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Turnover of Synaptic Membranes: Age-Related Changes and Modulation by Dietary Restriction

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We examined age-related changes in the turnover rates of synaptic membrane components that might underlie the decrease in synaptic functions in senescence. Synaptic membrane constituents were labeled in vivo with deuterium and the disappearance of the deuterated molecules from synaptic membranes was measured by mass spectrometry. The turnover rates of phosphatidylcholine, phosphatidylethanolamine, cholesterol, and synaptophysin were all shown to slow down with aging. Dietary restriction, which is known to retard various aging processes, was found to decrease the turnover rates of membrane lipid species. Consequently, the fatty acid composition in phospholipids remained unchanged in the synaptic plasma membranes of food restricted mice. In contrast, the turnover rate of synaptophysin was accelerated under dietary restriction. This may mean that increased turnover enhances the removal of damaged proteins from membranes. © 2002 Wiley-Liss, Inc.

Key words: synaptic membranes; aging brain; lipid turnover; phospholipids; cholesterol; synaptophysin; dietary restriction

Synaptic transmission is a principal activity in the information processing in the brain, and it is performed by a complex reaction called exocytosis in which synaptic vesicles are fused with synaptic plasma membranes to release neurotransmitters. We previously showed that exocytosis decreased with aging (Tanaka et al., 1996). As might be expected from synaptic dysfunction in senescence, cognitive functions also decline in senescence (Ando and Ohashi, 1991; Dorce and Palermo-Neto, 1994; Quirion et al., 1995). Many studies have shown compositional changes of synaptic membranes with aging and these changes are thought to correlate with decreased synaptic functions. Previous studies have reported age-related changes in lipid composition (Wood et al., 1984; Tanaka and Ando, 1990; Waki et al., 1994) and in protein composition (London et al., 1985; Shimohama et al., 1998). These compositional changes may lead to functional deterioration in synaptic membranes (Ando et al., 1986; Tanaka and Ando, 1990). Defects in synaptic membranes are known to be accelerated under pathological courses such as Alzheimer's disease (Nitsch et al., 1992; Mason et al., 1992).

On the other hand, neurons have a remarkable potential to regenerate and remodel synapses in damaged tissues (Stroemer et al., 1995). Reactive synaptogenesis has been observed to occur in aged rat brains as well as in young ones (Anderson et al., 1986; Kugler et al., 1993). These responses are due to the characteristics of neuronal plasticity. Synaptic contacts are known to develop in response to synaptic activity. Long-term potentiation was reported to be followed by synaptogenesis (Chang et al., 1991). We observed synaptic strengthening in the rat brain as one type of synaptic plasticity that occurred in response to an enriched environment as a rearing condition (Nakamura et al., 1999). In association with synaptic remodeling, increased metabolic turnover of membranes was observed (Poirier et al., 1993). Thus, synapses are actively and continuously remodeled by metabolic turnover throughout life. Therefore, one of the objectives of this study was to delineate the changes with aging in the turnover rates of synaptic membrane constituents. Dietary restriction has been shown to improve synaptic functions such as long-term potentiation (Eckles-Smith et al., 2000) and learning ability (Algeri et al., 1991) as well as to elongate life span (Masaro, 2000). Thus, a second objective was to determine whether some of the changes that

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occur during aging could be restored through dietary restriction.

MATERIALS AND METHODS

Animals

Three age groups of C57BL/6 mice (2, 6, and 22 months old) were obtained from the aging colony of Tokyo Metropolitan Institute of Gerontology. Mice were maintained under 12-hr light/12-hr dark conditions and were fed autoclaved CRF-1, Charles River Japan (Atsugi). Two- and 6- month-old mice were fed ad libitum. Twenty-two-month-old mice were divided into two groups, an ad libitum-fed control group and a dietary-restricted group. Dietary restriction consisted of 70% of ad libitum-fed calories. The mice were fed every other day starting at the age of 4 months. Body weights of the dietary-restricted group remained relatively constant until an age of about 13 months, and thereafter slightly increased in senescence, while body weights of ad libitum-fed group continuously increased during the whole period of experiment (Fig. 1). For the labeling experiments, 2-month-old mice were given drinking water containing 40% of D_2O for 10 days and 6- and 22-month-old mice were given drinking water containing 35% D_2O for 20 days.

