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Changes in the Levels of Neuropeptides and Their Metabolizing Enzymes in the Brain Regions of Nucleus Basalis Magnocellularis-Lesioned Rats

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Abstract. The regulation mechanism of the interrelation between neuropeptides and their metabolizing enzymes in in vivo tissues is still not clear. In the present report, we attempted to measure the levels of neuropeptides and their enzymes in the frontal cortex, hippocampus, and striatum of the rat that had been bilaterally lesioned by the infusion of ibotenic acid or amyloid β-peptide 25 – 35 (Aβ25 – 35) into the nucleus basalis magnocellularis. In the drug-treated rats, at two weeks after the infusion, the decrease of somatostatin-like immunoreactivity (SS-LI) and the increase of cholecystokinin-8S-LI were found in some brain regions relative to vehicle-treated rats. The immunoreactivities of endopeptidase 24.15 and puromycin-sensitive aminopeptidase and the leucine aminopeptidase- and aminopeptidase B-like enzyme activities did not change in the three brain regions, suggesting that the levels of those peptide-degrading enzymes do not correlate with the changes of the neuropeptide levels. The decrease of subtilisin-like proprotein convertase (SPC)-like enzyme activity was found in the hippocampus of the Aβ25 – 35-treated rats. The SS mRNA level decreased in the hippocampus in parallel with decreases in the SS-LI level and SPC-like enzyme activity. The present data indicate that some of the neuropeptide-processing enzymes may contribute to the control of neuropeptide levels.

Keywords: cholecystokinin, somatostatin, peptidase, ibotenic acid, amyloid β-peptide 25 – 35

Introduction

Much evidence have emerged that in addition to classical neurotransmitters, neuropeptides play a major role in neuronal transmission (1). It is known that neuropeptides play a variety of physiological roles including learning and memory. Recently, it has become increasingly important to clarify the in vivo regulation mechanisms of the neuropeptides in order to elucidate the neurotransmission process. However, biosynthesis and catabolic processes of bioactive neuropeptides differ greatly from those of classical neurotransmitters, which are biosynthesized by each specific enzyme.

Most neuropeptides and peptide hormones are initially synthesized as larger biologically inactive precursor proteins and then are processed into biologically active mature forms by tissue- and region-specific proteases. The precursor protein is initially cleaved at the carboxy (C)-terminus of a monobasic or dibasic amino acid site by a family of subtilisin-like proprotein convertase (SPC), which includes prohormone convertase (PC) 1/3 (EC 3.4.21.93) and PC2 (EC 3.4.21.94). Following endoproteolysis by SPC, carboxypeptidase E (CPE) (CPH, EC 3.4.17.10) is required to remove basic amino acids from the C-terminus of each peptide. After the processing by the CPE, the peptide with a C-terminal glycine residue is converted into a C-terminal amide by peptidylglycine α-amidating monooxygenase (PAM) (EC 1.14.17.3). A series of the above proteolytic cascades are a common mechanism for most neuropeptides and peptide hormones. Up to now, there is almost no evidence about the regulation mechanism controlling the biosynthesis of neuropeptides by those processing

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enzymes.

The peptide-degrading enzymes might also play an important role in the physiologically functional regulations of a number of neuropeptides and peptide hormones because it has been generally believed that neuropeptides are degraded by the enzymes located in extracellular spaces or synaptic clefts. These peptide-degrading enzymes include a number of exopeptidases, such as puromycin-sensitive aminopeptidase (PSA; EC 3.4.11.14) and leucine aminopeptidase (LAP; EC 3.4.11.1), and endopeptidases, such as endopeptidase 24.15 (EP24.15, PZ-peptidase, thimet oligopeptidase; EC 3.4.24.15) and prolyl endopeptidase (PEP, prolyl oligopeptidase, post-proline cleaving enzyme; EC 3.4.21.26). However, the regulation mechanism of the correlation between neuropeptide levels and peptide-degrading enzyme activities in in vivo tissues is not known well.

As classical neurotransmitters, some neuropeptides seem to be positively involved in cognitive regulation processes, possibly as neuromodulators. Somatostatin (SS) and cholecystokinin (CCK), neuropeptides highly concentrated in mammalian hippocampus and cerebral cortex, have been known to be involved in the regulation of learning and memory (2 – 4). Substance P (SP) also appears to be functionally involved in learning and memory because it has been reported that SP increases the duration of NMDA receptor channel opening (5) and enhances glutamate currents mediated by NMDA receptors (6 – 9). In fact, the reduction of SS (10 – 15) and SP (16 – 18) contents in the cortex and hippocampus of the patients with Alzheimer's disease (AD) have been reported.

Since the degeneration of human forebrain cholinergic neurons is a characteristic neuropathological feature of AD, the animal with neural lesions in the nucleus basalis magnocellularis (NBM) has been used as an animal model of AD-type amnesia. In the present study, therefore, we have investigated changes of SS, CCK, and SP contents and their metabolizing enzyme (peptide-degrading and processing enzymes) activities or immunoreactivities in rats that have been NBM-lesioned by ibotenic acid (IBO) and/or amyloid β-peptide 25 – 35 (Aβ25 – 35) and discussed the cross-relationship between the neuropeptides and their metabolizing enzymes in the brain regions of NBM-lesioned rats.

