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Developmental Regulation of SSeCKS Expression in Rat Brain

Li Chen · Jing Qin · Chun Cheng · Hai'ou Liu · Shuqiong Niu · Ji Qian · Linlin Sun · Feng Xiao · Shuxian Shi · Aiguo Shen

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Abstract SSeCKS (src suppressed C kinase substrate) was identified as a PKC substrate/PKC-binding protein, which plays a role in mitogenic regulatory activity and has a function in the control of cell signaling and cytoskeletal arrangement. Previous studies showed that expression of SSeCKS mRNA and protein levels were developmentally regulated in rat testis and the molecular might have some effects on the process of spermiogenesis. Here we carried out experiments to investigate the expression of SSeCKS in rat brain. Western blot analysis indicated that SSeCKS could be detected in the whole brain of developing rat embryos and reached its peak at 1 week after birth, while during mature period, its level was decreasing. Regional-distribution analysis showed that the expression pattern of SSeCKS in telencephalon, hippocampus and diencephalons was in accordance with the result from whole brain both in mRNA and protein level. However, in cerebellum, SSeCKS was almost in the same level, and in brainstem, the expression level was higher in 4-week-old rat brain than in 1-week-old one. Immunohistochemistry results showed SSeCKS was in diffused and granule-like distribution. Double immunofluorescence staining showed that it was expressed by some GFAP positive cells. All the results suggested that SSeCKS might affect brain development and further research is needed to have a good understanding of its function and mechanism.

Keywords SSeCKS · Brain development · Rat

Introduction

Protein kinase C (PKC) and many of its substrates play a central role in shaping actin-based cytoskeletal architecture during cell migration, cytokinesis and tissue development (Jaken 1996; Kiley et al. 1995). SSeCKS was identified by Jaken et al. (Chapline et al. 1996, 1998) and Gelman et al. (Lin et al. 1995, 1996) as a PKC substrate/PKC-binding protein. Evidence was presented that SSeCKS played a role in regulating the actin-based cytoskeletal network. Chapline et al. (1998) showed that SSeCKS was phosphorylated by PKC and that there was a strong correlation between phosphorylation of the protein and actin-based cytoskeletal rearrangements. Their results showed that phosphorylated SSeCKS, but not actin, accumulated in membrane protrusions and ruffles in REF52 rat embryo fibroblasts, indicating that PKC activation and SSeCKS may be involved in membrane-cytoskeletal remodeling in the cultured cells.

The studies from Jaken's laboratory indicated that PKC-interacting proteins were primary targets for PKC phosphorylation and that phosphorylation of these proteins might be related to the role of PKC in regulating cell

Chen and Qin contribute equally to this work.

L. Chen · J. Qin
Department of Pathology, Medical School of Nantong University (Former Nantong Medical College), Nantong 226001, People's Republic of China

C. Cheng · H. Liu · J. Qian · L. Sun · F. Xiao · S. Shi
Department of Microbiology and Immunology, Medical School of Nantong University (Former Nantong Medical College), Nantong 226001, People's Republic of China

S. Niu · A. Shen (✉)
The Jiangsu Province Key Laboratory of Neuroregeneration, Nantong University, Nantong 226001, People's Republic of China
e-mail: Shen_aiguo@yahoo.com

growth and differentiation (Chapline et al. 1998). It has been suggested that SSeCKS may effect growth regulation. SSeCKS mRNA and protein levels are down-regulated in ras-transformed and src-transformed cells. Moreover, the overexpression of clone SSeCKS in either untransformed or transformed fibroblasts resulted in a significant decrease in cellular proliferation rates.

