

**Enzyme Blockade:
A Nonradioactive Method to Determine the Absolute Rate of
Cholesterol Synthesis in the Brain[†]**

R. Kennedy Keller[‡], Michael Small[‡], and Steven J. Fliesler^{§*}

[‡]Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, FL and [§]Departments of Ophthalmology (Saint Louis University Eye Institute) and Pharmacological and Physiological Science, Saint Louis University School of Medicine, Saint Louis, MO

Running title: Brain Cholesterol Synthesis

*Correspondence to:

Steven J. Fliesler, Ph.D.

Saint Louis University Eye Institute

Saint Louis University School of Medicine

1755 S. Grand Blvd.

St. Louis, MO 63104-1540 U.S.A.

Email: Fliesler@slu.edu

Abbreviations: AD, Alzheimer's disease; ARMD, age-related macular degeneration; AY9944, *trans*-[1,4-*bis*(2-dichlorobenzylaminomethyl) cyclohexane] dihydrochloride; BS, brain stem; CB, cerebellum; CH, cholesterol; CNS, central nervous system; DES, desmosterol; DHC, 7-dehydrocholesterol; FC, frontal cortex; HC, hippocampus; NSL, nonsaponifiable lipid; PC, posterior cortex.

[†] Portions of this study were presented at the University of South Florida Graduate Research Symposium (Tampa, FL, Feb. 26, 2004)

Supplementary Keywords: CNS; sterol metabolism; Alzheimer's disease; 7-dehydrocholesterol; AY9944

SUMMARY

The standard *in vivo* method to determine rates of brain cholesterol synthesis involves systemic injection of $^3\text{H}_2\text{O}$ and measurement of incorporated radioactivity into 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 [^3H]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 to 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 $\mu\text{g/g/day}$) 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 and cerebellum having the lowest rate.

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; refs. (1-3)) and age-related macular degeneration (ARMD; refs. (4,5)). For instance, retrospective studies have suggested that statins, which act on the rate-limiting enzyme of cholesterol synthesis (HMG-CoA reductase), lower 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, while 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 drops precipitously after weaning (14), as the rate of myelination dramatically declines (for a 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 $t_{1/2}$ on the order of months (16). A portion of brain cholesterol is metabolized to 24S-hydroxycholesterol, which then exits across the blood-brain-barrier into the bloodstream (17). Over 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 metabolism of cholesterol via the 24S-hydroxy route.

Since 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 A β formation in guinea pigs. While this method does not involve the use of radioactive precursors, it only provides an estimate of the relative 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 employing $^3\text{H}_2\text{O}$ 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 $^3\text{H}_2\text{O}$ 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 handling and disposal of a volatile radioisotope and the resulting radioactive carcasses and tissue extracts. Other workers have employed $^2\text{H}_2\text{O}$, or ^{18}O (25-28), rather than $^3\text{H}_2\text{O}$; while 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 employed 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 (DHC reductase, E.C.1.3.1.21), 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 the dramatic elevation of 7-dehydrocholesterol (DHC) levels and lowering 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, since, as mentioned above, the brain synthesizes virtually all of its own cholesterol and turns it over slowly. In order 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, employing AY9944, meets all these criteria and that it yields values for brain cholesterol synthesis rates that agree well with those reported previously using the $^3\text{H}_2\text{O}$ 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 & Jackson, Fisher Scientific). [^3H]Acetic acid (sodium salt, 15 Ci/mmol) and [1,2- ^3H]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, in preparation) 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).¹

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 non-transgenic crosses of mice from C57B6, SJL, Swiss Webster, and B6D2 backgrounds (see (35)). All procedures involving animals were approved by the University of South Florida IUCAC 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 $\mu\text{l/h}$ (20 μg AY9944/h) for 3 days, or approximately 0.7 μg AY9944/h/g body weight (animals averaged 30g). At various times after implantation, mice were euthanatized by pentobarbital overdose (150 mg/kg) and tissues were taken for sterol analysis (see below). For the administration of radioactivity, 200 mCi of sodium [^3H]acetate (sodium salt, 15 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) in ethanol were taken to dryness under nitrogen, then dissolved in PBS, and mice were injected intraperitoneally (1 mCi/g body weight).

