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DEVELOPMENTAL CHANGE AND MOLECULAR PROPERTIES OF SOMATOSTATIN RECEPTORS IN THE RAT CEREBRAL CORTEX

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Summary: The postnatal development and molecular properties of somatostatin receptor were studied in rat cerebral cortex. With [^{125}I-Tyr^{11}]SRIF as a radiolabeled ligand, the specific ligand binding to crude membrane increased transiently in the early phase of postnatal development and then decreased. This increase of somatostatin binding was mainly due to the increased number of binding sites. The two subtypes classified by Tran et al., SS_A and SS_B, were confirmed and the studies on the relative amount of the subtypes revealed that more SS_A subtype was expressed compared with SS_B subtype during a week after birth, but, thereafter, both subtypes were almost equally expressed throughout the developmental stages tested. Molecular weight of the covalently labeled somatostatin receptor (SS_A subtype), which was determined with the aid of the cross- linking agents, was estimated to be approximately 71,000 with no intramolecular disulfide bond.

Somatostatin (SRIF) is a tetradecapeptide first isolated from mammalian hypothalamus as an inhibitor of growth hormone release from the pituitary (1). Subsequently, this peptide has been detected in gut and central nervous system (2). In extrahypothalamic regions of central nervous system, SRIF is localized in the synaptosome, affects electrical activity of neurones and binds to synaptosomal membranes. These observations make it likely that SRIF may play a role as a neurotransmitter or neuromodulator (2).

One approach to elucidate the physiological role of SRIF in the brain is to examine the developmental pattern of SRIF receptors. Recent reports have shown that neurons containing SRIF mRNA and its translated product is relatively enriched at early stages of postnatal development in rat neocortical neuronal system (3, 4). SRIF binding in cerebral cortex of rat brain was reduced in aged rats (5). However, it is unclear whether there is a correlation between developmental changes of SRIF neurons and those of SRIF receptor number.

The brain SRIF receptor has been divided into two subtypes with different affinities to a cyclic SRIF analog, SMS201-995: one, which is designated as SS_A receptor, has a high affinity binding property, whereas the other, which is termed as SS_B receptor, displays low affinity for the peptide (6). Proportion of these two subtypes varies with different brain regions (6). These characteristics of the brain SRIF receptor are in contrast with those of the anterior pituitary and the pancreas where only one type of high affinity SRIF receptor similar to SS_A receptor subtype has been reported in terms of binding properties for SMS201-995 (6). Our recent studies, however,

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showed using other SRIF analogs (7) that there may be a structural heterogeneity of somatostatin receptor in the pituitary. These observations suggest that there may be SRIF receptor subtypes in respective tissues in addition to the receptor heterogeneity among different tissues (7, 8, 9, 10).

The present study was undertaken to investigate developmental changes of the SRIF receptor and relative amount of the receptor subtypes in the rat cerebral cortex. The molecular weight of the covalently labeled SRIF receptor (SS_A subtype) of rat brain was estimated to be approximately 71,000, being in contrast with those from the rat pituitary (Mr= 82,000 and 94,000 (7) or Mr= 88,000 (9)) and pancreas (Mr= 92,000) (8).

Materials and Methods

Wistar rats were obtained from Shizuoka Laboratory (Hamamatsu, Japan) and bred in an air-conditioned room. The day of birth was designated as day 0. Male rats were used for studies of 61- to 361-day old rats, while both male and female rats were used for studies of 1- to 30-day old rats. Rats were killed, the brain was placed on ice and cerebral cortex (the frontalcortex and parietalcortex were used) was dissected. The tissues were pooled from 4, 3 and 2 animals, for day 1 to 8, day 10 to 30 , and day 61, respectively.

Crude membranes were prepared according the method described previously (11) using buffers containing protease inhibitors composed of 200 KIU/ml Trasylol, 50 μM phenylmethylsulfonyl fluoride and 50 μg/ml bacitracin. Tissues were homogenized in 10 volumes of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EGTA) with 100 strokes in a Dounce homogenizer at 4°C, followed by centrifugation for 5 min at 150 xg. The resultant supernatant was further centrifuged for 30 min at 15,000 xg, and the pellet was collected, washed twice with TME buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 1 mM EGTA), resuspended in TME buffer, and stored at -80°C until used.