SSeCKS shows significant sequence homology with Gravin (Nauert 1997), an intracellular protein that is widely expressed in various tissues including fibroblasts, neural crest derived cells, and the central and peripheral nervous systems (Grove et al. 1994). Labeling of SSeCKS in central axonal collaterals of primary sensory neurons was observed in the dorsal horn at all spinal levels (Siegel et al. 2002). Sperm maturation in the adluminal crypts of Sertoli cells is controlled by the PKC-dependent organization of actin and tubulin networks in both sperm and Sertoli cells (Zini et al. 1997). SSeCKS is a high-molecular-mass heat-stable protein present in rat testis (Chapline et al. 1996). Western blot from previous researchers showed that it was especially highly expressed in rat testis. However, in adult rat brain, its level was low. But in unmature rat brain, its expression pattern is unknown. So we carried out the experiments to study the protein molecular in rat brain and found SSeCKS was expressed at high levels in the developing rat brain while the expression decreased in the mature rat brain. Double immunofluorescence staining indicated that it partly colocalized with GFAP. All these suggested SSeCKS might have an effect on brain development.

Materials and Methods

Experimental Animals

All experiments were conducted on SD (Sprague-Dawley) rats (Department of Animal Center, Medical College of Nantong University). Animals were kept under standardized laboratory conditions in an airconditioned room with free access to food and water. For the whole brain Western blot analysis, rats were killed under combined anesthesia. Subsequently, the skull was opened and the whole brain was removed, and for regional-distribution Western blot, fresh brains were dissected in five regions: telencephalon, hippocampus, diencephalon, cerebellum and brainstem. The tissues of corresponding regions from three to four animals were pooled. All samples were kept on ice until total protein isolation. For regional-distribution real-time PCR analysis, fresh brains were also dissected in five regions we said above. For immunohistochemistry staining, rats were anesthetized and perfused intracardially with 0.9% saline, followed by fixative (4% paraformaldehyde). The brains were immediately removed from the cranium, immersion-fixed overnight at 4°C in the same fixative and subsequently processed for sectioning.

RNA Isolation and Reverse Transcription (RT)

Total RNA was isolated from 100 mg of rat brain tissue by Trizol. RNA concentration was determined by absorption at 260 nm, and the 260/280 nm absorption ratio of the samples was >1.9. The ThermoScript RT System (Fermentas) was used for the RT reactions, each sample contained approximately 5 μg RNA. The cDNA was diluted 1:1 and 2 μl was used in each 20 μl PCR reaction.

Real-time PCR

The total RNA was extracted by Trizol. PCR primers for SSeCKS, and β_2-microglobulin (β_2-M) were designed corresponding to the coding region of the genes as follows: SSeCKS primers, sense 5′-AAGTGCTGGCTTCGGAGAA AG-3′ and antisense 5′-TGACTTCAGGAACTTCAAGGC TC-3′; β_2-M primers, sense 5′-GTCTTTCTACATCCTGG CTCACA-3′ and antisense 5′-GACGGTTTTGGGCTCCT TCA-3′. TaqMan probes for SSeCKS, and β_2-M were designed corresponding to the coding region of the genes as follows: SSeCKS probe, 5′(FAM)-AGCCTGTCCAGT-CT CAGAGCCCTGTG-(TAMRA)3′ and β_2-M probe, 5′ (FAM)-CAC-CCACCGAGACCGATGTATATGCTTGC-(TAMRA)3′. The reaction mixes included 1 × PCR buffer, 20 mM magnesium chloride, 0.2 mM deoxyNTP, and 10 nmol TaqMan probe with a pair of 10 nmol SSeCKS primers, 10 nmol β_2-M primers and probe of each molecule. Real-time PCR was performed in a Rotor Gene 3000 Detector (Perkin-Elmer/Applied Biosystems, Foster City, CA). The thermal cycling program consisted of 3 min at 94°C, followed by 40 cycles of 20 s at 94°C and 1 min at 60°C. To account for variability in total RNA input, the expression of the transporters was normalized to β_2-M in the samples.