Materials and Methods

Preparation of drug solution

IBO (Sigma, Tokyo) was dissolved in 10 mM phosphate-buffered saline (PBS, pH 7.4) at a concentration of 8 μg/μl. Aβ25 – 35 (Sigma) was prepared according to the reported method (19). In brief, Aβ25 – 35 was dissolved in 2 mM HCl, lyophilized under freeze-drying conditions to convert it to the HCl salt form, and stored at −80°C. One day before use, the peptide was dissolved in PBS (pH 7.4) at a concentration of 10 μg/μl and incubated for 24 h at room temperature.

Animals and surgery

Male Wistar rats, weighing 250 – 300 g, were purchased from Sankyo Laboratory (Tokyo) and housed with free access to food and water. They were maintained in a 12-h light/dark cycle with lights on at 6:00 a.m. at constant temperature (22 ± 1°C) for one week. All animal experiments were carried out in accordance with the Animal Experiment Guidelines published by the Japanese Government in 1980 and have been approved by the Animal Experiment Committee of Yokohama City University Graduate School of Integrated Science.

On the day of surgery, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. The incisor bar was set −1.0 mm posterior and ±2.6 mm lateral to the bregma and 7.8 mm below the top of the skull. Rats received bilateral infusions at 1 μl volume of vehicle (PBS), IBO, or Aβ25 – 35 using a 5-μl Hamilton syringe. The injection was made over a period of 5 min and the needle was left in the injection site for a further 5 min.

Sample preparation

Two weeks after the surgery, the animals were separated to 2 groups. One group of the rats was used for enzyme activity and immunoreactivity. The rats were killed by decapitation, and the brain tissues were rapidly separated on ice to give the following regions: frontal cortex, hippocampus, and striatum. The left-side of each brain region was homogenized by sonication in 1 ml of 10 mM Tris-HCl buffer (pH 7.0). The homogenate was centrifuged at 50,000 ×g for 1 h and the supernatant obtained was stored at −80°C until measurement of the enzyme activities and Western blot analysis. On the other hand, the right-side of each brain region was homogenized by sonication in 500 μl of 2 M acetic acid. After centrifugation for 1 h at 50,000 ×g, the supernatant was lyophilized and reconstituted in enzymeimmunoassay (EIA) buffer [10 mM PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA), 0.05% Tween-20, and 200 KIU/ml aprotinin]. The solution was frozen at −80°C until EIA analysis.

Another group of the rats was killed and dissected to obtain each brain region as mentioned above for Northern blot analysis. These tissues were immediately frozen in liquid nitrogen. Total RNAs were extracted

from the frozen tissues with TRIZOL (Invitrogen, Tokyo) as reported previously (20) and stored at −80°C until use.

Antibodies

Anti-SS-14 and anti-glial fibrillary acidic protein (GFAP) antibodies were purchased commercially [Peptide Institute (Osaka) and DAKO (Kyoto)], respectively. Rabbit anti-PSA serum was kindly provided by Dr. Y. Yamamoto (Shiga University of Medical Science, Otsu) (21). Anti-rat EP24.15 rabbit serum recognizing the C-terminus (521 – 537 amino acids) of EP24.15 (22) was prepared from female white rabbits as reported previously (23). Anti-CCK-8S and anti-SP antibody were prepared in the same way. The specificity of the anti-CCK-8S antibody has been previously reported (23). At 50% of CCK-8S bound, the cross-reactivity to CCK-4, CCK-8 (nonsulfated), CCK-33, and human gastrin I (1 – 17) was 0.3%, 1.3%, 105%, and 66%, respectively. The cross reactivity of anti-SP antibody was 0.3% for neurokinin A and SP with a C-terminal free acid, and for the other tachykinins, it was less than 0.1%.

EIA procedures

EIA for SS, CCK-8S, and SP were based on the double antibody method (23). In brief, the total assay volume of 0.3 ml contained 0.1 ml of the diluted primary antiserum (SS antiserum, 1/1000; CCK-8S, 1/3000; SP, 1/20000 in EIA buffer); 0.1 ml of the enzyme-conjugated solution [SS-horseradish peroxidase (HRP), 1/1000; CCK-8S-HRP, 1/3000; SP-HRP, 1/5000]; and 0.1 ml of the sample or standard peptide solution. The reaction mixture was incubated for 24 h at 4°C. To each tube was added 0.5 ml of the anti-rabbit γ-globulin goat serum (it was diluted 1/50 with 0.1 M borate buffer, pH 8.6, containing 0.1% BSA and 4% polyethylene glycol 6000) and 0.1 ml of rabbit γ-globulin (0.5 mg/ml). After a 10-min incubation at room temperature, free and bound enzyme conjugates were separated by centrifugation at 1,500 ×*g* for 40 min at 4°C. The supernatant containing free enzyme conjugate was decanted and to the precipitate was added 0.2 ml of a substrate solution (13 mg *o*-phenylenediamine/5 ml of 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.01% H_2O_2). After a 20-min incubation at room temperature, 1 ml of 0.5 M H_2SO_4 was added to stop the enzyme reaction, and the absorbance at 492 nm was read in a Quantum II dual wavelength analyzer (Abbott Labs, Chicago, IL, USA).

