EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 33, No. 4, 220-225, December 2001

The proteins of synaptic vesicle membranes are affected during ageing of rat brain

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Accepted 20 November 2001

Abbreviations: CaM, calmodulin; GDI, guanine nucleotide dissociation inhibitor; SV, synaptic vesicle; LP2 membrane, crude synaptic vesicle membrane; GEP, guanine nucleotide exchange protein; CaMK, calmodulin-dependent protein kinase; PKA, protein kinase A; PKC, protein kinase C; PA, phosphatidic acid; PI, phosphatidyl inositol

Abstract

Low molecular weight GTP-binding proteins are molecular switches that are believed to play pivotal roles in cell growth, differentiation, cytoskeletal organization, and vesicular trafficking. Rab proteins are key players in the regulation of vesicular transport, while Rho family members control actin-dependent cell functions, *i.e.* the regulation of cytoskeletal organization in response to extracelluar growth factors and in dendritic neuron development. In this study, we have examined the regulation of small GTP-binding proteins that are implicated in neurosecretion and differentiation of neuron during ageing processes. Comparison of small GTP-binding proteins from the synaptosome and crude synaptic vesicles (LP2 membranes) of 2 months and 20 months old rat brain respectively showed no difference in the level of Rab family proteins (Rab3A and Rab5A). However, Rho family proteins such as RhoA and Cdc42 were elevated in LP2 membranes of the aged brain. The dissociation of Rab3A by Ca^{2+}/calmodulin (CaM) from SV membranes was not changed during aging. Ca^{2+}/CaM stimulated phosphorylation of the 22 and 55-kDa proteins in SV membranes from the aged rat brain, and inhibited phosporylation of 30-kDa proteins. GTPγS inhibited phosphorylation of the 100-kDa proteins and stimulated phosphorylation of the 70 kDa in LP2 membranes from both the young and aged rat brains, whereas GDPβS caused just the opposite reaction. These results suggest that protein phosphorylation and regulation of Rho family GTPases in rat brain appears to be altered during ageing processes.

Keywords: Small GTP-binding proteins, Rab3A, Rab5, Cdc42, RhoA, guanine nucleotides, Phosphorylation, Synaptic vesicle

Introduction

Synaptic transmission is the exchange of information in the brain, which is performed by the release of neurotransmitters from its presynaptic nerve endings. The neurotransmitters are stored in small organelles of the synaptic vesicles (SVs) in the resting stage. When the neuron is stimulated, an action potential is generated and arrives in the nerve terminal, the membrane depolarizes and voltage-gated Ca^{2+} channels open. The resulting Ca^{2+} influx triggers exocytosis of synaptic vesicles, leading to the release of neurotransmitter (Jahn and Südhof, 1994; Bauerfeind *et al.,* 1996). SVs have many important protein sets such as synapsins, synaptotagmin, synaptobrevin/vesicle associated membrane protein (VAMP), synaptophysin, synaptophorin, SCAMPs, neurotransmitter transporters, proton pump, SV2, and Rab3A (Südhof, 1995). Exocytosis of synaptic vesicles requires the formation of fusion complex consisting of synaptobrevin, which is localized in synaptic vesicle, and syntaxin and SNAP-25, which is localized in presynaptic plasma membranes (Söllner *et al.,* 1993).

Rab3A is Ras-related small GTP-binding protein, and is most abundant in brain, especially on SVs (Jahn and Südhof, 1994). It was reported that Rab3A plays as a regulator for the neurosecretion (Holz *et al.,* 1994; Johannes *et al.,* 1994; Johannes *et al.,* 1998). Both Rab3A and Rab3C dissociate quantitatively from the vesicle membrane after Ca^{2+}-dependent exocytosis and this dissociation is partially reversible during recovery after stimulation (Fischer von Mollard *et al.,* 1991; 1994). A considerable portion of Rab3A in brain is cytosolic, and this cytosolic Rab3A is complexed to guanine nucleotide dissociation inhibitor (GDI), a protein capable of dissociating Rab proteins from membranes (Ullrich, 1993). It was reported that Ca^{2+}/calmodulin (CaM) could

also dissociate Rab3A from SV, and forming a 1:1 complex of Rab3A-CaM (Park *et al.,* 1997). RalA, another Ras-related small GTP-binding protein, was reported to be also an abundant protein in SV (Bielinski *et al.,* 1993). RalA forms also a complex with CaM in a Ca^{2+}-dependent manner (Wang *et al.,* 1997), and is dissociated by Ca^{2+}/CaM from LP2 membranes (Park *et al.,* 1999). In addition, Ca^{2+} and CaM regulate the GTP-binding state of RalA, thereby they are supposed to regulate the activity of RalA (Park, 2001).