Preparation of Synaptosomes and Synaptic Plasma Membranes

Synaptosomes were isolated from the cerebral cortex based on the method of Booth and Clark (1978) as modified by Tanaka and Ando (1990). In brief, synaptosomes were collected at the interface between the 7.5% and 12% layers of Ficoll after centrifugation at 99,000 × g for 1 hr. The synaptosomes were then disrupted by homogenization in 1 mM sodium phosphate containing 0.1 mM EDTA (pH 7.5) to obtain synaptic plasma membranes (Mena et al., 1980). The homogenate was kept on ice for 1 hr and then centrifuged at 22,000 × g for 20 min. The pellet was subjected to centrifugation at 82,000 × g for 1 hr in discontinuous gradients consisting of 0.6, 0.8, and 1.0 M sucrose. A synaptic plasma membrane-enriched fraction was obtained at the interface between 0.1 and 1.0 M sucrose. Protein contents in the membrane fractions were determined by the method of Lowry et al. (1951).

Preparation of Lipids

Total lipids were extracted from the synaptic plasma membrane fractions with chloroform/methanol (1:1) and individual lipid species were isolated as previously reported (Ando and Saito, 1987). The total lipids were applied to a DEAE-Toyopearl 650 M column (acetate form; Tosoh, Tokyo) and neutral lipids were eluted with chloroform/methanol/water (30:60:8). The neutral lipid fraction was subjected to preparative high-performance thin-layer chromatography using nanoplates (E. Merck, Germany) that were developed stepwise with chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1) and *n*-hexane/*i*-propanol/acetic acid (65:35:2). The bands corresponding to cholesterol, phosphatidylcholine and phosphatidylethanolamine were scraped off to give the individual lipid species.

Preparation of Synaptophysin

Synaptosomes were solubilized in 6 M urea and synaptic proteins were separated by polyacrylamide gel electrophoresis (PAGE). The band corresponding to synaptophysin that was located by Western blotting was cut off. The gel band obtained was subjected to sodium dodecylsulfate (SDS)-PAGE and a single band identified as synaptophysin was cut out. Synaptophysin was electrophoretically eluted from the gel band and desalted.

Derivatization of Lipids and Amino Acids

Cholesterol was reacted with trifluoroacetic anhydride to give trifluoroacetyl cholesterol. Phospholipids were degraded in 3 % HCl-methanol at 100°C for 3 hr to produce fatty acid methyl esters. Fatty acid profiles of phospholipids were determined by gas-liquid chromatography using a 20% polyethylene glycol-succinate column. Synaptophysin was hydrolyzed with 6 N HCl. The amino acids produced were converted to their butyl esters by heating in 0.3% HCl-butanol at 100°C for 2 hr and then reacted with trifluoroacetic anhydride to give their N-trifluoroacetyl butyl ester derivatives.

Determination of Deuterated Molecules

Volatile derivatives of lipids and lipid degradation products were introduced into a gas chromatograph-mass spectrometer (JMS-DX304/304, JEOL, Tokyo). Cholesterol was separated with an OV-17 capillary column (0.25 mm i.d. × 10 m) and fatty acids were separated with an Omegawax capillary column (0.25 mm i.d.× 10 m). Amino acid derivatives were separated by an OV-17 capillary column (0.25 mm i.d. × 10 m). Electron impact ionization was used for the mass spectrometric determination of the deuterated compounds. Trifluoroacetyl cholesterol, fatty acid methyl esters, and amino acid N-trifluoroacetyl butyl esters were monitored using their prominent ions at $[M-113]^+$, $[M]^+$ and $[M-58]^+$, respectively. These ions of deuterated and nondeuterated compounds were determined by the selected ion-monitoring technique (Tanaka and Ando, 1981) to delineate the molar distributions of deuterated molecular species with each compound. The molar distribution patterns of deuterated molecules were analyzed by curve fitting using the Bernoulli distribution equation to determine the fraction of newly synthesized molecules by subtracting the contribution of recycling fraction from the total labeled fraction observed (Ando et al., 2002).

