methods

Enzyme blockade: a nonradioactive method to determine the absolute rate of cholesterol synthesis in the brain

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Abstract The standard in vivo method to determine rates of brain cholesterol synthesis involves systemic injection of ^3H_2O and measurement of incorporated radioactivity in sterols. Herein, we describe an alternative method ("enzyme blockade") that obviates the use of radioactivity. The method relies on the ability of AY9944, a potent and relatively selective inhibitor of cholesterol synthesis, to cause the time-dependent accumulation of 7-dehydrocholesterol (DHC), a cholesterol precursor detected with sensitivity and specificity by reverse-phase HPLC-coupled spectrophotometry at 282 nm. To validate the method, adult AY9944-treated and control mice were injected with [^{3}H]acetate. After 24 h, most of the radioactivity in brain sterols from treated mice accumulated in DHC, without significantly perturbing overall sterol pathway activity, compared with controls (where cholesterol was the dominant radiolabeled sterol, with no label found in DHC). When adult mice were treated continuously with AY9944, the time-dependent accumulation of DHC in brain was linear (after ~8 h) for 3 days. The rate of brain cholesterol synthesis determined by this method (~30 μg/g/day) closely agrees with that determined by the radioactive method. We also determined the cholesterol synthesis rate in different regions of adult mouse brain, with frontal cortex having the highest rate and cerebellum having the lowest rate.—Keller, R. K., M. Small, and S. J. Fliesler. **Enzyme blockade: a nonradioactive method to determine the absolute rate of cholesterol synthesis in the brain.** ***J. Lipid Res.*** **2004.** 45: **1952–1957.**

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There is growing interest in, and experimental documentation of, the relationship between central nervous system (CNS) cholesterol metabolism and neurodegeneration, particularly in age-related diseases such as Alzheimer's disease (AD) (1–3) and age-related macular degeneration (ARMD) (4, 5). For instance, retrospective studies have suggested that statins, which act on the rate-limiting enzyme of cholesterol synthesis (HMG-CoA reductase), decrease the risk of developing AD (6), and expression of specific allelic isoforms of apolipoprotein E, a cholesterol transport protein, is associated with an increased risk of developing AD (7). The relationship between statin use, apolipoprotein E isoform expression, and ARMD development or progression, although suggestive, is somewhat more speculative (8–11). These findings provide the impetus for developing techniques to investigate cholesterol metabolism in the CNS. In general, the rate of de novo cholesterol synthesis in the mammalian brain is relatively high in the fetus and newborn, where it is synthesized de novo, with little or no contribution from maternal sources (12, 13). Brain cholesterol synthesis decreases precipitously after weaning (14), as the rate of myelination dramatically declines [for review, see ref. (15)]. The fate of cholesterol in the adult CNS is uncertain, but all indications are that the turnover is relatively slow, with a half-life on the order of months (16). A portion of brain cholesterol is metabolized to 24*S*-hydroxycholesterol, which then exits across the blood-brain barrier into the bloodstream (17). More than 50% of cholesterol release from the brain may occur via this route (18). Neurodegeneration in both humans (19) and an animal model (20) leads to changes in the metabolism of cholesterol via the 24*S*-hydroxy route.

Because cholesterol synthesis in the adult CNS is slow relative to other bodily tissues, quantification of its absolute rate presents methodological problems. Fassbender and coworkers (21, 22) measured steady-state levels of lathosterol (5α-cholest-7-en-3β-ol), a cholesterol precursor, in their study of amyloid Aβ formation in guinea pigs. Although this method does not involve the use of radioactive precursors, it only provides an estimate of the relative

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Abbreviations: AD, Alzheimer's disease; ARMD, age-related macular degeneration; CNS, central nervous system; DHC, 7-dehydrocholesterol; FC, frontal cortex; NSL, nonsaponifiable lipid; SLOS, Smith-Lemli-Opitz syndrome.

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rates of synthesis and relies on the assumption that the concentration of precursor is a valid indication of brain sterol synthetic rate. Dietschy and Spady (23) have provided extensive evidence documenting the validity of using ^3H_2O as a radiolabeled precursor to measure the absolute rate of cholesterol synthesis in different organs in vivo. Recently, Quan et al. (24) used this approach to determine rates of cholesterol synthesis in the mouse CNS. The major drawback to the ^3H_2O method is that it requires large amounts of radioactivity (typically on the order of 0.5–1.0 mCi/g body weight), especially when used to measure CNS cholesterol synthesis. This is both expensive and raises environmental issues concerning the handling and disposal of a volatile radioisotope and the resulting radioactive carcasses and tissue extracts. Other workers have used ^2H_2O or ^{18}O (25–28) rather than ^3H_2O; although these procedures eliminate the radioactive waste problem associated with ^3H, they require analysis using a mass spectrometer.

