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Developmental patterns in rat brain of phosphatidylinositol synthetic enzymes and phosphatidylinositol transfer protein

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Phosphatidylinositol synthetic and intermembrane transfer activities were studied in rat in the developing whole brain and isolated cerebellum. Specific activities of CTP: phosphatidate cytidylyltransferase and CDPdiacylglycerol: inositol phosphatidyltransferase were found to have similar developmental patterns. Levels of phosphatidyltransferase seen in fetal animals (whole brain only) and neonatal (whole brain and cerebellum) were maintained through approximately postnatal day 15, peaked at day 28, and then declined to somewhat higher than fetal levels at day 60. Cytidylyltransferase activity varied from the phosphatidylinositol synthesizing enzyme in that specific activity continued to increase up to day 60. Whole brain phosphatidylinositol transfer specific activity showed a sharp peak at postnatal day 9 after which activity was maintained at or above the fetal levels to day 60. Cerebellum phosphatidylinositol transfer specific activity had a similar peak which was delayed 7–10 days compared to the whole brain. Phosphatidylinositol transfer protein was also determined immunologically: whole brain levels increased dramatically from fetal day 16 to 18 and then remained relatively constant, while cerebellum levels (measured from postnatal day 7) displayed a variable profile between days 7 and 28. The developmental pattern of CTP: phosphatidate cytidylyltransferase in rat brain is reported here for the first time.

Introduction

A low aqueous solubility makes phospholipid molecules poor candidates for spontaneous transit among intracellular membranes [1]. In contrast, the flux of phospholipids through the endoplasmic reticulum-Golgi-vesicular network is well documented [2–4]. The role of cytoplasmic proteins, which bind and transport phospholipids in a variety of in vitro situations, while attractive and in some cases even likely as an in vivo mechanism [5], has yet to be established unequivocally. Proteins which catalyze the intermembrane transfer of phosphatidylinositol have been characterized by structure, catalytic activity, tissue distribution, and physiological function [6]. Molecular weights from 34000 to 36000 and isoelectric points from pH 5.0 to 5.6 have been found in a broad spectrum of eukaryotic cells, including yeast [7], bovine brain and heart [8,9], human platelets [10] and, more recently, rat tissue [11]. Phosphatidylinositol is preferentially transported by these phosphatidylinositol transfer proteins; phosphatidylcholine is a weaker substrate.

Changes in phosphatidylinositol transfer specific activity in developing rat brain have been previously studied in vitro using several transfer (donor-acceptor membrane) systems and various protein preparations [12–14]. These studies attempted to link early postnatal myelination with catalytic activities. The present study was undertaken to describe and compare in vitro specific activities of two key phosphatidylinositol synthetic enzymes, CDPdiacylglycerol: inositol phosphatidyltransferase (EC 2.7.7.11) and CTP: phosphatidate cytidylyltransferase (EC 2.7.7.41), and phosphatidylinositol transfer specific activity in whole brain and cerebellum during the phases of pre- and postnatal development. In addition, tissue levels of transfer protein during development were compared by immunoblot analysis.

Materials and methods

Materials

myo-[2-^{3}H]Inositol (555 GBq · mmol^{-1}) was purchased from Amersham (Arlington Heights, IL); cholesteryl[1-^{14}C]oleate (2.1 GBq · mmol^{-1}) and [5-^{3}H]cytidine triphosphate (877 GBq · mmol^{-1}) were

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purchased from New England Nuclear (Boston, MA). Bovine plasma albumin and goat anti-rabbit IgG antibody-alkaline phosphatase conjugate, lactosylceramide, and CTP were purchased from Sigma (St. Louis, MO); CDPdioleoylglycerol was purchased from Serdary Research Laboratories (London, Ontario); and egg phosphatidate was obtained from Avanti (Birmingham, AL). All other reagents were the highest quality available.

Animals, tissue preparation

Pregnant Sprague-Dawley rats, with known vaginal plug date, were obtained from Sasco (Omaha, NE). All animals were fed ad libitum. Beginning at day 16 of term (fetal day 16), mothers were killed by CO_2 asphyxiation and the fetuses were removed and quickly decapitated. The brain was excised and placed in a glass Petri dish over ice; it was rapidly processed whole or further dissected to yield the cerebellum. For postnatal studies, pups were housed with their dams until weaning. Animals were pooled from several litters, without distinction as to sex.

