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EXPRESSION OF α-SYNUCLEIN IN DIFFERENT BRAIN PARTS OF ADULT AND AGED RATS.

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The synucleins are a family of presynaptic proteins that are abundant in neurons and include α-, β-, and γ-synuclein. α-Synuclein (ASN) is involved in several neurodegenerative age-related disorders but its relevance in physiological aging is unknown. In the present study we investigated the expression of ASN mRNA and protein in the different brain parts of the adult (4-month-old) and aged (24-month-old) rats by using RT-PCR technique and Western blot, respectively. Our results indicated that mRNA expression and immunoreactivity of ASN is similar in brain cortex, hippocampus and striatum but markedly lower in cerebellum comparing to the other brain parts. Aging lowers ASN mRNA expression in striatum and cerebellum by about 40%. The immunoreactivity of ASN in synaptic plasma membranes (SPM) from aged brain cortex, hippocampus and cerebellum is significantly lower comparing to adult by 39%, 24% and 65%, respectively. β-synuclein (BSN) was not changed in aged brain comparing to adult. Age-related alteration of ASN may affect the nerve terminals structure and function.

Keywords: *α-synuclein, mRNA expression, immunoreactivity, brain, aging*

INTRODUCTION

α-Synuclein (ASN) belongs to a larger family of molecules, including β-synuclein (BSN) (or phosphoneuroprotein 14) (1), γ-synuclein (or breast carcinoma-specific factor) and synoretin (2, 3). ASN, a 140 amino acid synaptic

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molecule, was originally identified in human brain as the precursor protein of the non-amyloid β-protein (Aβ) component of Alzheimer's disease (AD) amyloid (NAC). NAC is a highly hydrophobic 35 amino acid central domain (residues 61-95) within the ASN molecule liberated by till unknown mechanism and it is the component of senile plaques in AD (4-6). Highly conserved amino-terminal domain (residues 1-60) of ASN is responsible for association with phospholipid bilayers and C-terminal region (residues 96-140) is rich in Pro, and the acidic residues Glu and Asp (7-9). The functions of the ASN and other members of this family of proteins are poorly understood, however several lines of evidence suggest potential roles in synaptic and neuronal plasticity. ASN is involved in regulation of several enzymes, transporters, presynaptic vesicle dynamic and neurotransmitter release. Moreover, ASN interacts with a variety of proteins including 14-3-3 protein, PKC isozymes, BAD, ERK, MAPKs, Aβ and tau and with several divalent cations (10, 11). It is suggested that soluble nonaggregated ASN decreases caspase-3 activity, modulates bcl2 expression and inactivats the c-Jun N-terminal kinase stress-signaling pathway and through this action it may play antiapoptotic function (12). However, the ASN molecule is capable of self-aggregating to form oligomers and polymers. Polymerization includes formation of protofibrils, nucleation (13), and fibril formation (14). Oxidative stress mediated by iron, cytochrome c and copper promoted ASN aggregation (15-20). ASN polymerization could be trigger by mutations associated with familial parkinsonism, by binding to lipid membrane vesicles and by interactions with Aβ peptide (21-30). Aggregated form of ASN leading to the Lewy bodies formation has been implicated in the pathophysiology of neurodegenerative disorders including Parkinson's disease, dementia with Lewy bodies, Lewy body variant of AD and other α-synucleinopathies (9, 31). In Lewy bodies the ratio of BSN to ASN is altered (32) and it is suggested that a balance between these two synucleins might be critical in neurodegenerative disorders. Hashimoto et al. (17) indicated that BSN might block ASN accumulation and amyloidogenesis. Till now, little is known about the expression of ASN and BSN in physiological aging of the brain. Our preliminary data indicated that aging significantly decrease ASN but not BSN protein amount in the brain (33, 34). In this study we investigated ASN and BSN mRNA and protein expression in different brain parts of adult and aged rats.

MATERIALS AND METHODS

Animals

Males adult (4-month-old) and aged (24-month-old) Wistar rats were supplied from the Animal Breeding House of the Medical Research Center, Warsaw, Poland. The Institutional Ethics Committee accepted the research project.

Preparation of homogenate and synaptic plasma membranes

Animals were decapitated and the brain cortex, hippocampus, striatum and cerebellum immediately isolated. A 10% homogenate from each brain parts was obtained by homogenization in a Dounce glass-glass homogenizer in 0.32 M sucrose with 10 mM Tris-HCl buffer, pH 7.4. Then homogenate was used for preparation of synaptic plasma membranes (SPM) as described previously (35).

