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## Postnatal changes in Rho and Rho-related proteins in the mouse brain

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### Abstract

To provide information on the role of Rho, a GTP-binding protein, in postnatal development of the brain cells, the change in the levels of Rho protein and Rho-related proteins was examined in the brain of mice for two weeks after birth, in parallel with the changes in the activity of marker enzymes for neuronal and glial cells. The activities of acetylcholine esterase and choline acetyltransferase of whole brain homogenate, both of which are neuronal marker enzymes, were progressively increased in an age-dependent manner. The activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase, a glial marker enzyme, increased markedly between one and two weeks after birth. In contrast, the levels of RhoA and RhoB in the membrane fraction were decreased during the postnatal period. The amount of Rho GDP dissociation inhibitor, a regulatory protein for Rho, was unchanged, while those of Rho target proteins, Rock-2 and citron, were gradually increased. Since the inactivation of Rho is known to induce neurite extension and neuronal and glial differentiation *in vitro*, our results suggest that the Rho signalling pathway plays a regulatory role in the postnatal differentiation of neuronal and glial cells *in vivo*.

Key Words : brain, postnatal development, Rho, Rho GDI, Rock-2

### Introduction

Rho is a small GTP-binding protein to regulate actin cytoskeleton. The activation of Rho leads to assembly of contractile actin-myosin filaments (stress fiber) and associated focal adhesion complexes<sup>3)</sup>. Rho is known to be present abundantly in the brain<sup>17)</sup>, but its role has been poorly understood. It was reported in neuroblastoma cells cultured *in vitro* that the activation of Rho induces neurite retraction<sup>8)</sup>.

Inactivation of Rho by a bacterial ADP-ribosyltransferase (C3) induces neurite extension of neuroblastoma cells and an increase in some marker enzyme activities such as choline esterase and choline acetyltransferase<sup>5,9,16)</sup>. A similar *in vitro* effect of C3 on cell differentiation was also formed in cultured glioma cells<sup>9,18)</sup>. All these results suggest a vital role of Rho in neural cell differentiation, and an involvement in the *in vivo* neuro-glial development, such as those during postnatal period.

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As far as we know, however, there has been no report dealing with the quantification of Rho in the mammalian brain during postnatal development. In the present study, we examined the postnatal change in the amounts of Rho and Rho-related proteins in the mouse brain in 0-2 weeks after birth.

## Materials and Methods

### *Materials and Animals*

Acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), cyclic NADP, glucose-6-phosphate dehydrogenase and glucose-6-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [1-<sup>14</sup>C] acetyl-CoA was obtained from American Radiolabeled Chemicals. (St. Louis, MO, USA). Antibodies against RhoA, RhoB, Rho GDP dissociation inhibitor (GDI), Rock-2 and citron were obtained from Santa Cruz Biotechnology. (Santa Cruz, CA, USA). Horseradish peroxidase conjugated anti-rabbit IgG goat antibody and anti-goat IgG rabbit antibody were obtained from Zymed Laboratories. (South San Francisco, CA, USA) and Jackson ImmunoResearch Laboratories. (West Grove, PA, USA), respectively. PVDF (Immobilon-P) membrane was obtained from Millipore Co. (Bedford, MA, USA). ECL Western blotting detection reagent was purchased from Amersham Pharmacia Biotech (Tokyo, Japan).

Pregnant female and adult female (12-14 weeks old) ICR mice were obtained from Sankyo Labo Service. (Tokyo, Japan).

### *Preparation of subcellular fractions of mouse brain*

Whole brain was obtained from adult mice (12-14 weeks old) and also from infant mice 0, 1 and 2 weeks after birth under chloroform anesthesia. The brain was homogenized in a buffer [0.3 M sucrose, 20 mM Tris-HCl (pH 7.5), 1mM EDTA, 2  $\mu$ g/ml aprotinin], and centrifuged at 1,000 $\times$ g for 20 min to remove nuclei and debris. The post nuclear supernatant

was centrifuged at 100,000 $\times$ g for 60 min, and the resulting supernatant was used as a cytosol fraction. The pellet was resuspended in a half volume of buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2  $\mu$ g/ml aprotinin] containing 1% sodium cholate, and centrifuged at 100,000 $\times$ g for 60 min to precipitate detergent-insoluble materials. The supernatant was used as a membrane fraction. Protein concentration in each fraction was determined by the method of Bradford<sup>1)</sup>.