Tissue samples were weighed and then homogenized in 10 mM Hepes-Na, 1 mM Na_2EDTA, 0.32 M sucrose (pH 7.4) at a ratio of 1 g tissue per 4 ml buffer. The tissue/buffer mixture was subjected to six strokes of a Teflon pestle in a Potter-Elvehjem apparatus at 800 rpm. Homogenates were then centrifuged at $11\,500 \times g$ for 15 min. The supernatant was then centrifuged at $11\,500 \times g$ for 20 min and then at $150\,000 \times g$ for 1 h to yield a crude microsomal pellet and cytosolic fraction. The pellet was resuspended in 10 mM Hepes-Na, 50 mM NaCl, 1 mM Na_2EDTA (pH 7.4). The cytosolic fraction was dialyzed against 200 volumes of this buffer for 12 h. The preparations were stored at −75°C before use.

Activity measurements

(i) CTP:phosphatidate cytidylyltransferase. CDPdiacylglycerol synthesis was assayed according to Bishop and Strickland [15]. In a final volume of 0.8 ml of 50 mM potassium phosphate (pH 6.8), were mixed 5 μmol [^{3}H]CTP ($1 \cdot 10^6$ dpm), 80 μmol $MgCl_2$, 0.25–0.30 mg of microsomal protein and 400 nmol of egg phosphatidate. The phosphatidate was prepared by brief sonication with 1 mg bovine serum albumin, 65 μg G3635 cationic detergent (ICI, Wilmington, DE), and 30 μl diethyl ether in 300 μl buffer before mixing with other components. The $MgCl_2$ was added after a 3 min incubation at 37°C. The reaction was continued for another 10 min, after which the liponucleotide was extracted under acidic conditions and quantitated by liquid scintiliation counting. Enzyme specific activity was expressed as nmol CTP incorporated per min per mg protein. Boiled protein blanks were used to correct for endogenous phosphatidate.

(ii) CDPdiacylglycerol : inositol phosphatidyltransferase. By adaptation of protocols described by Kumara-Siri and Gould [16] and Parries and Hokin-Neaverson [17], the formation of phosphatidylinositol was measured. To a reaction mixture of 50–200 μg microsomal protein, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 0.5 mM EGTA, 2.5 mg · ml^{-1} bovine plasma albumin, and 5 mM 2-mercaptoethanol, all in 10 mM Hepes-Na (pH 8.4), were added 100 nmol of CDPdioleoylglycerol and, in a final concentration of 2 mM, [^{3}H]inositol ($5–8 \cdot 10^6$ dpm), with a final reaction mixture volume of 250 μl. The reaction proceeded for 45 min at 37°C and was terminated by adding 3 ml of ice-cold chlorofom/methanol/12 M HCl (200:100:1, by vol.) and vortexing. After 5 min, 5 ml of 2 M KCl and 20 mM inositol (pH 3.0) was added and the solution vortexed. After low-speed centrifugation for 5 min, the upper phase was aspirated and the bottom phase washed twice with 3 ml of 2 M KCl and 20 mM inositol/methanol (1:1, by vol.), and then once with 3 ml of methanol/water (1:1, by vol.). Mixtures were vortexed, centrifuged, and aspirated between washes. The final organic phase, containing > 95% of the radiolabelled phospholipid, was dried under a stream of N_2 and analyzed by liquid scintillation spectrometry. Boiled microsomes were used as blanks; the specific activity of phosphatidylinositol synthesis was expressed as nmol phosphatidylinositol formed per h per mg protein.