In the experiment using [^3H]acetate, to ensure that brain tissue was not contaminated with radioactive sterols from extracellular fluids (*e.g.*, blood), the brains were homogenized in 20 vols of 10 mM Tris-Cl, pH 7.4, and the homogenate was centrifuged 100,000 $\times g$ 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 was repeated four more times, after which the supernatant contained less <10% of the total radioactivity in the resuspended pellet. In all other experiments, whole brain tissues or brain regions were used directly, since 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).

In order to determine cholesterol synthesis in different regions of the brain, four male mice (~30 g each) were treated with AY9944 as above and euthanatized after three 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 MeOH 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 under nitrogen. The residues were dissolved in mobile phase solvent (MeOH-isopropanol, 7:1, by vol.) and aliquots were subjected to reverse-phase HPLC (Spheri-5 RP C18 column, 4.6 x 150 mm, Brownlee Laboratories; flow rate, 1 ml/min) with on-line UV 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 on-line 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 2-tailed Student's *t*-test, assuming equal variances (homoscedastic).

RESULTS

Chromatographic resolution and detection of sterols

Figure 1 shows the HPLC elution profile obtained for desmosterol (DES), DHC, and cholesterol (CH), comparing detection at 205 nm vs. 282 nm, using an internal standard of [³H]cholesterol with scintillation detection. As shown in the lower panel, only DHC is detected at 282 nm (the absorption spectrum for DHC has a relative maximum at 282 nm, due to the presence of conjugated double bonds in ring B of the sterol nucleus; see insert, Fig. 1). Empirically, we determined its molar extinction coefficient at this wavelength to be 13,100 M⁻¹cm⁻¹ (in MeOH-isopropanol, 7:1, by vol). Hence, detection at 282 nm offers a selective and sensitive means for quantifying DHC mass. Also, under the chromatographic conditions employed, baseline resolution of all three sterol standards was achieved (middle panel, Fig. 1).

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

In order for AY9944 to be a useful tool to quantify sterol synthesis in the brain, it must potently and selectively inhibit 3 β -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 μ g/h) subcutaneously to 4 adult mice by Alzet[®] pump. Four sham-operated mice served as controls. After 48 h, all mice were injected with [³H]acetate (1 mCi/g body weight). One day later, all animals were euthanized and tissues were taken for analysis of nonsaponifiable lipids (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 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 UV-detectable mass with retention time corresponding to DHC (Fig. 2, right-hand 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, ca. 4-8 min). Although the identity of these compounds is not certain, their polarity, relative abundance, and incorporation of [³H]acetate is 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 approximately 85% ($\pm 4\%$), with only a slight, but not significant, decrease in overall sterol synthesis (Table I). It is to be noted that the 85% inhibition represents that observed between 2 and 3 days accumulation of DHC. Although the exact nature of AY9944 inhibition of DHC reductase has not been demonstrated, assuming that it is competitive in nature, then the percent 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 potentially 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 employed. We implanted Alzet pumps containing AY9944 subcutaneously in nine mice. At each of three time intervals (1, 2, and 3 days post-implantation), three mice were subsequently euthanized, the brains were removed, saponified, and the nonsaponifiable lipids 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 wt. in these animals. Figure 3 shows that, following 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 three-day time period examined. [Subsequently, we have found that this lag period can be reduced by almost 6 h if the Alzet pump is pre-equilibrated in buffer at 37 °C overnight prior to 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/CH/day). Since the mouse brains used in this study contained approximately 10 mg cholesterol, this corresponds to an accumulation of 30 μ g of sterol synthesized per brain per day. This value is in good agreement with the sterol synthesis value of 35 μ g/day for mouse CNS obtained by Quan *et al.* (24), who used the $^3\text{H}_2\text{O}$ method for determining the absolute rate of brain sterol synthesis in the mouse.

