Summary: Purpose: The AY-9944 (AY)-treated rat is a reproducible and clinically relevant animal model of atypical absence seizures. AY inhibits cholesterol synthesis, but the relation between brain sterol levels and the spontaneously recurrent absence seizures has not been determined.

Methods: Long-Evans hooded rats were treated every 6 days from postnatal day (P)2 to P20 with AY (7.5 mg/kg, s.c.) or saline. Electrodes were permanently implanted under pentobarbital anesthesia at P50. Spike-and-wave discharge (SWD) duration and amplitude were quantified at P55. Changes in brain sterols after AY were examined in three different experiments, looking at brain regions (experiment 1), recovery after stopping AY (experiment 2), or gender differences (experiment 3).

Results: Experiment 1: AY caused spontaneously recurrent slow SWD that lasted 59 times longer and had a 3.2-fold higher amplitude than that in controls. At P55, brain cholesterol was reduced and 7-dehydrocholesterol was increased in all brain regions (p < 0.0001). Experiment 2: Four hundred days after stopping AY-9944 treatment (P420), brain sterol levels had returned to normal levels, but the AY-induced SWD lasted twice as long as at P55. Experiment 3: At P55, AY-induced changes in plasma and liver (but not brain) sterols were significantly more severe in females compared with males.

Conclusions: AY-induced seizures appear to be related to AY-induced changes in brain sterols but persisted long after the sterols had returned to normal after the last AY injection. Hence, there appears to be a critical developmental window during which the AY must be given but after which the AY-induced change in brain sterols is no longer essential to sustaining the seizures. Key Words: AY-9944—Cholesterol—7-Dehydrocholesterol—Electroencephalogram—Atypical absence seizure—Brain.
for Sick Children (Toronto, Ontario, Canada). The suckling rats were weaned at P21 and then housed in groups of three animals of the same gender, until the day of surgery for electrode implantation. All animals were maintained in a controlled environment at 12-h light, 12-h dark cycle with lights on at 0600 h. They were given ad lib access to food and water. AY-9944 (trans-1,4-bis [2-chloro-benzylaminomethyl] cyclohexane dihydrochloride) was a gift from Wyeth-Ayerst (Philadelphia, PA, U.S.A.) and was administered subcutaneously at a dose of 7.5 mg/kg, with saline as the vehicle.

Three groups of experiments were done. In experiment 1, the regional analysis of sterols in brain was performed. Rats were injected either with AY (n = 10) or the equivalent volume of saline (controls, n = 10) every 6 days from P2 to P20. The principal brain regions were then dissected before sterol analysis. In experiment 2, we sought to determine the sustainability of the seizures and sterol changes induced by AY over time. Two AY rats were maintained for 400 days after the last AY injection at P20. In experiment 3, we ascertained gender differences in AY-induced alterations in sterols in brain, blood, and liver. The seizure and sterol responses of male (n = 6) or female (n = 5) rats to the same AY protocol were examined.

Surgery and electrocorticography (ECOG)

Before electrode implantation, anesthesia was achieved with a single i.p. injection of pentobarbital (PTB; 35 mg/kg), which lasted for 1.5–2 h. Surgeries consisted of permanent implantation of two frontal and two parietal monopolar epidural electrodes and were done at P50, at which time the average body weight was 250–350 g for males and 200–300 g for females. Electrodes were secured with dental cement, and two screws previously attached to the parietal regions of the scalp without touching the dura mater (7). To define the source and extent of the SWD in the AY model, two frontal monopolar electrodes were placed 1 mm deep, 2.20 mm anterior to bregma, and 3 mm lateral from midline. Referential, unilateral left and right bipolar, and linked monopolar recordings from ipsilateral regions were compared to determine the phase relation, amplitudes, and source of the SWD (3). After surgery, animals were returned to the animal facility for a 4-day recovery period.

On the day of recordings (P55), the rats were freely moving in individual, warm Plexiglas chambers (Harvard Apparatus, Holliston, MA, U.S.A.). A 20-min adaptation period before ECoG recordings minimized movement artifact. ECoG recordings were made on paper by using a Grass Polysonomograph (Grass Instruments, Quincy, MA, U.S.A.). All baseline and test recordings were performed from 10:00 to 14:00 h to minimize circadian variations (8).

Brain lipid analysis

Immediately after the ECoGs, the rats used for brain sterol analysis were killed with PTB (35 mg/kg), and the brains quickly removed. In experiments 1 and 2, brains were dissected into five separate regions: cortex, thalamus, hippocampus, brainstem, and cerebellum. In experiment 3, plasma, brain, and liver were collected. Tissue or plasma samples were homogenized in an equal volume of saline, and total lipids were extracted by using 20 volumes of 2:1 chloroform: methanol. For fatty acid analysis of brain phospholipids, fatty acid methyl esters were prepared by using boron trifluoride in methanol, as previously described (9). Fatty acid analysis was done by capillary gas chromatography on a 30-m column (9,10). Brain total phospholipids were quantified during fatty acid analysis by using an internal standard, heptadecanoic acid, which was added at the stage of total lipid extraction. Tissue sterols were obtained after saponification of the total lipid extracts in 1N methanolic KOH. Sterols were derivatized by using tert-butyldimethylsilyl chloride, and analyzed with capillary gas chromatography using 5-α-cholestanate as an internal standard.