RNA extraction and RT-PCR

Total RNA was extracted from brain cortex, hippocampus, striatum and cerebellum from adult and aged animals using a TRI REAGENT isolation kit (Sigma, St. Louis, MO, U.S.A.) according to manufacturer's procedure. The yield and quality of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from 8µg of total RNA by reverse transcription (RT), using Reverse Transcription System (Promega Corporation, Madison, WI, U.S.A.). Reaction was performed in final volume of 20µl using 1.500 units of AMV reverse transcriptase, 0.5µg oligo$(dT)_5$', 2500 units of Rnasin(inh), 1mM each deoxyribonucleotide, 5mM $MgCl_2$ and RT buffer in one cycle: 42°C for 1h and 99°C for 5 min with subsequent cooling to 4°C.

Polymerase chain reaction (PCR) was carried out using *Taq* PCR Master Mix Kit (Qiagen, GmbH, Germany) according to the manufacturer's procedure in total volume of 50µl with 20pmol of each primer. The primer sequences for ASN were 5'-TGCTGTGGATATTGTTGTGG-3' (forward) and 5'-AGGTGCGTAGTCTCATGCTC-3' (reverse). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primer sequences 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (forward) and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 20 cycles for both ASN and GAPDH using a Perkin-Elmer GeneAmp 2400 thermal cycler. The conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. After amplification, samples were separated on 2% agarose gel containing 200µg/l ethidium bromide in 0.5x Tris-borate-EDTA buffer. The intensity of ASN and GAPDH bands was estimated by densitometric analysis of the gel in UV light using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Western blot analysis

Aliquots containing equal amounts of protein from homogenate and SPM were diluted 1:1 in 2 x electrophoresis sample buffer (2xSB) and incubated at 95°C for 5 min. Then, proteins were loaded onto 15% polyacrylamide denaturing gels (40µg per lane) and subjected to electrophoresis (applied constant voltage, 100 V). After protein separation, each gel was placed on a nitrocellulose membrane (Amersham, Hybond-Extra C) in transfer buffer, and the proteins were transfered to the membrane by the application of 100 V for 2 h. After the transfer was completed, equal protein loading and protein transfer was confirmed by Ponceau S staining. In order to block non-specific binding, the membrane was incubated at room temperature (RT) for 1 h in 5% dried skimmed milk and 0.3% Tween 20 in phosphate-buffered saline (PBS), pH 7.4. Incubation with primary antibody (anti-synuclein α developped in rabbit, 1:1000 dilution, anti-β-synuclein (PNP-14), 2µg/ml dilution, Sigma Immunochemicals, St. Louis, MO, U.S.A.) in PBS with 0.1% Tween 20 and 1% bovine serum albumin (BSA) was performed overnight at 4°C. Then blots were washed three times for 15 min at RT in PBS with 0.1% Tween 20. The next membranes were incubated with

horseradish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody diluted 1:8000 (Sigma, St. Louis, MO, U.S.A.) for 1 h at RT. After that blots were washed as described above and bound antibodies visualized by enhanced chemiluminescence (Amersham, U.K.). Incubation with anti-β actin antibody confirmed additionally equal protein loading. Quantification of immunoblots was performed with the NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

RESULTS

To determine the expression of ASN mRNA and protein in rat brain parts RT-PCR and Western blot in brain cortex, hippocampus, striatum and cerebellum was performed. The results indicated that ASN mRNA expression in adult brain was similar in cerebral cortex and hippocampus, slightly lower in striatum and significantly lower in cerebellum comparing to brain cortex and hippocampus (*Fig. 1*). ASN protein expression in the homogenate from brain cortex, hippocampus and striatum was similar but was significantly lower in cerebellum by about 70% comparing to the other investigated brain parts (*Fig. 1*). Brain aging significantly decreased ASN mRNA expression in striatum and cerebellum by 39% and 47%, respectively (*Fig. 2*). ASN protein level in SPM from aged brain cortex, hippocampus and cerebellum decreased by 39%, 24% and 65%, respectively comparing to adult (*Fig 3*). BSN immunoreactivity was not altered by brain aging (data not shown).