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 three days as described above, and then dissected out various brain regions and quantified their sterol content. The rate of sterol synthesis (expressed as μ g DHC accumulated per g wet wt. of brain per 3 days)

was calculated based on the equation derived from the data presented in Fig. 3. As shown in Figure 4, frontal cortex (FC) had the highest rate of cholesterol synthesis, different from all other regions examined ($0.01 < p < 0.05$), except posterior cortex (PC; $p < 0.14$). Cerebellum (CB) had the lowest rate, significantly lower than any other brain region examined (*e.g.*, 35.9% of the FC rate, $p < 0.001$). Hippocampus (HC), posterior cortex (PC), and brain stem (BS) exhibited rates comparable to (and not statistically different from) each other and intermediate to the rates observed for FC and CB. The cholesterol concentrations in the major regions of the brain were determined (in mg/g wet wt, mean \pm SD, $n=4$, except hippocampus, $n=1$): frontal cortex, 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 using 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 employed 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 three days of administration. Strictly speaking, the general approach of enzyme blockade is not novel. Over 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 affect on total sterol synthesis was not rigorously assessed.

The results obtained using this non-radioisotopic, 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 disposal of radioactive tissues and extracts. AY9944, as the hydrochloride salt, is aqueous-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 lab (S.J. Fliesler and R.K. Keller, unpublished) 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 employed (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, due to 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).

ACKNOWLEDGMENTS

This study was supported, in part, by U.S.P.H.S. grant EY07361 (SJF), by March of Dimes grant 1-FY01-339 (SJF), and by an unrestricted departmental grant from Research to Prevent Blindness (SJF). The authors thank Michael J. Richards and Samuel Clarke for technical assistance. The gift of mice from Drs. David Morgan and Marcia Gorton (University of South Florida) and an authentic sample of AY9944 from Wyeth-Ayerst Research are gratefully acknowledged.

FOOTNOTES

¹ AY9944 now is commercially available (Calbiochem, Cat. #190080).

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FIGURE LEGENDS

Fig. 1. Reverse-phase HPLC analysis of sterol standards. CH, cholesterol; DHC, 7-dehydrocholesterol; DES, desmosterol. Mobile phase: MeOH-isopropanol, 7:1 (v/v), at 1 ml/min. Relative response, normalized to dominant sterol component. *Upper panel:* [³H]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 unique detection of DHC at this wavelength. *Inset, bottom panel:* UV absorption spectrum of DHC in mobile phase, illustrating maximal absorbance at 282 nm.

Fig. 2. Reverse-phase, radio-HPLC analysis of brain NSLs from control (*left panels*) and AY9944-treated mice (*right panels*), 2 days after systemic injection with [³H]acetate (1 mCi/g body wt.). *Upper panels:* mass, detected at 282 nm; *lower panels:* radioactivity detected by flow-through scintillation spectrometry. Elution positions corresponding to DHC and CH are indicated.

Fig. 3. Time course for accumulation of DHC in brains of AY9944-treated mice. Following an initial lag of ~0.3 day, accumulation was linear over 3 days, fitting a simple, linear algebraic equation with a correlation coefficient (R^2) of 0.9705.

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

TABLE 1. Effect of AY9944 on incorporation of [³H]acetate into 7-dehydrocholesterol (DHC) and cholesterol (CH) in mouse brains.

SAMPLE	³ H]ACETATE INCORPORATION				
	DHC (dpm/nmol CH)	CH (dpm/nmol CH)	DHC+CH (dpm/nmol CH)	% Inhibition ^a DHC:CH	%Inhibition ^b [³ H]Ac: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

On day 1, Alzet pumps (3-day duration, 1 ul/hr) containing AY9944 (20 mg/ml) were implanted into mice (N=4). Four mice served as untreated controls. On day 2, all mice were administered [³H]acetate (1 mCi/g body wt.) by intraperitoneal injection. On day 3, all animals were euthanatized and tissues were harvested for analysis of NSLs.

^a Inhibition of conversion of DHC to CH.

^b Inhibition of total sterol synthesis (conversion of [³H]acetate to sterols) by AY9944. Equal to ave. radioactivity (dpm) in DH+CH in AY9944-treated brain divided by ave. dpm in CH from control brain.