The synapse and SVs including membrane proteins are changed along with aging. Synaptic density is decreased during aging, and specifically, there is a significant decline in presumptive inhibitory synaptic terminals as well as a significant decline in synapses that contact dendritic spines (Brunso-Bechtold *et al.,* 2000). Furthermore, dystrophic neurites of senile plaques are defective in proteins that control exocytosis and neurotransmission. Most neuritic senile plaques have a decreased level of synaptophysin, synaptotagmin and Rab3A, whereas SNAP-25 exists at the level of normal neurites (Ferrer *et al.,* 1998). It was found that synaptobrevin also binds to synaptophysin and that synaptophysin-bound synaptobrevin cannot form the fusion complex (Calakos and Scheller, 1994; Edelmann *et al.,* 1995). In recent years, it was found that synaptophysin-synaptobrevin complex is upregulated during neuronal development (Becher *et al.,* 1999).

Protein phosphorylation is an important regulator of long-term synaptic plasticity and is also a direct and rapid modulator of exocytosis in neurons and most other cell types (Turner *et al.,* 1999). Synapsin (Benfenati *et al.,* 1992), 25-kDa synaptosomal associated protein (SNAP-25), syntaxin 1, VAMP (Söllner *et al.,* 1993), two soluble proteins such as SNAP and NSF (Hirling and Scheller, 1996), synaptotagmin (Bennett *et al.,* 1993), and synaptophysin (Rubenstein *et al.,* 1993) are phosphorylated. Raphyllin3A is also phosphorylated *in vitro* by CaMKII and PKA (Fykse and Südhof, 1995). Phosphorylated proteins were associated with altered functional properties, hence it could be thought that protein phosphorylation of SV might be altered during ageing leading to the malfunction of brain.

Thus, we tried to elucidate the possibility that the behavior of Rab3A by Ca^{2+}/CaM may be changed during ageing, which is anticipated to be a malfunction of brain in aged brain. In addition, we analyzed the phosphorylation of proteins in LP2 membranes during ageing in rat brain.

Materials and Methods

Materials

CaM and RabGDI were purified from bovine brain. $CaCl_2$, suprapure grade, was from EM Science. GDP, GDPβS, and GTPγS were from Calbiochem. Glycine, SDS, Tween-20 and Tris/base, electrophoresis grade, were from Bio-Rad. PVDF membrane was from Millipore. Anti-Rab3A, -RalA, -Cdc42, and -RhoA antibodies and peroxidase-conjugated anti-rabbit IgG antibody were purchased from Santa-Cruz. Aprotinin, leupeptin, pepstatin A, PMSF, BSA, and other reagents were purchased from Sigma.

Preparation of LP2 membranes from rat brain

Synaptosomes were prepared from the brain of the young (2 months) and the old (20 months) SD rat by using Ficoll gradient method (Fisher von Mollard *et al.,* 1991; Park *et al.,* 1997). Crude SV, which contains Rab3A-enriched membranes, was prepared through lysis of synaptosome in hypotonic solution, and it has been also referred to as LP2 membranes (Huttner *et al.,* 1983; Park *et al.,* 1997).

