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Differential Incorporation of Precursor Moieties into Cerebral Cortex and Cerebellum Glycerophospholipids During Aging

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The incorporation of polar and non-polar moieties into cerebral cortex (CC) and cerebellum (CRBL) phospholipids of adult (3.5-month-old) and aged (21.5-month-old) rats was studied in a minced tissue suspension. The biosynthesis of acidic phospholipids through [^{3}H]glycerol appears to be slightly increased with respect to that of zwitterionic or neutral lipids in CC of aged rats with respect to adult rats. On the contrary, the synthesis of phosphatidylcholine (PC) from [^{3}H]choline was inhibited. However, the incorporation of [^{14}C]serine into phosphatidylserine (PS) was higher in CC and CRBL in aged rats with respect to adult rats. The synthesis of phosphatidylethanolamine (PE) from PS was not modified during aging. Saturated ([^{3}H]palmitic) and polyunsaturated ([^{3}H]arachidonic) acids were incorporated successfully by adult and aged brain lipids. In addition [^{3}H]palmitic, [^{3}H]oleic and [^{3}H]arachidonic acid were employed as glycerolipid precursors in brain homogenate from aged (28.5 month old) and adult (3.5 month old) rats. [^{3}H]oleic acid incorporation into neutral lipids (NL) and [^{3}H]arachidonic acid incorporation into PC, PE and phosphatidylinositol (PI) were increased in aged rats with respect to adult rats. Present results show the ability and avidity of aged brain tissue in vitro to incorporate unsaturated fatty acids when they are supplied exogenously. They also suggest a different handling of choline and serine by base exchange enzyme activities to synthesize PC and PS during aging.

KEY WORDS: Aging; brain; cerebral cortex; cerebellum; lipid metabolism; phospholipid metabolism.

INTRODUCTION

The aging brain is characterized by a measurable decline of physiological functions. Even if causes are still unknown, several hypotheses have been advanced in an attempt to explain them. For example, the membrane deterioration theory is considered one of the most important in explaining the aging phenomena. As phospholipids are an integral part of membranes they undoubtedly play an important role in regulating cell membrane function and therefore, any modification in their metabolism may influence cellular function.

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Abbreviations used: CC, cerebral cortex; CRBL, cerebellum; DAG, diacylglycerol; FA, fatty acids; HPTLC, high performance thin layer chromatography; NL, neutral lipids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PSS1, phosphatidylserine synthase 1; PSS2, phosphatidylserine synthase 2; SPH, sphingomyelin; TAG, triacylglycerols; TLC, thin layer chromatography; TPL, total glycerophospholipids.

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It is known that brain aging is accompanied by changes in the overall composition of membrane lipids (1–4). Qualitative or quantitative changes in the ratios of phospholipid headgroup classes and/or in the proportion of their fatty acyl moieties are known to start alterations in the physicochemical properties of biological membranes, along with many of their functions. Our previous studies on different brain areas from aged rats document a phospholipid-specific fatty acid composition alteration produced by aging (2). However, no significant changes in the content of different phospholipid classes were observed, except for one previously reported for sphingomyelin (SPH) (5) and cardiolipin (6).

In this report we present data describing de novo synthesis as well as polar and acyl moiety turnover of phospholipids in brain tissue in vitro. The purpose of this study is to make a correlation between lipid compositional studies and metabolism in our aging experimental model (Wistar rats of 21.5 and 28.5 months of age as "aged" and 3.5 months of age as "adult"). We studied the in vitro metabolism of choline and serine, and the glycerol incorporation into the backbone of glycerolipids in a minced tissue suspension of cerebral cortex (CC) and cerebellum (CRBL) from 3.5- and 21.5-month-old rats. These data were taken as a metabolic pattern of a first stage in our experimental system of normal aging in rats where minimal changes in acyl composition of glycerophospholipids are present (2). In previous studies the fatty acid composition of total lipids from rats of 21.5 and 28.5 months of age showed an increase in monounsaturated (18:1 and 20:1) and a decrease in polyunsaturated (20:4, 22:4 and 22:6) content with respect to adult rats (2). In the present study, [^{3}H]palmitic, [^{3}H]oleic and [^{3}H]arachidonic acid were used as radioactive precursors to examine the saturated and polyunsaturated acyl chain turnover in the phospholipids of rat brain. These labeling studies were carried out in a minced tissue suspension of CC and CRBL from 21.5- and 3.5-month-old rats as well as in brain homogenates from 28.5- and 3.5-month-old rats.

EXPERIMENTAL PROCEDURES

General. Wistar-strain rats were kept under constant environmental conditions and were fed on a standard pellet diet. Rat ages were carefully assessed: adult rats were 3.5 months old and aged rats were 21.5 and 28.5 months old, respectively. Animals were sacrificed by decapitation, the brain was immediately dissected (2–4 min of decapitation), rinsed with ice-cold buffer, and the different regions were subsequently obtained.

[2-^{3}H]Glycerol (specific activity 5–10 Ci/mmol), [^{14}C(U)] L-Serine (specific activity 168 mCi/mmol), [methyl-^{3}H]choline-chloride (specific activity 69.5 Ci/mmol), [9,10-^{3}H(N)] palmitic acid (specific activity 47 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H(N)] arachidonic acid (specific activity 83.8 Ci/mmol), [9,10-^{3}H(N)]oleic acid (specific activity 2–10 Ci/mmol) and Omnifluor were purchased from New England Nuclear-Dupont (Boston, MA). All other reagents were of analytical grade.