RESULTS

Mice were given diluted D_2O for 10–20 days, and thereafter every five mice were killed at various intervals from day zero (the third day after the cessation of D_2O administration) to day 70. Phosphatidylcholine, phosphatidylethanolamine, and cholesterol were prepared from the synaptic plasma membranes. Synaptophysin was isolated from synaptosomes and degraded to produce amino acids. Deuterated lipids and amino acids were determined by mass spectrometry. Disappearance curves of the deuterated compounds from synaptic membranes were drawn according to the method previously reported (Ando et al., 2002).

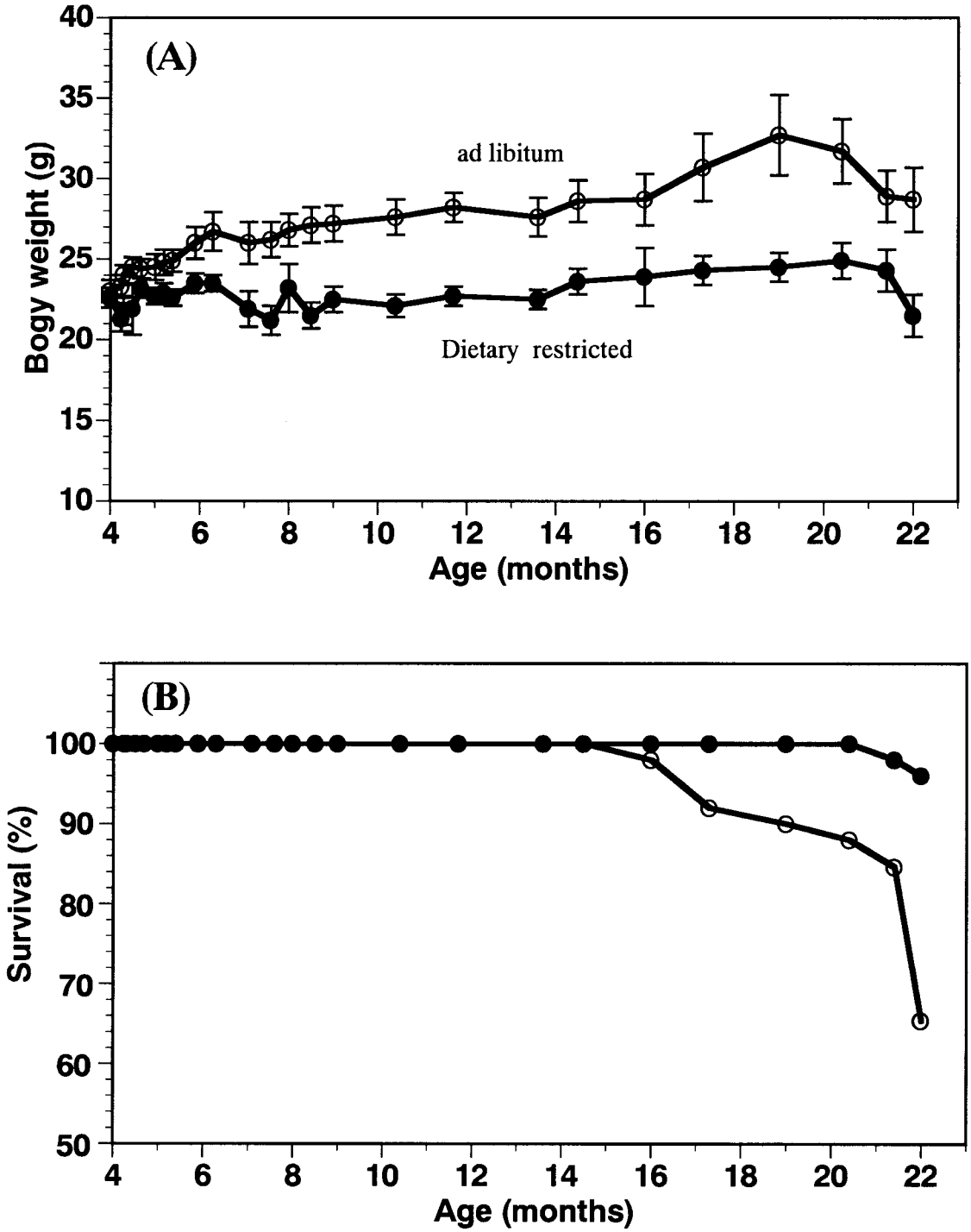

Fig. 1. Time courses of body weight (**A**) and survival curves (**B**) of C57BL/6 mice. Open circles, ad libitum fed mice; closed circles, dietary-restricted mice.

Figure 2 shows the apparent disappearance curves observed for different fatty acid constituents of phosphatidylethanolamine in synaptic plasma membranes. Palmitic acid had the shortest half-life, and stearic acid, palmitoaldehyde, arachidonic acid, and docosatetraenoic acid showed longer half-lives in that order. Because the half-lives appeared to be longer than they actually were as a result of the recycling of the residues (Benjamins and McKhann, 1973), the palmitic acid residue having the shortest half-life was employed as a minimal recycling