### *Western blot analysis*

The membrane and cytosol fractions containing 10  $\mu$ g protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 13% gel for RhoA and RhoB and a 6% gel for citron and Rock-2. For the detection of Rho GDI, 0.5  $\mu$ g protein of each sample was electrophoresed on the 13% gel. After blotting the separated proteins onto a PVDF membrane, the membrane was immersed into phosphate buffered saline with 0.1% Tween 20 (PBST) containing 5% skim milk for 30 min, and subsequently incubated in PBST containing 5% skim milk and 1  $\mu$ g/ml respective antibodies for 1 hour. After washing twice with PBST, the membrane was incubated for 30 min with anti-rabbit IgG goat antibody or anti-goat IgG rabbit antibody, both of which were conjugated with horseradish peroxidase. After washing with PBST four times, bound antibodies were detected by the ECL kit. Exposed X ray film was scanned and band intensities corresponding to each protein were analyzed using NIH image. Data are expressed as relative intensity to those of a control sample (rat brain homogenate 30  $\mu$ g protein) which was analyzed on the same membrane.

### *Enzyme assays*

The membrane and cytosol fraction were mixed at a ratio of 1 : 2, and used for the following enzyme assays<sup>9)</sup>. For acetylcholine esterase (AChE) assay, 20  $\mu$ l enzyme sample

was mixed with 130  $\mu$ l of 0.1 M phosphate buffer (pH 8.0), containing 3.3 mM DTNB, and after 10 minutes, acetylthiocholine iodide was added to a final concentration of 3 mM. The rate of acetylcholine hydrolysis was determined from the change in the absorbance at 412 nm for a few minutes.

For measurement of choline acetyltransferase (ChAT), 20  $\mu$ l enzyme sample was mixed with 20  $\mu$ l of 50 mM phosphate buffer (pH 7.4), containing 0.1 mM [1- $^{14}$ C] acetyl-CoA, 10 mM choline chloride, 0.2 mM eserine sulfate, and 0.3 M NaCl, and incubated at 37°C for 30 min. Reaction was stopped by the addition of 400  $\mu$ l of 14.3 mM phosphate buffer (pH 7.4), containing 71  $\mu$ M p-chloromercuribenzoate, 29% acetonitrile, and 1.43 mg/ml sodium tetraphenylboron. [ $^{14}$ C] acetylcholine was extracted with 800  $\mu$ l toluene scintillator and its radioactivity was measured by a liquid scintillation counter.

For measurement of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP), 10  $\mu$ l enzyme sample was mixed with 90  $\mu$ l 0.2 M MES (pH 6.0), containing 0.06U glucose-6-phosphate dehydrogenase, 1 mM cyclic NADP, 5 mM glucose-6-phosphate, 30 mM MgCl<sub>2</sub>, and CNP activity was determined from the change in the absorbance at 340 nm for a few minutes.

#### Statistical Procedures

The significance of the difference between group means was analyzed by one-way ANOVA and Fisher's protected least significant difference test.

### Results

To ascertain the postnatal development of neural and glial cells in the mouse brain, the activities of AChE and ChAT (marker enzymes for neuronal cell), and that of CNP (marker enzyme for glial cell) were measured with brain homogenate of mice at 0, 1 and 2 weeks of age. As shown in Fig. 1, the AChE activity was increased after birth, and reached in two weeks