Metabolic studies. They were carried out in two different preparations:

a) Minced Tissue Suspension: It was prepared from cerebral cortex (CC) and cerebellum (CRBL) of 3.5- and 21.5-month-old rats. Tissues were minced to obtain about 1 mm^3 fragments and transferred to conical tubes containing minimum essential medium (Gibco Lab, Grand Island, N.Y.) supplemented with 2.2 g/l of $NaHCO_3$, 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO) and L-glutamine, at a final concentration of 2 mM (10 ml of medium per CC or CRBL). Tissue fragments were aspirated and expelled 10 times using a 1-mm diameter Pasteur pipette. After sedimentation, particles were resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4). Samples (about 100 μg of protein) were incubated for 15, 30, 60 and 90 min at 37 °C in the same medium using 5 μCi of [2-^{3}H] glycerol or [^{14}C]serine or [^{3}H]choline or 2.5 μCi of [^{3}H] palmitic acid or [^{3}H]arachidonic acid in a final volume of 0.2 ml. Fatty acids (FA) were resuspended in a fatty acid-free bovine serum albumin solution with buffer (4:1, mol:mol). This mixture with FAs was sonicated for 3 min in a MicroTip Branson Sonifier. An aliquot was routinely taken to measure radioactivity before adding the labeled FA to the incubation medium. In all cases, lipids were extracted from the tissue as described by Bligh and Dyer (7) and the chloroform layer was taken to dryness under N_2. Lipid samples were resuspended in chloroform/methanol (C:M 2:1, v/v) and extracts were subsequently purified as previously described (8). Unlabeled glycerol (1%), DL-serine (1%) or choline (1%) was added to the upper phases to wash lipid extracts labeled with [2-^{3}H]glycerol, [^{14}C]serine or [^{3}H]choline respectively.

b) Homogenates: They were prepared from the whole brain of 3.5- and 28.5-month-old rats as adult- and aged rats, respectively. Homogenates were made at 30 % (w/v) in 0.25 mM sucrose, 62 mM phosphate buffer pH 7.4, 0.15 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 4.2 mM N-acetylcysteine. The assay was started by adding 1 mg brain homogenate protein (100 μl) to 42 mM phosphate buffer, pH 7.4, supplemented with 3.5 mM ATP, 0.2 mM CoA, 1.5 mM NADH, 1.6 mM N-acetylcysteine, 0.33 mM nicotinamide, 42 mM NaF and 2 mM $MgCl_2$ in a total volume of 2 ml. The free FA was also present in the medium at 50 μM concentration (1 μCi of [^{3}H]palmitic acid) or 10 μM concentration (1 μCi of [^{3}H]arachidonic acid or 0.5 μCi of [^{3}H]oleic acid). After evaporation to dryness under nitrogen, FAs were sonicated in propylene glycol in a water bath for 5 min and added to the medium (0.25 % v/v). An aliquot was routinely taken to measure radioactivity before adding the labeled FA to the incubation medium. Samples were incubated at 37 °C during 30 or 60 min. The assay was stopped and lipids were extracted by addition of C:M (2:1, v/v), as previously described (8).

Lipid Isolation and Analytical Methods. Phospholipids were isolated by two-dimensional TLC (9) or by monodimensional pre-coated HPTLC (10). Neutral lipids were isolated by monodimensional TLC on Silicagel G plates developed in hexane/diethylether/acetic acid (70:30:2, v/v). After development, lipid spots were located on plates by exposure to iodine vapors. Spots were scraped off and subjected to phosphorus analysis (9) or transferred to vials containing 0.4 ml water and 10 ml of 0.5% omnifluor in toluene/Triton X-100 (4:1, v/v) to measure radioactivity by liquid scintillation counting. When HPTLC plates were used the adsorbent

was transferred to vials containing 0.4 ml water and kept overnight before scintillator addition. Protein content was determined by the method of Lowry et al. (11).

Statistical Analysis. Statistical analysis was performed using Student's *t* test, with the values representing the mean ± S.D. (standard deviation) of the total number of samples indicated in each legend. In the case of ratios or percentages (Fig. 4-6 and Table I) the SD was calculated according to the ad-hoc statistical treatment (12).

RESULTS

In Vitro Metabolic Studies by Using [^{3}H]Glycerol, [^{3}H]Choline and [^{14}C]Serine in Tissue Fragments. In vitro metabolic studies were carried out using tissue fragments obtained from CC and CRBL in oxygenated Krebs Ringer - bicarbonate supplemented with glucose (experimental procedures have been described in Methods). This medium appears to be adequate for free bases, glycerol and also for FA incorporation studies.

[2-^{3}H]Glycerol was incorporated as a function of time into glycerolipids. The pattern of incorporation showed to be PA>CDP-DG>PI>PC and PE during the first 15 min of incubation (data not shown). Phosphatidic acid (PA) synthesized from this precursor represented 42 % of the total phospholipid (TPL) labeling at this incubation time (data not shown). Fig. 1a shows that [2-^{3}H]glycerol was actively incorporated into phospholipids (57 %) and NL (43 %) from CC at 60 min of incubation in adult rats. Results from individual phospholipids are shown in Fig. 1b and indicate that PI, PC and PE were the predominantly synthesized phospholipids (28, 22 and 19 % of TPL labeling respectively). DAG was the predominantly synthesized NL (50% with respect to total NL, data not shown). Glycerolipid precursors as PA and CDP-DG plus PS were also determined and represented only about 26% at 60 min incubation.