In the course of our studies concerning cholesterol metabolism in the CNS, particularly the retina (29, 30), we have used the drug AY9944 to develop an animal model (31, 32) of the Smith-Lemli-Opitz syndrome (SLOS), a human autosomal recessive disease caused by defective cholesterol synthesis. AY9944 potently and relatively selectively (33) inhibits 3β-hydroxysterol-Δ^7-reductase (EC1.3.1.21), which catalyzes the final step of cholesterol synthesis along the Kandutsch-Russell pathway and which also is defective in SLOS (34). Long-term treatment with AY9944 results in a dramatic increase of 7-dehydrocholesterol (DHC) levels and a decrease of cholesterol levels in all bodily tissues (30, 31). During these studies, it occurred to us that the accumulation of DHC in the presence of AY9944 might be useful for quantifying the rate of CNS cholesterol biosynthesis. The brain, unlike the liver, is more amenable to such an approach, because, as mentioned above, the brain synthesizes virtually all of its own cholesterol and turns it over slowly. For this "enzyme blockade" method to be valid, however, several criteria must be met: *1*) the enzyme inhibitor must cross the blood-brain barrier readily; *2*) enzyme inhibition must be selective and essentially complete; *3*) detection of the accumulated precursor (DHC, in this case) must be sensitive and specific; and *4*) the block must not appreciably alter overall CNS sterol synthesis. Herein, we demonstrate that the enzyme blockade method, using AY9944, meets all of these criteria and that it yields values for brain cholesterol synthesis rates that agree well with those reported previously using the ^3H_2O method.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all biochemical reagents were from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Authentic sterol standards were purchased from Steraloids (Newport, RI). All organic solvents were HPLC grade (Burdick and Jackson, Fisher Scientific). [^{3}H]acetic acid (sodium salt; 15 Ci/mmol) and [1,2-^{3}H]cholesterol (40 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). AY9944 (*trans*-[1,4-*bis*(2-dichlorobenzylaminomethyl)cyclohexane]dihydrochloride) was prepared by custom organic synthesis (A. H. Fauq and S. J. Fliesler, unpublished data) and purified by recrystallization to >99% homogeneity. The chemical, physical, and spectroscopic properties were confirmed by comparison with an authentic sample of AY9944 (a generous gift of Wyeth-Ayerst Research, Princeton, NJ). AY9944 now is commercially available (Calbiochem; catalog number 190080).

Procedures involving animals

Adult male mice, 26 weeks of age (a gift from Drs. David Morgan and Marcia Gorton, University of South Florida, Department of Pharmacology), were of mixed genetic background, derived from the nontransgenic crosses of mice from C57B6, SJL, Swiss Webster, and B6D2 backgrounds (35). All procedures involving animals were approved by the University of South Florida Institutional Animal Care and Use Committee and Radiation Safety Office and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Alzet® osmotic pumps (model 1003D; Durect Corporation, Cupertino, CA) containing a solution of AY9944 (20 mg/ml in sterile water) were implanted under the dorsal skin of mice; sham-operated mice served as controls. Per the manufacturer's product literature, these pumps deliver drug at a constant flow rate of 1 μl/h (20 μg AY9944/h) for 3 days, or ~0.7 μg AY9944/h/g body weight (animals averaged 30 g). At various times after implantation, mice were euthanized by pentobarbital overdose (150 mg/kg) and tissues were taken for sterol analysis (see below). For the administration of radioactivity, 200 mCi of sodium [^{3}H]acetate (15 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) in ethanol was taken to dryness under nitrogen and then dissolved in PBS, and mice were injected intraperitoneally (1 mCi/g body weight).

In the experiment using [^{3}H]acetate, to ensure that brain tissue was not contaminated with radioactive sterols from extracellular fluids (e.g., blood), the brains were homogenized in 20 volumes of 10 mM Tris-Cl, pH 7.4, and the homogenate was centrifuged at 100,000 *g* for 1 h. The resulting supernatant was assayed for total radioactivity, and the membranous pellet was resuspended in Tris buffer as before. Centrifugation and washing were repeated four more times, after which the supernatant contained less than 10% of the total radioactivity in the resuspended pellet. In all other experiments, whole brain tissues or brain regions were used directly, because preliminary experiments indicated that whole-body perfusion did not affect the levels of DHC accumulated in the brain (indicating that there was no contribution of DHC mass from blood).

To determine cholesterol synthesis in different regions of the brain, four male mice (~30 g each) were treated with AY9944 as above and euthanized after 3 days. Brains were removed and quickly dissected in the cold into frontal (anterior) cortex, posterior cortex, cerebellum, hippocampus, and brain stem. The various regions were then weighed and analyzed for concentration of DHC and cholesterol as described below.