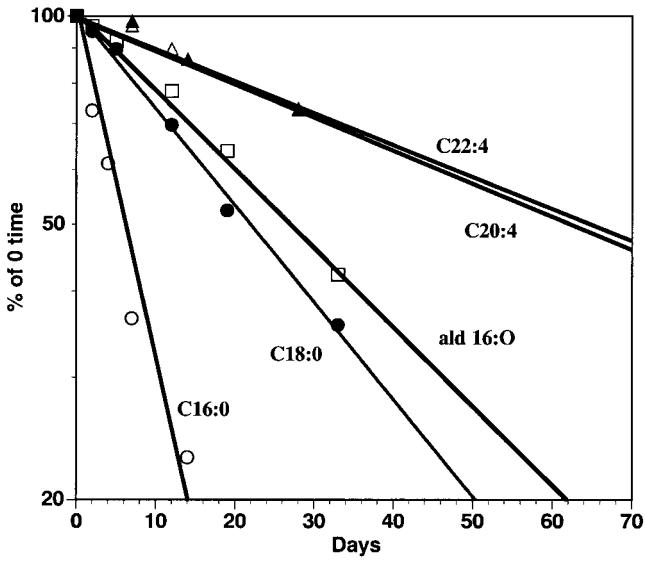

Fig. 2. Disappearance curves for phosphatidylethanolamine molecules containing different fatty acid residues in synaptic plasma membranes. Constituent fatty acids are palmitic acid (open circles), stearic acid (closed circles), arachidonic acid (open triangles), docosatetraenoic acid (closed triangles) and palmitoaldehyde (open squares).

marker to obtain the most probable turnover rates of phospholipids. Therefore, the disappearance curves for phospholipid species containing palmitic acid residues are compared among different ages and between ad libitum and dietary restricted conditions as shown in Figure 3. The half-lives of the phospholipids are listed in Table I. Phosphatidylethanolamine appeared to be more rapidly metabolized than phosphatidylcholine. The turnover rates of both phospholipids were slightly faster in the brains of young mice (2 months old) than in those of adult mice (6 months old) and aged mice (22 months old). Under the dietary-restricted conditions, the turnover rates of both phospholipids were retarded. The fatty acid composition in phospholipids was partially altered with aging in the ad libitum-fed mice, but was not much affected in the dietary restricted mice (Table II).