In the minced tissue suspension of CC from aged rats the synthesis of TPL was higher with respect to adult rats (Fig. 1a). Data from individual phospholipids revealed that the de novo synthesis of acidic phospholipids was the one affected, PA being the most augmented phospholipid with respect to the value from adult rats (Fig. 1b). Also CDP-DG plus PS, PI and to a lesser extent PE labeling showed higher levels in aged than in adult rats. PC synthesis from [2-^{3}H] glycerol was similar in CC from aged and adult animals. On the contrary, NL synthesis from [2-^{3}H]glycerol was slightly inhibited in aged rats with respect to adult rats, TAG being the most affected (25 % inhibition, data not shown).

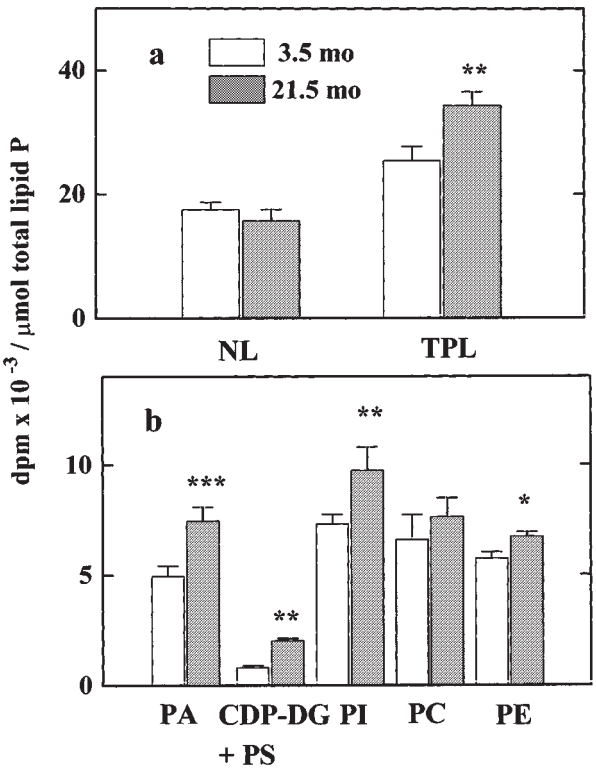

Fig. 1. Glycerolipid synthesis in minced tissue suspensions of cerebral cortex from adult (□) and aged (■) rats incubated with [^{3}H]glycerol. Minced tissue suspensions were obtained from CC of 3.5- and 21.5-month-old rats as adult and aged rats, respectively. Tissues were minced to obtain about 1 mm^3 fragments and processed as indicated in Experimental Procedures. Particles were resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4). Samples (about 100 µg of protein/sample) were incubated for 60 min at 37°C in a final volume of 0.2 ml of the same medium using 2 µCi [^{3}H]glycerol (specific activity 5–10 Ci/mmol). Data are expressed as incorporation in dpm/µmol of total lipid P and represent the mean ± S.D. of 3 adult and 3 aged rats. Panel a: neutral and total phospholipid synthesis; panel b: individual phospholipid synthesis. *: $p < 0.05$; **: $p < 0.025$; ***:$p < 0.01$.

The [^{3}H]choline incorporation into lipids was also studied in CC suspension from adult and aged rats. PC synthesis by base exchange reaction from free choline was shown to be active in adult rats and increased as a function of incubation time up to 90 min, the longest incubation time studied (Fig. 2). It represented about 90–95% of the total [^{3}H]choline incorporated into choline-containing pospholipids at this incubation time. Sphingomyelin and lysophosphatidylcholine (LPC) incorporated about 3–5% of total lipid radioactivity. LysoPC production also increased up to 90 min in adult rats (insert, Fig. 2). PC synthesis from choline was strongly inhibited (by 50 %) in the CC suspension from aged rats with respect to adult rats (Fig. 2). This

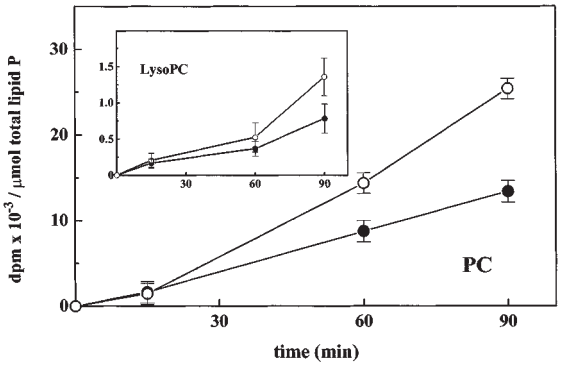

Fig. 2. PC and LysoPC synthesis in minced tsisue suspensions of cerebral cortex from adult (○) and aged (●) rats incubated with [methyl-^{3}H]choline. Minced tissue suspensions were obtained from CC of 3.5- and 21.5-month-old rats as adult and aged rats, respectively as described in Fig. 1. Samples (about 100 µg of protein/sample) were incubated for 15, 30, 60 and 90 min at 37°C in a final volume of 0.2 ml of the same medium using [methyl-^{3}H]choline chloride (specific activity 69.5 Ci/mmol). Data are expressed as incorporation in dpm/ µmol of total lipid P and represent the mean ± S.D. of 3 adult and 3 aged rats.