Data analysis

AY-induced absence seizures were quantified by measuring SWD duration from onset to offset for three consecutive 20-min periods (11). SWDs were scored in each animal only if two frontal and parietal ECoGs demonstrated the distinct 5-Hz SWD morphology with at least a fourfold higher amplitude compared with control (12). All seizure data are expressed as the arithmetic mean ± standard error of mean (SEM). Brain lipid data are expressed as the mean ± SD. Comparison of means between two groups were done by two-tailed Student's t test. Analysis of variance (ANOVA) for repeated measures was used to quantify the amplitude differences between ECoG baseline, spontaneous bursts, and SWDs as a function of AY and time after injection. Effectiveness for age-pairing results was evaluated statistically by using the nonparametric Spearman correlation test, with a probability value of p < 0.05 chosen as an index of statistical significance.

RESULTS

Electrocorticography characteristics of AY seizures

At P55, AY-induced spontaneously recurrent SWDs lasted for 1,447 ± 303 s/h and had a mean amplitude of 465 ± 99 μV compared with 25 ± 26 s/h and 142 ± 70 μV, respectively, in controls (Fig. 1A), proved to be a robust and long-lived phenomenon that became more severe with aging. Four hundred days after the last AY injection at P20, AY-induced SWDs lasted twice as long as those recorded at P55 in the same animals (2,980 s/h; p < 0.0005; Fig. 1B).
Brain Sterols in the AY-9944 Model

BRAIN STEROLS IN THE AY-9944 MODEL

FIG. 1. A: Electrocorticographic (EcoG) recording at P55 in the AY model illustrates the spontaneously recurrent spike-and-wave discharge (SWD; arrows) after cholesterol biosynthesis inhibition during development from P2 to P20. The ictal behavior consisted of frozen staring, vibrissae twitching and facial myoclonus, and intermittent ability to move during seizures. B: EcoG recording at P420 shows a more severe and prolonged SWD (arrow), which also was associated with head clonus occurring at the same SWD frequency of 5–6 Hz. F-F, Frontal-Frontal; P-P, Parietal-Parietal differential recordings.

Brain lipids

Capillary gas chromatograms showed two distinct features in AY-treated rats: a smaller cholesterol peak compared with controls and an additional 7-dehydrocholesterol peak, which was not detected in controls (Fig. 2).

In experiment 1, brain cholesterol in controls was 15–17 mg/g in all regions except the brainstem, in which it was 22 mg/g. Cholesterol was reduced in all separated brain regions (cerebral cortex, hippocampus, thalamus, cerebellum, and brainstem) by an average of 39–42% or from ~16 mg/g to ~10 mg/g in AY-9944–treated rats (all values of p < 0.0001; Fig. 3A). 7-Dehydrocholesterol was increased from undetectable levels to ~4.5 mg/g in all regions except the brainstem, where it was 7.6 mg/g (p < 0.01; Fig. 3B).

Brain phospholipid concentration remained unchanged after AY treatment, so the cholesterol/phospholipid ratio in AY-9944 subjects was reduced by 36–40%. Fatty acid profiles were unaffected by AY-9944 in all of the five brain regions examined (data not shown). Four hundred days after discontinuing AY (experiment 2), brain sterols had returned to normal levels (Fig. 3). AY had a similar effect on brain, liver, and plasma sterols in male and female rats (Table 1). In experiment 3, AY reduced tissue and plasma cholesterol and increased 7-dehydrocholesterol in both males and females, but the 7-dehydrocholesterol/cholesterol ratio was 25–64% lower in plasma, brain, and liver of male compared with female rats. This difference achieved statistical significance (p < 0.01) in plasma and liver only.

DISCUSSION

To our knowledge, this is the first report on brain sterol measurement in the AY model of atypical absence seizures. Our results indicate that in this model, there is a significant reduction in cholesterol and increase in 7-dehydrocholesterol in all brain regions examined. Because brain total phospholipids and fatty acid profiles remained unaffected, this study confirms that, at least within the brain of the developing rat, AY-9944 appears to have a specific effect on cholesterol biosynthesis. However, whether cholesterol reduction or accumulation
Before AY-9944

After AY-9944

FIG. 2. Capillary gas chromatogram of brain sterols from 55-day-old control (upper) or AY-9944-treated (lower) rats. In both chromatograms, the peaks are the internal standard 5α-cholestane (5C), cholesterol (CH), which is the only detectable sterol in controls, and 7-dehydrocholesterol (7-DHC), which is the only additional sterol present after AY-9944 treatment.

of 7-dehydrocholesterol during development is directly responsible for the generation of atypical absence seizures remains to be determined.