Figure 4 shows the decay curves of deuterated cholesterol. The half-lives of cholesterol are shown in Table I. The turnover rate of cholesterol was fast in young brains. In adult brains, it slowed to about half of the level of the young, and then rebounded in senescence to a level near that in the young. Under dietary restriction, the turnover rate was retarded.

The disappearance rates of synaptophysin were determined by measuring the amounts of deuterated glutamate as shown in Figure 5. The half-lives were slightly longer in senescence than in adults (Table I). The turnover rate was accelerated under dietary restriction.

DISCUSSION

We focused on the metabolic activities of the major lipids of synaptic plasma membranes and of synaptophysin, one of major synaptic membrane proteins. The quantities of deuterated lipids were determined by mass spectrometry according to our previous method (Ando et al., 2002). This method can eliminate the contribution due to recycling of labeled molecules so that true disappearance curves of initially incorporated membrane constituents are delineated. Radiolabeled precursors were reported to give widely different values of turnover rates for membrane lipids (Miller et al., 1977). In contrast, the present method using D_2O seemed to have an advantage in tracing initially labeled molecules for long periods. The in vivo labeling with D_2O was successfully applied to proteins as well as to lipids. As glutamate was efficiently labeled among amino acid residues, deuterated glutamate was employed for monitoring the disappearance of synaptophysin from synaptic membranes. In theory, cholesterol and palmitic acid are labeled with deuterium during their de novo synthesis, and other fatty acids having longer chains than palmitic acid are labeled in the process of chain-elongation. Thus, these molecules were well determined by mass spectrometry and their decay curves were separately drawn.

The turnover rate of phosphatidylethanolamine varied depending upon the fatty acid residues monitored (Fig. 2). Recycling or reuse of precursors in membrane metabolism was shown to give half-lives that were longer than the true ones (Benjamins and McKhann, 1973). Accordingly, palmitic acid, which had the shortest half-life among fatty acid constituents, was employed as a minimal recycling tracer in the present study. In 17-day-old rat (Miller et al., 1977) and adult mouse (Sun and Su, 1979), the half-lives for phosphatidylethanolamine in membrane fractions were found to be 33 days and 11.8 days, respectively. In contrast, we obtained shorter half-lives: 5.7 days at 2 months of age and 7.6 days at 6 months of age (Table I). This indicates that the present method using D_2O provides more accurate turnover rates affected by minimum recycling in vivo. On the other hand, the data in Figure 2 clearly show that stearic acid, arachidonic acid, and other fatty acids except palmitic acid were largely or efficiently reused for the synthesis of phosphatidylethanolamine in synaptic plasma membranes.

Therefore, the disappearance curves for phospholipids were based on the disappearance of palmitic residues (Fig. 3). The half-lives of the phospholipids were calculated from these curves (Table I). Phosphatidylethanolamine appeared to be metabolized a little more rapidly than phosphatidylcholine. Previously reported half-lives of phospholipids are widely scattered. For instance, the half-life for phosphatidylcholine varied from 2.7 to 19 days (Abdel-Latif and Smith, 1970; Miller et al., 1977) and the half-life for phosphatidylethanolamine varied from 12 to 33 days (Miller et al., 1977; Sun and Su, 1979). Those values are not consistent with ours. The turnover rates of phosphatidylcholine were slightly faster in the brains of young mice than those in adult and ad libitum-fed aged mice (Fig. 3A and Table I). A similar tendency was observed with phosphatidylethanolamine (Fig. 3B).