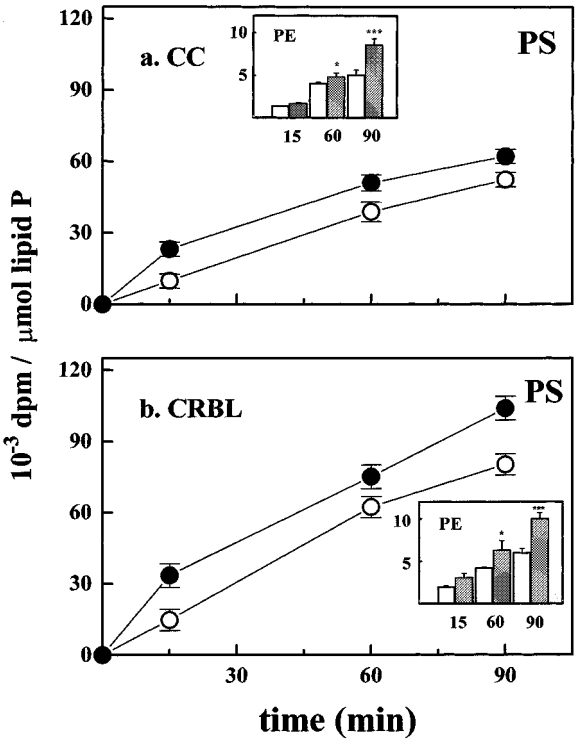

Fig. 3. PS and PE synthesis in minced tissue suspensions of cerebral cortex and cerebellum from adult (○, □) and aged (●, ■) rats incubated with [^{14}C(U)]L-serine. Minced tissue suspensions were obtained from CC and CRBL of 3.5- and 21.5-month-old rats as adult and aged rats, respectively as described in Fig. 1. Samples (about 100 µg of protein/sample) were incubated for 15, 30, 60 and 90 min at 37°C in a final volume of 0.2 ml of the same medium using 5 µCi of [^{14}C(U)]L-serine (specific activity 168 mCi/mmol). Data are expressed as PS or PE (insert) incorporation in dpm/µmol of total lipid P and represent the mean ± S.D. of 3 adult and 3 aged rats. *: $p < 0.05$; ***:$p < 0.01$.

effect was observed at 60 and 90 min of incubation. Whereas LPC synthesis was also decreased, its proportion with respect to PC proved to be similar (3.92 ± 0.34% with respect to 3.58 ± 0.46% in adult and aged rats, respectively).

Fig. 3a shows the time course of the incorporation of [^{14}C]serine into phospholipids of CC and CRBL of adult and aged rats as a function of the incubation time which was linear up to 90 min of incubation. Labeled lipids isolated by two-dimensional TLC indicated that up to 85% of the incorporated serine was recovered into PS, while a small percentage (8–10%) was detected in PE. SPH synthesis was also observed but the percentage label in SPH was lower than in PE (4–6 %) (data not shown). It is known that serine enters into many lipid pathways (synthesis of glycerol, FAs, and sphingosine). However, we have obtained indirect evidence that the radioactivity present in PS was localized in its base moiety. If previous [^{14}C]glycerol synthesis from [^{14}C]serine took place, the label would be present in PA, PI or PC, the latter being lipids that were previously shown to be mainly synthesized in this tissue by the de novo synthesis pathway from glycerol (Fig. 1). Fig. 3 also shows that [^{14}C]L-serine incorporation into PS from CC and CRBL was higher in aged rats than in adult rats (10% and 30% increases in CC and CRBL, respectively, at 90 min of incubation). In addition, incorporation of labeled serine into PS was higher in CRBL than in CC of adult rats (Fig. 3). Phosphatidylethanolamine was also synthesized by decarboxylation of labeled PS in both neural tissues (Insert Fig, 3, CC and CRBL). PE synthesis using serine as a precursor in CC and CRBL showed time dependence and it also showed that PE synthesized from serine by PS decarboxylase was augmented in aged with respect to adult rat tissues. The proportion of labeled PE with respect to labeled PS in aged with respect to adult rats shows a net increase at 90 min of incubation.

[^{3}H]Arachidonic Acid and [^{3}H]Palmitic Acid as Glycerolipid Precursors in a Minced Tissue Suspension of Cerebral Cortex and Cerebellum of Adult and Aged Rats. Fatty acids available from the blood stream and those released from brain lipids constitute the pool of free FAs capable of being esterified into neural tissue phospholipids. Fatty acid oxidation and acyl-CoA synthesis are also active in brain cells and might play a role in the steady state of the metabolically active pool of FAs. The approach in the previously described

experiments with a minced tissue suspension that maintains cellular integrity makes it possible to study all the above mentioned mechanisms.

Fig. 4 shows the [^{3}H]palmitic acid (16:0) incorporation into TPL and NL in CC and CRBL from 3.5- and 21.5-month-old rats, respectively. FA incorporation with respect to total radioactivity (FA present in the medium plus FA incorporated into lipids) in both lipid classes is shown in Fig. 4a. It is interesting to note that the incorporation into NL (DAG plus TAG) in both tissues, was higher than in TPL in 3.5-month-old rats, the major difference being observed in the CRBL. As indicated in Fig. 4a, in CC this proportion proved to be greater in NL (12 %) with respect to TPL (7.4 %) whereas in CRBL it was even greater (14 % in NL with respect to 3.5 % in TPL).

Fig. 4b shows the distribution of the [^{3}H]16:0 incorporation into individual glycerophospholipids at 60 min incubation. At this time, 16:0 in PC from CC, represented 50% of the total FA incorporated into TPL, followed by PE, which represented 23% of the total. Phosphatidic acid, PI and CDP-DG plus PS represented 12, 9 and 3% respectively. [^{3}H]Palmitic acid incorporation into PC from CRBL also represented 50% of the TPL. In addition, PE presented a labeling level similar to that of PC (40% of TPL). CRBL, in contrast to CC, presented only up to 6% of the total incorporation into other glycerophospholipids (PA, PI and CDP-DG plus PS).

As shown in Fig. 4a and b, acylation with the saturated FA in the minced tissue suspension of CC and CRBL from 21.5-month-old rats was similar to that observed in adult rats. No significant differences were observed in TPL or NL nor in individual glycerophospholipids in the two regions studied.