Western Blot Analysis

Proteins were mixed with 2 × loading and DTT (4:5:1), boiled in water for 5–10 min, cooled in ice and then resolved on 6% SDS-PAGE gels and transfered to PVDF membranes. The membranes were blocked with 5% dried skim milk in TBST (2.42 g Tris, 8 g NaCl, 0.05%Tween 20). After 2 h at room temperature, the filters were washed by TBST for three times and then incubated overnight with affinity purified polyclonal antibody against SSeCKS (Sigma, 1:2,000) at 4°C. Finally, Rabbit-anti-Sheep IgG conjugated to horseradish peroxidase (SouthernBiotech, 1:2,000) was added for an additional 2 h and the blots were developed using ECL (Pierce company, USA).

Immunohistochemistry

Immunohistochemistry was performed on 8 μm sections of 1- and 4-week-old rat brain. The standard two steps staining method was adopted. Briefly, the sections were immersed in 0.3% (v/v) H_2O_2/methanol for 30 min at 37°C to inhibit endogenous peroxidase. After that, sections were washed three times with 0.01 mol/l PBS and then incubated in 10% normal serum blocking solution—species same as secondary antibody, containing 3% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100, prior to incubation with polyclonal antibody specific for SSeCKS (Sigma, 1:100) in a moist chamber for 6 h at 37°C. Incubation of primary antibody was omitted in several sections for control. The sections were washed in PBS three times and treated for 2 h with Rabbit-anti-Sheep IgG (SouthernBiotech, 1:200) conjugated to horseradish peroxidase. After washing three times, the sections were stained in DAB containing 0.5% H_2O_2 for about 2 min, then counterstained with hematoxylin, dehydrated, cleared and mounted in Permount.

For double immunofluorescent staining, sections were first blocked with 10% normal serum blocking solution—species same as secondary antibody, containing 3% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100 and 0.05% Tween 20 overnight at 4°Cin order to avoid unspecific staining. Then the sections were incubated with both polyclonal antibody specific for SSeCKS (Sigma, 1:100) and monoclonal primary antibody for different markers as follows: GFAP (Sigma, 1:200), NeuN (Chemicon, 1:600), OX42 (Serotec, 1:50), Galac (Chemicon, 1:100) for 48–60 h at 4°C. After wash in PBS for three times, each time 10 min, the secondary antibodies (FITC-Rabbit-anti-Sheep, Santa Cruz, 1:50; TRITC-Donkey-anti-Mouse, SBA, 1:50/1:100) were added in dark room and incubated for 2–3 h at 4°C. The images were captured by Leica fluorescence microscope (Germany).

Statistical Analysis

For all experiments, the relative expression values were calculated using the wstata statistics software. Data are expressed as mean +SD. Differences in changes of values between the groups were tested using a Student's t test. A difference was accepted as significant if $P < 0.05$. Each experiment consisted of at least three replicates per condition.

Results

The Expression of SSeCKS is Developmentally Changed in the Whole Rat Brain

From others' records, we knew that SSeCKS presented in rat testis, rat brain and several cell lines. Western blot analysis showed that SSeCKS protein was highly expressed in rat testis. Low levels of protein can be seen in rat brain and lung (Erlichman et al. 1999). Whether this phenomenon also exists in rat embryo or early stage of rat brain development needs further elucidation. Therefore, we first tested the expression of SSeCKS in whole rat brain. Our time points included different stages of rat embryo, newborn, 1-week-old, 2-week-old, 4-week-old and more mature rats. Western blot analysis was performed to examine the expression of SSeCKS. As we can see from Fig. 1a, the blot revealed a high-molecular-mass doublet (290/280 kD) expressed in the brains of different stages. It increased with time, began to upregulate in 18-day-old rat embryo and peaking at 1-week-old mice, then it began to decrease. We could also see that in adult rat brains, SSeCKS almost undetectable which is consistent with previous reports. These data indicates that SSeCKS is expressed at a much higher level in the newborn brain than in the adult one. Densitometry analysis indicated that the SSeCKS band was about 2-fold in the 1-week-old rat brain more than in the 4-week-old one, again indicating that SSeCKS was expressed at a higher level in the newborn brain than in the adult (Fig. 1b). The same results were obtained in two independent experiments. All the results indicate that SSeCKS indeed exists in rat brain, its expression is developmentally regulated and high expression can be seen in unmature rat brain.