(iii) Phosphatidylinositol transfer activity. Assay conditions for the protein-mediated transfer of phosphatidylinositol between small unilamellar vesicles have been detailed by Kasper and Helmkamp [18]. Briefly, phosphatidyl[^{3}H]inositol was prepared with rat liver microsomes and then purified by thin-layer chromatography. The assay solution contained 100 nmol donor vesicles (phosphatidylcholine/ phosphatidylinositol/ lactosylceramide, 87:5:8, mol%, and 5000 dpm phosphatidyl [^{3}H]inositol), 200 nmol acceptor vesicles (phosphatidylcholine/phosphatidylinositol, 95:5, mol%, and 2500 dpm cholesteryl] [^{14}C]oleate), 0.1 mg bovine plasma albumin, 20–100 μg cytosolic protein, 50 mM NaCl, 1 mM Na_2EDTA, and 10 mM Hepes-Na (pH 7.4) in a total volume of 500 μl. After a 30 min incubation at 37°C, the donor vesicles were agglutinated and precipitated in the presence of 0.25 mg *Ricinus communis* agglutinin. The supernatant, which contained only the acceptor vesicles, was analyzed by liquid scintillation spectrometry. Controls lacked the cytosolic protein. Specific activity was presented as nmol phosphatidylinositol transferred per h per mg protein.

Preparation of rabbit anti-phosphatidylinositol transfer protein antibody

Bovine brain phosphatidylinositol transfer protein was purified to homogeneity by published methods [19] and used to produce a polyclonal antibody in rabbit.

Excellent immunologic cross-reactivity between rabbit and rat phosphatidylinositol transfer proteins has been noted [11].

Electrophoresis and immunoblotting

Electrophoresis was carried out on gels of 12% polyacrylamide (0.75 mm thickness) in the presence of sodium dodecylsulfate and 2-mercaptoethanol. For immunological studies, up to 50 μg of lyophilized 150000 $\times g$ supernatant protein from brain tissue homogenates was electrophoresed. The proteins were then transferred electrophoretically to nitrocellulose membranes and developed immunochemically, as described elsewhere [11]. Film positives of the immunoblots were analyzed densitometrically (Zeineh Laser Scanning Densitometer, Fullerton, CA). Instrument response was calibrated with a standard curve of purified bovine phosphatidylinositol transfer protein, electrophoresed, blotted, and stained under similar conditions.

Other analytical methods

Protein was measured by the method of Lowry et at. [20] using bovine plasma albumin as standard. Lipid phosphorus was determined by the method of Rouser et at. [21]. Unpaired samples were compared statistically using Student's *t*-test.

Results

Activity of phosphatidylinositol synthetic enzymes

The specific activities of two major phosphatidylinositol synthetic enzymes were measured during periods of rapid brain growth (approx. prenatal day 16 through postnatal day 60 or adult). The synthesis of CDPdiacylglycerol catalyzed by CTP: phosphatidate cytidylyltransferase, depends upon the availability of phosphatidate and thusly the respective enzymes for phosphatidate production. In turn, CDPdiacylglycerol serves as the precursor for other anionic phospholipids.

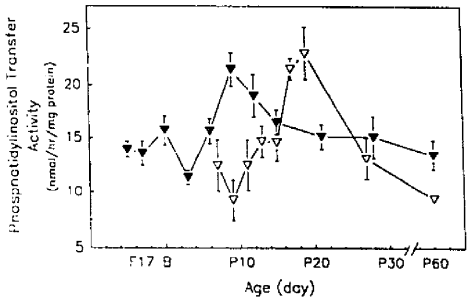

Fig. 2. Developmental patterns of phosphatidylinositol transfer activity in brain. Cytosolic fractions were prepared from brain homogenates as described in Materials and Methods. Phosphatidylinositol transfer specific activity was measured using 20–100 μg of protein. Age is represented as described in Fig. 1. Transfer values for whole brain (▼) are the mean ± S.E. for 7–12 determinations; values for cerebellum (▽) are mean ± S.D. for 4–6 determinations.

CDPdiacylglycerol : inositol phosphatidyltransferase, on the other hand, is the terminal enzyme in the synthesis of phosphatidylinositol. It was of interest to measure the specific activities of both enzymes.