Western blot analysis

Four micrograms of the total protein lysate of each sample was loaded onto a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk and then incubated with the diluted primary antiserum (anti-GFAP antibody, 1/10000; anti-EP24.15 antibody, 1/10000; anti-PSA antibody, 1/10000) for 2 h at 4°C. Protein bands were detected with the enhanced chemiluminescence system ECL plus (Amersham Biosciences, Piscataway, NJ, USA) using Image Reader LAS-1000 (Fujifilm, Tokyo) and was evaluated by densitometric image analysis using Image Gauge V4.0 software (Fujifilm). Optical densities (ODs) were background-subtracted and analyzed. Final ODs were expressed as pixel means ± S.E.M.

Enzyme activity

Assay for SPC-like activity: SPC-like activity was determined by the fluorometric method. The standard reaction mixture contained 100 mM sodium acetate buffer (pH 5.0); 1 mM $CaCl_2$; 0.1% Triton X-100; 100 μM pyroglutamyl-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (Pyr-RTKR-MCA); protease inhibitor mixture (1 μM E-64, 1 μM pepstatin, 10 μM leupeptin, 300 μM phenylmethylsulfonyl fluoride, 5.0 μg/ml aprotinin, 0.28 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.14 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 10 μM captopril); and 55 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for 4 h, and 1 ml of 1 M acetate buffer (pH 4.2) was added to stop the enzyme reaction. The release of 7-amino-4-methylcoumarin was measured using a spectrofluorometer (FP-777; JASCO, Tokyo) with excitation at 380 nm and emission at 460 nm.

Assay for CPE-like activity: CPE-like activity was detected by the HPLC-fluorescence assay method as described previously (24, 25). The standard reaction mixture contained 50 mM acetate buffer (pH 5.5), 0.2 M NaCl, 1 mM $CoCl_2$, 5 μM *N*-dansyl-Gly-Lys, and 20 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for 30 min, and stopped by heating at 95°C for 5 min. After centrifugation, 20 μl of the supernatant was applied on a C_{18} reversed-phase polymer column (2.0 × 110 mm; BASJ, Tokyo) HPLC with fluorometry. The fluorescence was monitored with excitation at 333 nm and emission at 533 nm.

Assay for PAM activity: PAM activity was determined by the HPLC-fluorescence assay method as described previously (26, 27). The reaction mixture consisted of 50 mM sodium acetate buffer (pH 5.5), 0.2 M NaCl, 5 μM $CuSO_4$, 100 μg/ml of catalase, 2.5 mM ascorbic acid, 0.5 mM *N*-ethylmaleimide, 10 μM *N*-dansyl-D-Tyr-Val-Gly, and 30 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for

24 h and stopped by heating at 95°C for 5 min. After centrifugation, 20 μl of the supernatant was assayed by C_{18} reversed-phase polymer column (4.0 × 120 mm, BASJ) HPLC with the fluorometry.

Assay for aminopeptidase B-like activity: Aminopeptidase B-like (APB) (EC 3.4.11.6) activity was determined by the fluorometric method. The standard reaction mixture contained 0.1 M PIPES-NaOH buffer (pH 7.0), 0.1 M NaCl, 10 μM puromycin, 10 μM E-64, 50 μM Arg-MCA, and 3 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for 10 min, and 1 ml of 1 M acetate buffer (pH 4.2) was added to stop the enzyme reaction.

Assay for LAP-like activity: LAP-like activity was determined by the fluorometric method. The standard reaction mixture contained 50 mM Tris-HCl buffer (pH 9.5), 0.2 M NaCl, 10 μM puromycin, 10 μM E-64, 0.1 mM $MgCl_2$, 50 μM Leu-MCA, and 40 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for 2 h, and 1 ml of 1 M sodium acetate buffer (pH 4.2) was added to stop the enzyme reaction.

Assay for PEP-like activity: PEP-like activity was determined by the fluorometric method as described previously (28). The standard reaction mixture contained 50 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM DTT, 0.1 mM succinyl-Gly-Pro-MCA, and 40 μl of enzyme in a total volume of 100 μl. The reaction was performed at 37°C for 1 h, and 1 ml of 1 M sodium acetate buffer (pH 4.2) was added to stop the enzyme reaction.

Assay for deamidase activity: Deamidase (EC 3.4.16.5) activity was determined by the HPLC-fluorescence assay method as described previously (29). The standard reaction mixture consisted of 50 mM sodium acetate buffer (pH 4.4), 0.016% β-cyclodextrin, 50 μM *N*-dansyl-D-Tyr-Val-NH_2, 5 mM reduced glutathione, and 40 μl of enzyme in a total reaction volume of 100 μl. The reaction was performed at 37°C for 8 h and stopped by heating at 95°C for 5 min. After centrifugation, 20 μl of the supernatant was assayed by C_{18} reversed-phase polymer column (4.0 × 120 mm) HPLC with the fluorometry.

Assay for β-glucuronidase activity: β-Glucuronidase (EC 3.2.1.21) activity was determined by the spectrophotometric method. The standard reaction mixture consisted of 0.1 M sodium acetate buffer (pH 4.0), 1 mM *p*-nitrophenyl-β-D-glucuronide, and 60 μl of enzyme in a total volume of 200 μl. The reaction was performed at 37°C for 3 h, and then 0.3 ml of 0.2% SDS, 0.4 M Tris was added to stop the enzyme reaction. The absorbance was measured at a wavelength of 410 nm with a U-2000 spectrophotometer (Hitachi, Tokyo).