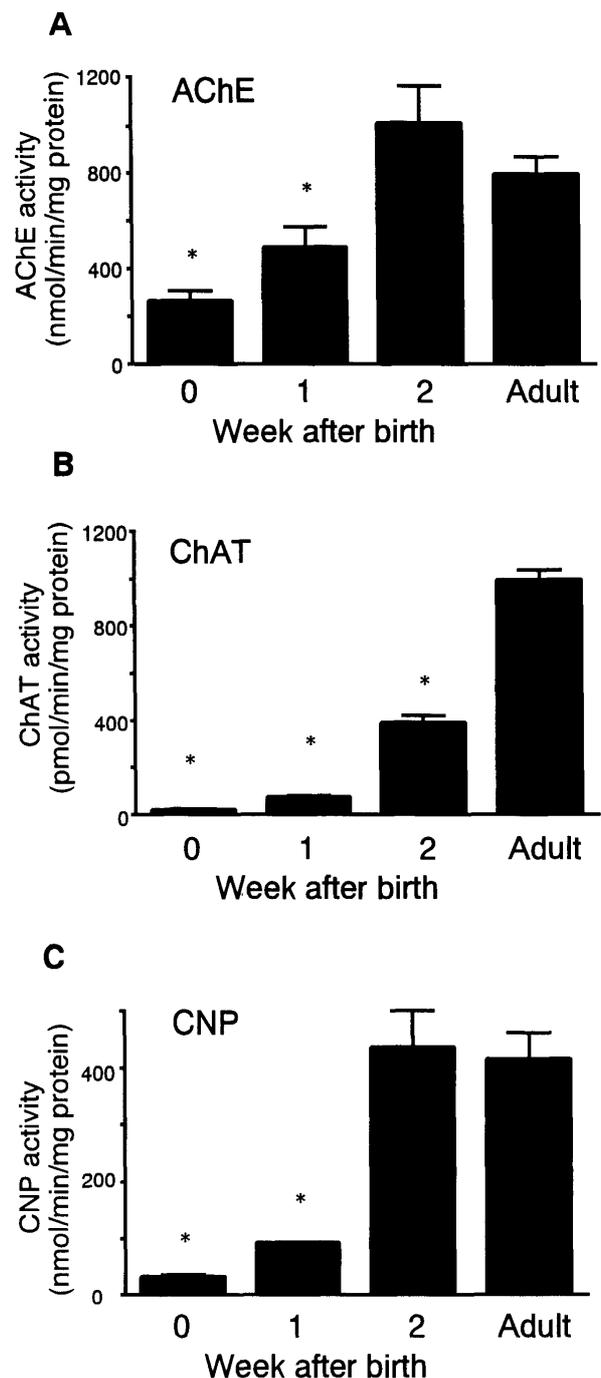


Fig. 1 Marker enzyme activities of differentiation of neural and glial cells in the mouse brain during postnatal development. Acetylcholine esterase (AChE), choline acetyltransferase (ChAT) and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) activities were measured for a mixture of cytosol and membrane fractions of mouse brain at 0, 1, 2 weeks old and adult. Data are means  $\pm$  SE of three independent preparations. \* $P$  < 0.05 compared with Adult.