Results of [^{3}H]arachidonic acid (20:4) incorporation in CC and CRBL preparations are shown in Fig. 5a and b. In the samples obtained from both adult and aged rats, the proportion of FA incorporated into total lipids with respect to total radioactivity (FA present in the medium plus FA incorporated into lipids) was slightly lower than that observed with 16:0 (Fig. 4). Although both FAs showed a similar incorporation into TPL (around 7%), 20:4 was incorporated to a lesser extent than 16:0 into NL. The distribution of 20:4 incorporation into individual phospholipids showed a specific tisular pattern. In CC, the order was PC > PI > PE representing about 40, 26 and 20% of the TPL respectively, whereas in CRBL the order was PC >> PE >= PI (Fig. 5b). In this tissue PC incorporated 20:4 to the same extent as 16:0, it accounting for about 50% of the label incorporated into TPL. On the contrary, the label into PI from CRBL represented only 15% of TPL incorporation.

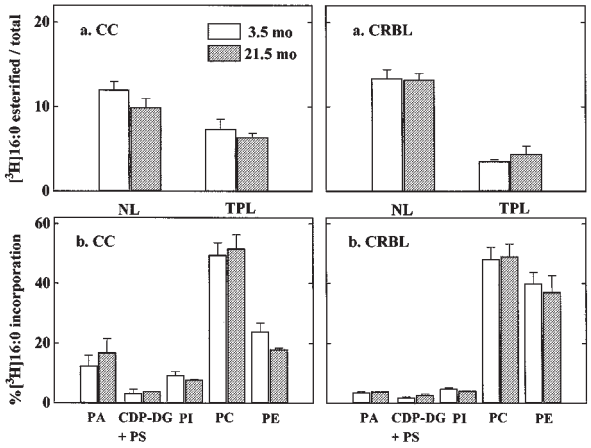

Fig. 4. Glycerolipid acylation in minced tissue suspensions of cerebral cortex and cerebellum from adult (□) and aged (■) rats incubated with [^{3}H] palmitic acid. Minced tissue suspensions were obtained from CC and CRBL of 3.5- and 21.5-month-old rats as adult and aged rats, respectively as described in Fig. 1. Samples (about 100 μg of protein/sample) were incubated for 15, 30, 60 and 90 min at 37°C in a final volume of 0.2 ml of the same medium using 2.5 μCi of [^{3}H]palmitic acid (specific activity 47 Ci/mmol). Data are expressed as the ratio of [^{3}H]palmitic acid esterified to total [^{3}H]palmitic acid present (esterified + free) in panel a and percentage [^{3}H]palmitic acid incorporated into phospholipids in panel b, and represent the mean ± SD of 3 adult and 3 aged rats. Panel **a:** neutral and total phospholipid synthesis; panel **b:** individual phospholipid synthesis.

Aging did not affect the incorporation of 20:4 into lipids from CC or CRBL of 21.5-month-old rats with respect to 3.5-month-old rats. It is interesting to point out that not only 16:0 but also 20:4, that was reported to be significantly decreased in total lipids of CRBL from 21.5-month-old rats (13), were incorporated into lipids as efficiently as in 3.5-month-old rats (Fig. 5).

[^{3}H]Palmitic Acid, [^{3}H]Oleic Acid and [^{3}H]Arachidonic Acid as Fatty Acid Precursors in Glycerolipid Biosynthesis in Brain Homogenates of Adult and Aged Rats. To avoid the uptake step of FAs by the tissue, that was operative in the experimental approach described above where cell to cell attachments and microvessels are present, experiments utilizing FA precursors in brain homogenates were carried out. In these experiments, attention was focused on the acyl activation and its incorporation into the brain homogenate glycerolipid acceptors. In addition, the experiment was designed with 28.5-month-old rats instead of 21.5-month-old rats because at this age there were more marked changes in the acyl group composition of different glycerophospholipid (2). [^{3}H] Palmitic acid, [^{3}H]oleic acid (18:1) and [^{3}H]arachidonic acid were used as precursors of glycerolipids in brain homogenates from adult (3.5-month-old) and

aged (28.5-month-old) rats. Table I shows the incorporation of fatty acids into TPL and NL in brain homogenates with respect to total fatty acids offered (FA in the medium plus FA incorporated into lipids). In contrast to what was observed in the minced tissue suspension (Fig. 4a and 5a), FAs were mainly incorporated into phospholipids instead of NL. In addition, the level of total FA incorporation into lipids (about 40% for 16:0 and 18:1 and 60% for 20:4) was higher than that observed in the incubation of minced neural tissue suspension. Although the proportion of FAs incorporated into NL with respect to the total label offered was similar for saturated, monounsaturated and polyunsaturated FAs, this ratio into TPL was arachidonic acid > oleic acid = palmitic acid. The homogenates from brain of 28.5-month-old rats also incorporated these FAs into NL and TPL, being the incorporation for all of them similar with respect to values observed in adult brain.