Protein assay

Protein concentration was measured by the Lowry method as modified by Hartree (30) using BSA as standard protein.

cRNA probe synthesis

The entire coding region for rat preprocholecystokinin mRNA was subcloned into pSPT19 (pCCK). The plasmid was linearized with *Hind*III and SP6 polymerase was used to generate the labeled cRNA using a DIG RNA Labeling Kit according to Roche Diagnostics' instructions. A partial coding region from 2,180 to 2,490 for rat β-actin mRNA (310 base pairs) and from 112 to 472 for rat preprosomatostatin (360 base pairs) (proSS) mRNA was inserted into pBluescript SK(+). These plasmids were linearized with *Bam*HI and T7 polymerase was used to generate the labeled β-actin and proSS cRNAs.

Northern blot analysis

Approximately 10 μg of the total RNA from each brain region was electrophoresed in a 1.0% agarose gel containing 2.2 M formaldehyde and 1 × MOPS. The electrophoretically-separated RNAs were transferred to a nylon membrane by capillary blotting. The membrane was prehybridized, hybridized and detected according to Roche Diagnostics' instructions with slight modification. For the quantification of chemiluminescent signals, Image Reader LAS-1000 and Image Gauge V4.0 software were used.

Statistics

Statistical analysis of the different treatment groups was carried out by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test for multiple comparisons. Data are given as the mean ± S.E.M. values.

Results

Changes of neuropeptide contents in the brain regions of the rat infused by IBO or Aβ25 – 35

It has been reported that IBO injection into rat NBM decreases 60 – 75% ChAT immunoreactivity between 1 and 4 weeks after the injection, and the memory impairment occurs at 1 week after the injection (31, 32). In the present study, therefore, we chose the 2 weeks after the infusion as the time for measuring the sample. As shown in Table 1, there was significant reduction of somatostatin-like immunoreactivity (SS-LI) level in both the frontal cortex (71%) and striatum (58%) of IBO-treated rats compared with the controls. The CCK-8S-LI level in the striatum showed a significant

Table 1. The measurements of neuropeptide levels in the frontal cortex, hippocampus, and striatum 2 weeks after IBO and Aβ25 – 35 infusion into rat NMB

	Frontal cortex	Hippocampus	Striatum
SP-LI (pg/mg protein)			
PBS	294.9 ± 10.1	77.2 ± 4.5	960.2 ± 19.2
IBO	281.3 ± 10.8	71.9 ± 5.0	842.5 ± 65.1
Aβ25 – 35	303.1 ± 20.5	74.5 ± 8.1	909.1 ± 29.5
CCK-LI (pg/mg protein)			
PBS	963.6 ± 56.8	277.9 ± 17.4	108.1 ± 14.5
IBO	1119.0 ± 77.0	283.9 ± 21.3	194.7 ± 32.4*
Aβ25 – 35	1430.0 ± 86.0**	321.5 ± 41.9	209.7 ± 18.0**
SS-Li (ng/mg protein)			
PBS	11.52 ± 0.64	1.62 ± 0.09	2.92 ± 0.26
IBO	8.13 ± 0.52**	1.58 ± 0.14	1.69 ± 0.38*
Aβ25 – 35	9.48 ± 0.73*	1.14 ± 0.12*	1.72 ± 0.24**

Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test compared with PBS groups. *P<0.05, **P<0.01.

increase (80%) in IBO-treated rats. In Aβ25 – 35-treated rats, the SS-LI level decreased 18%, 30%, and 41% in the frontal cortex, hippocampus, and striatum, respectively, compared with control animals. The level of CCK-8S-LI increased 49% and 94% in the frontal cortex and striatum, respectively, in Aβ-treated rats. In contrast, the level of SP-LI was not affected in any brain regions examined. Since each neuropeptide content was measured 2 weeks after the infusion and the brain regions measured were different from the injection site, changes of those neuropeptide levels in drug-treated rats might not be transient but a relatively stabilized state, meaning that the metabolism of the neural and glial cells in the brain regions is close to a steady state.

β-Glucuronidase activity and GFAP immunoreactivity

It has been reported that the hyper-reactivity of astrocytes are observed not only in the lesion sites,