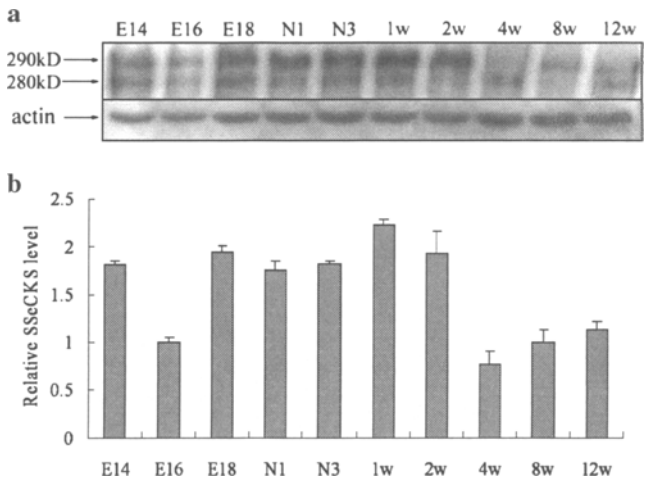

Figure 1 Developmental expression of SSeCKS by Western blot analysis. **a**: The 40 μg protein samples from total brain tissues of 14-day-old rat embryos (lane 1), 16-day-old rat embryos (lane 2), 18-day-old rat embryos (lane 3), 1-day-old (lane 4), 3-day-old (lane 5), 1-week-old (lane 6), 2-week-old (lane 7), 4-week-old (lane 8), 8-week-old (lane 9) and (lane 10) 12-week-old rats was subjected to SDS/PAGE (6% gels). E14, 14-day-old rat embryos; E16, 16-day-old rat embryos; E18, 18-day-old rat embryos; N1, newborn rat (1-day-old); N3, newborn rat (3-day-old); w, week. **b**: Each lane in A was scanned by laser densitometry and the absorbance of the appropriate bands was calculated. The concentration of SSeCKS protein relative to β-actin in each lane was determined from the ratio of their absorbance

Regional-distribution Analysis of SSeCKS mRNA Expression in Rat Brain by Real-time PCR

Since the expression of SSeCKS in rat brain was developmentally associated, in order to reveal the regional distribution of SSeCKS in rat brain, and to find out whether there is a difference in the regional distribution between the newborn and adult rats, we first chose real-time PCR to detect the molecular in mRNA level. The samples used were freshly isolated total RNA from telencephalon, hippocampus, diencephalon, cerebellum and brainstem of 1-and 4-week-old rat brains respectively. From Fig. 2, we could see that the relative quantity of SSeCKS was significantly high in telencephalon, hippocampus, diencephalon of 1-week-old rat brains but very low in the same region of 4-week-old rat brain., while there was no striking different in the cerebellum. In brainstem, the abundance of SSeCKS was high in 4-week-old rat brain and low in 1-week-old rat brain.

Differential Regional Distribution of SSeCKS Expression in Newborn and Adult Rat Brain

To further testify our findings, we did Western blot to investigate whether the expression pattern was consistent with the result of real-time PCR. The samples used were freshly isolated total protein from telencephalon, hippocampus, diencephalon, cerebellum and brainstem of 1- and 4-week-old rat brains respectively. In telencephalon, SSeCKS was expressed abundantly in newborn rats, with a significant decrease in adult rats, and a similar expression pattern could be detected in hippocampus and diencephalon. In contrast, the expression levels were low in cerebellum in both newborn and adult rats. The brainstem was the only area that differed significantly from the rest of the samples, with a relative high expression in newborn rats, and a higher expression in adult rats (Fig. 3a). Densitometry analysis indicated that the SSeCKS band was higher in telencephalon, hippocampus and diencephalon of the 1-week-old than in the 4-week-old mouse (Fig. 3b). These results were in good agreement with real-time PCR analysis of different brain regions.