Lipid extraction and analysis

Lipid extraction and analysis were performed essentially as described in detail previously (36). In brief, the resuspended membrane fractions were saponified with 20% (w/v) KOH in 66% aqueous methanol solution for 1 h at 100°C under argon in sealed glass tubes. After cooling, the hydrolyzed samples were extracted twice with equal volumes of petroleum ether, and the pooled nonsaponifiable lipid (NSL) extracts were backwashed with 5% (v/v) aqueous acetic acid and then taken to dryness un-

der nitrogen. The residues were dissolved in mobile phase solvent (methanol-isopropanol, 7:1, v/v), and aliquots were subjected to reverse-phase HPLC (Spheri-5 RP C18 column, 4.6 × 150 mm; Brownlee Laboratories; flow rate, 1 ml/min) with online ultraviolet detection at either 205 or 282 nm (Shimadzu CR-3A). Response factors at both wavelengths for authentic sterol standards were determined empirically, which then were used to quantify sterol mass in the tissue extracts. Radioactivity in the effluent was monitored online using a flow-through scintillation spectrometer (Radiomatic Flo-One/Beta; Packard Instruments), mixing the effluent with Packard Flo-Scint III cocktail.

Statistical analysis

Statistical comparison of data sets was performed using a two-tailed Student's *t*-test, assuming equal variances (homoscedastic).

RESULTS

Chromatographic resolution and detection of sterols

Figure 1 shows the HPLC elution profiles obtained for desmosterol, DHC, and cholesterol, comparing detection at 205 nm versus 282 nm, using an internal standard of [^{3}H]cholesterol with scintillation detection. As shown in the bottom panel, only DHC is detected at 282 nm (the absorption spectrum for DHC has a relative maximum at 282 nm, because of the presence of conjugated double bonds in ring B of the sterol nucleus; see inset). Empirically, we determined its molar extinction coefficient at this wavelength to be 13,100 M^{-1} cm^{-1} (in methanol-isopropanol, 7:1, v/v). Hence, detection at 282 nm offers a selective and sensitive means for quantifying DHC mass. Also, under the chromatographic conditions used, baseline resolution of all three sterol standards was achieved (Fig. 1, middle panel).

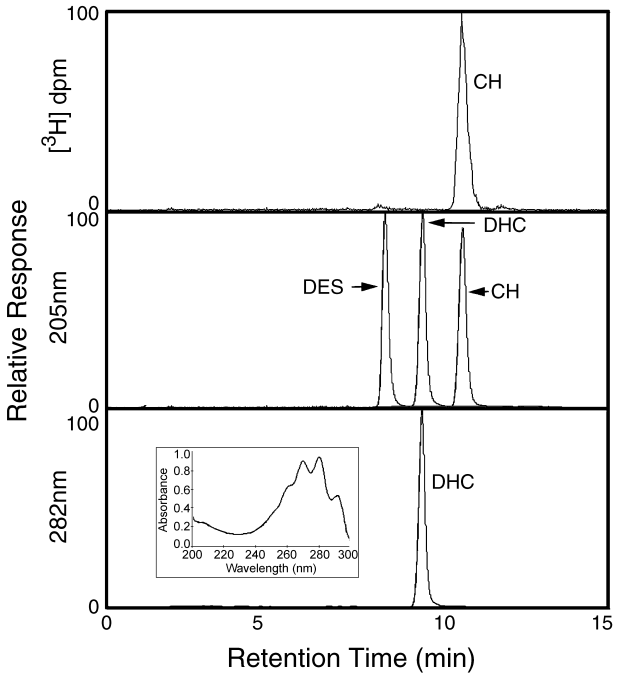

Fig. 1. Reverse-phase HPLC analysis of sterol standards. The mobile phase was methanol-isopropanol, 7:1 (v/v), at 1 ml/min. Relative response was normalized to the dominant sterol component. Top panel: [^{3}H]cholesterol internal standard, measured by flow-through scintillation spectrometry. Middle panel: Detection of sterol standards at 205 nm. Bottom panel: Detection of sterol standards at 282 nm, demonstrating the unique detection of DHC at this wavelength. Inset: Ultraviolet absorption spectrum of DHC in the mobile phase, illustrating maximal absorbance at 282 nm. CH, cholesterol; DES, desmosterol; DHC, 7-dehydrocholesterol.