In whole brain and cerebellum the activities of CTP : phosphatidate cytidylyltransferase and CDPdiacylglycerol : inositol phosphatidyltransferase displayed similar, though not identical developmental profiles (Fig. 1). The levels of CDPdiacylglycerol : inositol phosphatidyltransferase seen in the fetal animals (whole brain) and neonates (whole brain and cerebellum) were maintained through postnatal day 15, after which activity increased markedly to day 28. Thereafter, activity declined to the value observed at day 60. Day-60 levels were higher than fetal levels for whole brain and day-7 levels for cerebellum. The developmental patterns of whole brain and cerebellum CTP : phosphatidate cytidylyltransferase specific activity were different from the phosphatidylinositol synthesizing enzyme (Fig. 1). With the exception of a small, though reproducible peak at postnatal day 17 in cerebellum, both tissues had

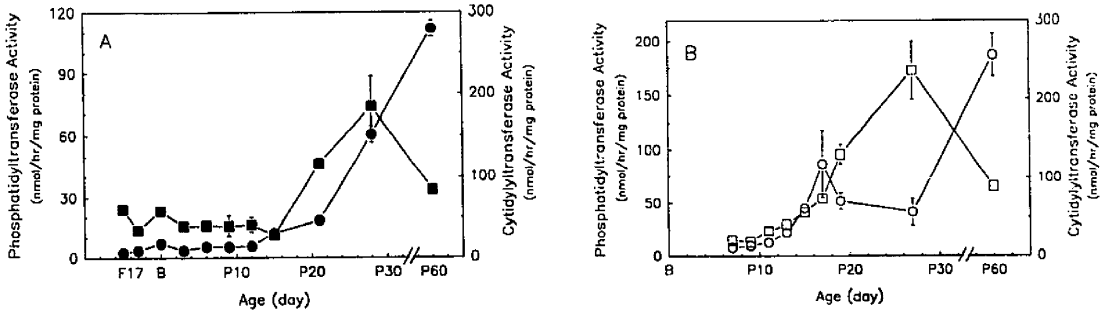

Fig. 1. Developmental patterns of phosphatidylinositol synthetic enzymes in brain. Microsomes were prepared from brain homogenates as described in Materials and Methods. For CTP: phosphatidate cytidylyltransferase specific activity (●, ○), 250–300 μg of microsomal protein was used; for CDPdiacylglycerol : inositol phosphatidyltransferase specific activity (■, □), 50–200 μg of microsomal protein was used. (A) Whole brain. (B) Cerebellum. Age is indicated as fetal(F), day of birth(B) or postnatal day(P); data points represent the mean S.D. of determinations.

specific activities which increased up to day 60. This increase was greater than 20-fold for cerebellum, compared to postnatal day 7, and 20–40-fold for whole brain, compared to postnatal day 6 or fetal day 16, respectively.

Phosphatidylinositol transfer activity

The specific activities of phosphatidylinositol transfer during late fetal and early postnatal development were measured in whole brain and cerebellum. Whole brain phosphatidylinositol transfer specific activity increased from fetal day 16 to postnatal day 9 (Fig. 2). Transfer activities then declined and were maintained at or slightly above fetal levels to day 60. Cerebellum phosphatidylinositol transfer specific activity, in contrast to whole brain, reached a peak at day 19. Day-60 levels were similar to those observed in the early postnatal period (Fig. 2).

Phosphatidylinositol transfer protein levels

Although specific activities based on total tissue protein concentration is an acceptable standard, further investigation was undertaken to develop a more quantitative approach. The availibilty of a polyclonal rabbit antibody proved useful in determining transfer protein levels in unfractionated tissue cytosol. Densitometric scans of immunoblots of dye-stained purified bovine brain phosphatidylinositol transfer protein on nitrocellulose indicated that immunoblot intensity was linear in the range 0–180 ng of protein with a least-squares regression correlation coefficient of 0.992 (data not shown). The levels of phosphatidylinositol transfer protein in whole brain displayed a sharp 150% increase from fetal day 16 tissue to fetal day 18 (Fig. 3). A more gradual rise of another 30% to day 12 was followed by relatively constant levels through day 60. Measurements of cerebellum phosphatidylinositol transfer protein levels, starting at day 7, indicated a somewhat variable profile up to day 19 (Fig. 3). After day 19, cerebellum levels slightly declined in the period out to day 60.