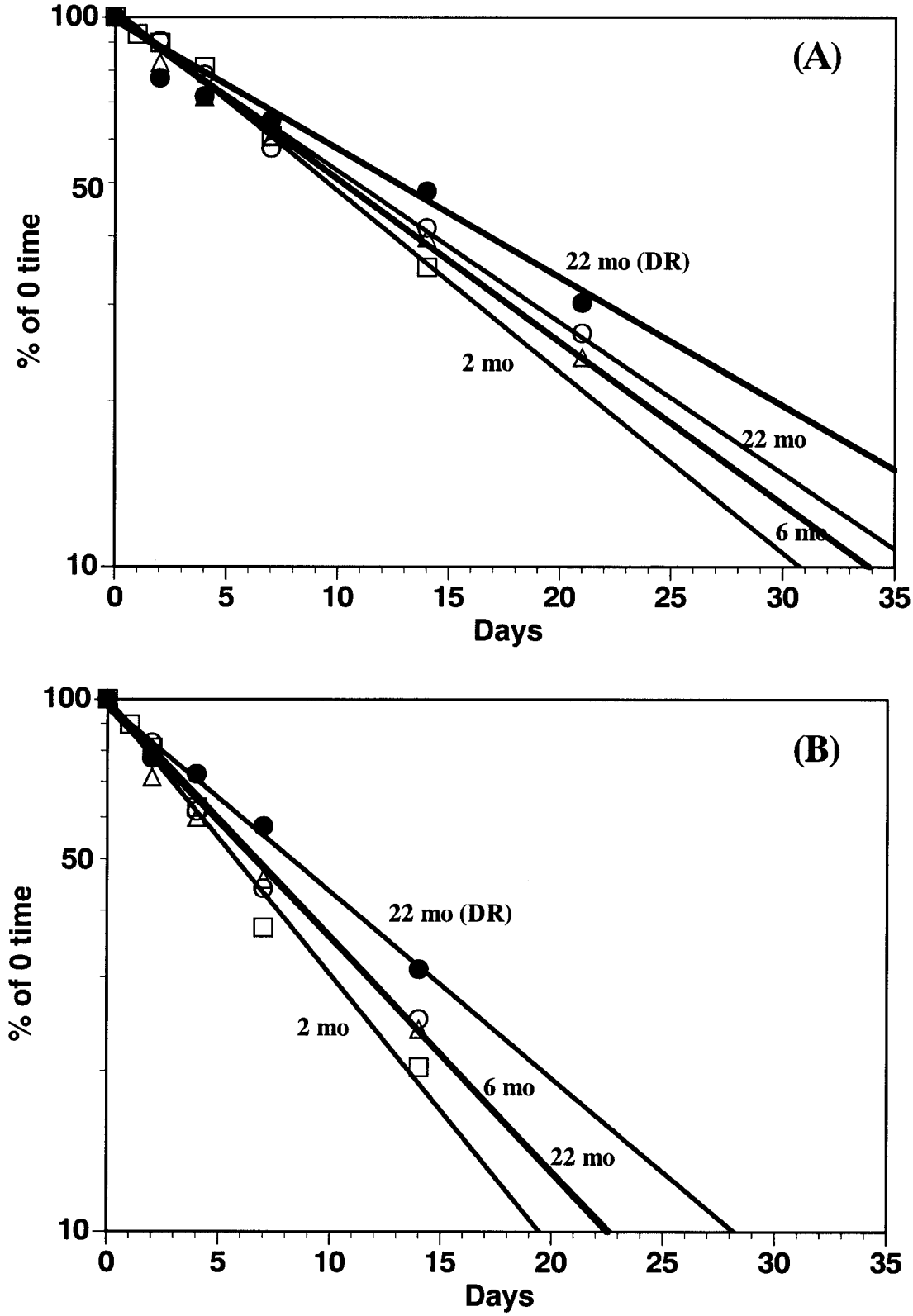

Fig. 3. Disappearance curves for phosphatidylcholine (**A**) and phosphatidylethanolamine (**B**) and effects of aging and dietary restriction on their turnovers. Open squares, 2 months old; open triangles, 6 months old; open circles, 22 months old; closed circles, 22 months old under dietary restriction.

To our knowledge, the turnover of cholesterol in synaptic membranes has not been reported. The disappearance curves and half-lives for cholesterol in synaptic plasma membranes of young, adult and aged brains were determined in this study (Fig. 4 and Table I). The turnover rate of cholesterol was fast in young brains (74 days) and slowed down in adult brains (158 days). The half-life of cholesterol was determined to be 359 days in the brain myelin of adult mice by the present method (Ando et al., 2002). Thus, our studies have revealed that synaptic plasma membranes and myelin membranes each have discrete metabolic pools for cholesterol. The turnover of cholesterol, unlike the turnover of phospholipids, appeared to be accelerated in the synaptic membranes of aged brains.