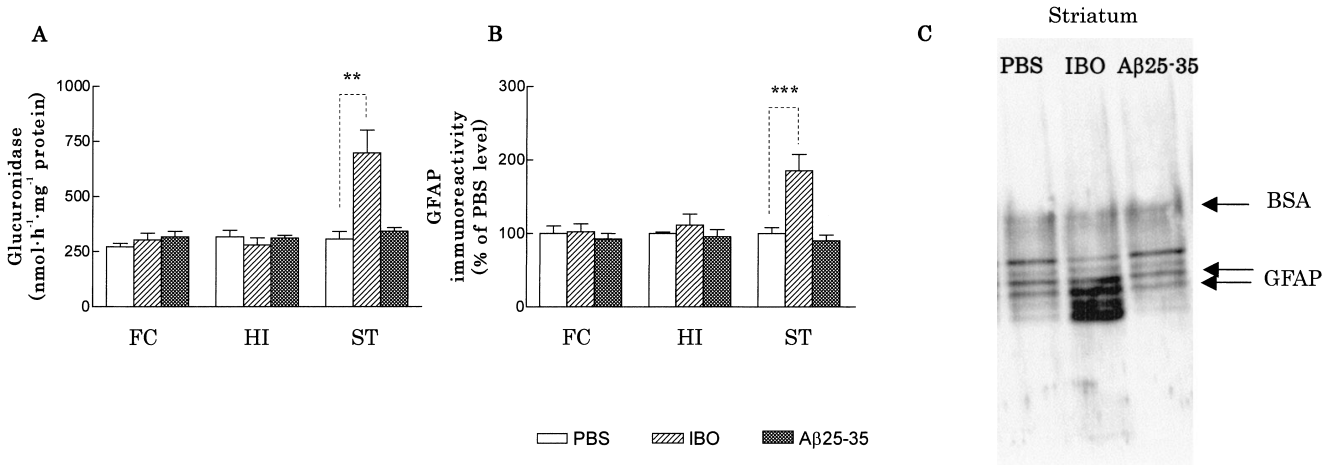

Fig. 1. β-Glucuronidase activity (A) and GFAP-immunoreactivity (B, C) in the brain regions of the rats 2 weeks after IBO and Aβ25 – 35 infusion into the NBM. A: The supernatant of each sample was applied to the enzyme assay. Data are mean ± S.E.M. (bars) values from six to seven animals. B: Four micrograms of the total protein lysate of each sample was applied for Western blot analysis. C: One example of the Western blot analysis in the striatum is shown. Data are the mean ± S.E.M. (bars) values from six animals. Values are expressed as a percentage of the PBS groups. Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test compared with the PBS groups. **P<0.01, ***P<0.001. Whole experiments were repeated twice with similar results. FC, frontal cortex; HI, hippocampus; ST, striatum. PBS, vehicle; IBO, 8 μg/μl ibotenic acid; Aβ25 – 35, 10 μg/μl amyloid β-25 – 35; BSA, bovine serum albumin.

but also in the efferent sites after the damage of the neurons that project on afferent fibers (33, 34). In our preliminary experiments, we also found the increase of GFAP immunoreactive astroglial cells in the NBM sites of the rats 2 weeks after the treatments of Aβ25 – 35 and IBO, respectively (data not shown). β-Glucuronidase, a lysosomal enzyme, is a marker enzyme of glial proliferation because endocytosis of β-glucuronidase into pri-