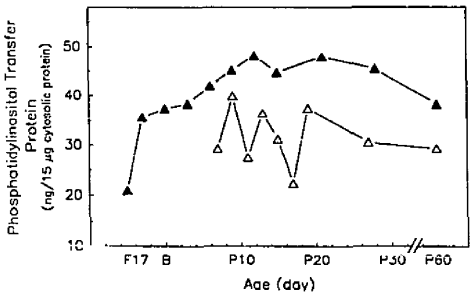

Fig. 3. Level of phosphatidylinositol transfer protein in brain cytosol preparations. Aliquots (15 µg) of high-speed supernatant fractions were analyzed by electrophoresis, immunoblotting, and densitometry. The intensity of the single immunoreactive band in each sample, with apparent M_r of 36000, was expressed as ng phosphatidylinositol transfer protein. Each point is the average of duplicate gels for whole brain (▲) and cerebellum (△).

Discussion

From the specific activity results it is clear that the developmental profiles of two key enzymes responsible for the synthesis of phosphatidylinositol are remarkably similar, except for the period beyond day 28. CTP : Phosphatidate cytidylyltransferase activity increased monotonically in whole brain as the animals aged, but displayed a small peak at day 17 and a steady increase beyond day 27 in cerebellum. In both tissue preparations, despite a difference in absolute activity values, CDPdiacylglycerol : inositol phosphatidyltransferase activity was found to peak around 28 days postnatal and then decline. Protein-catalyzed transfer of phosphatidylinositol was measured at a relatively high specific activity even in fetal whole brain and was characterized by a peak activity which preceded the rapid rise of the synthetic enzyme activities. The cerebellum peak of phosphatidylinositol transfer activity was delayed approximately 10 days compared to whole brain. The synthetic enzyme activity increases over fetal or neonatal levels were more dramatic (> 10-fold) than those seen in phospholipid transfer activities (about 2–3-fold). The levels of transfer protein in cerebellum measured immunologically from postnatal day 7 through day 60 displayed a variable pattern with no clear trend between day 7 and 19, but ultimately resembling the whole brain data in the gradual slight decline to day 60.

Developmental aspects of intermembrane phosphatidylinositol transfer in rat whole brain or cerebral hemispheres have been previously reported. Ruenwongsa et al. [12] measured phosphatidylinositol transfer activity in pH 5.1-treated post-mitochondrial supernatants of whole brain and found that transfer activity was not detectable at birth, first observed at day 4, and increased to a plateau around 35 days postnatal. Brophy and Aitken [13] analyzed rat cerebral cortex cytosolic fractions for their ability to transfer phosphatidylinositol. Specific activity, normalized to mg protein, exhibited a well-defined maximum at day 18 postnatal; however, specific activity, related to gram of tissue, was not as sharply delineated but was consistently higher at days 16–24. Day-27 animals were the oldest used. More recently, Carey and Foster [14] compared phosphatidylinositol transfer activity in whole brain cytosol preparations from animals aged 2, 9, 17, and approximately 60 days. They observed maximum activity at 9 days, followed by decreased levels at day 17 and in the adult samples.

Our findings for whole brain phosphatidylinositol transfer specific activity, using two populations of small unilamellar vesicles, agree with the detailed early post-

natal profile in cerebral cortex [13] and the less complete but more long-term data from whole brain [14]. In all three cases, transfer activity attained a maximum in the postnatal period between 9 and 18 days. Moreover, each of these studies demonstrated significant activity during late fetal and/or early postnatal growth and a return to these early levels as the animals proceeded into pubertal and adult development. Our experimental results with the cerebellum yielded a similar transfer activity-development pattern: comparable catalytic activity in early postnatal and adult samples with a sharp maximum at 17–19 days. For the present investigation and those of others [13,14], the values of phosphatidylinositol transfer specific activities rarely differed by more than 3-fold throughout the broad developmental period. This narrow range of catalytic activity is supported by a similarly narrow range in immunologically detectable phosphatidylinositol transfer protein in the tissue preparations.