Purification of CaM

Calmodulin was purified from bovine brain as described with a few modifications (Gopalakrishna and Anderson, 1982; Lee *et al.,* 1995) from 350 g of bovine brain. The homogenate of rat brain (SD) was prepared by Warring blender and Potter-Elvehjem homogenizer in one volume of 30 mM Hepes, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM $CaCl_2$, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml leupeptin. The supernatant of brain homogenate was obtained at 66,000 *g*, 4ºC for 30 min, and the supernatant solution was adjusted to a $CaCl_2$ concentration of 5 mM, heated at 85 ºC for 3 min, and cooled on ice. Heat-aggregated material was removed by centrifugation at 12,000 *g* for 30 min at 4ºC. The supernatant was loaded to a 50 ml bed volume of phenyl-Sepharose column pre-equilibrated in buffer I (30 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 0.1 mM DTT), and the gel was washed with 10 column volumes of buffer I and 5 column volumes of buffer II (30 mM Hepes, pH 7.5, 200 mM NaCl, 1 mM $CaCl_2$, 0.1 mM DTT). The bound CaM was eluted with buffer III (30 mM Hepes, pH 7.4, 2 mM EGTA, 0.1 mM DTT), and CaM fractions were pooled together and concentrated to 1.5 ml using ultrafiltration with PM10 membrane (Amicon). Glycerol was added to final concentration of 5% (v/v) as a stabilizer, and aliquots of CaM were stored at -70ºC. Coomassie staining of CaM-run SDS-PAGE gel showed a single band.

Purification of RabGDI

RabGDI was purified from bovine brain as described with a little modification from 450 g bovine brain (Sasaki *et al.,* 1990). The brain was homogenized by a Waring Blender and a Potter-Elvehjem Teflon-glass homogenizer in 2 volumes of 25 mM Tris, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 2 μg/ml aprotinin, 2

μg/ml leupeptin, and 1 μg/ml pepstatin A (Buffer A). Tissue debris was removed by ultracentrifugation at 100,000 *g*, 4°C for 1h. The clear supernatant was loaded on DEAE-sephacel column (5 x 40 cm). The column was washed with 8 liters of Buffer A and the GDI fraction was eluted with Buffer A containing 0.3 M NaCl. The eluted solution was treated with 40% ammonium sulfate and incubated on ice for 30 min. The 40% ammonium sulfate pellet was removed by centrifugation at 20,000 *g*, 4°C for 20 min and the supernatant was treated with 60% ammonium sulfate and incubated for 50 min on ice. The 60% ammonium sulfate pellet was saved by centrifugation at 20,000 *g*, 4°C for 20 min. The pellet was resuspended in Buffer B (25 mM Tris, pH 7.5, 1 mM DTT, 0.5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A) containing 10% glycerol and dialyzed against this buffer. Then sodium cholate was added to 1% (w/v) in the solution to break Rab/RabGDI complex and pH of the solution was adjusted to be 8.0 by adding 1 M Trizma/base. One third of the solution was loaded on Mono-Q column (1 x 10 cm) equilibrated with Buffer B containing 1% sodium cholate and 10% glycerol. The column was washed with 200 ml of the same buffer and the elution was performed with a 180 ml Buffer B and 180 ml Buffer B containing 0.5 M NaCl, 1% sodium cholate and 10% glycerol using a linear gradient. About 11 mg of pure GDI was obtained from 450 g of bovine brain. Aliquots of purified RabGDI solution were stored at -70°C before use.

Dissociation of Rab3A from LP2 membranes

LP2 membranes (60 μg protein) were incubated at 30°C for 30 min with various additives in 50 μl of buffer (50 mM Hepes, pH 7.4, 0.5 μM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). The reaction mixtures were centrifuged for 30 min at 100,000 *g* in a Beckman TLA45 rotor. The supernatants were stored at -70°C and the pellets were resuspended in 50 μl of the same buffer with brief sonication and vortexing. Western Blotting using anti-Rab3A or -RalA antibody and peroxidase-congugated anti-IgG antibody was performed to measure the levels of Rab3A or RalA from supernatants and membrane pellets (Park *et al.,* 1997; 1999).

Phosphorylation assay

Kinase reactions were performed using endogenous membrane kinase of LP2 membranes in 10 μl of the kinase buffer (5 mM Hepes, pH 7.4, 0.05 μM $MgCl_2$, 0.1 mM DTT, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, 100 μM Na_3VO_4, 3 mM β-glycerophosphate) in the presence of 0.1 mM GDPβS, or 0.1 μM GTPγS, and then 2 μCi [γ-^{32}P] ATP and 1 mM $MgCl_2$ were treated to the SV membranes. The mixture was incubated at 30°C for 30 min and the reaction was stopped by the addition of 2.5 μl of SDS-PAGE sample buffer. The sample was resolved on SDS-PAGE (15% or 7-15% gradient gel) and ^{32}P-labeled proteins were subsequently detected by autoradiography on the dried gel.