Binding assay was performed using [^{125}I-Tyr^{11}]SRIF (2,000 C_i/mmol, Amersham International PLC, Bucks, England) in siliconized microlabtubes (1.5 ml). Crude membranes (about 10 μg protein) were incubated at 30°C for 30 min in 50 mM HEPES-KOH buffer, pH 7.5 (100 μl) containing 2 mM $MgCl_2$, 10 mg/ml BSA, 200 KIU/ml Trasylol, 50 μM phenylmethylsulfonyl fluoride, 0.2 mg/ml bacitracin and [^{125}I-Tyr^{11}]SRIF. Bound and free peptides were separated by centrifugation at 15,000 xg for 3 min. Nonspecific binding was determined by further addition of excess SRIF (100 nM) in the assay buffer. Under these conditions, all of the radioactivity of [^{125}I-Tyr^{11}]SRIF was recovered by immunoprecipitation with SRIF antiserum (Amersham) after incubation. Protein concentration was determined by the method of Lowry et al. , using bovine serum albumin as a standard (12).

Cross-linking studies were carried out using crude membrane preparation of day 7 as described elsewhere (7). Briefly, membranes (50 μg protein) were incubated at 30°C for 30 min with [^{125}I-Tyr^{11}]SRIF in 500 μl of binding assay mixture and the reaction was stopped by adding equal volume of 50 mM HEPES-KOH, pH 7.5 containing 5 mM $MgCl_2$. Labeled membranes were separated by centrifugation, washed with 1 ml of PM buffer (10 mM potassium phosphate buffer, pH 7.4 containing 5 mM $MgCl_2$) and suspended in 250 μl of PM buffer in microlabotubes. Under the dark condition, 2.5 μl of 10 mM N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) or 10 mM N-hydro-succinimidyl-4-azidobenzoate (HSAB) dissolved in dimethylsulfoxide was added into the suspension. Following incubation at 4°C for 5 min, the samples were irradiated for 10 min at 4°C using 20-watt fluorescent sunlamp for ANB-NOS or 15-watt germicidal lamp for HSAB at the distance of 3 cm above their surfaces. Then reaction was quenched with 50 mM Tris-HCl buffer, pH 7.5. The labeled membranes were solubilized with 0.063 M Tris-HCl, pH 6.8 containing 4% SDS, 10% glycerol with or without 50 mM dithiothreitol, heated at 60°C for 30 min and then subjected to SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the gel was fixed, stained, destained and dried on a filter paper under vaccum. Autoradiograms were obtained after exposure to Kodak X-Omat AR film with intensifying screens at -70°C.

SMS201-995 was generously provided by Sandoz Pharmaceuticals, LTD., Tokyo, Japan. SRIF was purchased from Peptide Institute, Inc., Osaka, Japan and the cross-linking reagents were from Pierce Chemical Co., Illinois, USA.

Results

Developmental change of SRIF receptors

The specific binding of SRIF to crude membranes of cerebral cortex increased about 2 fold from day 1 to day 5, remained constant and then declined after day 13 (Fig. 1). These results demonstrated that the expression of SRIF receptors was relatively high at the early phase of postnatal development. We next examined whether the binding properties of [^{125}I-Tyr^{11}]SRIF binding were altered during postnatal development. Scatchard analysis of the results from saturation curves gave straight lines in cerebral cortex from both 8- and 61-day old rats (Fig. 2 A,B). The mean values of K_d and B_{max} of 8-day old rats were 81.1 ± 4.5 pM, and 300.0 ± 19.6 fmol /mg protein, respectively (n=3), whereas those of 61-old rats were 111.6 ± 7.7 pM and 106.9 ± 5.1 fmol/mg protein, respectively (n=3). Thus the increased binding activity observed in 8-day old rats seemed to be due to not only a large increase in the number of binding sites but also a slight increase in the binding affinity.