Administration of AY9944 to mice in vivo results in the accumulation of newly synthesized DHC in the brain without altering de novo sterol synthesis

For AY9944 to be a useful tool to quantify sterol synthesis in the brain, it must potently and selectively inhibit 3β-hydroxysterol-Δ^7-reductase and it should have a minimum effect on the overall rate of sterol synthesis. To test these possibilities, we administered AY9944 (20 μg/h) subcutaneously to four adult mice by Alzet® pump. Four sham-operated mice served as controls. After 48 h, all mice were injected with [^{3}H]acetate (1 mCi/g body weight). One day later, all animals were euthanized and tissues were taken for analysis of NSLs (see Materials and Methods). Brain NSL extracts were analyzed by reverse-phase HPLC with detection at 282 nm and simultaneous radioactivity detection. As shown in **Fig. 2**, extracts from control mice did not exhibit either a mass peak or a radiolabel peak with retention time in the region corresponding to DHC; however, a distinct peak of radioactivity corresponding to the retention time of authentic cholesterol was observed. In contrast, brain NSL extracts from AY9944-treated mice exhibited a prominent peak of radioactivity and ultravio-

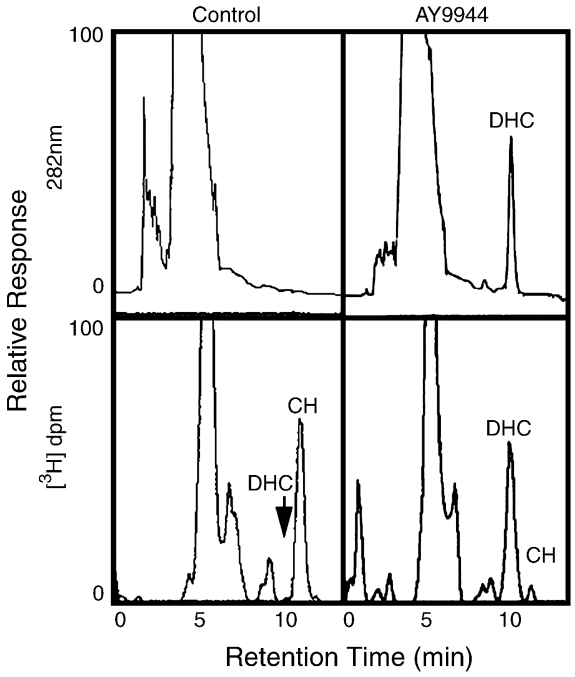

Fig. 2. Reverse-phase, radio-HPLC analysis of brain nonsaponifiable lipid from control mice (left panels) and AY9944-treated mice (right panels) 1 day after systemic injection with [^{3}H]acetate (1 mCi/g body weight). Top panels: Mass, detected at 282 nm. Bottom panels: Radioactivity, detected by flow-through scintillation spectrometry. Elution positions corresponding to DHC and cholesterol are indicated.

let-detectable mass with retention time corresponding to DHC (Fig. 2, right panels), and there was only a very small peak of radiolabel corresponding to cholesterol. In the chromatograms of NSLs from both treated and control animals, the majority of the radioactivity eluted from the reverse-phase column much earlier than sterols (retention time range, ~4–8 min). Although the identity of these compounds is not certain, their polarity, relative abundance, and incorporation of [^{3}H]acetate are consistent with newly synthesized, long-chain fatty acids.

Statistical analysis of the HPLC data from treated and control animals revealed that, after 3 days of AY9944 treatment, the efficiency of inhibition of the conversion of DHC to cholesterol was ~85 ± 4%, with only a slight, but not significant, decrease in overall sterol synthesis (**Table 1**). It is to be noted that the 85% inhibition represents that observed between 2 and 3 days of accumulation of DHC. Although the exact nature of AY9944 inhibition of 3β-hydroxysterol-Δ^7-reductase has not been demonstrated, assuming that it is competitive in nature, then the percentage inhibition at early time points (0–2 days) is likely considerably greater.

AY9944-dependent accumulation of DHC in brain is linear with time

Having demonstrated that AY9944 potently blocked the conversion of DHC to cholesterol in the brain without significantly affecting overall sterol synthesis, we next evaluated whether or not the rate of accumulation of DHC was linear under the conditions used. We implanted Alzet® pumps containing AY9944 subcutaneously in nine mice. At each of three time intervals (1, 2, and 3 days postimplantation), three mice were subsequently euthanatized, their brains were removed and saponified, and the NSLs were subjected to reversed-phase HPLC analysis, monitoring the effluent at 282 nm to measure the accumulation of DHC. Data were normalized to the level of brain cholesterol, which was determined using a cholesterol oxidase kit (Sigma) and confirmed by HPLC (detection at 205 nm). The concentration of whole brain cholesterol averaged 20 ± 1 mg/g wet weight in these animals. **Figure 3** shows that, after a lag period of 8 h (i.e., the time required for the drug to reach a pharmacologically active concentration in the brain), accumulation was linear over the 3 day time period examined. (Subsequently, we have found that this lag period can be reduced by almost 6 h if the Alzet® pump is preequilibrated in buffer at 37°C overnight before implantation. However, this concomitantly reduces the effective lifetime of the pump.) The slope of the line, obtained by least-squares analysis, was determined to be 3.0×10^{-3} (DHC/cholesterol/day). Because the mouse brains used in this study contained ~10 mg of cholesterol, this corresponds to an accumulation of 30 μg of sterol synthesized per brain per day. This value is in good agreement with the sterol synthesis value of 35 μg/day for mouse CNS obtained by Quan et al. (24), who used the ^3H_2O method to determine the absolute rate of brain sterol synthesis in the mouse.

