig. 3B and D).

However, the speed of swimming (velocity) did not differ between non-Tg and NSE/hPen-2 Tg mice (Fig. 3C). Thus, these results show that overexpression of the hPen-2 gene in the brain of the NSE/hPen-2 Tg mice led to an accelerated brain deficit on learning and memory.

**Alterations of motor activity in NSE/hPen-2 Tg mice.** The motor activity was assessed by measurement of infrared-beam breaks in a food intake/water intake/motor activity monitoring system (21). Motor activity of NSE/hPen-2 Tg mice was significantly increased compared to non-Tg mice (Fig. 4A). This hyperactivity was predominant in the dark phase for 12 h (Fig. 4B). Especially,
Figure 4. Alteration of the motor activity of NSE/hPen-2 Tg mice. (A) Total motor activity for 24 h. (B) Ratio of light to dark motor activity. (C) Spontaneous locomotor activity of NSE/hPen-2 Tg mice at 12-months of age expressed as the count of infrared-beam splits during the light and dark phases. *P<0.05; significant differences between NSE/hPen-2 Tg and non-Tg mice.

Figure 5. Alteration of (A) daily food and (B) water intake of NSE/hPen-2 Tg mice. Each column expresses (Aa and Ba) the total amount of feeding for 24 h, and (Ab and Bb) the ratio of light/dark on the amount of feeding. Each bar in (Ac and c' and Bc and c') indicate the amount of food intake and water intake for 1 h of the light phase or dark phase. *P<0.05; significant differences between NSE/hPen-2 Tg and non-Tg mice.
in the late stage of the dark phase, the motor activity of NSE/hPen-2 Tg mice was higher, while non-Tg mice showed higher motor activity in the early phase of the dark phase (Fig. 4C). These results suggest that Pen-2 overexpression in NSE/hPen-2 Tg mice could contribute to the hyperactivity in the dark phase.

Alterations of the feeding behavior of NSE/hPen-2 Tg mice. Changes in the eating behavior or diet are common in dementia such as frontotemporal dementia, AD and Parkinson's disease (22,23). To evaluate and characterize the difference in the feeding behavior between non-Tg and NSE/hPen-2 Tg mice, the amount of food and water consumption were detected by the food intake/water intake/motor activity monitoring system. The feeding behavior of NSE/hPen-2 Tg mice differed markedly from that of non-Tg mice. Daily food consumption was significantly greater in NSE/hPen-2 Tg mice compared to non-Tg mice (Fig. 5Aa). The ratio of food consumption in the light versus dark phase was dramatically increased in NSE/hPen-2 Tg mice compared to non-Tg mice (Fig. 5Ab and c). Especially, NSE/hPen-2 Tg mice took lots of pellets at the early stage of light phase (Fig. 5Ac). However, the water intake by NSE/hPen-2 Tg mice was decreased by 50% compared with the water intake of non-Tg mice (Fig. 5Ba). These decreases of water consumption were mainly observed at the late stage of the light phase than the dark phase (Fig. 5Ab, c and c'). These results showed that Pen-2 overexpression of NSE/hPen-2 Tg in 12-month-old mice may induce changes in eating and drinking behavior.

Aβ-42 peptide deposition in the brain of NSE/hPen-2 Tg mice. To determine whether hPen-2 overexpression in NSE/hPen-2 Tg mice is accompanied by elevated levels of Aβ-42 peptides produced by γ-secretase including APH-1, NCT and full length PS2 (PS2-FL) as well as the PS2 fragment (PS2-CTF), immunostaining analysis was conducted for the hippocampus region of the brain using an Aβ-42 peptide-specific antibody. Levels of Aβ-42 peptide were higher in the CA1-3 of hippocampus (Fig. 6Bb), DG (Fig. 6Bb) and cortex region (Fig. 6Bd) of NSE/hPen-2 Tg mice than those of non-Tg mice (Fig. 6Aa and c). Also, a significant increase of Aβ-42 peptides in soluble mixture of NSE/hPen-2 Tg mice brain was detected by ELISA (Fig. 6C). These results showed that Pen-2 protein overexpression in the brain of NSE/hPen-2 Tg mice may induce the production and deposition of Aβ-42 peptide.

Effect of feeding behavior dysfunction induced by Pen-2 overexpression on diabetes and obesity. In humans, the alteration of feeding behavior is considered to be the major cause for diseases such as diabetes, obesity, cardiovascular disease and hypertension (24). To explore whether the feeding behavior dysfunction could induce the alteration of an indicator for diabetes and obesity, the level of some parameters including glucose, insulin, cholesterol and triglyceride were measured in the serum of NSE/hPen-2 Tg mice. As shown in Fig. 7A and B, cholesterol and triglyceride as an indicator of obesity were higher in NSE/hPen-2 Tg mice than in non-Tg mice. Especially, the triglyceride concentration in NSE/hPen-2 Tg mice was markedly higher (2-fold) compared with non-Tg mice. In case of a diabetes indicator, the serum glucose concentration was significantly increased in the NSE/hPen-2 Tg mice compared to non-Tg mice (Fig. 7C). Furthermore, the insulin concentrations showed a pattern opposite to that of serum glucose concentrations. NSE/hPen-2 Tg mice showed a significant decrease in insulin concentration, while that of non-Tg mice as maintained at 10.54 ng/ml (Fig. 7D). Furthermore, to investigate the effect of the alteration of glucose concentration induced by feeding behavior on Glut-1 and Glut-3 expression, the Glut-1
and Glut-3 membrane content in the brain were measured. Glut-1 and Glut-3 expression in NSE/hPen-2 Tg mice were significantly decreased by 30-40% compared to non-Tg mice (Fig. 7E). Taken together, these results indicate that the alteration of feeding behavior induced by Pen-2 overexpression may induce the key symptoms of diabetes and obesity.