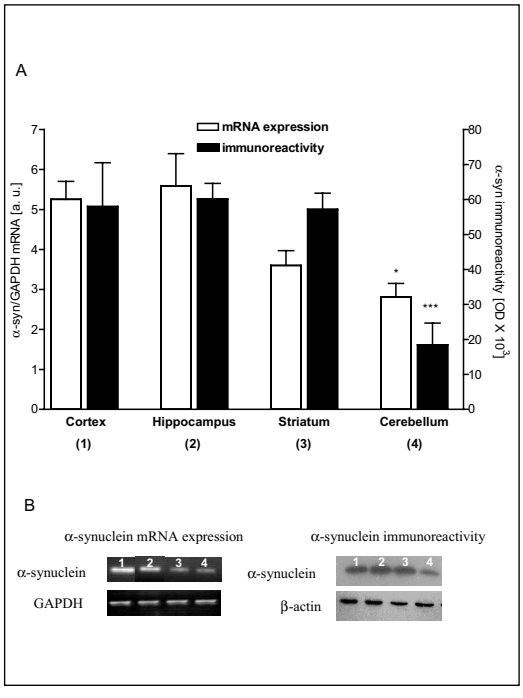

Fig. 1 ASN mRNA and protein expression in the adult rat brain parts.
(A) ASN mRNA expression and its immunoreactivity in brain cortex (1), hippocampus (2), striatum (3) and cerebellum (4) isolated from adult animals. The mRNA ratios were calculated by dividing ASN mRNA levels with the corresponding GAPDH mRNA levels measured in the same experiment. For each experiment, four rats per group were used and all values are expressed as the means ± SEM. Differences were analyzed using one-way ANOVA with the different brain parts as the independent factor with Tukey post-hoc test, ***$p<0.001$, *$p<0,05$ compared with other brain parts.
(B) A representative gel showing the expression of ASN mRNA and GAPDH as an internal control (left panel), and a immunoblots showing ASN immunoreactivity and β-actin as a control (right panel) in brain parts; 1 - brain cortex, 2 - hippocampus, 3 - striatum, 4 - cerebellum.

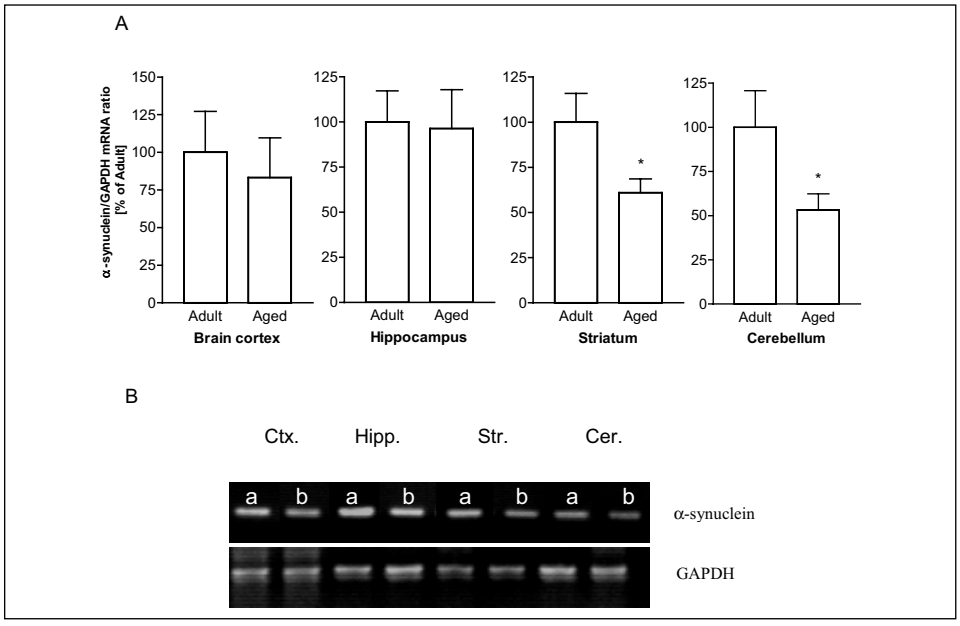

Fig. 2 The effect of aging on ASN mRNA expression in the rat brain parts.
(A) ASN mRNA expression in brain cortex, hippocampus, striatum and cerebellum from adult and aged animals. Adult animals were used as a control. For the experiment, six to eight rats per group were used and all values are expressed as the means ± SEM. Differences between adult and aged animals were analyzed using paired *t-test*. *$p<0.05$, compared with adult.
(B) A representative gel showing the expression of ASN mRNA and GAPDH as an internal control in brain cortex (Ctx.), hippocampus (Hipp.), striatum (Str.), and cerebellum (Cer.) from adult (a) and aged (b) animals.

DISCUSSION

Our study indicated similar ASN expression on mRNA and protein level in brain cortex, hippocampus and striatum with a markedly lower level in cerebellum comparing to the other brain parts. The difference in protein level in this brain structure versus the others is much more pronounced than in mRNA level. These data suggested that not only lower mRNA expression but also other factors might be responsible for the low ASN protein concentration in cerebellum. It could be caused by the lack of the dopaminergic and cholinergic synapses in this structure, cerebellum is enriched only in noradrenergic neurons containing ASN. Axons of dopaminergic, cholinergic and noradrenergic neurons form synaptic connections in brain cortex and hippocampus where ASN protein level is very high. In striatum ASN is probably localized in dopaminergic endings of neurons from substantia nigra and in short internal cholinergic neurons. Moreover, post-translational modification of ASN protein in cerebellum including proteolysis or conformational changes should be taken into