Tran et al (6) demonstrated that there are two types of SRIF receptor in the brain, which display different binding affinity for SMS201-995. We confirmed their observation in the present study. As shown in Fig. 3 A, SMS201-995 produced biphasic displacement curves against [^{125}I-Tyr^{11}]SRIF binding. The SS_Areceptor subtype was characterized by demonstrating that the high affinity [^{125}I-Tyr^{11}]SRIF binding was displaced by 40 nM SMS201-995. The residual binding acitivity, which represents SS_B receptor subtype, was totally displaced by 10 μM SMS201-995. The results shown in Fig. 3 A also show that the relative amount of SS_A and SS_B subtypes varies with membrane preparations with different ages (day 8 versus day 61). It should be noted that displacement curves of [^{125}I-Tyr^{11}]SRIF binding to the membranes from day 8 and day 61 rats by native SRIF displayed no shoulder and were essentially indistinguishable (data not shown). When relative amount of SS_A and SS_B subtypes was examined with membranes from various ages using SMS201-995 (Fig. 3 B), SS_A subtype accounted for about 65% of the total number

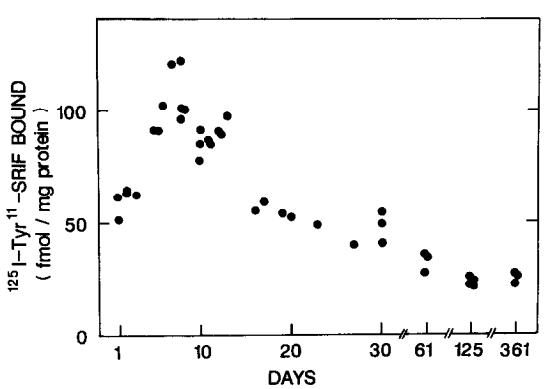

Figure 1. Developmental change of the specific binding of [^{125}I-Tyr^{11}]SRIF to the crude membranes of the rat cerebral cortex. The membranes (about 10 μg) from rats with different ages were incubated with [^{125}I-Tyr^{11}]SRIF (60 pM) as described under Materials and Methods. Each point represents the mean of triplicate determinations.

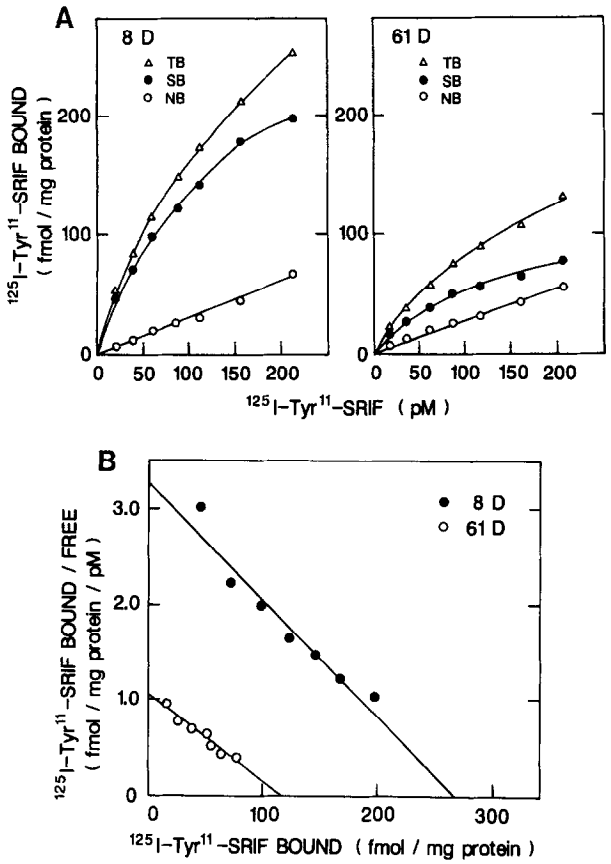

Figure 2. Saturation curves (A) and Scatchard plots (B) of the binding of [^{125}I-Tyr^{11}]SRIF to the cerebral cortex membranes from 8-day and 61-day old rats. [^{125}I-Tyr^{11}]SRIF at different concentrations was incubated with membranes (10 μg protein) as described under Materials and Methods. TB, NB and SB indicate the total binding, nonspecific binding and specific binding, respectively. Each point represents the mean of duplicate determinations.

of SRIF receptors from day 1 to day 8, but thereafter amount of both subtypes was almost equal throughout the developmental stages tested.

Cross-linking studies of SRIF receptors.