**Discussion**

To investigate the gene function and screen for novel drugs, various Tg mice models were developed by microinjection of one gene of γ-secretase components under the appropriate promoter into a fertilized egg. These mice showed the AD-like phenotypes including Aβ-42 production, behavioral defects and an increase of γ-secretase activity. PS1 and PS2 mutant Tg mice showed a range of pathological and physiological alterations that mimic many aspects of AD and merit its more thorough examination as a model of AD (25,26). Also, in a study using APH-1 knock-out mice, the deletion of APH-1-induced the reduction of γ-secretase complex formation and β-amyloid secretion (27,28). Nicastrin (NCT) conditional knockout mice show a progressive impairment in learning and memory, and display age-dependent cortical neuronal loss (29,30). However, no studies have developed and characterized the novel Tg mice overexpressing the hPen-2 gene under the control of the NSE promoter. Presently, we developed novel

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**Figure 7.** Alterations of indicators for diabetes and obesity, and glucose transporter expression. (A-D) Blood was collected from the abdominal vein of the mice, and serum contents of cholesterol, triglyceride and glucose were analyzed using serum biochemical analyzer. Insulin concentration was determined with an ELISA kit containing insulin specific antibody. Three experiments were assayed in triplicate using serum biochemical analysis and ELISA. (E) Glut-1 and Glut-3 protein expression in the brain were detected with anti-Glut-1 and Glut-3 primary antibody and HRP-conjugated goat anti-rabbit IgG. The densitometric intensity of the Glut-1 and Glut-3 proteins was calculated. The values represent the mean ± SD. *P<0.05; significant difference between NSE/hPen-2 Tg and non-Tg mice.
Tg mice showing Aβ-42 deposition and behavioral defects. Specifically, our study explored novel characteristics, such as motor activity enhancement, feeding behavior dysfunction and the induction of diabetes in NSE/hPen-2 Tg mice. The present data are the first to indicate that hPen-2 could induce the AD-like phenotype in vivo.

Recently, the alteration of the feeding behavior in an AD model animal was reported for the first time (31,32). APP23 Tg mice overexpressing human APP cDNA encoding the Swedish double mutation (K670N/M671L) under the control of the neuron-specific murine Thy-1.2 promoter showed the pathological features, learning and memory deficits analogous to AD patients (33). Especially, in a feeding behavior study using APP23 Tg mice, the mice drank more and took more pellets compared with non-Tg mice, although the olfactory function was normal (34). In the present study, the food intake of NSE/hPen-2 Tg mice was significantly increased, similar to the results reported in APP23 Tg mice. However, the water intake was strikingly different between NSE/hPen-2 Tg mice and APP23 Tg mice. The NSE/hPen-2 Tg mice developed herein drank less water than non-Tg mice. Furthermore, the data showed an alteration of the ratio of food and water consumption in the light vs. dark phase using the food intake/water intake/motor activity monitoring system. The results suggest that the dysfunction of feeding behavior in NSE/hPen-2 Tg mice was more affected in the light than the dark phase.

In humans, alteration in feeding behavior is common in dementia and helps distinguish between the different types of dementia. Several reports compared and analyzed the difference in dietary and eating behavior among patients with neurodegenerative disorders such as AD, early frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB). The change in the eating behavior was significantly more common in the FTD patients than in the AD patients (22,23). Also, DLB patients showed remarkable high scores than AD patients for eating and swallowing problems (35). However, dysfunction of the feeding behavior in AD Tg mice has not been hitherto reported. Our results showed the possibility that the AD-like phenotypes induced by Pen-2 overexpression may regulate the feeding behavior in NSE/hPen-2 Tg mice. Studies on the correlation between feeding behavior and dementia in humans may benefit from the present findings.

A number of studies suggested that the pathogenesis of AD is highly correlated with diabetes and obesity. After induced onset diabetes by administration of streptozotocin (STZ), rPr5 Tg mice expressing P301L mutant Tau showed hyperphosphorylation of Tau protein in the brain (36). Also, APP/PS1 double Tg mice displayed increased severity in AD pathology involving Aβ generation, neuritic plaque formation and spatial memory deficits (37,38). These reports have suggested that experimental diabetes could exacerbate the pathology of AD in various animal models. Meanwhile, the mouse model of the combined AD phenotype and diabetes phenotype was used to investigate the pathological interaction between these diseases. In a study using APP23-ob/ob double mutant mice, the onset of diabetes exacerbated AD-like cognitive dysfunction, without Aβ peptide deposition (39). Also, mice overexpressing APP and displaying type 1 diabetes showed a decrease of insulin receptor activity, increase of GSK3β activity, increase of Tau phosphorylation and Aβ plaque number (40). In the present study, the increase of pathological features of diabetes and obesity were detected in the NSE/hPen-2 Tg mice compared with non-Tg mice. These results suggest that the pathological features of AD induced by overexpression of hPen-2 could exaggerate diabetes and obesity in NSE/hPen-2 Tg mice. Also, our study may provide other evidence of the pathophysiological correlation between diabetes and AD.

All of the above-mentioned results support the suggestion that Pen-2 in NSE/hPen-2 Tg mice may play an important role in developing the pathological characteristics of AD. Therefore, NSE/hPen-2 Tg mice can serve as an animal model for AD to understand the basal mechanism of AD pathology and screen novel therapeutic drugs.

Acknowledgements

This research was supported by Bio-Scientific Research Grants funded by the Pusan National University (PNU, Bio-Scientific Research Grant) (PNU-2010-101-236).

References