Results and Discussion

Synaptic density, especially presumptive inhibitory synaptic terminals and synapses that contact dendritic spines, are changed during ageing (Brunso-Bechtold *et al.,* 2000). In addition, dystrophic neurites of senile plaques have decreased the level of proteins that controls exocytosis and neurotransmission, such as synaptophysin, synaptotagmin and Rab3A (Ferrer *et al.,* 1998).

Thus, we determined the level of Rab3A and Rab5A in the aged rat brain by Western blotting, showing that Rab3A and Rab5 of LP2 membranes were not significantly changed during ageing. On the contrary, RhoA and Cdc42 were increased in SV of the aged rat brain (Figure 1), and RhoA was also increased in the aged human fibroblast (data not shown). These results indicate that Rab family proteins are relatively constant, while Rho family proteins are increased during aging. Rho family proteins may be related to the morphological change of senescent cells and neurite deformation of the aged brain. The expression of other synaptic proteins is differentially regulated during development and ageing. Synaptophysin, SNAP-25, synaptobrevin, and synaptotagmin are increased, whereas syntaxin and drebrin are decreased during ageing (Shimohama *et al.,* 1998). Thus the decrease of Rho proteins in SV of the aged rat brain may be a specific phenomenon.

Recently, it was reported that CaM/Rab3A complex

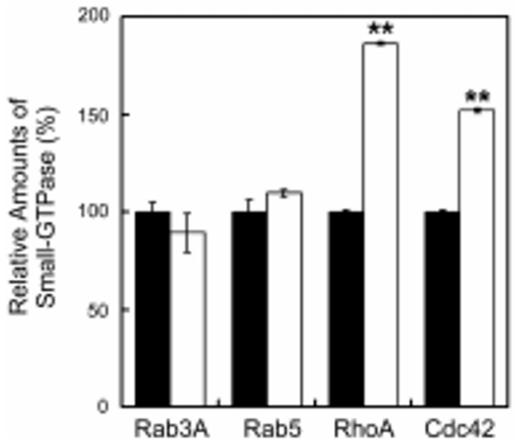

Figure 1. Amounts of small GTP-binding proteins of LP2 membranes during ageing. SV membranes (18 μg protein) of young (■) and old (□) rat brain was loaded on SDS-PAGE, and Rab3A, Rab5, RhoA, and Cdc42 were analyzed with Western blotting by using anti-Rab3A, -Rab5, -RhoA, and Cdc42 antibodies. The values expressed as means ±S.E (n=3) shows statistically significant difference (**$p < 0.01$).