Immunohistochemical Localization of SSeCKS in Rat Brain

In order to observe the localization of SSeCKS in different regions of rat brain, we used immunohistochemistry to definite whether it was consistent with the result of Western blot. The overall expression of SSeCKS was widespread and the most prominent expression was seen in cerebral cortex and hippocampus. In cerebral cortex, the expression pattern of SSeCKS was diffused and granular-like (Fig. 4d). While in hippocampus, strong signals were presented in the hippocampal formation and we just showed a typical region (Fig. 4c) of hippocampus from 1-week-old rat brain. The apparent difference of expression in cortex and hippocampus was detected between the 1- and 4-week-old rats. The expression of SSeCKS decreased markedly in the 4-week-old rat brain (Fig. 4e,f). We used the hippocampus and cortex from 1-week-old rats as a negative control (Fig. 4a,b).

Double Immunofluorescent Staining for SSeCKS and GFAP, NeuN, OX42 and Galac

To further determin SSeCKS was expressed in what kind of cells, we used double immunofluorescent staining to

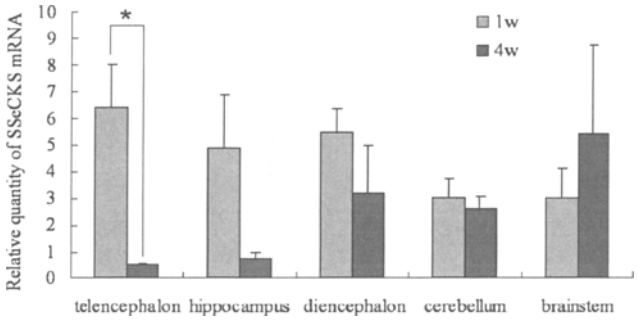

Figure 2 Regional distribution of SSeCKS expression by real-time PCR analysis. Significantly high expression of SSeCKS was seen in telencephalon, hippocampus, diencephalon of 1-week-old rat brains, but in the same region of 4-week-old rat brain, the level was low. In cerebellum, the difference wasn't very obvious and in brainstem, it was opposite. The ratios acquired from quantity of SSeCKS to β_2-M were defined as the relative SSeCKS mRNA levels. Asterisk, $P < 0.05$

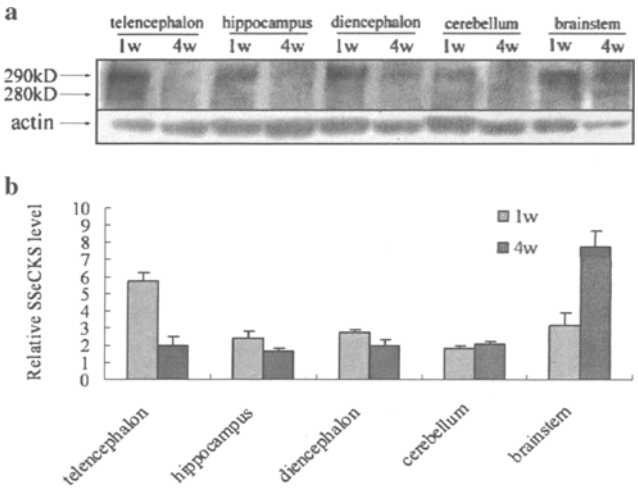

Figure 3 Regional distribution of SSeCKS expression by Western blot analysis. **a**: 40 μg total protein samples from tissues of telencephalon, hippocampus, diencephalon, cerebellum and brainstem of 1-and 4-week-old rats were used. w, week. **b**: Each lane in A was scanned by laser densitometry and the relative SSeCKS protein levels were determined from the ratio of their absorbance relative to β-actin in each lane