With respect to the turnover of proteins in the brain, most previous studies did not specify protein species and presented average metabolic rates for total proteins in subcellular or membrane fractions. We focused on synaptophysin, a membrane-bound protein localized in synaptic vesicles, because it is known as a specific marker for synapses (Wiedenmann and Franke, 1985), and determined its turnover in adult and aged mouse brains (Fig. 5 and Table I). Various values have been reported for the average half-lives for membrane proteins: 20–24 days in mitochondrial fractions containing synaptosomes (Lajtha and Marks, 1971) and 7 days in synaptosomes (Dunlop, 1983), both of which were calculated from the incorporation rate, and 5.8 days in synaptic membranes as calculated from the decay curve (Chee and Dahl, 1978). The half-lives of synaptophysin obtained in the present study lie in the range of those reported values. The turnover rate of synaptophysin appeared to be slower in senescence than in the adult stage (Table I). This finding seems to be consistent with the results reported by Smith et al. (1995) that the average rate of protein synthesis in the brain decreased by 11% in aged rats. An alternative possibility is that the age-related decline in turnover rate of synaptophysin is due to a decrease in the proteolytic activity (Rattan, 1996).

Dietary restriction, i.e., a significant reduction in calorie intake, is known to slow the rate of aging in organisms (Tavernarakis and Driscoll, 2002). The survival rate of dietary-restricted mice was actually higher than that of ad libitum-fed mice as shown in Figure 1B. The turnover rate of synaptophysin that decreased in senescence was accelerated by dietary restriction as can be seen in Figure 5 and Table I. The metabolic acceleration of protein by dietary restriction was frequently observed in extraneural tissues such as hepatocytes (Ishigami and Goto, 1990; Lambert and Merry, 2000) and other tissues (Taylor et al., 1995). Although similar modulatory effects of dietary restriction on protein turnover in the brain have not been reported in the literature, enhanced removal of damaged proteins in the brain of dietary-restricted rats was found by Takahashi and Goto (1987). The latter finding seems to be consistent with our result and to be beneficial in preventing age-related deficits in synaptic functions. In

TABLE I. Half-Lives (Days) of Synaptic Membrane Components in Mouse Brains

Age (months)	PC (16:0)	PE (16:0)	Cholesterol	Synaptophysin
2	8.7	5.7	74	n.d.
6	10.8	7.6	158	12.7
22 (ad libitum)	11.0	7.2	87	15.1
22 (dietary restricted)	14.2	9.4	120	10.4

PC, phosphatidylcholine; PE, phosphatidylethanolamine; n.d., not determined.

TABLE II. Fatty Acid Composition (%) of Phosphatidylcholine and Phosphatidylethanolamine in Mouse Brain Synaptic Plasma Membranes†

	Age (months)		
	6	22 (ad libitum)	22 (dietary restricted)
Phosphatidylcholine			
C16:0	38.4 ± 4.4	45.4 ± 5.9	32.9 ± 1.9*
C18:0	19.8 ± 1.4	18.6 ± 1.9	23.3 ± 1.3*
C18:1	33.7 ± 1.1	32.1 ± 1.8	36.5 ± 1.0*
C20:4	5.7 ± 1.1	3.0 ± 1.2#	5.4 ± 0.2*
C22:6	2.4 ± 1.2	1.0 ± 1.4	1.6 ± 1.2
Phosphatidylethanolamine			
C16:0	12.7 ± 1.5	11.3 ± 1.0	11.0 ± 1.8
C18:0	30.2 ± 3.0	22.8 ± 5.0#	29.7 ± 3.3*
C18:1	9.0 ± 4.2	19.3 ± 2.6##	8.1 ± 1.9**
C20:4	14.9 ± 0.9	14.1 ± 0.8	14.9 ± 0.3
C22:4	6.1 ± 1.8	6.5 ± 1.0	7.5 ± 1.0
C22:6	21.1 ± 4.0	14.4 ± 3.5#	18.9 ± 5.9*
C16:0 ald	3.1 ± 0.4	5.3 ± 1.3##	4.1 ± 0.9
C18:0 ald	3.0 ± 0.3	6.1 ± 2.4#	5.9 ± 1.6