To understand the molecular properties of rat brain SRIF receptors, we first attempted to know the molecular weight by means of photoaffinity cross-linking reagents which we successfully used to demonstrate estradiol-dependent and -independent SRIF receptor subtypes in the rat pituitary gland (7). When the cerebral cortex membranes from 7-day old rats were incubated with [^{125}I-Tyr^{11}]SRIF and then cross-linked with 0.1 mM ANB-NOS (Fig.4) or 0.1mM HSAB (data not shown), one single band with the molecular weight of approximately 71,000 was apparent after SDS-polyacrylamide gel electrophoresis under nonreducing conditions, whereas two bands with molecular weight of 71,000 and 250,000 were detected in the presence of 50 mM dithiothreitol (Fig. 4 A). The radioactivity corresponding to 71,000 dalton protein band was reduced by the addition of 4 nM SMS201-995 and totally disappeared by 40 nM SMS201-995, suggesting that the 71,000 dalton protein represents SS_A receptor subtype (Fig. 4 B). In contrast,

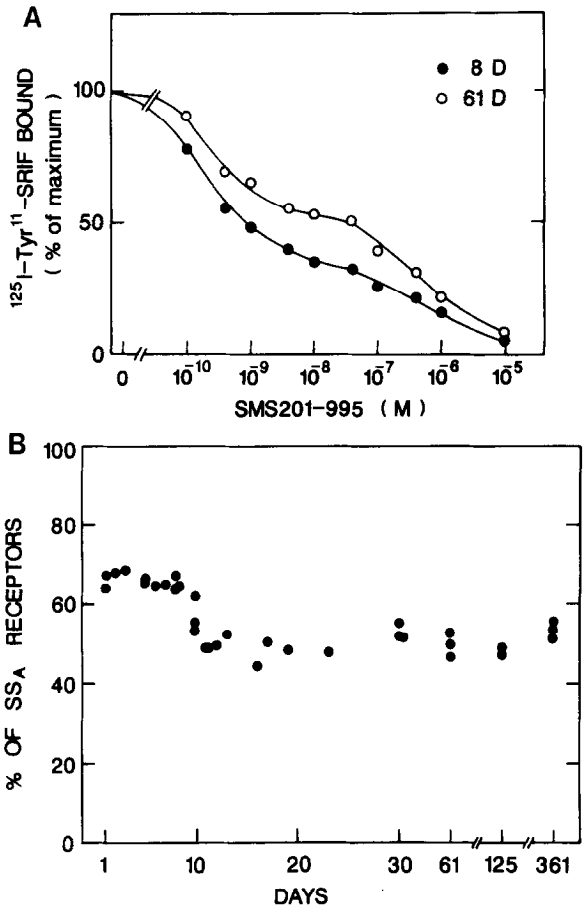

Figure 3. (A) Displacement curves of the binding of [^{125}I-Tyr^{11}]SRIF by SMS201-995. The maximum binding in the absence of competitor was taken as 100%. Crude membranes from 8-day old (●) and 61-day old rats (○) were incubated with [^{125}I-Tyr^{11}]SRIF (50 pM) in the absence and presence of different concentrations of SMS201-995. Data points represent duplicate or triplicate determinations from a representative experiment. (B) Change of the proportion of SS_A receptor subtype of the rat cerebral cortex during development. The binding activity which was displaceable by the presence of 40 nM SMS201-995 was taken as the SS_A subtype. Each point represents the mean of triplicate determinations.

the radioactivity corresponding to the 250,000 dalton protein band still remained by the addition of 40 nM SMS201-995, suggesting that the 250,000 dalton protein may be SS_B subtype. However, since the ligand binding to the 250,000 dalton protein was also reduced by inclusion of 4 and 40 nM SMS201-995, it remains to be determined further whether the 250,000 dalton protein represents SS_B receptor subtype or an artifactual product. Similar results were obtained with another heterobifunctional cross-linking reagent, HSAB (data not shown).

Discussion

In this study, we first reported that the specific binding activity of both SS_A and SS_B receptors in the rat cerebral cortex increased transiently in the early phase of postnatal development. The