Our developmental profile for whole brain phosphatidylinositol transfer protein specific activity deserves several comments. First, peak transfer activity is seen immediately prior to the onset of myelination, toward the end of the second postnatal week. Without examination of specific myelin-producing glial cells, we cannot say whether this coincidence implicates phosphatidylinositol transport in the biogenesis of this unusual membrane. Alternatively, a wider spectrum of neurological developments may contribute to our observations. Secondly, the immunologically determined levels of transfer protein project values that were already high in fetal animals, in contrast to activity measurements. This anomaly reflects a difference between the detection of an epitope but not necessarily an indication of catalytic activity. Differences between whole brain and cerebellum phosphatidylinositol transfer specific activities and developmental profiles extend our earlier description of regional distribution of transfer activity in mature rat brain [22]. Interestingly, the study of the cerebellum has been extensive and fruitful for a number of reasons. The anatomical architecture has been elucidated more completely than other central nervous system structures [23]. This organization has a small number of neuronal cell types (Purkinje, granule, basket, stellate), and the developmental time frame is well characterized [24]. Cerebellar development in the rat is primarily a postnatal event, and neuronal maturation, including myelination and synaptogenesis, follows a highly ordered space and time program. Our data demonstrate an excellent correlation between cerebellar development and phosphatidylinositol transfer specific activity. A final comment is our decision to choose, arbitrarily, to normalize our phosphatidylinositol synthetic and transfer data to mg protein in membrane or cytosol preparations. We appreciate the fact that this parameter may undergo dramatic changes during specific developmental periods and accordingly cannot provide an altogether accurate representation of specific activity profiles.

Whole brain specific activity of CDPdiacylglycerol : inositol phosphatidyltransferase was found by Salway et al. [25] to increase about 10-fold from day 1 prepartum to day 25 and then decline to levels which were moderately lower. Our data not only corroborate these findings for whole brain, but they also establish a similar trend in cerebellum where the overall change from early postnatal through days 27 and 60 is 2-fold greater. To our knowledge the developmental patterns of CTP : phosphatidate cytidylyltransferase specific activities in rat brain are reported here for the first time. Activities remained relatively low and constant up to day 21 for whole brain and day 27 for cerebellum, followed by a pronounced increase to adult levels. The difference in developmental pattern for these two enzymes may reflect the known role of these in phospholipid biosynthesis. CDPdiacylglycerol : inositol phosphatidyltransferase is involved solely in phosphatidylinositol synthesis, while CTP : phosphatidate cytidylyltransferase catalyzes the synthesis of the precursor for many anionic phosphoglycerides. The product molecule, CDPdiacylglycerol, is subsequently utilized in phosphatidylglycerol, cardiolipin, and phosphatidylinositol synthesis [26]. Increased synthetic activity may be necessary in the adult brain to maintain the concentrations of these phospholipids, and the phosphatidylinositol biosynthetic capability may be most critical during rapid myelination. Indeed, phosphatidylinositol levels expressed as percent of total whole brain lipid remain quite constant from day 10 through day 60 [27]. The content of phosphatidylinositol also remained essentially constant in myelin when expressed as the mol fraction of total myelin lipid from day 15 to day 60. The postnatal period between days 14 and 28 is usually associated with maximal rates of myelin deposition, even though myelin concentrations continue to increase throughout the life of the animal [27]. Concentrations of phosphatidylinositol metabolites rose rapidly during the period of extensive myelin deposition [28–31]. Phosphatidylinositol is the precursor to the polyphosphoinositides, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. From birth to day 12 the polyphosphoinositides represent $< 5\%$ of adult levels; they further increase to about 10% of adult levels between day 12 and 18 [28]. A peak in the tissue content of phosphatidylinositol 4,5-bisphosphate has been reported between day 21 and 34 [31], with neonatal levels being about 20% of adult levels. These relatively small increases probably do not tax the phosphatidylinositol pool and do not demand any significant increase in phosphatidylinositol synthesis. On the other hand, there may well be a modest adjustment in the synthesis (and transfer?) of phos-

phatidylinositol which is efficiently converted to phosphatidylinositol 4-phosphate by the concommitant increase in phosphatidylinositol kinase activity around day 12 [25]. Our results, nonetheless, indicate a positive correlation between polyphosphoinositide levels and CTP : phosphatidate cytidylyltransferase and CDPdiacylglycerol : inositol phosphatidyltransferase specific activities at different stages of brain development.

Conclusions

In both whole brain and cerebellum, the developmentally regulated increase in phosphatidylinositol transfer protein and catalytic activity clearly preceded the tissue's increased capacity to synthesize this anionic phospholipid. The temporal profiles of synthetic and transfer specific activity were generally comparable in the two brain regions. However, the peak of phosphatidylinositol transfer activity was delayed 7–10 days in cerebellum, in keeping with the rostral-caudal development pattern characteristic of rat brain.

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