Our recovery study (experiment 2) indicates that the reduction of brain cholesterol and the elevation of 7-dehydrocholesterol in AY-treated rats is transitory once the AY treatment is stopped. However, the adult rats (P420) continued to experience atypical absence seizures, which lasted twice as long as at P55. Thus the AY-induced epileptiform discharges are spontaneously recurrent and appear to be life long, but the brain sterol changes are not. There seems to be a critical developmental window for AY induction of SWDs, because early treatment with AY-9944 disclosed a more severe SWD duration on the ECoG compared with that of rats treated with AY later in life (13).

Cholesterol biosynthesis inhibitors were first shown to induce chronic epileptiform activity in 1978 (14). This phenomenon was further characterized 12 years later when it was proposed that the AY-treated rat could represent a model of absence epilepsy (i.e., spontaneous, recurrent, absence seizures) (15). Subsequently it was demonstrated that a γ-aminobutyric acid (GABA)B-receptor antagonist, CGP-35348 (16), reduced absence seizures in AY-treated rats. We have confirmed these findings, and provided EEG, pharmacologic, and developmental characterization of this model. We also demonstrated the increased sensitivity of Long-Evans strain of rats to AY and showed a distinctly greater susceptibility to AY-induced SWDs in female rats (1). Our present data show that the same dose of AY increases the 7-dehydrocholesterol-to-cholesterol ratio more in plasma and liver (but not brain) of females than in males, which helps link the brain sterol changes to seizure severity in this model (1,2,10). Sex differences were reported recently in total cholesterol, low-density and high-density lipoprotein cholesterol in response to antiepileptic medication in male and female patients (17). Therefore it is conceivable that the greater seizure susceptibility to AY in female rats may relate to gender differences in plasma lipoprotein metabolism or to the different susceptibility to altered brain sterol levels (18).

Two diseases are associated with perturbed brain cholesterol (i.e., Niemann–Pick type C and Smith–Lemli–Opitz syndrome). Niemann–Pick type C is an abnormality of intracellular cholesterol transport (19), and Smith–Lemli–Opitz syndrome is a genetic deficiency of cholesterol synthesis that adversely affects fetal and neonatal development (20). However, neither of

TABLE I. Brain, liver, and plasma sterols in Long-Evans rats treated with AY-9944

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>7-DHC</th>
<th>7-DHC/CH</th>
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</thead>
<tbody>
<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10.1 ± 1.2*</td>
<td>3.9 ± 0.8</td>
<td>0.39 ± 0.11</td>
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<tr>
<td>Female</td>
<td>9.7 ± 0.7</td>
<td>5.0 ± 0.8</td>
<td>0.52 ± 0.10</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>1.3 ± 0.2</td>
<td>0.0 ± 0.3</td>
<td>0.46 ± 0.15</td>
</tr>
<tr>
<td>Female</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.00 ± 0.27*</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.4 ± 0.03</td>
<td>0.1 ± 0.02</td>
<td>0.18 ± 0.10</td>
</tr>
<tr>
<td>Female</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.01</td>
<td>0.52 ± 0.19*</td>
</tr>
</tbody>
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CH, cholesterol; 7-DHC, 7-dehydrocholesterol.

*mg/g or mg/ml for plasma; mean ± SD (n = 5 for females and n = 6 for males).

* p < 0.01 versus males.
BRAIN STEROLS IN THE AY-9944 MODEL

FIG. 3. Sterol measurements from five separate brain regions showing an average 40% reduction of cholesterol (A) and five- to sevenfold increase in 7-dehydrocholesterol (B) in all regions in AY-9944-treated Long-Evans hooded rats compared with controls (n = 10/group; experiment 1). Recovery values are for rats at P420 (n = 2, experiment 2). Data are expressed in mean ± SD. Spike-and-wave discharge duration at P420 was 2,980 s/h or twice as long as that at P55 (p < 0.0005). Ctx, Cortex; Hi, hippocampus; Th, thalamus; Cbl, cerebellum; Bst, brainstem.

these diseases has atypical absence seizures as part of the clinical phenotype. From a neurobiologic perspective, 25-hydroxycholesterol has been shown to reduce GABA_B but not GABA_A receptor-mediated inhibition selectively (21). However, nothing is known of the neurologic effects of the brain sterol raised by AY (7-dehydrocholesterol). Furthermore, cholesterol-derived neurosteroids augment GABA_A receptor-mediated and inhibit GABA_B receptor-mediated inhibition (22). Membrane cholesterol has been shown to alter the sensitivity of the GABA_A receptor to GABA (23). In addition, there is a cholesterol-recognition site in the peripheral-type benzodiazepine receptor on mitochondria, which could be altered by postnatal AY treatment (24). Alternatively, AY could exert an effect on brain unrelated to the ability of this compound to inhibit cholesterol synthesis. Brain cholesterol is made locally only in the brain (25) and can be experimentally depleted by cholesterol biosynthesis inhibitors other than AY 9944 (6,13,26). We are presently comparing these inhibitors to assess the relative importance of total brain sterol reduction versus a change in the 7-dehydrocholesterol/cholesterol ratio in seizures induced by cholesterol biosynthesis inhibitors other than AY.

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