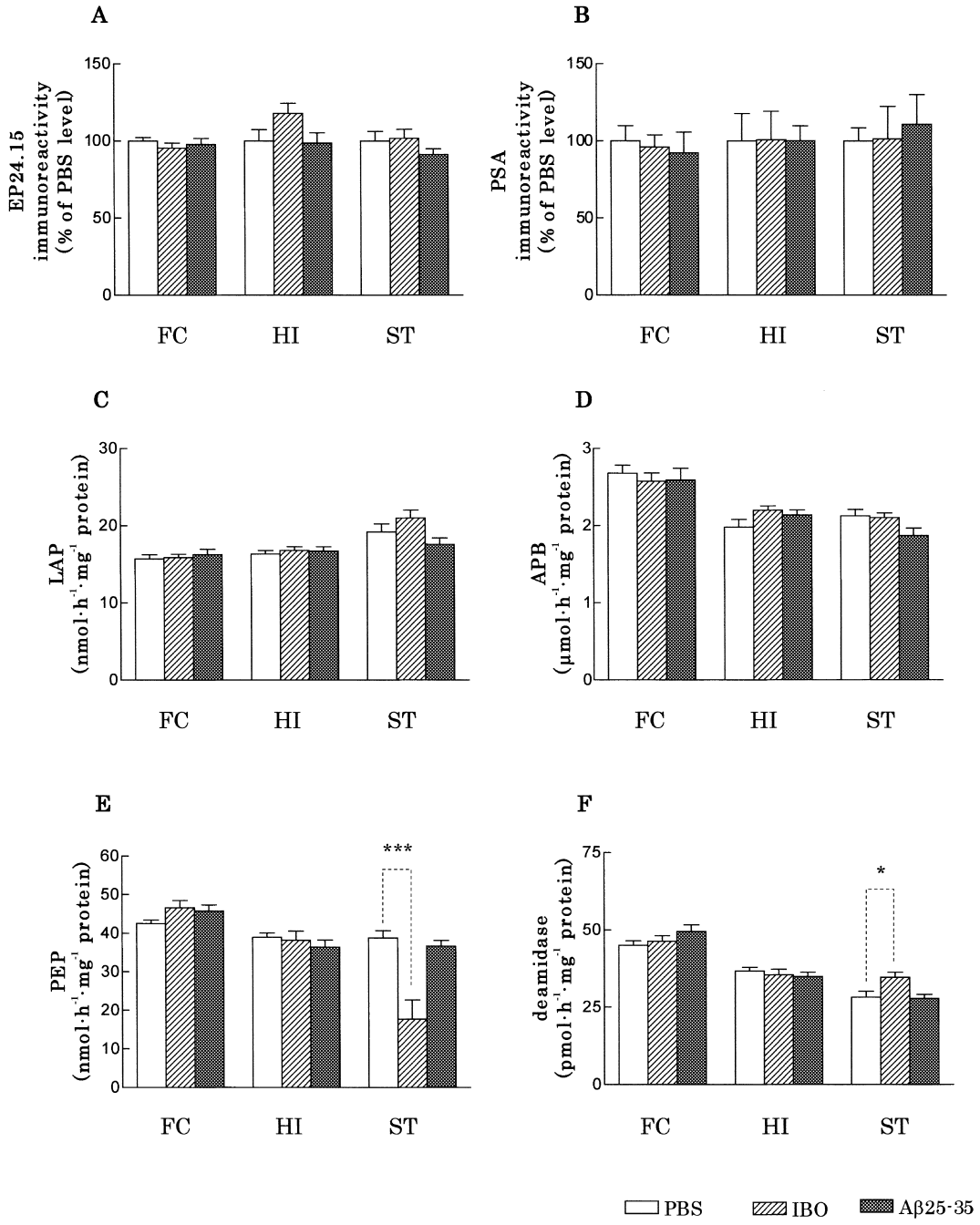

Fig. 2. Immunoreactivity (A, B) and enzyme activity (C – F) of the neuropeptide degrading enzymes in the brain regions of the rats 2 weeks after IBO and Aβ25 – 35 infusion into the NBM. A and B: 4 μg of the total protein lysate of each sample was applied for Western blot analysis. Data are the mean ± S.E.M. (bars) values from six animals. Values are expressed as a percentage of the PBS groups. C – F: The supernatant of each sample was applied to the enzyme assay. Data are the mean ± S.E.M. (bars) values from 6 to 7 animals. Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test compared with the PBS groups. *P<0.05, ***P<0.001. Whole experiments were repeated twice with similar results. FC, frontal cortex; HI, hippocampus; ST, striatum.

mary astrocytes has been reported (35). In the present study, therefore, we investigated changes of the GFAP immunoreactivity and β-glucuronidase activity in the three areas of the rats treated with IBO and Aβ25 – 35. As shown in Fig. 1, IBO infusion increased 127% and 85% the β-glucuronidase activity and GFAP immunoreactivity, respectively, only in the striatum, while Aβ25 – 35 and vehicle infusions did not show any of these changes.

Changes of peptide-degrading enzyme activities and immunoreactivities

Proteins of EP24.15 and PSA were determined by Western blot analysis (Fig. 2: A and B), and other peptide-degrading enzymes, LAP, APB, PEP, and deamidase, were measured by their enzyme activities (Fig. 2: C – F). In the in vitro experiments, EP24.15, PSA, and LAP degrade CCK and SS (36 – 38); deamidase might inactivate CCK and SP; and APB is involved not only in the degradation but also in the biosynthesis of neuropeptides (39). In the Aβ25 – 35-treated rats, however, all of the degrading enzymes measured did not change their immunoreactivities and enzyme activities, while the CCK-LI and SS-LI levels increased and decreased, respectively, (Table 1). In the IBO-treated rats, the striatal PEP-like and deamidase activities were 45% and 123% of the vehicle-treated rats, respectively, but no statistical significance was found in the immunoreactivities of PSA and EP24.15 and in the