definite more accurate localization of the molecular. Since previous research showed that astrocyte which involved in constituted blood-brain barrier expressed SSeCKS, we first examined the immunohistochemical colocalization of SSeCKS and GFAP, a marker for astrocyte. The results of the colocalization of SSeCKS and GFAP in the 1-week-old brain are shown in Fig. 5a. In cortex, little mature astrocyte was seen while finely granular and diffuse expression of SSeCKS generally existed and the colocalization was not apparent. In hippocampus, the molecular was also in a granular-like distribution. We could see a few more astrocytes in the same area and there was some staining of SSeCKS in glial cells that stained positively for GFAP, indicating that SSeCKS was expressed by part of the astrocytes. Next, to determine whether SSeCKS also exist in neurons, colocalization of the neuron cell marker NeuN with SSeCKS was performed (Fig. 5b). In cortex, we could see a lot of neurons, while SSeCKS was small dots around the cell body. But it is not clear that whether the signal is in the cytoplasm of the cell. In hippocampus, neuron cells were mainly in CA1–CA3 regions and dentate gyrus and there was almost no staining of SSeCKS in cells that stained positively for NeuN. Furthermore, we performed double immunofluorescence staining with SSeCKS and OX42, the marker of microglia, in the brain. But in our experiment, we hadn't detected any cell stained positively for OX42, so the colocalization was not definite (data not shown). The staining result of Galac was similar to that of OX42.

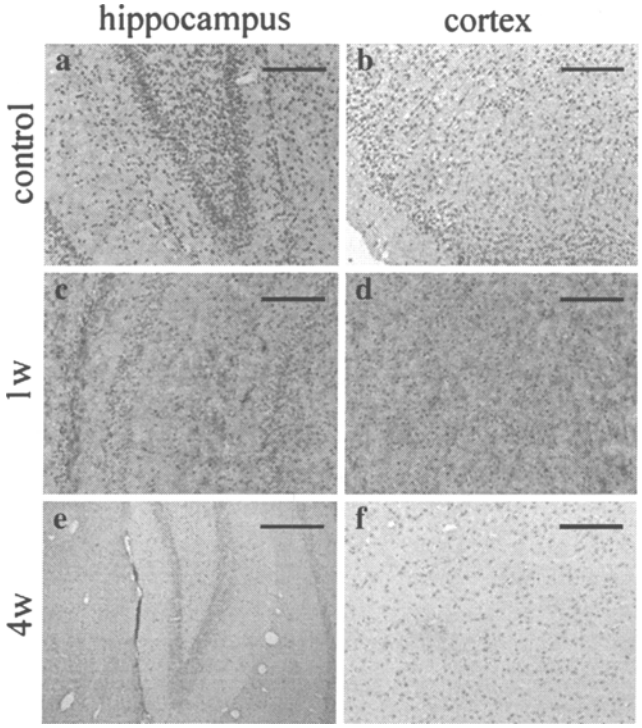

Figure 4 Immunohistochemical localization of SSeCKS in rat brain. **a**, **b**: 1-week-old rat hippocampus and cortex as a control; **c**, **d**: 1-week-old rat hippocampus and cortex; the immunopositive staining was diffused. **e**, **f**: 4-week-old rat hippocampus; few expression was detected. *Bars*, 100 μm