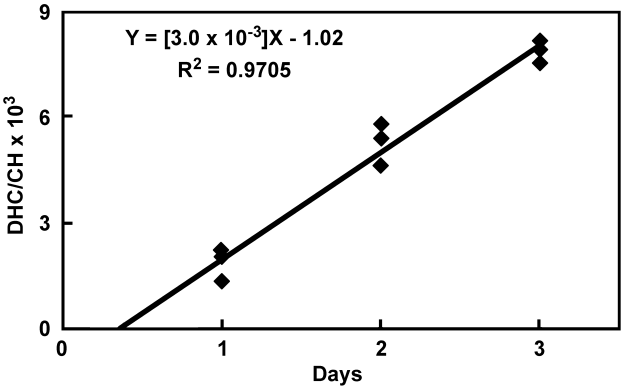

Fig. 3. Time course for the accumulation of DHC in brains of AY9944-treated mice. After an initial lag of 8 h, accumulation was linear over 3 days, fitting a simple, linear algebraic equation with a correlation coefficient (R^2) of 0.9705.

Use of AY9944 to determine regional cholesterol synthesis in the adult mouse brain

To quantify cholesterol synthesis in different regions of the brain, we administered AY9944 to four mice for 3 days as described above and then dissected out various brain regions and quantified their sterol content. The rate of sterol synthesis (expressed as micrograms of DHC accumulated per gram wet weight of brain per 3 days) was calculated based on the equation derived from the data presented in Fig. 3. As shown in **Fig. 4**, frontal cortex (FC) had the highest rate of cholesterol synthesis, different

TABLE 1. Effect of AY9944 on the incorporation of [^{3}H]acetate into DHC and CH in mouse brains

	[^{3}H]acetate Incorporation				
Sample	DHC	CH	DHC + CH	Percent Inhibition[a]	Percent Inhibition[b]
	dpm/nmol CH	*dpm/nmol CH*	*dpm/nmol CH*	*DHC/CH*	*[^{3}H]acetate/sterols*
Control	Not detected	19.1 ± 3.7	19.1 ± 3.7	—	—
AY9944	15.1 ± 2.8	2.7 ± 1.0	17.8 ± 4.0	85 ± 3.9	6.8

CH, cholesterol; DHC, 7-dehydrocholesterol. On day 1, Alzet® pumps (3 day duration, 1 μl/h) containing AY9944 (20 mg/ml) were implanted into mice (n = 4). Four mice served as untreated controls. On day 2, all mice were administered [^{3}H]acetate (1 mCi/g body weight) by intraperitoneal injection. On day 3, all animals were euthanized and tissues were harvested for analysis of nonsaponifiable lipids.

[a] Inhibition of the conversion of DHC to CH.

[b] Inhibition of total sterol synthesis (conversion of [^{3}H]acetate to sterols) by AY9944. Equal to {1.00 − [average radioactivity (dpm) in DH + CH in AY9944-treated brain divided by average dpm in CH in control brain]} × 100.

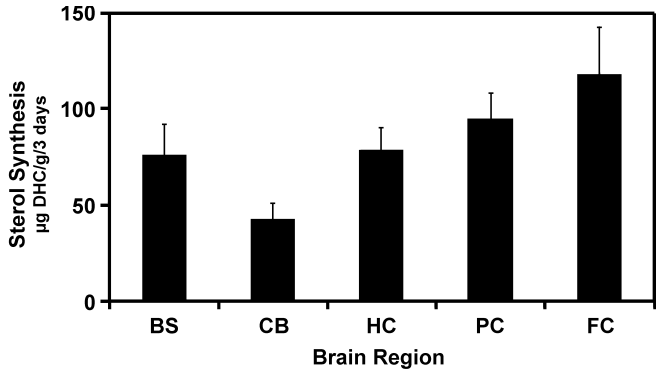

Fig. 4. Rate of cholesterol synthesis in different regions of mouse brain, determined by the enzyme blockade method. Values are given as means ± SD (n = 4), expressed as accumulation of DHC per gram of tissue per 3 days (see linear equation, Fig. 3). BS, brain stem; CB, cerebellum; FC, frontal cortex; HC, hippocampus; PC, posterior cortex.