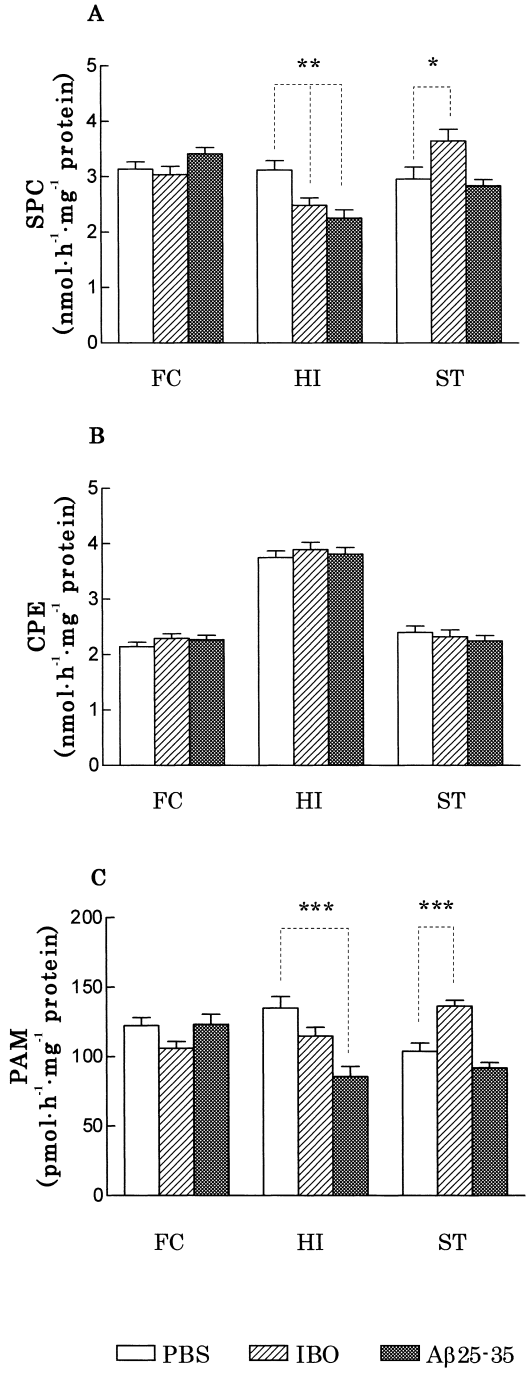

Fig. 3. The enzyme activity of SPC (A), CPE (B), and PAM (C) in the brain regions of the rats 2 weeks after IBO and Aβ25 – 35 infusion into the NBM. The supernatant of each sample was applied to the enzyme assay. Data are the mean ± S.E.M. (bars) values from six to seven animals. Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test compared with the PBS groups. *P<0.05, **P<0.01, ***P<0.001. Whole experiments were repeated twice with similar results. FC, frontal cortex; HI, hippocampus; ST, striatum.

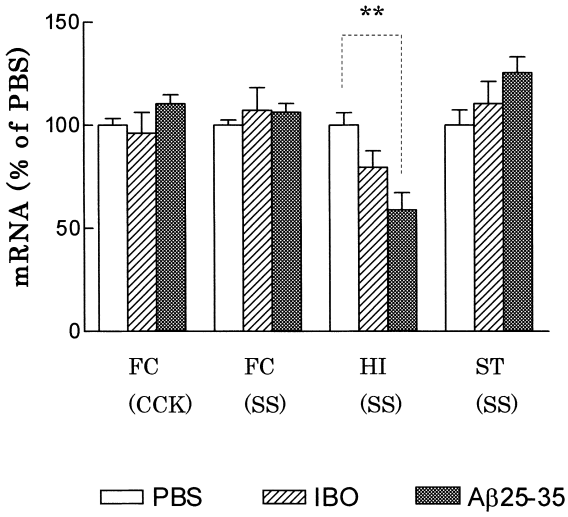

Fig. 4. Quantitation of proCCK and proSS mRNAs in the brain regions of the rats 2 weeks after IBO and Aβ25 – 35 infusion into the NBM. Total RNA (10 μg) was applied on all lanes. Specific proCCK and proSS mRNA levels were normalized to β-actin mRNA densitometric values and expressed as the mean ± S.E.M. of 100% PBS groups from five rats. Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test compared with PBS groups. **P<0.01. FC, frontal cortex; HI, hippocampus; ST, striatum.

APB-like and LAP-like enzyme activities. These results indicate that the changes of those peptide-degrading enzymes have less effect than those of the neuropeptides in the IBO- and Aβ25 – 35-treated rat brains.

Changes of neuropeptide-processing enzyme activities

As shown in Fig. 3, the SPC-like and PAM activities increased 23% and 31%, respectively, in the striatum of IBO-treated rats in comparison with vehicle-treated rats, whereas SPC-like activity in the hippocampus significantly decreased and was 80% of the controls. The hippocampal SPC-like and PAM activities were 28% and 37%, respectively, lower in Aβ-treated rats than in vehicle-treated rats. No change of CPE-like activity was observed in the three brain regions examined. These results suggest that the changes between neuropeptide and their processing enzyme levels are not parallel.

Changes of neuropeptide precursor mRNA levels in the brain regions of IBO- or Aβ25 – 35-infused rats

To investigate whether the changes of neuropeptide levels are derived from their mRNA levels, Northern blot analysis of their mRNAs was performed in the brain regions of vehicle- and drug-treated rats. proSS mRNA level in the hippocampus was reduced and was consistent with the change of SS-LI level in the Aβ25 – 35-treated rats, whereas no change was observed in the proCCK and proSS mRNA levels in the frontal cortex and striatum in which the changes of CCK-8S-LI and SS-LI levels were found (Fig. 4). proCCK mRNA in the striatum was not detected because its concentration was lower than its detection limit (data not shown).

Discussion

Changes of neuropeptide levels in the brain regions of the rat infused by IBO and Aβ25 – 35

The animal lesioned by the infusion of IBO and Aβ25 – 35 into rat NBM has been obtained as an animal model of the AD patient. Some of the behaviors such as spatial memory impairment in the AD model rat are similar to the behaviors in AD patients, because it has been reported that the infusion of IBO (40 – 43) or Aβ25 – 35 (44) into the NBM results in cognitive impairment accompanied with the depletion of choline acetyltransferase. We determined whether the cortical SS-LI level in both IBO- and Aβ25 – 35-treated rats was reduced (Table 1). The decrease of SS in the cortex might be due to the increase of SS release because NBM lesions cause the decrease of excitatory NBM-cortical cholinergic inputs and then decrease GABAergic inhibitory neuronal activity in the frontal cortex. The reason for the decrease of SS in the striatum, however, is not clear, but may be due to the more complicated cortico-striatal neural networks. It is likely that the reduction of SS-LI in NBM-lesioned rats contributes to mnemonic deficits because cysteamine, a depletor of SS, induces memory deficits in experimental rats (2, 3). In addition, the reduction of hippocampal SS-LI level in the Aβ25 – 35-treated rats might contribute to mnemonic deficits. In the present study, however, SP-LI level in the three brain regions was unaffected, which is opposite to the observation in the brain of AD patients (16 – 18). We speculate that the reduction of cortical SS in the brain of AD patients might be correlated with the reduction of cholinergic neurons in NBM, whereas the reduction of the cortical SP might be correlated with the death of cortical neurons. Little is reported about the change of CCK-8S in the brain of AD patients. In the present study, the increase of CCK-8S-LI was found in the frontal cortex and striatum. The increase of CCK content in the cortex after the lesions may be the decrease of the excitatory cholinergic input onto the cortical CCK interneurons. The reason for the increase of CCK in the striatum is not clear, but may be due to the decrease of CCK release via the decrease of cortico-striatal glutamatergic input. Although the increase of CCK-8S in the striatum remains unclear, the increase of the cortical CCK-8S may contribute to improve memory deficits because local application of CCK-8S into the frontal cortex enhances extracellular gulutamate levels (45).