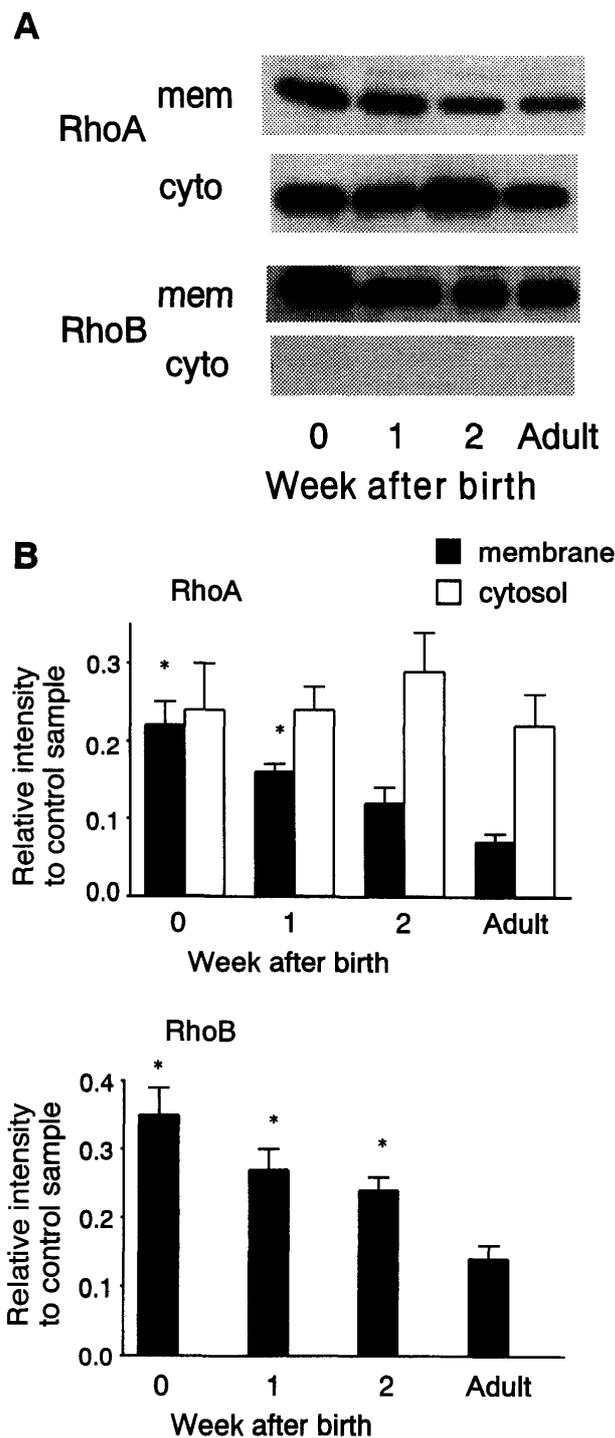


Fig. 2 Western blot analysis of RhoA and RhoB in the mouse brain. A ; Membrane and cytosol fractions (10  $\mu$ g protein) of the mouse brain were electrophoresed in 13% gel, and RhoA and RhoB were detected by specific antibodies against respective proteins. Representative results of three experiments are shown. B ; The amounts of RhoA and RhoB were quantified by scanning the intensity of respective protein bands in Western blots (n=3), and expressed as relative to those of a control sample (rat brain homogenate, 30  $\mu$ g protein). Data are means  $\pm$  SE of three independent preparations. \* $P$ <0.05 compared with Adult.

to a comparable level to adults. The ChAT activity was very low at birth, and increased gradually, but was still lower at 2 weeks than the adult level. The CNP activity was also low at birth, but increased markedly in 1-2 weeks, reaching the adult level.

The postnatal change in the Rho level was examined by Western blot analysis using specific antibodies against RhoA and RhoB. The amount of RhoA in the whole homogenate did not change during the postnatal period (data not shown). After subcellular fractionation, RhoA was detected in both the cytosol and membrane fractions (Fig. 2A). The quantitative analysis of each protein band revealed that the RhoA level in the cytosol fraction did not change during the postnatal period, while those in the membrane fraction was highest at birth and gradually decreased in a age-dependent manner (Fig. 2B). RhoB was found only in the membrane fraction, and changed in a similar way to RhoA after birth (Figs.2A and 2B).

The postnatal changes in the level of some Rho-related proteins were also examined. Rho GDI, a regulatory protein for Rho activity, was detected in both the cytosol and membrane fractions, and its level showed no significant change during the postnatal period (Fig. 3A). Citron and Rock-2, both of which are target proteins for Rho, were also detected by Western blot analysis only in the membrane fraction and in the both fractions, respectively. In contrast to Rho and Rho GDI, citron and Rock-2 increased markedly 0-2 weeks after birth (Figs. 3B and 3C).