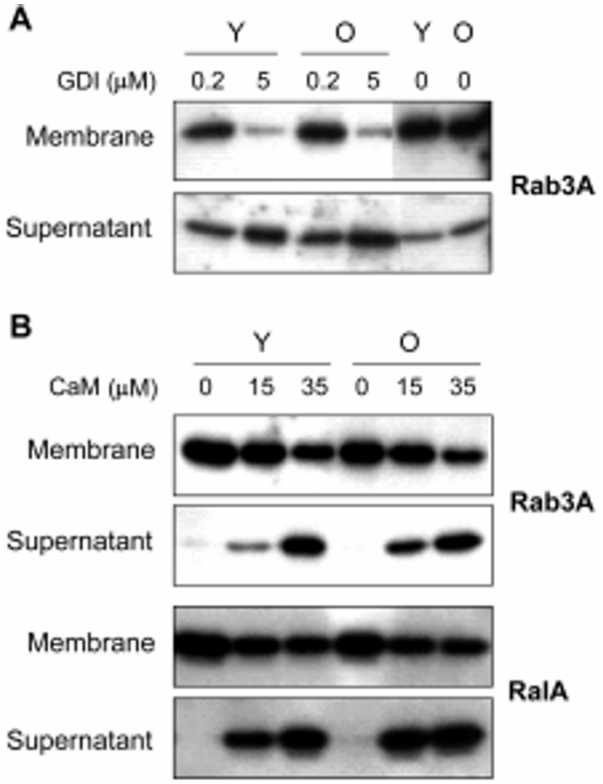

Figure 2. Dissociation of Rab3A from LP2 membranes by GDI and Ca^{2+}/CaM. SV membranes (15 μg protein) of young (Y) and old (O) rat brain were incubated with 0.2 and 5 μM GDI (A), and with 15 and 35 μM CaM in the presence of 0.1 mM $CaCl_2$ (B) at 30°C for 30 min, respectively. Membrane fraction was collected by ultracentrifugation at 100,000 *g*, 4°C for 30 min, and Rab3A and RalA of the membrane pellet and the supernatant were quantitated by Western blotting by using anti-Rab3A and -RalA antibodies. The results were representatives of at least three independent experiments.

formation and translocation of Rab3A are very important for the secretion. Ca^{2+}-dependent association of CaM with Rab3A correlates with insulin exocytosis in pancreatic β cells (Kajio *et al.,* 2001), and Rab3A is absent in endocytic SV indicating that Rab3A is dissociated from SV during endocytosis (Maycox *et al.,* 1992). Although the amount of Rab3A was not changed during ageing, there was a possibility that the behavior of Rab3A may be changed during ageing. To test this possibility, the dissociation of Rab3A from LP2 membranes by Ca^{2+}/CaM or by RabGDI was examined. The purified GDI and CaM were incubated with SV membranes to allow dissociation of Rab3A and RalA, and the dissociated Rab3A and RalA were determined by Western blot analysis. GDI dissociated Rab3A from LP2 membranes, while Ca^{2+}/CaM did both Rab3A and RalA. However, the extent of Rab3A and RalA dissociation from LP2 membranes were found to be similar from the young and aged rat brains (Figure 2). Rab3A from the LP2 membranes of the aged rat brain appeared to dissociate at a lower concentration of CaM (15 μM) than the young rat brain but it was not significant. The results indicate that the dissociation of Rab3A and RalA from LP2 membranes by Ca^{2+}/CaM or RabGDI is not a main factor for the malfunction of brain in the aged rat.

The translocation of Rab3A from Rab3A-RabGDI complex to the membrane is stimulated by phosphatidic acid (PA) in membrane (Jung *et al.,* 1999). The activation of small GTP-binding proteins has been generally executed through GTP binding with the aid of GDP/GTP exchange factor. A GDP/GTP exchange protein (GEP) specific to the Rab3A subfamily has been purified and characterized in recent years (Wada *et al.,* 1997). Rab3 GEP, however, is inactive on Rab3 complexed with RabGDI. In addition, the interaction of Rac-RhoGDI complex is disrupted in the presence of various lipids like arachidonic acid, PA, and phosphatidylinositol (PI), which were shown to possess biological activities in a variety of systems (Chuang *et al.,* 1993). Phosphorylation of RhoGDI stabilizes the RhoA-RhoGDI complex in neutrophil cytosol, and RhoA and RhoGDI components are released to their free form upon treatment of this complex with alkaline phosphatase (Bourmeyster and Vignais, 1996). As shown in the previous reports, the translocation of small GTP-binding proteins is regulated by several factors. However, the regulation of translocation of Rab3A from Ca^{2+}/CaM-Rab3A complex has to be elucidated. Taken together, these results suggest LP2 membranes of both young and old rat brain may not have changed in the regulatory factors of translocation of Rab3A and RalA.

The protein phosphorylation is important for the regulation of neurosecretion. Ca^{2+} caused an elevation of 50 kDa protein (p50) phosphorylation in LP2 membrane of the young rat brain but the addition of CaM reduced phosphorylation of the same protein, while the phosphorylation of 55 kDa protein (p55) was activated by Ca^{2+}/CaM. It is still to be clarified whether Ca^{2+}/CaM relates the activation to the dissociation of Rab3A. Guanine nucleotides have some effects on the phosphorylation of the proteins in LP2 membranes. GTPγS stimulated the phosphorylation of 70 kDa protein (p70), but inhibited 100 kDa protein (p100) phosporylation in both LP2 membranes from the young and aged rat brain, whereas GDPβS caused just the opposite reaction (Figure 3A). This indicates that GTP-binding proteins may regulate the phosphorylation of the proteins in SV membranes.