In the present study, different changes in the neuropeptide levels between IBO- and Aβ25 – 35-infused rat brain regions were observed. Although these two drugs impair the cholinergic neurons in NBM, the impairment may be attributable to different action mechanisms. It has been reported that IBO (46) and Aβ (47, 48) impair the cholinergic neurons non-selectively and selectively, respectively. However, the changes of β-glucuronidase activity and GFAP immunoreactivity between IBO- and Aβ25 – 35-treated rats (Fig. 1: A and B) were observed only in the striatum but not in the frontal cortex and hippocampus. In addition, PEP activity decreased in the striatum of IBO-treated rats (Fig. 2E). The increases of β-glucuronidase activity and GFAP immunoreactivity and the decrease of PEP activity in the striatum are probably due to the protective responses of the striatal neurons because it has been reported that in Aβ-resistant cells lysosomal enzymes including β-glucuronidase are up-regulated after the treatment with toxic Aβ (49) and inhibition of PEP activity protects against cell death (50, 51).

The relation between neuropeptide and their degrading enzyme levels

The changes of some peptidase levels have been observed in the brain of AD patients (52, 53). Although CCK-8S-LI and SS-LI levels were influenced in the drug-treated rats (Table 1), no change was observed in the immunoreactivities of PSA and EP24.15 and the APB-like and LAP-like enzyme activities in the present study (Fig. 2). These results suggest that the changes of those peptide-degrading enzymes are less affective than the changes of the neuropeptide levels in in vivo tissues. This was not a surprising phenomenon because those peptide-degrading enzymes have broad substrate specificity and hydrolyze many peptides. Therefore, it is safe to assume that the changes of peptidases in the brain of AD patients are caused by other factors such as cell death and glial proliferation. In fact, it is probable that the increase of the striatal deamidase activity (Fig. 2F) in the IBO-treated rats results from the glial proliferation because deamidase is one of the lysosomal enzymes as well as β-glucuronidase. As a consequence, the increase of striatal deamidase activity may promote the metabolizing rate of neuropeptides.

The relation between neuropeptide and their processing enzyme levels

In the present study, mRNA levels of neuropeptides were unaffected except for the hippocampal SS mRNA level in the Aβ25 – 35-treated rats (Fig. 4). There are many reports on the discordance between the amount of mature neuropeptides and their mRNA expression levels (20, 54, 55). Until now it had been believed that neuropeptide-processing enzymes regulate the amount of mature neuropeptide. In fact, the increases of SPC and PAM activities in the striatum correlate with the increase of CCK-8S-LI in the IBO-treated rats, and the level of SS-LI was decreased in parallel with the changes of SPC in the Aβ25 – 35-treated rats (Table 1 and Fig. 3). In the present study, however, there were some discordance with the changes of the neuropeptide and their processing enzyme levels. For example, in the striatum of IBO-treated rats, the decrease of SS-LI level is inconsistent with the increase of SPC activity, and the decreases of SPC and PAM activities are opposite results to the increase of CCK-8S-LI. There are two possible reasons for these results.

One possible reason for the discordance is due to the different distribution and expression levels between neuropeptides and their processing enzymes. The other possible reason is that SPC, CPE, and PAM, a series of neuropeptide-processing enzymes, are essential for producing biologically active neuropeptides, but their processing enzyme activities may not play a role in rate-limiting regulation of the amount of mature neuropeptides. Moreover, we cannot rule out the non-specific activities of those enzymes.

In some cases, however, changes of processing enzyme levels affect neuropeptide levels. For example, it is probable that a drastic alteration of the processing enzymes such as PC1/3 and PC2 affects the production rate of the amount of neuropeptide if their enzyme expression levels become extremely augmented in regions where their low expression levels are in a steady state, and vice versa because PC1/3 and PC2 produce the different neuropeptides from a precursor. In fact, an increased expression of PC2 in islet α cells was observed to lead to an increase in the level of glucagon-like peptide-1 from proglucagon (56).

In conclusion, the changes of SS-LI and CCK-8S-LI were found 2 weeks after the infusion of IBO and Aβ25 – 35 into the NBM. However, the changes of the peptide-degrading enzymes do not correlate with the changes of the neuropeptide levels, suggesting that these peptide-degrading enzymes may constitutively degrade neuropeptides in the extracellular space with their constant expression. The level of SS-LI changed in parallel with not only SPC activity but also its mRNA level, but the changes of the CCK-LI and SP-LI levels were inconsistent with the changes in the activities of their processing enzymes in some brain regions.

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