from all other regions examined ($0.01 < P < 0.05$), except posterior cortex ($P < 0.14$). Cerebellum had the lowest rate, significantly lower than any other brain region examined (e.g., 35.9% of the FC rate; $P < 0.001$). Hippocampus, posterior cortex, and brain stem exhibited rates comparable to (and not statistically different from) each other and intermediate to the rates observed for FC and cerebellum. The cholesterol concentrations in the major regions of the brain were determined (in milligrams per gram wet weight, mean ± SD, n = 4, except hippocampus, n = 1): FC, 15.4 ± 1.0; cerebellum, 13.4 ± 0.9; brainstem, 26.7 ± 1.0; hippocampus, 15. The relative rates of synthesis and concentrations of cholesterol are in good agreement with those reported by Quan et al. (24) for cholesterol synthesis in different brain regions of the 26 week old mouse. It is to be noted that if the cholesterol concentrations in the different regions were at steady state, we could make estimates of turnover rates using the synthetic rates calculated here, assuming a single pool of cholesterol in the different regions (probably an oversimplification). However, as shown by Quan et al. (24), there is still some accretion of cholesterol at 26 weeks, so newly synthesized cholesterol is accumulating while at the same time it is being exported from the brain (facilitated, in part, by hydroxylation of the side chain).

DISCUSSION

In the present study, we have used the enzyme blockade method to measure the rate of cholesterol synthesis in the adult mouse brain, without the need for radioisotopes. We only used radioisotopic compounds to validate the procedure. This method takes advantage of the fact that cholesterol is synthesized in situ and turns over relatively slowly in the adult brain. We have used a drug, AY9944, that readily crosses the blood-brain barrier, strongly inhibits cholesterol synthesis with specificity, and results in the accumulation of a precursor (DHC) that is readily detectable by HPLC at a wavelength (282 nm) that is specific for DHC. In addition, we have shown that AY9944 has no significant effect on the rate of the sterol pathway, even after 3 days of administration. Strictly speaking, the general approach of enzyme blockade is not novel. More than 25 years ago, Kaiser and Stocker (37) used AY9944 to study cholesterol metabolism in mini-pigs without the use of radioisotopes. However, the four criteria stipulated herein (see introduction) were neither fully addressed nor fully met. Similarly, Gibbons and Pullinger (38) used triparanol, another inhibitor of cholesterol synthesis, to determine the absolute rate of cholesterol synthesis in isolated liver cells, measuring the accumulation of the precursor desmosterol as a function of time. However, the quantification of desmosterol involved derivatization and gas chromatography with electron capture detection, the metabolic block was not complete, and the overall effect on total sterol synthesis was not rigorously assessed.

The results obtained using this nonradioisotopic, enzyme blockade method agree well with those obtained using previously established, standard methodology, which obligatorily employs substantial amounts of radioactive water. However, the enzyme blockade method obviates the expense and environmental hazards of handling relatively large amounts of volatile radioactivity, as well as the disposal of radioactive tissues and extracts. AY9944, as the hydrochloride salt, is water soluble, even beyond 20 mg/ml, and readily crosses the blood-brain barrier. Previous studies (31) indicate that AY9944 also crosses the blood-retina barrier, and preliminary results from our own laboratory (S. J. Fliesler and R. K. Keller, unpublished data) suggest that this method can be used to quantify the absolute rate of sterol synthesis in mouse retina. Given the simplicity of this procedure, it now should be straightforward to evaluate and compare the ability of other hypolipidemic drugs (e.g., statins) to cross the blood-brain barrier and interfere with cholesterol synthesis.

As mentioned at the beginning of this article, there is strong evidence that neurodegeneration is accompanied by alterations in brain cholesterol metabolism, as demonstrated conclusively in the mouse model of Niemann Pick type C. Many other mouse models of neurodegeneration are now being used (39–41), and hence the new technique described herein should prove to be a valuable tool. One possible drawback of the technique is that Alzet® pumps cannot be used in newborn mice, because of the small size of the animal relative to that of the pump (1.5 cm in length); however, there is a report (42) of osmotic pumps being used in mice as young as 4 weeks of age (~15 g). For younger mice, particularly neonates, direct systemic injection of AY9944 would be necessary, a technique we have used with success previously (31).