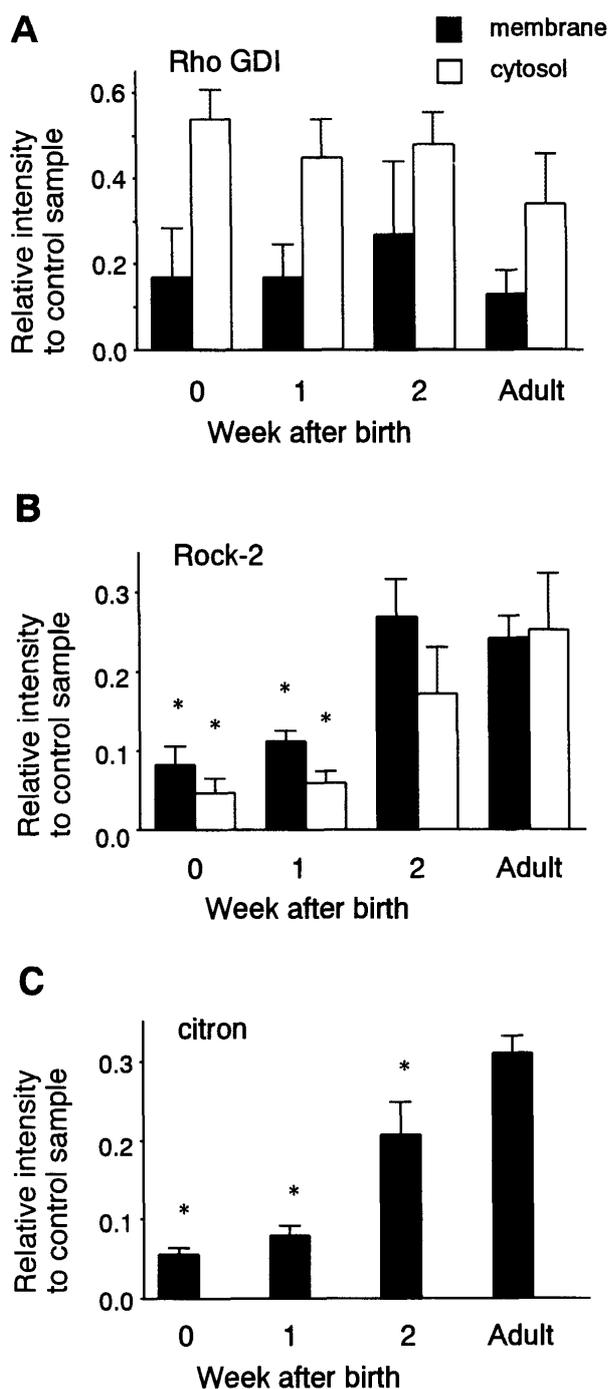


Fig. 3 Western blot analysis of Rho-associated proteins in the mouse brain. Membrane and cytosol fractions (10  $\mu$ g protein for Rock-2 and citron, 0.5  $\mu$ g protein for Rho GDI) of mouse brain were electrophoresed by SDS-PAGE (6% gel for Rock-2 and citron, 13% gel for Rho GDI). Data are means  $\pm$  SEM of three experiments. \* $P$  < 0.05 compared with Adult.

## Discussion

In the present study we examined postnatal changes in the activities of marker enzymes for neural and glial differentiation and the amounts of Rho, Rho GDI and Rho target molecules in the mouse brain. As expected, the enzymes activities were increased in 1-2 weeks after birth, reflecting the rapid postnatal growth and differentiation of neural and glial cells in the brain.

It is known that at least two isoforms of Rho family, RhoA and RhoB, are expressed in the brain<sup>17</sup>). This was confirmed in the present study by Western blot analysis using the respective antibodies specific to RhoA and RhoB. RhoA was detected in both the cytosol and the membrane fractions, whereas RhoB only in the membrane fraction. The amounts of RhoA and RhoB in the membrane fraction were decreased, while those of RhoA in the cytosol fraction was unchanged during the postnatal period of 1-2 weeks. There have been reports that Rho is translocated from cytosol to membrane when stimulated<sup>7</sup>) and returns to cytosol when repressed<sup>10,12</sup>), indicating that Rho associated with membrane is an active form. Interestingly, the postnatal decrease in Rho in the membrane fraction seems to be inversely related to the induction of the marker enzyme for neuronal and glial differentiation. This seems compatible with our previous *in vitro* findings that inactivation of Rho could cause an increase in the marker enzymes for neuronal and glial cells<sup>9</sup>). Thus, it is suggested that Rho might play a crucial role in neural and glial differentiation during postnatal development of the mouse brain.

The mechanisms and factors involving in the postnatal decrease in RhoA and RhoB in the membrane fraction remain to be obscure. Rho GDI, a regulatory protein for Rho, is known to transfer Rho from membrane to cytoplasm, re-

sulting in inactivation of Rho<sup>4,12)</sup>. In the present study, Rho GDI content was not changed in both membrane and cytosol fractions during the postnatal period. In PC12 neuroblastoma cells, nerve growth factor and dibutyryl cyclic AMP have been shown to remove RhoA from membrane<sup>19)</sup> and to induce cell differentiation. Moreover, platelet-derived growth factor also induces membrane localization of RhoA<sup>11)</sup> and RhoB expression in rat fibroblasts<sup>6)</sup>. It seems thus possible that these factors may be involved in the postnatal changes in RhoA and RhoB. Further studies are needed to clarify the role of these factors.

In the present study, we also examined the postnatal changes in the amounts of Rock-2 and citron, which are target proteins of Rho and are activated by Rho<sup>13,14,15)</sup>. Activation of Rock-2 causes neurite retraction<sup>8)</sup> and citron may regulate NMDA receptor signaling in the post-synaptic membrane of the thalamus<sup>2,20)</sup>. The present results showed that Rock-2 and citron were increased while RhoA and RhoB decreased after birth, suggesting that some Rho-related proteins other than Rock-2 and citron may be involved in the postnatal development of mouse brain.

In summary, the amounts of Rho and Rho associated molecules changed dramatically during postnatal development of the mouse brain, probably participating to neuronal and glial differentiation.

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