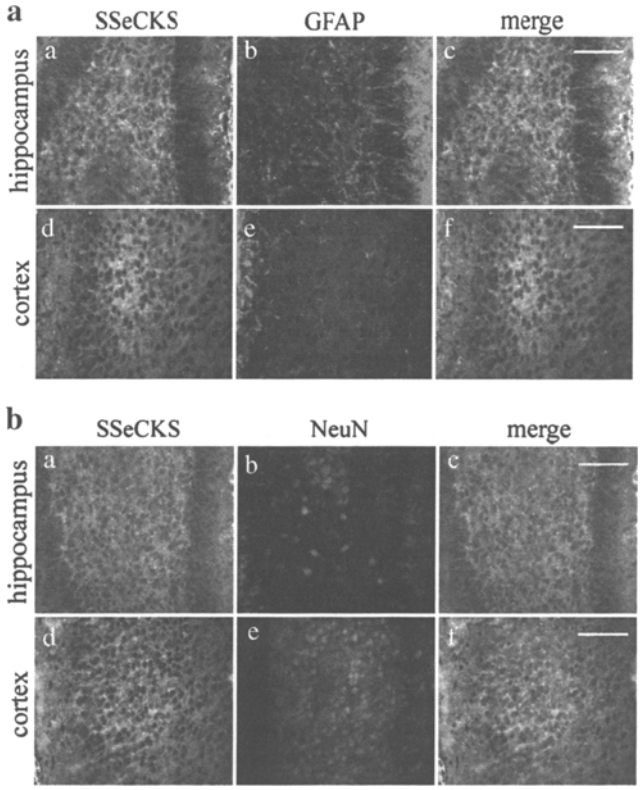

Figure 5 Colocalization of SSeCKS with GFAP, and NeuN in rat brain. Hippocampus and cortex from 1-week-old rats were stained for SSeCKS and GFAP/NeuN. We could see partial colocalization between SSeCKS and GFAP in hippocampus, while in cortex the colocalization was not significant. We hadn't found obvious colocalization between SSeCKS and NeuN. **a–c**, hippocampus; **d–f**, cortex. *Bars*, 50 μm. *Green*, SSeCKS; *Red*, GFAP/NeuN. **c** and **f**, merge

Discussion

In this paper, we first examined the SSeCKS in the whole brain of development stages and found it could be detected in embryonic stage and was especially high in 1-week-old rat brain while in 4-week-old rat brain it was in its lowest level. Then we tested the mRNA in different brain regions from the rats of these two time points and found that the expression pattern in telencephalon, hippocampus and diencephalons was in accordance with the result we had acquired. At the same time, real-time PCR also showed the expression patterns in cerebellum and brainstem were different from above three different brain areas: in cerebellum, the difference between 1-week-old and 4-week-old rat brain was not significant. In brainstem, the result was opposite. Protein level detection again

confirmed this result. From all these results, we know that SSeCKS is expressed in unmature rat brain in early develop stage. This is similar to the expression pattern of β-1,4-GalT I which is mainly detected in mid-embryonic stages. Nakamura et al. (2001) did research on gene expression of β-1,4-galactosyltransferase I, II and V during mouse brain development. The results showed that a higher expression level of the β-1,4-galactosyltransferase I (β-1, 4-GalT I) gene was detected only at the mid-embryonic stage and the level decreased thereafter. As a previously defined kinase and cytoskeleton scaffolding protein, SSeCKS subcellular distribution and functions are remarkably similar to those attributed to β-1,4-GalT I: (1) both are localized to the perinuclear/Golgi complex and to tips of lamellipodia; (2) both associate with the cytoskeleton; and (3) both inhibit cell growth when overexpressed (Gelman et al. 1998, 2000; Hinton et al. 1995; Nelson et al. 1999; Wassler et al. 2001). Since we have known that SSeCKS and β-1,4-GalT I interact functionally with each other, it is reasonable to presume that SSeCKS may play a role in brain development.