Fig. 6 shows the percentage incorporation into individual phosphoglycerides with respect to the incorporation into TPL (100 %) for 16:0, 18:1 and 20:4. A different pattern of incorporation was ob-

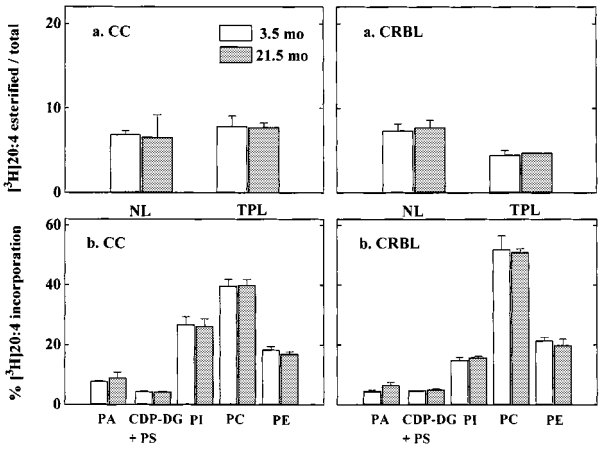

Fig. 5. Glycerolipid acylation in minced tissue suspensions of cerebral cortex and cerebellum from adult (□) and aged (■) rats incubated with [^{3}H]arachidonic acid. Minced tissue suspensions were obtained from CC and CRBL of 3.5- and 21.5-month-old rats as adult and aged rats, respectively as described in Fig. 1. Samples (about 100 μg of protein/sample) were incubated for 15, 30, 60 and 90 min at 37°C in a final volume of 0.2 ml of the same medium using 2.5 μCi of [^{3}H]arachidonic acid specific activity 83.8 Ci/mmol. Data are expressed as the ratio of [^{3}H]arachidonic acid esterified to total [^{3}H]arachidonic acid present (esterified + free) in panel a and percentage [^{3}H]arachidonic acid incorporated into phospholipids in panel b, and represent the mean ± SD of 3 adult and 3 aged rats. Panel **a:** neutral and total phospholipid synthesis; panel **b:** individual phospholipid synthesis.

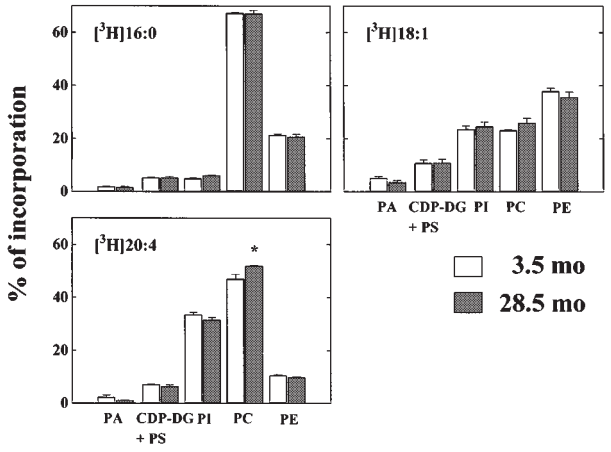

Fig. 6. Glycerolipid acylation in homogenates of cerebral hemispheres from adult (□) and aged (■) rats incubated with [^{3}H]palmitic acid or [^{3}H]oleic acid or [^{3}H]arachidonic acid. Homogenates were prepared from total hemispheres of 3.5- and 28.5-month-old rats as adult and aged rats, respectively. Homogenates were prepared and assays were performed as described in Experimental Procedures. The free fatty acid was also present in the medium at 50 μM concentration (1μCi of [^{3}H]palmitic acid) or 10μM concentration (1μCi of [^{3}H]arachidonic acid and 0.5 μCi of [^{3}H]oleic acid). Samples were incubated at 37°C during 60 min. Data show the fatty acid incorporation into individual glycerophospholipids (as the mean of the percentage incorporation ± SD from three animals) in brain homogenates. (*: $p < 0.05$).

TABLE I. Ratios of Fatty Acid Incorporated into Neutral Lipids and Total Phospholipids with Respect to Total Fatty Acids × 100 in Brain Homogenates from Adult and Aged Rats

	Palmitic Acid		Oleic Acid		Arachidonic Acid	
Fatty Acid	NL	TPL	NL	TPL	NL	TPL
ADULT	5.84 ± 0.74	36.12 ± 4.20	5.57 ± 1.21	35.66 ± 5.40	5.74 ± 0.71	53.25 ± 5.83
AGED	6.14 ± 0.56	35.28 ± 9.64	7.07 ± 0.76	35.94 ± 1.74	6.16 ± 1.43	55.84 ± 4.20

Homogenates were prepared from total hemispheres of 3.5- and 28.5-month-old rats as adult and aged rats, respectively. Homogenates and assays were carried out as described under Experimental Procedures. The free fatty acid was also present in the medium at 50 μM concentration (1 μCi of [^{3}H]palmitic acid) or 10 μM concentration (1 μCi of [^{3}H]arachidonic acid and 0.5 μCi of [^{3}H]oleic acid). Samples were incubated at 37 °C during 60 min. Values represent the mean ± SD from three animals and are expressed as ratios between fatty acid into glycerophospholipids (TPL) and neutral lipids (NL) in brain homogenates with respect to total (fatty acid in the medium plus fatty acid incorporated into lipids).

TABLE II. Fatty Acid Incorporation into Individual Glycerophospholipids in Brain Homogenates from Adult and Aged Rats

	Palmitic Acid		Oleic Acid		Arachidonic Acid	
Fatty Acid	ADULT	AGED	ADULT	AGED	ADULT	AGED
			10^{-3} dpm/mg prot			
DAG	64.0 ± 5.9	79.9 ± 7.3	34.5 ± 4.2	55.4 ± 7.7[b]	30.7 ± 1.3	32.3 ± 2.9
TAG	59.0 ± 3.3	82.0 ± 13.2[a]	21.2 ± 1.1	39.4 ± 5.9[b]	71.5 ± 3.6	81.4 ± 4.0[a]
PE	65.9 ± 3.3	62.3 ± 6.8	44.5 ± 5.6	48.3 ± 5.1	53.7 ± 1.2	63.1 ± 2.4[c]
PC	210.0 ± 7.9	205.3 ± 34.6	27.1 ± 3.5	32.6 ± 5.0	243.1 ± 18.5	337.9 ± 21.4[c]
CDP-DG+PS	15.8 ± 0.8	15.6 ± 2.6	12.4 ± 0.4	13.7 ± 2.6	36.2 ± 0.0	40.6 ± 2.6
PI	15.2 ± 0.8	18.2 ± 3.1	27.8 ± 4.9	34.7 ± 9.5	173.4 ± 0.4	205.1 ± 21.9[a]
PA	5.6 ± 0.7	4.7 ± 1.8	6.1 ± 1.5	4.5 ± 0.3	7.8 ± 0.3	6.2 ± 1.7