In addition, 18 kDa (p18) protein was phosphorylated in LP2 membranes of the young rat brain, whereas 22 kDa (p22) and 30 kDa (p30) proteins were phosphorylated in the old rat brain (Figure 3B). The phosphorylation of p18 and p30 in the young and old brain respectively was inhibited in the presence of Ca^{2+}/CaM. In this step, it is still to be elucidated whether the inhibition of phosphorylation is due to the dissociation of small GTP-binding proteins or due to the activation of Ca^{2+}/CaM-dependent phosphatase like calcineurin. However, the possible involvement of nonspecific Ca^{2+}/

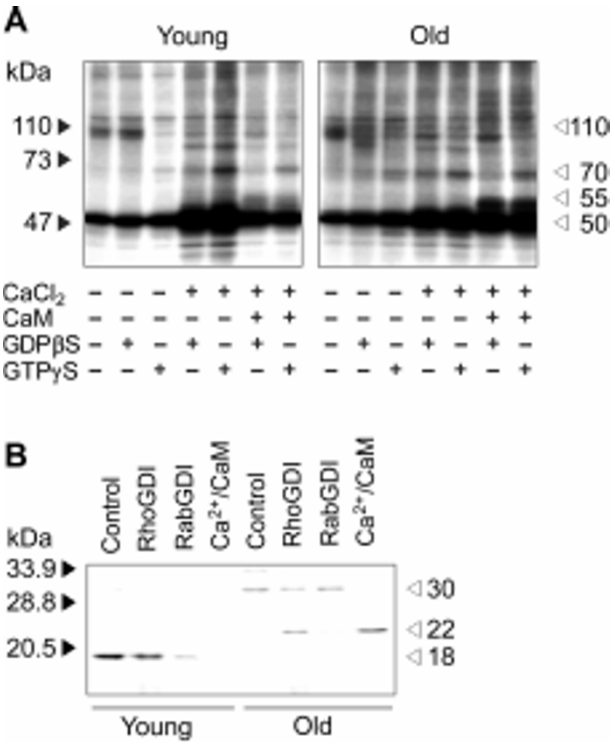

Figure 3. Phosphorylation of proteins in SV membranes. SV membranes (15 μg protein) of young (Y) and old (O) rat brain were incubated with 35 μM CaM, 0.1 mM $CaCl_2$, 0.1 mM GDPβS, GTPγS, and [γ-^{32}P]ATP at 30°C for 30 min (A), and with, 1 μg RhoGDI, 1 μg RabGDI, and 35 μM CaM in the presence of 0.1 mM $CaCl_2$, and then [γ-^{32}P]ATP was added for the protein phosphorylation (B). The reaction samples were loaded on 7-15% gradient gel (A) or 15% SDS-PAGE gel (B), and the gel was dried and the autoradiography was performed. The results were representatives of at least three independent experiments.

CaM dependent phosphatase in the reduction of protein phosphorylation can be ruled out, where the phosphorylation of p22 was activated in the aged rat brain (Figure 3B). RabGDI, which dissociates Rab proteins, inhibited that of p18 in LP2 membranes of the young rat brain and RhoGDI, which dissociates Rho proteins, stimulated the phosphorylation of p22 in LP2 membranes of the old rat brain. These results indicate that dissociation of small GTP-binding proteins affect the phosphorylation of membrane proteins of SV, and that the species of small GTP-binding proteins involved in the regulation of protein phosphorylation in SV are different between the young and old rat brain.

Munc18, one of SV proteins, is phosphorylated *in vitro* by protein kinase C (PKC) and cyclin-dependent kinase 5 (Cdk5). Phosphorylated Munc18 have shown the reduced affinity for syntaxin1A but with some difference where phosphorylation by Cdk5 resulted in a greater reduction in syntaxin1A binding affinity (Shuang *et al.,* 1996; Fletcher *et al.,* 1999). The phosphorylation of SV proteins may alter physiological function. The mechanism and the physiological meaning on the change of phosphorylation of SV from the aged rat brain is an interesting and anticipated observation and our results may provide some biochemical bases for further study.

Acknowledgements

The authors wish to acknowledge the financial support of the Korean Research Foundation in Program Year 1997 (#1997-005-F00222).

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