Furthermore, we did the research to determine that what kind of cell express the molecular. We chose four cell markers as follows: GFAP, NeuN, OX42 and Galac. First, the partial colocalization between SSeCKS and GFAP, a marker for astrocyte, indicates that astrocytes express SSeCKS during brain development. It is known that astrocytes and their processes play a role in guiding migration and axonal growth of neurons during embryonic development (Abe and Saito 2000; Mason et al. 1988) or in establishing the blood-brain barrier (Beck et al. 1986; Janzer and Raff 1987; Tao-Cheng et al. 1987). Previous data has shown that SSeCKS-overexpressed astrocyte is propitious to the mature and stabilization of blood-brain barrier and constitutive expression of SSeCKS in the adult brain may provide a stabilizing signal for blood-brain barrier integrity under physiological conditions (Lee et al. 2003). Since now our experiments showed that part of astrocytes in the early rat brain expressed SSeCKS, we hypothesized that SSeCKS might involved in the process that astrocytes guiding migration and axonal growth of neurons during embryonic development. Because SSeCKS is a cytoskeleton scaffolding protein, upon PKC activation, SSeCKS relocates from the cell cortex to perinuclear sites. Inducible overexpression of SSeCKS results in cell flattening, elaboration of the cortical cytoskeleton and an increase in integrin-independent FAK phosphorylation and growth arrest (Gelman et al. 1998). These investigations suggest that SSeCKS may exert its effects through modulation of the cytoskeleton. Meanwhile, scaffolding proteins capable of organizing the appropriate regulatory proteins and enzymes are involved in various signaling cascades. These scaffolding proteins are responsible for forming the framework of multiprotein complexes (Elion 1998) that can ensure precise regulation of the signaling cascade and allow the cell to respond specifically to a variety of hormones or extracellular stresses. During develop, migration and axonal growth of neurons involve series changes including transduction of different extracellular and intracellular signals, the change of cell morphology, remodel of cytoskeleton and so on. SSeCKS may play a role in this complicated process. At the same time, Gelman et al.'s research suggests roles for SSeCKS in the control of cytoskeletal and tissue architecture, formation of migratory processes and cell migration during embryogenesis, which just supports our view.

Then we observed the localization of NeuN and SSeCKS. But there wasn't any obvious colocalization between SSeCKS and NeuN. We could see SSeCKS mainly in the area around the nucleus of neurons. It is known to all that in early develop stage, neurons are small and large proportion of the cell body is nucleus, the quantity of cytoplasm is little. So the possibility that SSeCKS might in the cytoplasma of neuron could not be excluded and it still needed a further detection.

There is also another possibility that SSeCKS is expressed by neuron progenitor cell. Some investigators had shown that radial glia might serve as progenitors for the majority of neurons in the CNS (Casper and McCarthy 2006). Emerging evidence suggests that cells expressing a glia specific protein marker, glial fibrillary acidic protein (GFAP), give rise to specific populations of neurons during development (Kriegstein and Gotz 2003) and several different experimental approaches have contributed to this evidence (Anthony et al. 2004; Malatesta et al. 2000, 2003; Miyata et al. 2001; Noctor et al. 2002) and the cumulative evidence of these studies clearly indicate that GFAP-positive cells give rise to neurons. Studies published by Malatesta et al. suggest that GFAP-positive radial glia vary markedly in their ability to give rise to different populations of neurons. Neural progenitor cells that transiently express GFAP give rise to a large and diverse population of neurons and oligodendrocytes. In our experiments, we found some cells staining positively for GFAP also expressed SSeCKS, despite these cells were from glial, we presumed that maybe part of them were neural progenitor cells and SSeCKS might be involved in the neuron mature process.

Next, we labeled OX42, a marker for microglia and Galac, oligodendrocyte marker and we hadn't got any positive signal. Neither of them were found having colocalization with SSeCKS. The negative result may be ascribed to that our experiments were carried on 1-week-old rat brains which were in the early stage of brain development and many different kinds of cells are in their precurosor form and their epitopes are not formed or just in their forming process.

From all above, we know that SSeCKS exists in the rat brain during early development stage and it may play a role in this process. However, its physiological function as well as the mechanism and significance during brain development remain to be further elucidated.

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