Homogenates were prepared from total hemispheres of 3.5- and 28.5-month-old rats as adult and aged rats, respectively. Homogenates and assays were carried out as described under Experimental Procedures. The free fatty acid was also present in the medium at 50 μM concentration (1 μCi of [^{3}H]palmitic acid) or 10 μM concentration (1 μCi of [^{3}H]arachidonic acid and 0.5 μCi of [^{3}H]oleic acid). Samples were incubated at 37 °C during 60 min. Data show the fatty acid incorporation into individual glycerophospholipids (as the mean ± SD of 10^{-3} dpm/(h × mg of protein) from three animals) in brain homogenates. (a: $p < 0.05$; b: $p < 0.025$; c: $p < 0.005$).

served for each FA. While PC acylation with 16:0 and 20:4 represented about 60 and 50% respectively, acylation with 18:1 was about 25%. On the contrary, PE was mainly acylated with 18:1 (40% of the total label incorporated into glycerolipids). In addition, PI acylation with 20:4 was higher than with 18:1 and 16:0 (the percentage of incorporation was about 30, 20 and 5%, respectively). Fig. 6 also shows the percentage incorporation into individual phospholipids from the brain homogenate of 28.5-month-old rats. Values for 16:0 and 18:1 percentage incorporation were similar with respect to homogenates from adult rats. It is interesting to note that only the percentage incorporation of 20:4 into phospholipids was slightly modified. An increased percentage with respect to control was observed in PC. In addition, Table II shows the incorporation into individual glycerolipids in brain homogenates from aged and adult rats. It revealed that 20:4 was more efficiently incorporated into lipids from aged than from adult rats. While incorporation of 20:4 into PI was slightly increased (by 18%), this value for PC and PE was 30 and 20% higher than in adult rats. In addition, 18:1 and 16:0 were more efficiently utilized by the brain homogenate of 28.5-month-old rats in the DAG and TAG synthesis, than in 3.5-month-old rats, though no significant increase in individual phospholipids was observed.

DISCUSSION

Our results obtained from the incorporation of [^{3}H]choline into CC lipids of 21.5- with respect to 3.5-month-old rats (Fig. 2) showed an inhibition of PC labeling and revealed that the data obtained in a minced tissue suspension of CC are consistent with previously reported data (14–16). Phosphatidylcholine may be labeled with [^{3}H]choline by two mechanisms: a) de novo synthesis by the Kennedy pathway and b) PS synthase 1 (PSS1) activity that exchanges serine by choline in PS to synthesize PC (17). As the specific activity of the radiolabeled choline could be increasingly diluted along the reactions involved in a) in the different choline-containing precursors (i.e. phosphocholine and CDP-choline), the final product, PC, is unlikely to be detected. This suggests that PC labeling with [^{3}H]choline is mainly due to PSS1 activity. Decreased [^{3}H]choline incorporation into PC may be due to a) a dilution of the labeled precursor within the choline cell pool in the aged rat brain; b) an age-related redirection of choline uptake pathways; c) a decreased base exchange activity for the incorporation of choline into PC.

PC synthesis in CC from aged rat through the de novo pathway, measured by [^{3}H]glycerol incorporation into glycerolipids, was found to be unchanged. In addition, an increase in acidic phospholipid labeling was observed. However, it has been reported that PC enzymatic synthesis is decreased in brain during aging. These data were obtained from rat CC in in vivo experiments (16) and also in neuronal cell homogenates from rats up to 18 months of age (15). Further evidence employing labeled CDP-choline as precursor in brain microsomes indicates that metabolism was mainly impaired not by a decrease in CDP-base availability but by the modification of diacylglycerol fatty acid

composition (14). The differences between the above mentioned work and our work could be due to the different tissue preparations used. The minced tissue suspensions that were used in the experiments described in this work maintain the cellular compartments intact while allowing ready access of precursor molecules. It is interesting to note that the formation of the lyso-compound from labeled PC in our neural tissue preparation was minimal and that aging did not alter it. The inhibition of lysoPC labeling observed in CC from aged with respect to adult rats correlates with the inhibition observed in PC labeling using [^{3}H]choline.

According to previous work, the PC content in CC of the 28-month-old rats was similar to the PC content from adult rats (2). This unchanged content in PC may be due to a balance between synthesizing and degrading enzymatic activities. Experimental approaches using exogenous labeled glycerophospholipids as substrates show that the effect of age on phospholipase A activity differs greatly according to the substrate used (PC or PE) and the cerebral area tested (18). Although there is a decrease or slight stimulation when PE containing arachidonic acid is used as substrate, a 2-fold stimulation is seen in some brain areas of aged rats when the activity is tested with PC as substrate (18). The low content of lysoPC in our system points to an efficient reutilization of this compound in the reacylation reaction. This hypothesis is also supported by the results obtained when arachidonic acid was employed as precursor in brain homogenates as discussed below.

Although PS content in CC and CRBL was not significantly modified by aging (2), we have found an increased [^{14}C]serine incorporation into PS in both neural tissues (Fig. 3). Increased PS content in neural membranes, investigated through PS liposomes addition, has been implied in the increase of Ca^{2+} uptake into brain synaptosomes (19), norepinephrine turnover in hypothalamus (20) and ACh output in CC (21).