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REFERENCES

1. Miller, L. J., and R. Chacko. 2004. The role of cholesterol and statins in Alzheimer's disease. *Ann. Pharmacother.* **38:** 91–98.
2. Michikawa, M. 2003. The role of cholesterol in pathogenesis of Alzheimer's disease: dual metabolic interaction between amyloid beta-protein and cholesterol. *Mol. Neurobiol.* **27:** 1–12.
3. Puglielli, L., R. E. Tanzi, and D. M. Kovacs. 2003. Alzheimer's disease: the cholesterol connection. *Nat. Neurosci.* **6:** 345–351.
4. Handi, K., and C. Kenney. 2003. Age-related macular degeneration: a new viewpoint. *Front. Biosci.* **8:** e305–e314.
5. Gorin, M. B., J. C. Breitner, P. T. De Jong, G. S. Hageman, C. C. Klaver, M. H. Kuehn, and J. M. Seddon. 1999. The genetics of age-related macular degeneration. *Mol. Vis.* **5:** 29.
6. Wolozin, B., W. Kellman, P. Ruosseau, G. G. Celesia, and G. Siegel. 2000. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arch. Neurol.* **57:** 1439–1443.
7. Corder, E. H., A. M. Saunders, W. J. Strittmatter, D. E. Schmechel, P. C. Gaskell, G. W. Small, A. D. Roses, J. L. Haines, and M. A. Pericak-Vance. 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science.* **261:** 921–923.
8. Hall, N. F., and C. N. Martin. 2002. Could statins prevent age-related macular degeneration? *Expert Opin. Pharmacother.* **3:** 803–807.
9. Klein, R., and B. E. Klein. 2004. Do statins prevent age-related macular degeneration? *Am. J. Ophthalmol.* **137:** 744–746.
10. van Leeuwen, R., C. C. Klaver, Jr., A. Vingerling, A. Hofman, C. M. van Duijn, B. H. Stricker, and P. T. de Jong. 2004. Cholesterol and age-related macular degeneration: is there a link? *Am. J. Ophthalmol.* **137:** 750–752.
11. Baird, P. N., E. Guida, D. T. Chu, H. T. Vu, and R. H. Guymer. 2004. The epsilon2 and epsilon4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **45:** 1311–1315.
12. Jurevics, H. A., F. Z. Kidwai, and P. Morell. 1997. Sources of cholesterol during development of the rat fetus and fetal organs. *J. Lipid Res.* **38:** 723–733.
13. Woollett, L. A. 2001. The origins and roles of cholesterol and fatty acids in the fetus. *Curr. Opin. Lipidol.* **12:** 305–312.
14. Ness, G. C., J. P. Miller, M. H. Moffler, L. S. Woods, and H. B. Harris. 1979. Perinatal development of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat lung, liver and brain. *Lipids.* **14:** 447–450.
15. Dietschy, J. M., and S. D. Turley. 2004. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J. Lipid Res.* **45:** 1375–1397.
16. Andersson, M., P. G. Elmberger, C. Edlund, K. Kristensson, and G. Dallner. 1990. Rates of cholesterol, ubiquinone, dolichol and dolichyl-P biosynthesis in rat brain slices. *FEBS Lett.* **269:** 15–18.
17. Björkhem, I., D. Lütjohann, U. Diczfalusy, L. Ståhle, G. Ahlborg, and J. Wahren. 1998. Cholesterol homeostasis in the human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. *J. Lipid Res.* **39:** 594–600.
18. Lund, E. G., C. Xie, T. Kotti, S. D. Turley, J. M. Dietschy, and D. W. Russell. 2003. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. *J. Biol. Chem.* **278:** 22980–22988.
19. Bretillon, L., A. Siden, L. O. Wahlund, D. Lutjohann, L. Minthon, M. Crisby, J. Hillert, C. G. Groth, U. Diczfalusy, and I. Bjorkhem. 2000. Plasma levels of 24S-hydroxycholesterol in patients with neurological diseases. *Neurosci. Lett.* **293:** 87–90.
20. Xie, C., E. G. Lund, S. D. Turley, D. W. Russell, and J. M. Dietschy. 2003. Quantitation of two pathways for cholesterol excretion from the brain in normal mice and mice with neurodegeneration. *J. Lipid Res.* **44:** 1780–1789.
21. Fassbender, K., M. Simons, C. Bergmann, M. Stroick, D. Lutjohann, P. Keller, H. Runz, S. Kuhl, T. Bertsch, K. von Bergmann, M. Hennerici, K. Beyreuther, and T. Hartmann. 2001. Simvastatin strongly reduces levels of Alzheimer's disease β-amyloid peptides Aβ42 and Aβ40 *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA.* **98:** 5856–5861.
22. Lutjohann, D., M. Stroick, T. Bertsch, S. Kuhl, B. Lindenthal, K. Thelen, U. Andersson, I. Bjorkhem, K. von Bergmann, and K. Fassbender. 2004. High doses of simvastatin, pravastatin, and cholesterol reduce brain cholesterol synthesis in guinea pigs. *Steroids.* **69:** 431–438.
23. Dietschy, J. M., and D. K. Spady. 1984. Measurement of rates of cholesterol synthesis using tritiated water. *J. Lipid Res.* **25:** 1469–1476.