†Values are the mean ± S.D. (n = 5).
#$P < 0.05$; ##$P < 0.01$ (vs. 6-month-old mice) *$P < 0.05$; **$P < 0.01$ (vs. ad libitum-fed 22-month-old mice).

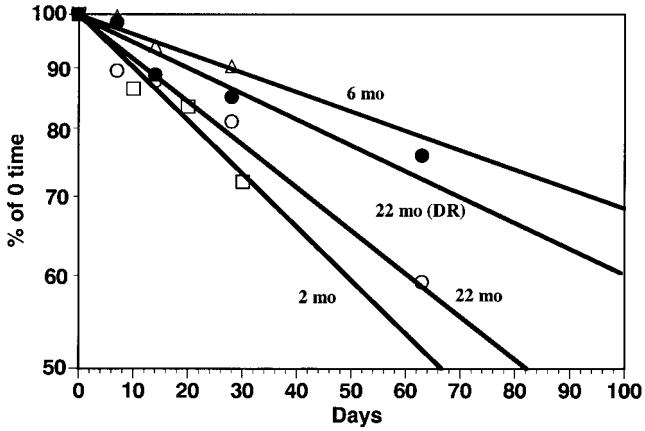

Fig. 4. Changes in the disappearance curves for cholesterol with aging and effects by dietary restriction. Open squares, 2 months old; open triangles, 6 months old; open circles, 22 months old; closed circles, 22 months old under dietary restriction.

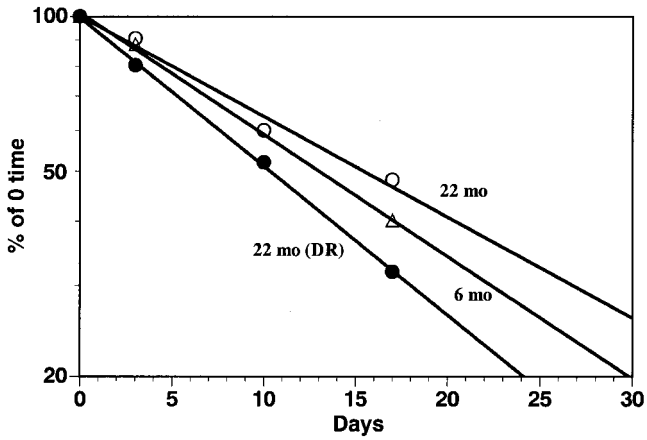

Fig. 5. Disappearance curves for synaptophysin in synaptic membranes. Open triangles, 6 months old; open circles, 22 months old; closed circles, 22 months old under dietary restriction.

this connection, much evidence has accumulated that dietary restriction suppresses the decline of dendritic spines (Moroi-Fetters et al., 1989), attenuates neuronal loss (Calingasan and Gibson, 2000), and prevents deficits in synaptic plasticity (Eckles-Smith et al., 2000).

In contrast to the accelerating effect of dietary restriction on protein turnover, some opposite effects were observed in the lipid turnover in synaptic membranes. As shown in Figures 3 and 4 and Table I, the turnover rates of two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, and cholesterol were all slower in dietary-restricted mice than in ad libitum-fed mice. The slowdown of the lipid turnover may be beneficial by helping to maintain the membrane structure. This is supported by the data in Table II in which the fatty acid composition of phospholipids in aged mice reared under dietary-restricted conditions is more similar to that in adult mice than to that in ad libitum-fed aged mice. Further studies are needed to determine why dietary restriction has such opposite effects on protein and lipid.

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