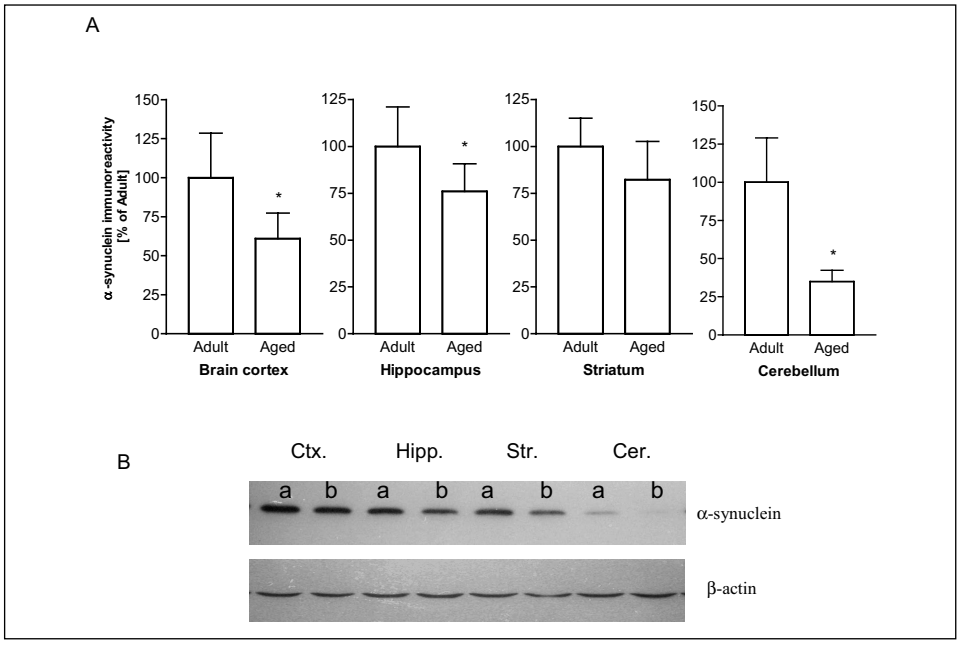

Fig. 3 Aging significantly decreased ASN immunoreactivity in rat brain.
(A) ASN immunoreactivity in brain cortex, hippocampus, striatum, and cerebellum from adult and aged animals. Adult animals were used as a control. For each experiment, six rats per group were used and all values are expressed as the means ± SEM. Differences between adult and aged animals were analyzed using paired *t-test*, *$p<0.05$, compared with adult.
(B) A representative immunoblot showing ASN immunoreactivity and β-actin as a control in brain cortex (Ctx.), hippocampus (Hipp.), striatum (Str.), and cerebellum (Cer.) from adult (a) and aged (b) animals.

consideration. A discrepancy between mRNA and protein levels were described for the other presynaptic proteins, including synaptotagmin I, synapsin I and synaptophysin (36). Our results indicated that aging affected ASN expression in the brain. The most significant alteration of ASN mRNA expression and its immunoreactivity was observed in aged cerebellum comparing to adult. These data indicated that cerebellum is mainly affected by age-related events. Expression of ASN in this part of the brain is altered on the gene and protein level. Future studies will reveal the mechanism(s) of this modification. In brain cortex and hippocampus aging significantly decreased ASN immunoreactivity with no effect on its gene expression. This alteration could be caused by several factors like higher degradation, lower biosynthesis or conformational changes. The low efficiency of translation process and in consequence decrease of protein amount could be also responsible for these changes. The last data on transgenic mice indicated that axonal transport of ASN slows with aging and could result in longer half-life of this protein creating greater opportunities for aggregation-

promoting modifications (37). It is possible that oxidative stress generated in aged brain cortex and hippocampus lead to ASN aggregation and to the lowering of soluble form of ASN. It was observed that oxidative stress is generated during brain aging and that protein oxidation significantly increased in aged brain. Moreover, it was founded that oxidized proteins lose their physiological function (38, 39). Several studies have shown a correlation between generation of reactive oxygen species (ROS) and aggregation of ASN (16, 20, 23, 24, 25). Oxidative stress could be responsible for ASN alteration in aged brain. In aged striatum a lower ASN mRNA expression not correlated with the protein changes. This discrepancy between mRNA and protein level is not fully understand and need future investigation. Aging had no effect on BSN indicated the higher ASN vulnerability for the age-related processes. Our results indicated for the first time that aging significantly decreased ASN expression in the investigated brain parts. This alteration could significantly affect synaptic function and could promote the aged brain for α-synucleinopathy and neurodegeneration.

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