24. Quan, G., C. Xie, J. M. Dietschy, and S. D. Turley. 2003. Ontogenesis and regulation of cholesterol metabolism in the central nervous system of the mouse. *Brain Res. Dev. Brain Res.* **146:** 87–98.
25. Jones, P. J., C. A. Leitch, Z. C. Li, and W. E. Connor. 1993. Human cholesterol synthesis measurement using deuterated water. Theoretical and procedural considerations. *Arterioscler. Thromb.* **13:** 247–253.
26. Lee, W. N., S. Bassilian, H. O. Ajie, D. A. Schoeller, J. Edmond, E. A. Bergner, and L. O. Byerley. 1994. *In vivo* measurement of fatty acids and cholesterol synthesis using D_2O and mass isotopomer analysis. *Am. J. Physiol.* **266:** E699–E708.
27. Diraison, F., C. Pachiaudi, and M. Beylot. 1997. Measuring lipogenesis and cholesterol synthesis in humans with deuterated water. *J. Mass Spectrom.* **32:** 81–86.
28. Bjorkhem, I., D. Lutjohann, O. Breuer, A. Sakinis, and A. Wennmalm. 1997. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with $^{18}O_2$ techniques in vivo and in vitro. *J. Biol. Chem.* **272:** 30178–30184.
29. Keller, R. K., and S. J. Fliesler. 1997. Isoprenoid metabolism in the vertebrate retina. *Int. J. Biochem. Cell Biol.* **29:** 877–894.
30. Fliesler, S. J. 2002. Effects of cholesterol biosynthesis inhibitors on retinal development, structure, and function. *In* Sterols and Oxysterols: Chemistry, Biology, and Pathobiology. S. J. Fliesler, editor. Research Signpost, Kerala, India. 77–109.
31. Fliesler, S. J., M. J. Richards, C-Y. Miller, and N. S. Peachey. 1999. Marked alteration of sterol metabolism and composition without compromising retinal development or function. *Invest. Ophthalmol. Vis. Sci.* **40:** 1792–1801.
32. Keller, R. K., T. P. Arnold, and S. J. Fliesler. 2004. Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **45:** 347–355.
33. Cenedella, R. J. 1980. Concentration-dependent effects of AY-9944 and U18666A on sterol synthesis in brain. Variable sensitivities of metabolic steps. *Biochem. Pharmacol.* **29:** 2751–2754.
34. Tint, G. S., M. Irons, E. R. Elias, A. K. Batta, R. Frieden, T. S. Chen, and G. Salen. 1994. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330:** 107–113.
35. Austin, L., G. W. Arendash, M. N. Gordon, D. M. Diamond, G. DiCarlo, C. Dickey, K. Ugen, and D. Morgan. 2003. Short-term beta-amyloid vaccinations do not improve cognitive performance in cognitively impaired APP + PS1 mice. *Behav. Neurosci.* **117:** 478–484.
36. Keller, R. K., and S. J. Fliesler. 1990. Incorporation of squalene into rod outer segments. *J. Biol. Chem.* **265:** 13709–13712.
37. Kaiser, W., and K. Stocker. 1978. Evaluation of a nonisotopic technique for studies of in vivo cholesterol metabolism in mini-pigs using inhibition of 7-dehydrocholesterol reductase by AY 9944. *Res. Exp. Med. (Berl.).* **174:** 79–108.
38. Gibbons, G. F., and C. R. Pullinger. 1977. Measurement of the absolute rates of cholesterol biosynthesis in isolated rat liver cells. *Biochem. J.* **162:** 321–330.
39. Ahmad-Annuar, A., S. J. Tabrizi, and E. M. Fisher. 2003. Mouse models as a tool for understanding neurodegenerative diseases. *Curr. Opin. Neurol.* **16:** 451–458.
40. Watase, K., and H. Y. Zoghbi. 2003. Modelling brain diseases in mice: the challenges of design and analysis. *Nat. Rev. Genet.* **4:** 296–307.
41. Shibata, N., H. Oda, A. Hirano, Y. Kato, M. Kawaguchi, M. C. Dal Canto, K. Uchida, T. Sawada, and M. Kobayashi. 2002. Molecular biological approaches to neurological disorders including knockout and transgenic mouse models. *Neuropathology.* **22:** 337–349.
42. Meyer, M. H., and R. A. Meyer, Jr. 1998. Increased intestinal absorption of calcium in young and adult X-linked hypophosphatemic mice after the administration of 1,25-dihydroxyvitamin D3. *J. Bone Miner. Res.* **3:** 151–157.

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ERRATA

In the article "Enzyme blockade: a nonradioactive method to determine the absolute rate of cholesterol synthesis in the brain" by Keller et al., published in the October 2004 issue of the *Journal of Lipid Research* (Volume **45**, pages 1952–1957), lines 20–22 of the Abstract should now read: "The rate of brain cholesterol synthesis determined by this method (~30 μg/day) closely agrees with that determined by the radioactive method."