It is known that in mammals base-exchange reaction represents, so far, the main pathway for PS synthesis in the nervous system and in all tissues (22,23). In this base-exchange reaction L-serine replaces PC and PE choline and ethanolamine moieties to produce PS and free choline or ethanolamine by PSS1 and PSS2 enzymatic activities, respectively (17). This enzymatic system is able to change the hydrophilic head of phosphoglyceride molecules without energy requirements. It has been previously reported from in vivo experiments that the [^{3}H]serine incorporation into lipids from aged CC (24-month-old rats) appears to be unaffected by age (16). The in vitro test, made with microsomes from whole brain, also indicated that the base-exchange activity of choline and ethanolamine free bases was not altered by aging (14). More recently, a slight increase of serine base-exchange has been reported in microsomes of CC and striatum (24). In our experimental approach all cellular compartments were preserved and conditions guaranteed the accessibility of free bases to them. This was not the case of ventricular injection of precursors where other transport mechanism could have been involved (16) or in experiments with microsomes where their obtention could produce the loss of cytoplasmatic components important for carrying out these reactions (14). It has been reported that aging induces a reduced energy availability in rat CC (25) and base-exchange is an interesting mechanism to change the phospholipid composition in membranes without energy requirements. Further studies on PS synthesis will be necessary taking into account that this is the first report that reveals a significant increased synthesis of this phospholipid in CC and CRBL by aging. Differential handling of choline and serine in PC and PS synthesis through base exchange activities by aged neural tissue merits additional studies.

Previous reports from our laboratory have shown that in aging there is a decrease in the content of polyenoic and an increase in the content of monoenoic FAs esterified to phospholipids (2), and that these changes are specific for different phospholipids and also for different areas and subcellular fractions of the brain. It is known that the source of FAs to be esterified in brain phospholipids are blood-circulating FAs (26,27) and part from those arising from deacylation of brain glycerolipids (28). In our experimental approach with minced tissue suspensions from CC and CRBL, where natural membrane barriers and intracellular components at physiological concentrations are present, we demonstrated that saturated and polyunsaturated FAs were successfully incorporated into neutral and polar glycerolipids. The incorporation of FAs into individual lipids from CC and CRBL of 21.5-month-old rats showed that aging at this time did not affect the incorporation of FAs into glycerolipids. In these experiments we have also found that FAs are incorporated more efficiently into NL than into phospholipids. However, when 20:4 was used the incorporation into both lipids in CC or CRBL was similar.

In previous in vivo studies the incorporation of labeled 20:4 into polar lipids injected in the lateral ventricle of the brain of aged (24 months of age) rats was significantly lower (by about 40%) when compared to adult animals (4 months of age). A similar but less marked behavior of this nature was observed for 16:0

(29). However, the incubation of a cellular subfraction from brain cortex in the presence of labeled arachidonoyl-CoA and 2-lysophosphoglycerides showed a moderate stimulation of the reacylation reaction rate in aged rats (30). It has been suggested that aging inhibits the incorporation of 20:4 via de novo phospholipid biosynthesis pathway but has no effect on the deacylation-reacylation cycle (31).

In this paper, we also show data on the incorporation of saturated and unsaturated FA into glycerolipids from brain homogenates of 3.5-month-old and 28.5-month-old rats. It has been previously observed that glycerophospholipid composition is not changed during aging (2). The cell free system used showed the incorporation of saturated, monoenoic and polyenoic FAs into endogenous glycerolipid acceptors after acyl activation (by the deacylation-reacylation cycle). Results from 16:0 and 18:1 expressed as ratios of esterified to total FA demonstrate that neural membranes from adult and aged rats utilize these precursors in NL and TPL synthesis in a similar way (Table I). Our data expressed as the percentage distribution of 20:4 into phosphoglycerides, also revealed the ability of neural tissue from 28.5-month-old rats to incorporate this polyunsaturated FA (Fig. 6). In addition, there is a net increase in the incorporation of 18:1 and 20:4 into individual glycerolipids; this clearly reveals that the aged brain is able to use both FAs more efficiently than the adult rat brain, being PC the phospholipid that incorporated most of the label. There is also a significant increase in 16:0, 18:1 and 20:4 into TAG. It is known that TAG is a highly active metabolic pool in neural tissue and it has been suggested as a FA reservoir (32,33). Present data reveal that in normal aging this function is still present and that available FAs are actively taken up into TAG. PE and PI showed an increased acylation with 20:4 but to a lesser extent, than PC (Table II). This was not the case for DAG. It is not surprising that an active deacylation-acylation mechanism is present for the renewal of arachidonic acid-enriched PC molecular species as it has been proposed that the deacylation-reacylation cycle is probably one of the most important events controlling the metabolic activity of 20:4 in the brain (4).

In addition, the increased incorporation of 18:1 into DAG could explain previously reported changes in DAG composition (14,30).

Previous studies demonstrate an increased incorporation of docosahexaenoic acid (22:6) into 28-month-old rat retinas with respect to 3-4 month-old rats (35). In agreement with our results, it has also been shown that in vitro incorporation of [2-^{3}H]glycerol into glycerolipids showed no changes between adult and aged rat retinas. In these studies 22:6 incorporation into all lipids of aged retinas was significantly higher (35). These results, together with the ones presented herein, allow us to suggest that neither an impairment in the de novo biosynthetic machinery nor in the enzymatic mechanism involved in the turnover of fatty acids in preexisting phospholipid molecules are responsible for the decrease in the content of polyunsaturated FA esterified to phospholipids observed during aging (35).

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