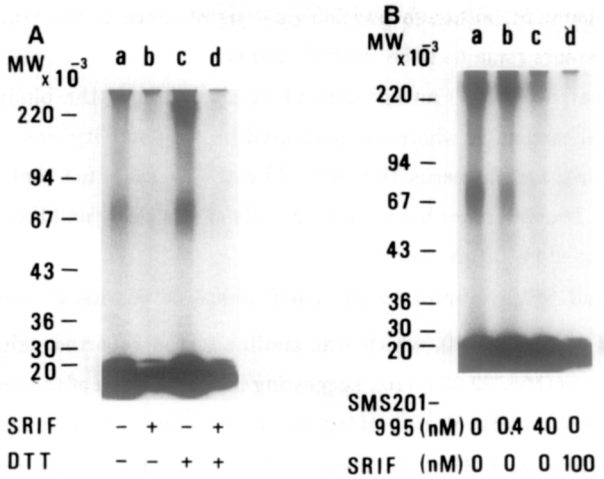

Figure 4. Cross-linking of [^{125}I-Tyr^{11}]SRIF to the cerebral cortex membrane receptors from 7-day old rats. (A) The crude membranes (50 μg protein) were incubated with [^{125}I-Tyr^{11}]SRIF (150 pM) in the absence and presence of 100 nM SRIF, cross-linked with ANB-NOS, solublized in the absence and presence of 50 mM dithiothreitol (DTT) and electrophoresed on a 10% polyacrylamide slab gel as described under Materials and Methods. (B) The crude membranes (50 μg protein) were incubated with the ligand in the absence and presence of indicated concentrations of SMS201-995 and SRIF, and cross-linked as described in (A). The membranes were treated with 50 mM dithiothreitol and subjected to SDS-polyacrylamide gel electrophoresis.

relative amount of SS_A receptor varied during development and was higher than that of SS_B receptor during a week after birth. Moreover, we demonstrated that the molecular size of the cross-linked SS_A receptor subtype of rat brain was estimated to be approximately 71,000 .

A recent review describes that in neurons in the developing cortex there is a transient expression of putative neurotransmitter substances, which may play an important role in establishment of neuronal circuits in the brain (13). Transient increases of both SRIF content and the number of SRIF neurons in early phase of postnatal development, showing peaks of expression at about two weeks after birth, have been reported (3, 4). The present observation that the transient increase of SRIF receptor sites occurs from day 5 to day 13 after birth shows a good correlation with those previous ones. In fact, localization of SRIF receptors has been suggested to show many parallels with the distribution of SRIF immunoreactive neurons (14). Therefore, formation of the SRIF receptor may coincide with the appearance of neurones containing immunoreactive SRIF in the neocortex. There has been a paper, however, which describes that SRIF receptor and immunoreactive SRIF in rat cerebellum were expressed transiently in the early phase of postnatal development but with different localization (15).

The transient expression of neurotensin (16) and N-methyl-D-aspartic acid receptors (17) at the early stage after birth was recently reported in the rat brain. The receptor sites of other classic neurotransmitters such as noradrenaline (18), acetylcholine (19), dopamine (20) and GABA (21) have been shown to increase after birth but attain the maximum levels at later stage of brain development. Thus, there seems to be a time-difference in the development of each neurotransmitter and its receptor system in various neurons. This is not surprising since it is plausible that each neurotransmitter and its receptor system play a particular role at various

stages of brain development, although physiological significance of the transient expression of neurotransmitter receptors remains to be studied further.

A previous paper described an age-dependent alteration of the binding activity of SRIF receptor in rat frontal cortex (5), where the decreased binding activity was ascribed to decreased affinity of the receptor (1 month versus 18 month old rat). We could not confirm their observation in the present study. The difference between the results of this study and those of the previous one remains to be determined in future studies.

The cross-linked SS_A receptor in the early phase of postnatal development showed a molecular weight of 71,000 (Fig.4), which was similar to the reported value of cerebrocortical membranes of adult rats (Mr= 72,000) (10), suggesting that alteration of the molecular size of SRIF receptor seems unlikely to occur during ontogenic development. An attempt to dissociate SRIF receptor subtypes (SS_A and SS_B) by means of photoreactive cross-linking reagents was unsuccessful. We observed 250,000 dalton protein in addition to 71,000 dalton protein on SDS-polyacrylamide gel electrophoresis under reducing condition. The latter protein was identified as SS_A subtype by comparing its affinity for SRIF and its analog. On the other hand, whether the former protein is SS_B subtype or an artifactual product remains unsettled in this study. In our recent study (7), we observed 250,000 dalton band using crude membranes but not purified membranes from the rat anterior pituitary. Therefore, there exists a possibility that the 250,000 dalton protein might have been produced by further cross-linking of the labeled receptor to other contaminating protein(s) in the crude membrane preparation.

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